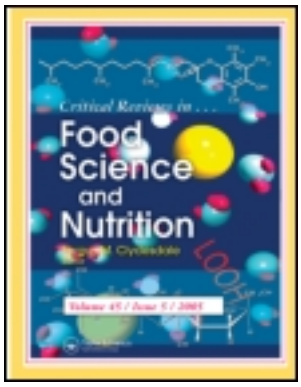

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The Role of Folic Acid Fortification in Neural Tube Defects: A Review

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The Role of Folic Acid Fortification in Neural Tube Defects: A Review

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The worldwide prevalence of neural tube defects (NTDs) has fallen noticeably during the past 30 years, but the specific etiology and causative mechanism of NTDs remain unknown. Since introduction of mandatory fortification of grains with folic acid, a further decrease in NTD prevalence has been reported in North America and other countries with large variations among ethnic subgroups. However, a significant portion of NTDs still persists. Population data suggest that women of childbearing age may not yet be adequately targeted, while the general population may be overfortified with folic acid. While an excessive folate intake may be associated with adverse effects, there remains uncertainty about the minimum effective folate intake and status required for NTD prevention, and the safe upper folate level. Besides folate, several other lifestyle and environmental factors as well as genetic variations may influence NTD development, possibly by affecting one-carbon metabolism and thus epigenetic events.

In conclusion, mandatory folic acid fortification plays a significant part in the reduction of NTD prevalence, but possibly at a cost and with a portion of NTDs remaining. More effective preventive strategies require better understanding of the etiology of this group of birth defects.

Keywords Prevention, etiology, nutrition, epigenetics, epidemiology

NEURAL TUBE DEFECTS: ETIOLOGY AND PREVALENCE

Neural tube defects (NTDs) refer to a cluster of neurodevelopmental conditions associated with the failure in the closure of the neural tube in the fetus within about 28 days following conception (Botto et al., 1999). They comprise spina bifida, anencephaly, encephalocele, craniorachischisis, and iniencephaly (Kondo et al., 2009). Spina bifida and anencephaly are the most common forms, with anencephaly being fatal after the first few hours of life and spina bifida having ranges in the severity of phenotypic defects (Botto et al., 1999). Treatment of spina bifida does not restore normal life as many patients continue to have motor and sensory dysfunction along with failure of sphincter control (Padmanabhan, 2006). Approximately 20% of affected infants have coexisting congenital anomalies, which often lead to limited survival in many cases and high cost burden due to treatment options and complications (Botto et al., 1999).

Each year, NTDs affect approximately more than 300,000 newborns worldwide (Christianson et al., 2006), with about 3000 of these in the United States (CDC, 2010). However, the worldwide prevalence of NTDs has fallen noticeably during the past 30 years (Kondo et al., 2009). Reasons for this trend are advancements in diagnostic imaging; use of clinical markers, such as serum alpha-fetoprotein to detect disease presence; and availability and increasing social acceptance of pregnancy termination, along with the introduction of preventive measures such as fortification with folic acid of staple foods in several countries (Kondo et al., 2009).

The development of the normal human neural tube is a multistep process that is genetically controlled and modulated by many environmental factors, especially maternal health and nutritional status (Padmanabhan, 2006). Epidemiologic studies have found an association between NTD prevalence and geographic variations, temporal variability, and ethnic differences

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as well as socioeconomic status (Botto et al., 1999). However, the specific etiology and exact causative mechanisms of NTDs, which is a prerequisite for effective preventive strategies, still remain unknown (Beaudin and Stover, 2009; Copp and Greene, 2010).

In this review, we will examine evidence on causal mechanisms; current prevention efforts, in particular folic acid fortification of staple foods; their success rates; and the necessary steps toward the ultimate goal of eliminating NTDs.

THE ROLE OF FOLIC ACID AND EFFECTS OF FORTIFICATION

History of Fortification

The food fortification policy in the United States has been in effect for over 75 years in an effort to bridge the gap between poor diets and disease leading to disability and mortality (Backstrand, 2002). Fortification of food with vitamins or minerals has been effective in the prevention of several micronutrient deficiencies and associated diseases, as in the case of iodine and goiter, niacin and pellagra (Backstrand, 2002), or vitamin D and rickets (Holick, 1994). These successes in disease elimination led epidemiologists to continue the search for more nutrient–disease relations. Over the past 30 years, a number of studies, both observational and randomized, have indicated that an increase in folic acid intake of women before and during the first 28 days following conception reduces the risk of NTDs in their children (MRC Vitamin Study Research Group, 1991; Czeizel and Dudas, 1992; Hall and Solehdin, 1998). It is well known that folate plays a decisive role in cell division and growth. Because the critical period is so early in the pregnancy, accompanied by the fact that >50% of all pregnancies in the United States are unplanned and NTDs most often affect couples with no family history of the disease (Backstrand, 2002), public health programs were implemented to increase the folate status of all women of reproductive age (Heseker et al., 2009).

The first attempts included public education campaigns and recommendation of periconceptional folic acid supplementation to women of childbearing age (Eichholzer et al., 2006; Heseker et al., 2009). However, as these efforts were only partially effective, public health authorities in North America and other countries such as Chile moved forward in attempts toward mandatory fortification of staple foods, such as flour or grains, with folic acid from the late-1990s onward (Eichholzer et al., 2006; Heseker et al., 2009). Mandatory folic acid fortification as a "passive" public health intervention (Backstrand, 2002) seemed to be the best way to reach the target group (Eichholzer et al., 2006) and therefore to prevent NTDs (Backstrand, 2002). Thus, folic acid fortification represents a decisive shift in longstanding public health policies that had claimed the necessity of widespread micronutrient deficiency in a well-defined population group as a prerequisite for fortification (Backstrand, 2002).

Bioavailability of Food Folate Versus Synthetic Folic Acid

Folate is the generic term for all forms of this water-soluble vitamin B complex and includes both naturally occurring food folate and the synthetic folic acid that is added to supplements and fortified foods. The terms "folic acid" and "folate" are often used interchangeably. However, because of substantial structural differences, it is important to differentiate between natural folate and synthetic folic acid, when considering the range of possible effects of folate. Generally, most studies do not consider food folate when tallying total intakes due to the difficulty in its quantification and the belief that there is no toxic amount of the natural form. The synthetic form is almost two times more bioavailable than natural food folate (Eichholzer et al., 2006). The concept of the dietary folate equivalent (DFE) takes into account a mixed intake of folic acid and natural folate (Choumenkovitch et al., 2002): 1 μg of DFE is equivalent to 1 μg of natural food folate, to 0.6 μg of synthetic folic acid from fortified foods or as a supplement taken with meals, and to 0.5 μg folic acid taken on an empty stomach (Eichholzer et al., 2006). The synthetic oxidized form of folic acid is more stable, whereas the naturally occurring reduced forms of food folate are more labile (Eichholzer et al., 2006; Lucock and Yates, 2009). Because it is more stable, increased synthetic folic acid intake leads to higher blood concentrations of the unmetabolized analyte in the bloodstream for an unknown duration of time, with unknown health consequences (Kelly et al., 1997; Smith et al., 2008; Dary, 2009; Berry et al., 2010).

Folate Recommendations Versus Folate Needs

The current recommended dietary allowance (RDA) for folate for the general US adult population is 400 μg of DFE per day (IOM, 1998). For primary prevention of NTDs, the US Public Health Service (PHS) recommended in 1992 that women of childbearing age who are capable of becoming pregnant should consume 400 μg of folic acid daily (CDC, 1992). Since 400 μg of synthetic folic acid is equivalent to approximately 800 μg of DFE, this is about twice the RDA for the general population. Women who have previously suffered a NTD-affected pregnancy are advised to take 4 mg of folic acid daily before conception and during the first months of pregnancy (CDC, 1992). These recommendations were mainly based on the results of the MRC Vitamin Study published in 1991, which was a randomized, double-blind, placebo-controlled trial of periconceptional folic acid and a combination of B-vitamins for the prevention of NTDs (MRC Vitamin Study Research Group, 1991). Only women with previous pregnancies with NTD outcomes were included, and they were randomly assigned to one of four groups: 4 mg folic acid alone, 4 mg folic acid plus a multivitamin, both, or neither. Folic acid supplementation ($\pm$ a multivitamin) led to a 72% decrease in NTDs (MRC Vitamin Study Research Group, 1991; Gaull et al., 1996). Later that year, another randomized trial was conducted by Czeizel and Dudas to determine whether

periconceptional vitamin supplementation could prevent a first occurrence of NTDs (Czeizel and Dudas, 1992). Women with no past histories of NTD-affected pregnancies were randomly assigned to a preconceptional multivitamin supplement containing 800 μg of folic acid or a trace-element control supplement without folic acid. Despite the fact that a multivitamin was used instead of folic acid alone, it was concluded that the 800 μg of folic acid exerted the observed 70% reduction in NTDs, confirming results of the MRC study (Beaudin and Stover, 2007).

In January 1998, mandatory folic acid fortification was implemented in the US by the Food and Drug Administration (FDA) (Backstrand, 2002; Eichholzer et al., 2006). It was considered as the most effective approach to increase the folate intake of women of childbearing age and therefore to reduce their risk of having a child with an NTD. Based on the PHS recommendation of 400 μg of folic acid daily, the amount of folic acid for fortification was chosen to maximize the intake of the target group (FDA, 1993; FDA, 1996). Through daily consumption of 400 μg of folic acid, the NTD risk could be reduced by 50% or more (CDC, 1992; FDA, 1993; Boulet et al., 2008). However, this was no firm estimate (CDC, 1992; FDA, 1993). The level of fortification was also modeled in such a way that almost nobody would receive more than 1 mg of total folate (food folate and folic acid from fortified foods and supplements) per day to avoid any adverse health effects, such as masking of vitamin B12 deficiency, primarily in elderly individuals (FDA, 1993; FDA, 1996). At that time, the reported median intake of folate from food in the US adult population was approximately 250 μg per day (IOM, 1998). In determining appropriate fortification levels with folic acid, the intakes of total daily folate and simulated fortifications were estimated for the target group as well as for the general population on the basis of national food consumption data (FDA, 1993, 1996; Yetley and Rader, 2004). Other options were considered, such as the folic acid fortification of cereal grains, fruit juices, and dairy products. As a result of the modeling procedures, the FDA determined that mandatory fortification of foods with folic acid should be limited to cereal grain products (FDA, 1993; FDA, 1996), which are appropriate vehicles for fortification and are consumed by more than 90% of women of childbearing age on a daily basis (Crane et al., 1995). The fortification level was set at 140 μg of folic acid per 100 g of cereal grain product (FDA, 1993, 1996; Yetley and Rader, 2004). This value, which is twice the amount of 70 μg per 100 g recommended in 1974 to restore the folate lost in the milling of cereal grain products (FDA, 1993; FDA, 1996), should provide on average 100 μg of additional folic acid daily (Quinlivan and Gregory, 2003; Yetley and Rader, 2004; Cornel et al., 2005). It was regarded as optimal fortification level in terms of safety for the general, nontargeted population. However, the FDA was aware that a certain percentage of the target group may not achieve the recommended 400 μg of folic acid per day through the folic acid intake from fortified foods combined with the consumption of naturally occurring food folate from a varied diet. Therefore, women of childbearing age are advised to additionally take folic acid tablets to meet the recommendation (Eichholzer et al., 2006).

The fundamental question of the minimum effective blood folate level required for the prevention of NTDs (Daly et al., 1995; Berry et al., 2010) and consequently also of the optimal amount of folate intake (Gaull et al., 1996; Daly et al., 1995; Pfeiffer et al., 2007; De-Regil et al., 2010) remains unresolved.

For ethical reasons, human data on a direct dose–response relation between folate intake and NTD risk cannot be obtained and is lacking up to now (Daly et al., 1997; Pfeiffer et al., 2007).

Although the FDA supports the PHS recommendation of 400 μg folic acid per day for women of childbearing age, which served as the basis for fortification modeling, this value was considered an "artificial goal" by some (FDA, 1993; Dary, 2009). At the time the decision was made to implement mandatory folic acid fortification, some argued that there was no data demonstrating that 400 μg per day is the optimal folic acid intake, and that this amount may be more than required for the reduction of NTD risk (FDA, 1993). In addition, the FDA believed "that it may be helpful . . . to include information that . . . 400 μg (of folic acid)/day is the target intake goal" (FDA, 1993).

The results by Daly et al. were the first demonstrating an inverse association between a woman's risk of having a child with NTD and early pregnancy red blood cell (RBC) folate levels in a continuous dose–response relation (Daly et al., 1995). The investigators evaluated the data of a case–control study by Kirke et al. (1993), where blood samples were taken from more than 56,000 pregnant women at a median gestational age of 15 weeks. From all stored samples, blood was retrieved for 84 NTD cases and 266 controls, and RBC folate, plasma folate, and plasma B12 were measured. As RBC folate reflects the folate turnover during the previous approximate 4 months, it may prove more pertinent to characterize the folate status of the participating women around and after conception than to characterize plasma folate. According to the results of Daly et al., women with RBC folate concentrations less than 340 nmol/L had a greater than eight-fold risk for an NTD-affected pregnancy than women with blood levels of 906 nmol/L or higher (Daly et al., 1995). Based on these findings, RBC levels of 906 nmol/L or greater were determined as most effective in minimizing NTD risk (Dary, 2009; Colapinto et al., 2011). However, a well-defined cutoff for an optimal folate status for NTD prevention does not exist (Daly et al., 1995).

As NTD risk and women's early pregnancy RBC folate levels were found linearly correlated, Daly et al. estimated the decrease in NTD rate based upon their results using two strategies to increase folate concentrations (Daly et al., 1995). Based on the first strategy, the high-risk approach to prevention, women with low RBC folate level under 340 nmol/L would be identified, followed by raising their blood level through folic acid supplementation above 960 nmol/L. Consequently, their NTD risk should be decreased by almost 90%, which would have significant benefit for the individual, but the effect on the population NTD prevalence may be limited. Conversely, based on the second strategy, the population approach, such as food

fortification, the benefit for each individual in the population is small, but the population effect may be large, because folate levels of most women of childbearing age would be raised independent of initial RBC folate levels. As data on the effect of folic acid intake on RBC folate levels of women of childbearing age was lacking, Daly et al. derived estimates based on the available literature (Daly et al., 1995). Thus, an increase in folic acid intake by 400 μg per day may correspond to an approximate doubling of RBC folate level. This would lead to an almost 50% reduction in the total NTD rate, confirming the estimates by the PHS (CDC, 1992; Daly et al., 1995). Because an increased intake of 1 mg of folic acid per day, which may raise RBC folate levels by 150%, would result in an only marginally greater reduction, the recommendation of 400 μg of folic acid daily may be near optimal (Daly et al., 1995).

Although there is uncertainty about the threshold for an optimal folate status and the corresponding optimal folate intake, targeting high-risk women of childbearing age is highly efficient in reducing an individual's risk. While mandatory folic acid fortification seems to be more effective in reducing the prevalence of NTDs, this shifts the distribution of folic acid exposure in the entire population to the right, with unknown health consequences.

Change in Folate Intake and Status Since the Implementation of Mandatory Folic Acid Fortification

Postfortification analyses in the United States revealed that the actual synthetic folic acid intake values were more than twice the expected levels (Choumenkovitch et al., 2002; Quinlivan and Gregory, 2003; Yetley and Rader, 2004; Dietrich et al., 2005). Results from the 2005 National Health and Nutrition Examination Survey (NHANES) study indicate that the population may be consuming approximately 260 μg of synthetic folic acid, as opposed to the 100 μg estimated (Dietrich et al., 2005). Several factors may explain this observation. First, the original folate intake estimates by the FDA, based on national food consumption data, may be too low. The traditional methods used for the analysis of food folate may underestimate the total amount of folate, as they do not result in complete release of folates from food matrices (FDA, 1993; Rader et al., 2000; Choumenkovitch et al., 2002). Second, fortified foods may contain a higher amount of folic acid than required by regulation. Fortified foods have been determined to contain 160% to 175% of their predicted folate content (Quinlivan and Gregory, 2003). Manufacturers may add overages to ensure that the fortified product contains the folic acid content required by the regulations and declared on the label throughout the shelf-life of the product (Rader et al., 2000; Choumenkovitch et al., 2002). Third, a wide range of products originally not included in the regulations are fortified with folic acid (Yetley and Rader, 2004). Following the mandatory fortification of foods with folic acid in the United States, Lewis et al. provided estimates of total folate intake (naturally occurring food folate plus folic acid from fortified foods and dietary supplements) in the entire population (Lewis et al., 1999). The estimates indicate that up to 25% of young children may have folic acid intakes above the tolerable upper intake level. Conversely, on average, about 80% of all women of childbearing age may not reach the recommended 400 μg of folic acid per day (Lewis et al., 1999).

Since the implementation of mandatory folic acid fortification in the United States, the folate status, determined by either serum or RBC folate level, has increased substantially in every age group (Pfeiffer et al., 2007). Serum folate concentrations more than doubled, whereas RBC folate levels increased by about 50% from before fortification to the first postfortification survey period (1999–2000) (Pfeiffer et al., 2007).

Only about 10% of women of childbearing age reached RBC folate levels of greater than 906 nmol/L that are considered highly efficient to prevent NTDs. Conversely, almost 100% of the entire US population has RBC folate concentrations of 305 nmol/L or greater that are considered adequate in the general adult population (Dietrich et al., 2005).

In Canada, folate deficiency has also been extremely rare in the general population since introduction of mandatory folic acid fortification (Dary, 2009; Colapinto et al., 2011). The analysis of data from the Canadian Health Measures Survey revealed that almost 80% of Canadian women of reproductive age show postfortification RBC levels of 906 nmol/L or greater (Colapinto et al., 2011). The considerable proportion of Canadians (40%) with high RBC folate levels above 1360 nmol/L raises concern. Although the RBC folate concentration associated with possible adverse effects is not known (Colapinto et al., 2011), the attempt to supply women of childbearing age with adequate folate levels may result in overfortification of the general population (Osterhues et al., 2009). Of particular concern is the long-term exposure of children to high total folate intake.

Effect of Mandatory Folic Acid Fortification on the Prevalence of Neural Tube Defects

There is no one precise figure indicating the exact decrease in worldwide, or even local, NTD incidence since introduction of mandatory folic acid fortification. Furthermore, the validity of some data estimating NTD incidence is questionable. NTD incidence often includes only data for live births and not for spontaneous or induced abortions. In countries like the United States, where there is no national birth defect registry, it is difficult to track national values of babies or aborted fetuses with NTDs or other birth defects (Williams et al., 2002).

In addition, even prior to folic acid fortification, the incidence of NTDs in the United States has been declining for years (Gaull et al., 1996; Eichholzer et al., 2006). Improved diets and the more widespread use of supplements are hypothesized to play a role in this trend (Gaull et al., 1996). Other reasons include advancements in prenatal examinations for the early detection of NTDs, followed by an increased number of terminations of affected pregnancies (Kondo et al., 2009). As NTD incidence had already declined before the implementation of folic acid

fortification, it is impossible to quantify the contribution of fortification on NTD incidence (Gaull et al., 1996).

Since implementation of mandatory folic acid fortification, NTD prevalence has declined by 37% in the United States according to CDC; in Canada, the prevalence has decreased by 46% after fortification (CDC, 2010). Interestingly, while birth certificate data support a decline in the rates of spina bifida since fortification, no decline in the prevalence of anencephaly is evident (Honein et al., 2001). A higher amount of folic acid may be required to prevent anencephaly or a smaller percentage of anencephaly cases may be preventable by folate compared with spina bifida (Williams et al., 2002). This raises questions about etiological similarities and differences between the two major types of NTDs and whether both can be classified under one umbrella term.

Evidentially, preconceptional folic acid intake prevents the first and second time occurrence of NTDs (De-Regil et al., 2010), but only to some extent. The decrease in NTD prevalence in the United States was lower than expected since introduction of folic acid fortification (Boulet et al., 2008). Furthermore, there are ethnic and racial differences in NTD prevalence (Boulet et al., 2008).

The relative decrease of NTD cases depends on the initial NTD rate (Heseker et al., 2009). In a Canadian study, the provinces with the highest baseline incidence experienced the largest percent decline in NTDs after fortification (De Wals et al., 2007). Although countries with mandatory folic acid fortification accomplished a higher folate intake on a large scale and a reduction in NTD prevalence, the decline was independent of the amount of folic acid administered (Heseker et al., 2009). A "bottom floor effect" is proposed to occur for folate-preventable NTDs (Heseker et al., 2009). Countries in which the NTD prevalence is close to the floor of NTDs preventable by folate may have much smaller reductions in NTD rates after fortification (Heseker et al., 2009; Mosley et al., 2009). Thus, there remain a substantial proportion of residual NTDs that appear unaffected by folic acid supplementation or fortification.

Ethnic communities vary in NTD incidence. Racial and ethnic differences in NTD incidence had been suggested prior to folic acid fortification, but were first confirmed by Williams et al. in 2005 (Williams et al., 2005). A statistically significant decrease in NTD incidence in the United States was found during the period of mandatory fortification among Hispanics and non-Hispanic whites, but not in non-Hispanic blacks. The baseline incidence was higher for the former two groups, with the greatest seen in Hispanic births (Williams et al., 2005). These data argue against raising the amount of folic acid currently used to fortify foods, as called for by some authors (Bentley et al., 1999; Wilson et al., 2007; Johnston, 2008), since this may not lead to any further reductions.

Pros and Cons of Mandatory Folic Acid Fortification

The decision about mandatory folic acid fortification of foods often involves discussions only relating to NTDs. Without question, an adequate total folate intake in women planning to conceive is important for the prevention of NTDs and possibly other congenital malformations. But does the benefit of folic acid fortification for women of childbearing age outweigh the risk of exposing millions of people of all ages and both sexes to higher folic acid levels?

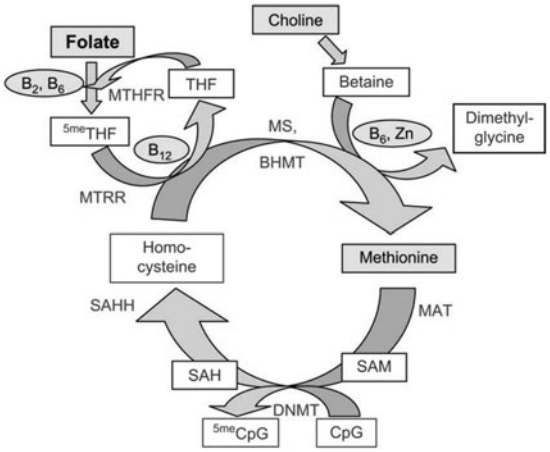

Figure 1 Simplified one-carbon metabolism converting homocysteine to methionine and involved dietary factors. Methyl donors are shown in boxes and cofactors in ellipses; dietary factors are shaded. CpG, cytosine (C), and guanine (G) linked by a phosphate molecule. Methyl donors: SAH = S-adenosylhomocysteine, SAM = S-adenosylmethionine, THF = tetrahydrofolate, 5meTHF = 5-methyl-THF. Enzymes: BHMT = betaine-homocysteine S-methyltransferase, B2 = vitamin B2, B6 = vitamin B6, B12 = vitamin B12, DNMT = DNA methyltransferase, MAT = methionine adenosyltransferase, MS = methionine synthase, MTHFR = 5,10-methylenetetrahydrofolate reductase, MTRR = 5-methyltetrahydrofolate-homocysteine methyltransferase reductase, SAHH = S-adenosylhomocysteine hydrolase, Zn = zinc. Used with permission and adapted from Foley et al. (2009).

An adequate folate status is essential for everyone. Folate is a crucial cofactor in one-carbon metabolism, mediating the conversion of homocysteine to methionine. Folate plays a decisive role in DNA synthesis and replication; see Fig. 1 (adapted from Foley et al. (2009)). Folate deficiency is associated with anemia and elevated plasma levels of homocysteine, a potential risk factor for cardiovascular disease (McNulty et al., 2008; Varela-Moreiras et al., 2009). A recent meta-analysis provides evidence that folic acid supplementation can significantly decrease the risk of a primary stroke (Wang et al., 2007). However, there is still inconsistency regarding a potential protective effect of folic acid supplementation on other cardiovascular endpoints (Wang et al., 2007). Evidence from observational and experimental studies suggests that folate depletion may also foster other diseases such as neuropsychiatric disorders and cancer, particularly colorectal cancer (Kim, 2007a, 2007b; Lucock and Yates, 2009).

Although folic acid was long regarded as safe and almost free of toxicity (Campbell, 1996), there are rising health concerns about its possible adverse effects and the consequences of a long-term high total folate intake and status. Prolonged periods of excess folic acid intake can mask symptoms of B12 deficiency in certain populations, which can lead to irreversible

neurological damage (Gaull et al., 1996; Eichholzer et al., 2006; Smith et al., 2008). By limiting total folate intake, we can avoid placing millions of elderly persons at an increased risk of cognitive impairment and anemia (Smith et al., 2008). Of concern is also an increasing body of evidence from both epidemiologic and experimental studies that suggest folate may play a dual role in carcinogenesis (Kim, 2007a, 2007b; Smith et al., 2008). While folate deficiency may be associated with the development of certain types of cancer, excess of folate may also promote the growth of existing preneoplastic lesions in both men and women (Kim, 2007a, 2007b). Excess folate levels have been implicated with the progression of preexisting neoplastic colonic polyps, especially in the elderly (Kim, 2007a; Solomons, 2007). In a 10-year randomized clinical trial including more than 1000 participants, Cole et al. found that 1 mg of folic acid assigned to patients with a history of colorectal adenomas did not reduce their risk of invasive neoplasia (Cole et al., 2007). In contrast, the results indicate an increase in risk for advanced lesions and adenoma multiplicity in the folic acid group. High-dose folate may provide nucleotide precursors to facilitate growth of preneoplastic cells or may act as an indirect methyl donor and cause inactivation of tumor suppressor genes, leading to accelerated tumor progression (Kim, 2006). In a recent randomized, double-blind, placebo-controlled trial, the effect of folic acid supplementation on methylation levels in normal colorectal tissue of subjects with a history of colorectal adenoma was examined (Wallace et al., 2010). The use of 1 mg of folic acid daily for 3 years was significantly associated with RBC folate levels. The investigators found a relation between higher levels of year 3-RBC folate and elevated methylation levels of estrogen receptor alpha (*ERα*) and secreted frizzled related protein-1 (*SFRP1*). Methylation of *ERα* and *SFRP1* may play an important role in tumorigenesis and has been observed in different types of cancer (Wallace et al., 2010). As aberrant DNA methylation in normal mucosa may be a predisposing event for neoplasia, higher intakes of folic acid through supplements or fortified foods give rise to substantial concern.

But what is the safe upper limit for folate intake and the safe upper folate level in blood?

A general problem in evaluating potential safety issues is that the terms "folic acid" and "folate" are often used interchangeably. While the PHS and the FDA decided in the early 1990s to use a 1 mg of total folate intake (food folate plus folic acid from fortified foods and supplements) as the safe upper intake limit (CDC, 1992; FDA, 1993), the Institute of Medicine set a tolerable upper limit of 1 mg daily folic acid intake for adults (IOM, 1998). The latter is the generally excepted upper intake limit. However, there is questionable reliability of this selected level (Shane, 2003). One argument against this upper intake level is that daily intakes of 400 μg of folic acid or more, which exceed a specific physiological threshold, lead to an increase of free, unmetabolized folic acid in the blood (Berry et al., 2010). The health consequences of higher levels of unmetabolized folic acid, however, are not known (Smith et al., 2008; Berry et al., 2010). There is also no universally accepted cutoff for the RBC folate concentration at which point adverse effects may begin to be observed (Colapinto et al., 2011). There is still a lack in population-based data assessing the safety of a high total folate intake and status (Pfeiffer et al., 2007; Osterhues et al., 2009; Colapinto et al., 2011), and so, long-term follow-up intervention studies are vitally needed.

REMAINING UNCERTAINTIES IN THE ETIOLOGY OF NEURAL TUBE DEFECTS

The effects of folic acid fortification on the prevalence of NTDs have not been equally distributed among various ethnic groups in the United States and elsewhere. This raises the question of the role of genetic variability as an effect modifier. While NTDs *initially* affect couples spontaneously, a family history of NTDs is one of the strongest risk factors for a *subsequent* NTD-affected pregnancy, suggesting a genetic component (van der Linden et al., 2006). Genetic variation can affect one-carbon metabolism. Methylenetetrahydrofolate reductase (*MTHFR*) encodes an enzyme that forms 5-methyltetrahydrofolate, the major circulating form of folate, which is involved in the remethylation of homocysteine to methionine (Botto and Yang, 2000; Wilcken et al., 2003); see Fig. 1.

The two major polymorphisms of the *MTHFR* gene, C677T and A1298C, reduce the activity of the gene. The A1298C polymorphism, most commonly found in Caucasian whites, does not correlate well with variation in NTD risk among ethnic groups (Wilcken et al., 2003). The frequency of the homozygous C677T genotype (TT), however, is highest among individuals of Hispanic ethnicity, followed by whites, with the lowest frequency found in blacks (Wilcken et al., 2003). This has been shown to be consistent with the variation in NTD prevalence among these three groups in the United States, where Hispanics have the greatest prevalence and blacks the lowest (Wilcken et al., 2003; Williams et al., 2005). However, the terms "Hispanic" and "black" are broad terms including multiple diverse ethnicities within each category. The C677T polymorphism may be associated with an elevated risk for developing NTDs (Botto and Yang, 2000; Kirke et al., 2004; Beaudin and Stover, 2009), as it may raise homocysteine levels, alter the distribution of folate cofactors in the cell, and/or depress folate status (Beaudin and Stover, 2009), leading to an impaired synthesis of the universal methyl donor S-adenosylmethionine (SAM). SAM mediates different cellular methylation reactions, such as DNA methylation as an epigenetic mechanism, which plays an important role in embryonic development (Beaudin and Stover, 2007). DNA methylation cannot only be affected by polymorphisms but also by the amount of methyl groups in the diet. Methionine, choline (via betaine) and folate (via methyltetrahydrofolate) are major sources of dietary methyl groups (Niculescu and Zeisel, 2002).

Epigenetic mechanisms may explain the folate–NTD association by the impairment of DNA methylation or histone modification during cranial neural tube closure (Beaudin and Stover, 2007; Weingartner et al., 2007; Blom, 2009; Kim et al., 2009).

Wang et al. investigated the methylation of long interspersed nuclear element-1 (LINE-1) sequences, which are considered to be predictive for global DNA methylation, in nervous tissues of NTD samples and respective controls free of NTD (Wang et al., 2010). The investigators found that LINE-1 methylation levels were about 4% lower in the NTD cases relative to unaffected control tissue samples ($p < 0.001$). Whether this difference in global methylation levels affects gene expression remains unclear, as expression profiles were not examined. Anencephalus NTDs had lower levels of global methylation than the spina bifida subgroup (Wang et al., 2010), confirming the proposed difference between these two major types of NTDs. Recently, hypomethylation of human brain NTD tissues was also observed by Chang et al. (Chang et al., 2011). Maternal serum folate levels were lower in NTD cases than in controls and correlated with DNA methylation.

OTHER IMPORTANT CONTRIBUTORS TO THE DEVELOPMENT OF NEURAL TUBE DEFECTS

B12 and Homocysteine

Vitamin B12, a cofactor for methionine synthase in one-carbon metabolism, has been often cited as a key player in the development of NTDs. A vitamin B12 deficiency is associated with impairment of the homocysteine remethylation cycle and of nucleotide biosynthesis, which may also lead to an increased risk of NTDs (Molloy et al., 2009a). Since 1980, several studies have demonstrated that lower levels of B12 are a significant risk factor for the development of NTDs (Schorah et al., 1980, Kirke et al., 1993; Suarez et al., 2003; Molloy et al., 2009b). The results by Molloy et al. indicate that the major risk may be related to maternal vitamin B12 levels below 250 ng/L (Molloy et al., 2009b). The need for B12-mediated end-products in rapidly dividing embryonic neural tube cells may be higher than the normal maternal requirements for B12 (Kirke et al., 1993; Suarez et al., 2003). Kirke et al. reported that NTD cases had both lower maternal plasma B12 and plasma folate status compared with controls (Kirke et al., 1993). In the early 1990s, Czeizel and Dudas used a multivitamin supplement including vitamin B12 and folic acid in their classic NTDs prevention trial (Czeizel and Dudas, 1992). Given the proximity of function of folate and vitamin B12 in the one-carbon metabolic pathway, a portion of the observed protection against NTDs could have been due to a synergistic effect between vitamin B12 and folic acid (Molloy et al., 2009a). A B12 deficiency may act in synergy with a low folate status to precipitate NTDs (Molloy et al., 2009b).

A high serum level of homocysteine is also a risk factor for the development of NTDs (Beaudin and Stover, 2009; Varela-Moreiras et al., 2009). Both folate and vitamin B12 deficiencies lead to increased levels of homocysteine (Varela-Moreiras et al., 2009). Therefore, homocysteine status is a sensitive indicator of inadequate levels of both vitamins (McMullin et al., 2001). Because of the proposed synergistic effects of folate and vitamin B12 levels (Molloy et al., 2009b), it may be a better marker of NTD risk than either folate or vitamin B12 status. However, the results by Felkner et al. also indicate an independent role of elevated homocysteine levels in the development of NTDs. Women with high homocysteine levels had markedly increased risks of having a child with NTD independent of folate or vitamin B12 levels (Felkner et al., 2009). This observation underlines the importance of measuring homocysteine in blood for NTD risk.

Choline

Lower periconceptional levels of choline are associated with an elevated NTD risk (Shaw et al., 2009). Choline is a dietary component that is essential for normal cell function (Zeisel, 2006). An adequate intake of choline might be of great importance during fetal development, when the organism is growing rapidly (Ozarda Ilcol et al., 2002). Choline is part of the one-carbon metabolism converting homocysteine to methionine (Zeisel, 2008); see Fig. 1. Individuals fed a choline-deficient diet had increased plasma levels of homocysteine (da Costa et al., 2005). However, any requirement for choline intake has to be considered in relation to other nutrients involved in one-carbon metabolism, such as folate, vitamin B12, and methionine (Zeisel, 2006; Zeisel, 2007). Within the one-carbon metabolism, there are two pathways mediating the methylation of homocysteine to form methionine. While folate and vitamin B12 are involved in the first pathway, betaine, which is derived from dietary choline, is the methyl donor in the alternative pathway (Zeisel, 2006; Zeisel and da Costa, 2009). Polymorphisms in the folate pathways could limit the availability of methyltetrahydrofolate, the methyl donor in the first pathway, thereby possibly increasing the use of choline (Zeisel, 2008). As mentioned above, the amount of methyl groups in the maternal diet affects embryonic development through methylation of DNA (Beaudin and Stover, 2007). Thus, methylation reactions may be one of the primary mechanisms that influence neural tube closure (Zeisel and da Costa, 2009). The protective effects of choline on NTD risk may also be due to its role as a precursor to phosphatidylcholine, a major component of cell membranes, which is hypothesized to affect early embryonic growth (Shaw et al., 2009). Additionally, choline influences apoptosis; its regulation is important for neural tube closure (Shaw et al., 2009).

Obesity/Diabetes Mellitus

Obesity is known to be associated with complications during and after pregnancy, along with other adverse reproductive outcomes, for both mother and child (Galtier-Dereure et al., 2000; McGuire et al., 2010). A recently published meta-analysis found that women who were obese at the start of pregnancy had an almost double risk of having a child with an NTD (Stothard et al., 2009). Interestingly, there was a greater effect of obesity on the incidence of spina bifida than on anencephaly (Stothard et al., 2009). The exact mechanism for the observed

association between obesity and congenital anomalies such as NTDs is not completely understood, but several possible explanations have been proposed (Moore et al., 2000; Watkins et al., 2003; Stothard et al., 2009). Obese women may suffer from nutritional deficiencies from poor diets with a lack of proper vitamin intake, suggesting that the obesity in itself may not be an independent risk factor for the development of NTDs (Hotzel, 1986; Watkins et al., 2003). Conversely, obese women might have an increased requirement for certain nutrients, including folate (Watkins et al., 2003). Werler et al. provided some evidence for this hypothesis as the reduction in risk of folate-dependent NTDs was not observed among heavier women (Werler et al., 1996). Furthermore, many obese women have type 2 diabetes, which is also a risk factor for birth defects, including NTDs (Becerra et al., 1990; Stothard et al., 2009). Like obesity, diabetes increases the NTD risk by two-fold (Hendricks et al., 2001). Both obesity and diabetes are characterized by similar metabolic disturbances, such as insulin resistance and hyperglycemia (Stothard et al., 2009). Hyperinsulinemia and/or hyperglycemia in early pregnancy may be associated with an increased risk for the fetus to suffer NTDs (Yazdy et al., 2010), because high levels of both glucose and insulin may exert teratogenic effects (Hendricks et al., 2001).

Further studies are needed to elucidate the exact mechanism of NTD development as the prevalence of obesity and diabetes continues to rise strongly worldwide.

Folic Acid Antagonists

Over the past decade, there have been several observational studies demonstrating an increased risk of a variety of birth defects due to gestational exposure of folic acid antagonists (Hernandez-Diaz et al., 2000; Meador et al., 2006; Matok et al., 2009). One group of folic acid antagonists include dihydrofolate reductase inhibitors, which consists of antibiotics and pharmacologic agents such as trimethoprim, sulfasalazine, aminopterin, and methotrexate. These agents displace folate from dihydrofolate reductase and thereby block the conversion of folate to its more active metabolites. The second group of folic acid antagonists, which are mainly antiepileptic drugs such as valproic acid or carbamazepine, may affect other enzymes in folate metabolism, impair the absorption of folate, or increase its degradation. Results from a large multicenter case–control study demonstrated an increased risk of a variety of birth defects due to folic acid antagonist intake during pregnancy (Hernandez-Diaz et al., 2000). Exposure to folic acid antagonists during the first or second month of gestation was associated with an almost three-fold increased risk of having a child with NTDs (Hernandez-Diaz et al., 2001). In a large retrospective cohort study (including live births and terminations), the odds ratio (OR) for NTDs was 6.3 [95% confidence interval (CI): 4.3–9.2] among women exposed to a folic acid antagonist during the first trimester of pregnancy (Matok et al., 2009). It is worth noting that simultaneous folic acid supplementation may decrease the risk of birth defects associated with the use of folic acid antagonists (Hernandez-Diaz et al., 2001; Matok et al., 2009). However, it is not completely understood whether simultaneous folic acid supplementation will diminish NTD risk and/or will cut efficacy of the antagonistic drug (Hernandez-Diaz et al., 2001).

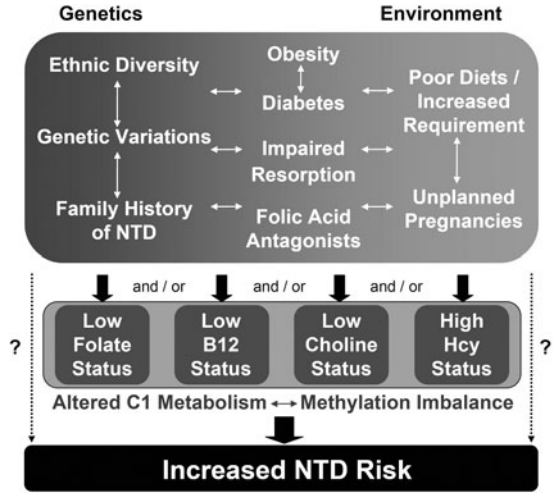

Figure 2 Multiple interrelated factors influencing the development of neural tube defects. NTD = neural tube defect, B12 = vitamin B12, Hcy = homocysteine, C1 = one-carbon.

While the number of pregnant women taking folic acid antagonists is relatively low and the absolute number of NTD cases within this group is small, folic acid antagonists pose a definite risk to the developing fetus.

CONCLUSIONS AND FUTURE PERSPECTIVES

While much progress has been made in the prevention of NTDs and in understanding of their causes, the exact molecular mechanisms remain unresolved. A multifactorial pathway is likely; see Fig. 2. Environmental factors and genetic variations may affect one-carbon metabolism and thus the susceptibility to the development of NTDs in early embryonic development. Maternal nutrition status, health and behavior during pregnancy, and maternal exposure to medication interact with developmentally regulated genes (Zhu et al., 2009). Imbalances in one-carbon metabolism and subsequently in DNA methylation may not only be of importance for the development of NTDs but also for other birth defects, including cardiovascular defects, oral clefts, and urinary tract abnormalities (Botto and Yang, 2000; Wilcken et al., 2003; Kirke et al., 2004; Beaudin and Stover, 2007; Weingartner et al., 2007; Kim et al., 2009; Lucock and Yates, 2009).

In countries that have adopted folic acid fortification, a considerable incidence of NTDs remains, which centers on certain ethnic groups (Suarez et al., 2003; van der Linden et al., 2006; Felkner et al., 2009; Molloy et al., 2009b; Zhang et al., 2009). Whether these population subgroups escape sufficient

fortification or whether other factors may explain these differences remains to be explored and may be addressed with a targeted intervention.

This raises the question of the sensibility of a population-based prevention approach. Exposing the majority of the population to folic acid, a micronutrient that increases substrate availability in the one-carbon metabolic pathway, may have unknown consequences for methylation stability and other adverse health effects. Effects on promotion of tumorigenesis have also been proposed.

Targeting subpopulations at elevated risk for NTDs may be preferable to an increase in fortification dose with uncertain preventive value (Kirke et al., 1993; Campbell, 1996; Smith et al., 2008; Zeisel et al., 2009). For example, since epidemiologic studies have shown that Hispanic women have higher rates of NTDs and lower rates of folic acid supplementation compared with other groups, it was suggested that fortifying corn masa flour with folic acid could help eliminate NTDs in that subpopulation (Hamner et al., 2009). In contrast to corn meal products manufactured in the United States, which are fortified with folic acid (SBA, 2010), corn masa flour does not have a standard of identity as developed by the FDA (Hamner et al., 2009). This means that corn masa flour has not been defined as a grain product requiring folic acid fortification (SBA, 2010). Currently, there are no existing federal regulations that permit folic acid fortification of corn masa flour (CDC, 2010).

However, before considering fortification, the minimum effective folate status as well as the optimal dose for NTD prevention needs to be evaluated. The safe upper limit of folate intake and the safe upper folate level in blood also need to be determined. Additionally, there is a lack of data on the long-term exposure to high total folate intakes. Blood levels of nutrients other than folate, such as Vitamin B12 and homocysteine, should also be considered as all of these substances contribute to the development of NTDs. Long-term follow-up intervention studies considering all of these issues are vitally needed.

Furthermore, the type of folate used for supplements or fortified foods needs to be addressed. L-5-methyltetrahydrofolate, the naturally occurring predominant form of folate, and thus directly bioavailable, may be an attractive alternative to the synthetic folic acid (Pietrzik et al., 2010). L-5-methyltetrahydrofolate, which is already being used as calcium salt in several supplements and pharmaceuticals, may have some advantages over folic acid since its potential for masking vitamin B12 deficiency symptoms may be lower and possible adverse effects associated with certain common polymorphisms in folate genes may be ameliorated. Additionally, the intake of L-5-methyltetrahydrofolate does not lead to an accumulation of unmetabolized folic acid in the body (Pietrzik et al., 2010).

In conclusion, mandatory folic acid fortification plays a significant part in contributing to the reduction in NTD prevalence, but possibly at a cost and with a substantial portion of NTDs persisting. The ultimate goal should be to target women at childbearing age rather than putting the general population on higher intakes of folic acid with unknown health consequences. A better understanding of the specific etiology and causative mechanisms of NTDs is required and is a prerequisite for effective preventive strategies.

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