

Folic acid in early pregnancy: a public health success story

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ABSTRACT Folate is a water-soluble B vitamin that must be obtained in the diet or through supplementation. For >50 yr, it has been known that folate plays an integral role in embryonic development. In mice, inactivation of genes in the folate pathway results in malformations of the neural tube, heart, and craniofacial structures. It has been shown that diets and blood levels of women who had a fetus with a neural tube defect are low for several micronutrients, particularly folate. Periconceptional use of folic acid containing supplements decreased recurrent neural tube defects in the offspring of women with a previously affected child and the occurrence of a neural tube defect and possibly other birth defects in the offspring of women with no prior history. Based on these findings, the U.S. Public Health Service recommended that all women at risk take folic acid supplements, but many did not. Mandatory food fortification programs were introduced in numerous countries, including the United States, to improve folate nutritional status and have resulted in a major decrease in neural tube defect prevalence. The success story of folate represents the cooperation of embryologists, experimentalists, epidemiologists, public health scientists, and policymakers.—Običan, S. G., Finnell, R. H., Mills, J. L., Shaw, G. M., Scialli, A. R. Folic acid in early pregnancy: a public health success story. *FASEB J.* 24, 000–000 (2010). www.fasebj.org

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THE TERATOLOGY SOCIETY was founded 50 yr ago, in 1960. Teratology is the study of birth defects, a global problem diagnosed in 2–3% of newborns, with a similar percentage diagnosed during the first year of life. These abnormalities of development are a leading cause of pediatric hospitalizations and result in 3.3 million deaths in children worldwide each year (1). The cause of most birth defects is unknown. Neural tube defects are among the most common category of birth defects worldwide, affecting 0.17–6.30/1000 live births, depending on race, geographic location, and

prevalence of antenatal diagnostic testing. Neural tube defects result from the incomplete closure of the neural tube during the fourth week of gestation (2). The most common neural tube defects are spina bifida, due to incomplete closure of the caudal neural tube, and anencephaly, due to incomplete closure of the rostral end of the neural tube. These malformations are fatal or result in significant lifelong disability.

For >50 yr, it has been known that folate plays an integral role in embryonic development. The investigation into the role of folate in neural tube defects and the use of folic acid supplementation to prevent these and perhaps other malformations has been an example of how scientists in diverse fields have worked together to favorably affect the public health. The Teratology Society is proud that many of these scientists are Society members, and many of the discussions leading to this major public health contribution took place at annual Society meetings.

We here review the basic science, experimental animal, and epidemiology studies that progressively led to an understanding of the importance of folate in embryonic development and to the development of a public health program to improve folate nutritional status in reproductive-age women. We will review the experimental animal studies that have examined the role of specific enzymes in the folate pathway and epidemiology studies that have shown the importance of adequate folate status for normal embryonic development. The fortification of enriched grain products with folic acid has led to a demonstrated decrease in the prevalence of neural tube defects in the United States and other countries with enrichment programs.

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BIOCHEMISTRY

Folate is a water-soluble B vitamin that cannot be synthesized by the body and therefore must be obtained in diet or through supplementation. Folate is found in green leafy vegetables, citrus fruit, legumes, and liver (3). Folic acid is a synthetic form of folate used in supplements because it is stable and is sometimes better absorbed than natural folates. Folic acid is converted *in vivo* to an active form of folate, tetrahydrofolate, and enters the folate pathway. The major metabolic role of folate is to transport 1-carbon units, which are used in 1-carbon transfer reactions that regulate DNA function and in DNA and RNA synthesis. Certain cell types are particularly folate-sensitive, accounting for the main deficits seen in folate deficiency. Embryonic neural crest cells have an extremely high expression of folate receptors, indicating a high folate demand in this particular cell type.

GENETIC MODELS AND HUMAN STUDIES

Mice in which the folate pathway genes have been experimentally inactivated (knocked out) have malformations of the neural tube, heart, and craniofacial structures (4).

Folr1

Folate-binding protein 1 (Folr1) is a membrane-bound receptor that transports folate monoglutamates. The essential nature of folate would suggest that the genes involved in folate uptake and transport would be expressed ubiquitously in every cell. Studies by Salbaum *et al.* (5) suggest that murine folate receptors are expressed with specific tissue distributions during development, including neuroepithelial and neural crest cells, in addition to the visceral endoderm. *Folr1* nullizygous embryos displayed severe abnormalities and died *in utero* by embryonic day 10 (6). At gestational day 8.5 in the midst of murine neural tube closure, the embryos had severe malformations, including neural, craniofacial, and cardiac malformations (6).

Supplementing pregnant *Folr1*^{+/-} mice with folic acid rescued nullizygous pups and resulted in a dose-dependent range of phenotypic appearances from normal to multiple defects. The malformations observed were wide ranging and involved neural tube, lip and palate, ventral body wall, limbs, digits, eyes, and cardiovascular system (7). Cardiovascular phenotypes were consistent with the reduction of neural crest cell migration. The hearts of these pups were significantly smaller than those of the wild type, mostly due to smaller ventricles. There was a wide range of cardiovascular malformations, including conotruncal defects, aortic arch abnormalities, ventricular septal defects, and isolated dextrocardia (8, 9). Conotruncal abnormalities included overriding aorta, transposition of the great arteries, and double outlet right ventricle.

Folr2

Folr2, like *Folr1*, codes for a protein that mediates folate binding and transport. Relative to *Folr1*, however, *Folr2* has a more global distribution and lower binding affinity for folate. Knocking out *Folr2* fails to adversely affect embryonic development, because the nullizygous pups appear phenotypically normal (6). However, nullizygous embryos appear to be more sensitive to select environmental agents, including arsenic and valproic acid, when compared with *Folr2* wild-type mice. Experimental exposure of pregnant mice to arsenic causes exencephaly, omphalocele, gastroschisis, craniofacial defects, and microphthalmia. These defects are exacerbated in the presence of a concomitant folate deficiency (10).

Rfc1/SLC19A1

Rfc1 is a transmembrane protein that is a major transporter of folate. Whereas *Folr1* and to some extent *Folr2* have tissue-specific expression patterns, *Rfc1* is ubiquitously expressed. *Rfc1*-knockout mice die early in development. If the *Rfc1*^{+/-} dams are supplemented with subcutaneously injected folic acid, their *Rfc1*^{-/-} pups survive longer but have renal impairment, bone marrow failure, and lymphoid depletion (11). Fewer than 10% of these pups survive to birth, and all liveborn pups died within the first few postnatal days.

Nullizygous *Rfc1*-knockout mice exhibit a range of congenital malformations related in a dose-dependent fashion to folic acid supplementation. *Rfc1*^{+/-} dams that are supplemented *via* subcutaneous injection with 25 mg/kg/d folic acid produce mice that survive longer (to about embryonic day 10) but had significant malformations (12). These malformations compromised craniofacial, cardiac, limb, and neural tube development. The affected fetuses also had decreased erythropoiesis. The placenta was abnormally formed; there was no chorioallantoic fusion, and the fetal trophoblasts failed to invade the maternal decidua. Higher dose supplementation of the *Rfc1*^{+/-} dams subcutaneously injected with 50 mg/kg/d folic acid enabled 22% of the conceptuses to come to term, although the pups were small and presented with craniofacial, heart, lung, and skin malformations. Heart defects include ventricular septal defects with thin myocardial walls and occasional overriding aorta. Lungs were underdeveloped and pale. Ectodermal manifestations include thickened and malformed cornea and open eyelid defect (12).

Pcft/Hcp1

Pcft/Hcp1 is an intestinal transporter involved in heme and folate uptake. A new murine model of megaloblastic anemia was developed through targeted disruption of the first 3 coding exons of the murine homologue of the *Pcft/Hcp1* gene. By 4 wk of age, Pcft/Hcp1-deficient (*Pcft/Hcp1*^{-/-}) mice developed severe macrocytic normochromic anemia and pancytopenia. Immature eryth-

roblasts accumulated in the bone marrow of *Pcft/Hcp1*^{-/-} mice and failed to differentiate further, showing an increased rate of apoptosis in intermediate erythroblasts and reduced release of reticulocytes. In response to the inefficient hematological development, the serum of the *Pcft/Hcp1*^{-/-} animals contained elevated concentration of erythropoietin, soluble transferrin receptor, and thrombopoietin. *In vivo* folate uptake experiments demonstrated a systemic folate deficiency caused by disruption of *Pcft/Hcp1*-mediated intestinal folate uptake, thus providing *in vivo* genetic evidence of a critical and nonredundant role of the *Pcft/Hcp1* in intestinal folate transport and erythropoiesis. Oral supplementation with folate in these knockout mice does not rescue the animals. Intraperitoneal injection of folate at 44 mg/kg, however, reverses the hematopoietic deficits (4). The *Pcft/Hcp1*-deficient mouse serves as a model for the hereditary folate malabsorption syndrome.

Mthfr

Mthfr-nullizygous mice lack functional activity of *Mthfr*; whereas the heterozygotes have activity of this enzyme reduced by 30–40% (13). *Mthfr*-knockout mice are hyperhomocysteinemic and express global DNA hypomethylation. As such, the *Mthfr*-deficient mice serve as a good model for a subgroup of humans with the common polymorphism in *MTHFR* causing decreased enzymatic activity. Plasma total homocysteine concentrations in heterozygous and nullizygous knockout mice are 1.6- and 10-fold higher than those in wild-type littermates, respectively. Both the heterozygous and null knockouts have either significantly decreased S-adenosylmethiothine concentrations, or significantly increased S-adenosylhomocysteine concentrations, or both. The heterozygous knockout mice appear normal, whereas the homozygotes are smaller and show developmental retardation with cerebellar pathology. Abnormal lipid deposition in the proximal portion of the aorta was observed in older heterozygotes and homozygotes, alluding to an atherogenic effect of hyperhomocysteinemia in the mutant mice. Although the original reports of the phenotype of these mutant mice failed to include neural tube defects, recent efforts involving transferring the null allele to a new genetic background have produced such malformations (Rima Rozen, Department of Pediatrics, McGill University, Montreal, QC, Canada; personal communication).

Mtr

The cytoplasmic enzyme 5-methyltetrahydrofolate-homocysteine S-methyltransferase, also known as methionine synthase, catalyzes the remethylation of homocysteine to methionine. This enzymatic reaction requires methylcobalamin, a derivative of cobalamin, or vitamin B₁₂, for its activity. Swanson *et al.* (14) used gene targeting technology to create an *Mtr*-deficient mouse. Mice that are nullizygous for the mutant allele die *in*

utero by embryonic day 7.5. The *Mtr* heterozygous-deficient mice were virtually indistinguishable from wild-type mice, despite a 40–50% decrease in *Mtr* activity. Plasma homocysteine was modestly increased in heterozygotes on a C57Bl/6J background maintained on a folate-replete diet.

Mtrr

As *Mtrr* is an enzyme essential for the activity of *Mtr*, ablation of *Mtrr* is embryolethal, consistent with the outcomes from the *Mtr*-knockout mouse (15). A gene-trapped embryonic stem cell clone was used to produce a mouse model for *Mtrr* deficiency in an effort to avoid the embryolethality observed in the gene-targeted nullizygous model. In *Mtrr*^{gt/gt} mice, the gene trap produced a hypomorphic expression pattern of wild-type *Mtrr* mRNA that varied by tissue, with the heart showing the lowest *Mtrr* levels. Although initial observations suggested a mild morphological phenotype in *Mtrr*^{gt/gt} mice, the metabolic phenotype was immediately recognized to be significantly altered. The gene trap was associated with a dose-dependent increase in homocysteine concentrations: *Mtrr*^{gt/gt} mice had plasma total homocysteine concentrations (mean±sd) of 18.4 ± 5.5 μM, heterozygotes with 5.5 ± 1.8 μM, and *Mtrr*^{+/+} with 4.6 ± 0.8 μM. Plasma methionine was decreased by 32% in *Mtrr*^{gt/gt} mice (40.4±6.6 μM for *Mtrr*^{gt/gt}, 48.9±10.5 μM for *Mtrr*^{+/gt}, and 59.4±7.4 μM for *Mtrr*^{+/+} mice). Although the mice homozygous for the gene-trapped allele have higher total plasma homocysteine and lower plasma methionine, *Mtrr*^{gt/gt} mice do not display decreases in the S-adenosylmethionine:S-adenosylhomocysteine ratio in most tissues. Further investigation of this *Mtrr* gene trap model (16) revealed that *Mtrr*-deficient dams had more resorptions and more “delayed embryos” per litter (resorptions per litter: 0.29±0.13; 1.21±0.41; 1.87±0.38 in *Mtrr*^{+/+}, *Mtrr*^{+/gt}, and *Mtrr*^{gt/gt} mothers, respectively) than did wild-type dams. *Mtrr*^{gt/gt} dams also displayed growth restricted placentas and embryos. Notably, litters of *Mtrr*^{gt/gt} dams had myocardial hypoplasia and displayed ventricular septal defects (0; 0.57±0.30; 1.57±0.67 in *Mtrr*^{+/+}, *Mtrr*^{+/gt}, and *Mtrr*^{gt/gt} groups, respectively). Risk of cardiac malformations, including ventricular septal defects (VSDs), was also conferred by embryonic genotype: *Mtrr*^{gt/gt} embryos had a higher percentage of those affected by VSDs (~28%) than *Mtrr*^{+/+} (~3%) or *Mtrr*^{+/gt} (~5%) embryos.

Shmt1

This enzyme exists as both cytoplasmic and mitochondrial *Shmt* isozymes: *Shmt1* encodes the cytoplasmic isozyme (*Shmt1*) and *Shmt2* is a nuclear-encoded mitochondrial isozyme (*Shmt2*). *Shmt1* and *Shmt2* share functional redundancy (17). The hydroxymethyl group of serine is a primary source of essential tetrahydrofolate-activated 1-carbon units that are required for synthesis of purines and thymidylate and for S-adenosylmethionine-dependent methylation reactions. Compartmentalization

of the 3 enzymes that participate in *de novo* thymidylate transport to the nucleus accounts for previous isotope tracer studies that demonstrate *Shmt1* preferentially shuttles 1-carbon units toward thymidylate biosynthesis (18). Nullizygous *Shmt1* mice are phenotypically normal, exhibiting no gross malformations, and are fertile. Clearly, *Shmt1* is not an essential gene required for normal morphogenesis (19). Disruption of *Shmt1* was observed to affect folate metabolism: *Shmt1*^{-/-} mice had elevated levels of S-adenosylmethionine and decreased levels of S-adenosylhomocysteine when compared to *Shmt1*^{+/+} mice. These differences were most pronounced in mice fed a folate/choline-deficient diet for 22 wk.

Mthfd1

The methylenetetrahydrofolate dehydrogenase 1 gene (*MTHFD1*) encodes a cytosolic trifunctional protein comprising 5,10-methylenetetrahydrofolate dehydrogenase, 5,10-methenyltetrahydrofolate cyclohydrolase, and 10-formyltetrahydrofolate synthetase. These reactions are involved in interconversion of 1-carbon derivatives of tetrahydrofolate (THF), substrates for methionine, thymidylate, and *de novo* purine syntheses. It has recently been demonstrated that the MTHFD1L enzyme is present in mitochondria obtained from normal embryonic tissues and from fibroblast cell lines (20). The embryonic mitochondria are able to fully synthesize formate from glycine. *MTHFD1L* gene expression was observed throughout embryogenesis. *In situ* hybridization experiments demonstrated that *MTHFD1L* was expressed ubiquitously throughout the embryo, with specific regions of higher expression. The expression patterns of *MTHFD1L* was essentially the same as that of *MTHFD2* and *MTHFD1* (cytoplasmic C1-THF synthase) at embryonic day 9.5, suggesting a highly coordinated regulation. When the gene is inactivated in mice, biochemical analysis revealed that the mutation conferred a 58.2% dehydrogenase activity in heterozygotes, with no detectable activity in nullizygotes. The total folate levels were significantly reduced in *Mthfd1*^{-/-}, 19.56, as compared to *Mthfd1*^{+/+}, 29.73, where values are expressed as an nmol/g of liver.

Mthfd2

Methylenetetrahydrofolate dehydrogenase 2 is a nuclear-encoded mitochondrial bifunctional enzyme with methylenetetrahydrofolate dehydrogenase and methenyltetrahydrofolate cyclohydrolase activities involved in interconversion of methylene, methenyl, and formyl derivatives of tetrahydrofolate. *Mthfd2* is unique in its absolute requirement for magnesium and inorganic phosphate. Di Pietro *et al.* (21) deleted the *Mthfd2* gene in mice, which resulted in phenotypically normal *Mthfd2*^{+/-}. Complete ablation was embryolethal, revealing the critical role of mitochondrial folate metabolism in development. *Mthfd2*^{-/-} embryos died after embryonic day 12.5, and although they appeared developmentally normal, they were smaller and hypopigmented when compared to normal littermates. Histopathological investigation of the

livers revealed that, although mitochondrial protein synthesis was not affected, the livers were hypopigmented and contained fewer nucleated cells, indicating a block in the establishment of erythropoiesis.

MTHFR in humans

Methylenetetrahydrofolate reductase (MTHFR) is an important enzyme responsible for the production of 5-methyltetrahydrofolate from 5,10-methylene tetrahydrofolate. It has been found that 5-methyltetrahydrofolate donates methyl groups to homocysteine to form methionine for methylation reactions. Polymorphisms of the *MTHFR* gene have been widely studied as genetic risk factors for neural tube defects as well as early fetal loss. Several polymorphisms of the gene have been evaluated in humans. The 2 most common are 677C→T and 1298A→C substitutions. Heterozygosity for either of these variants has been associated with a 50–60% reduction in enzyme activity, reduced folate concentrations, and hyperhomocysteinemia (22, 23). Most studies have observed an association between these 2 polymorphisms and neural tube defect risk (24). However, no study has demonstrated a modifying influence between either polymorphism and folic acid intake on neural tube defect risk. Risks associated with MTHFR polymorphisms have been inconsistently observed for other potential folic acid-responsive birth defects.

FOLIC ACID SUPPLEMENTATION IN HUMANS

More than 30 yr ago, seminal work by Smithells *et al.* (25) showed that diets and postpartum blood levels of women who had a fetus with a neural tube defect were low for several micronutrients, particularly folate. Subsequent small nonrandomized trials in women who had previous neural tube defect-affected pregnancies showed that folic acid or multivitamins taken in the periconceptional period reduced recurrence risks by ~4-fold (26–31). The double-blind, placebo-controlled, randomized trial rigorously conducted by the Medical Research Council (32) showed that supplementation with 4 mg of folic acid per day resulted in a 72% (95% confidence interval, 29–88%) reduction in neural tube defect recurrence risk. The trials, taken in composite, argue that supplementation of folic acid in the dose range of ~0.4 (27, 28) to 5 (31) mg/d prevents neural tube defect births among most women who have previously had affected pregnancies.

Evidence exists that folic acid supplementation reduces the first-time occurrence of neural tube defects as well. A trial performed in Hungary showed that a multivitamin preparation containing 0.8 mg of folic acid taken daily significantly reduced a woman's risk of having a fetus with a neural tube defect (33). Several epidemiologic observational studies have found that periconceptional use of multivitamins with folic acid or diets high in folate are associated with neural tube

defect risk reduction (34–38). In a community intervention in areas of north and south China, Berry *et al.* (39) observed a substantial reduction in neural tube defect occurrence from the use of a supplement containing only 400 µg folic acid. The risk reduction was 85% in north China, where the neural tube defect prevalence is high, and was 40% in south China, where neural tube defect prevalence is low.

Additional evidence indicating the importance of folate in neural tube defect risk reduction was provided by Hernandez-Diaz *et al.* (40). They observed that periconceptional intake of folic acid antagonist medications doubled the risk of neural tube defect-affected pregnancies and that risks for folate antagonists were attenuated in the presence of supplemental folic acid intake.

Other evidence that points toward altered folate metabolism as a contributor to abnormal neural tube development derives from measurements in postpartum sera. Numerous research efforts have been made to investigate pregnancy blood measures of folate and neural tube defect risks. Some investigators have observed lower folate levels in women with affected fetuses (41–43), but others have not (44–48).

Efforts to educate women of reproductive age to use folic acid supplements did not succeed in improving the folate status of women during early pregnancy (49). As a consequence, numerous countries instituted food fortification programs beginning in the 1990s. Since 1998 there has been compulsory fortification of enriched flour and other enriched grain products with folic acid in the United States (50). Food fortification with folic acid has been effective in increasing both serum and red blood cell folate levels among women of childbearing age in the United States (51). This fortification has coincided with decreases in prevalence of 19% for overall neural tube defects (52), of 31% for spina bifida (53, 54), and of 16% for anencephaly (53). By January 2008, 52 countries adopted wheat-flour fortification (55). Around the world, food fortification programs have produced an estimated 46% (95% CI, 37–54%) reduction in neural tube defect prevalence (56).

Thus, the preventive effects of folic acid on human neural tube defect risk have been well established, and the epidemiologic evidence is robust. Folic acid intake has also been linked to reduction in risk of other malformations with an embryogenesis that is dependent on cranial neural crest cells that derive from neuroepithelium. These malformations include conotruncal heart defects (57, 58) and orofacial clefts (59, 60–66).

The underlying mechanisms by which folic acid contributes to reduction in neural tube defect risks or other malformation risks are, however, unknown. Also unknown is why a substantial proportion of women who take folic acid supplements in the periconceptional period still deliver offspring with neural tube defects. Although Mendelian defects certainly explain some cases, the relationship between folate and other demonstrated risk factors such as obesity and diabetes mellitus remains to be clarified. These unknowns have

served to direct research inquiries toward genetic variation in folate metabolism and transport as potential underlying mechanisms and toward other nutrition hypotheses.

Folic acid fortification may offer other health benefits. Folic acid deficiency is a well-known cause of macrocytic anemia (67). Prior to folic acid fortification of food, ~25% of Americans were folate deficient (68). In addition, some, but not all, studies have suggested that folic acid supplementation may also reduce the risks of early preterm birth (69), preeclampsia (70), placental abruption, intrauterine growth restriction, and fetal death (71). Because women are instructed to take prenatal vitamins in the United States, it can be difficult to separate the effect of folic acid from other vitamins and minerals as well as from health-conscious behavior.

CONCERNS ABOUT FOLIC ACID FORTIFICATION

There has been concern that because folic acid is beneficial for rapidly dividing cells, folic acid fortification of food may increase the incidence or virulence of cancer. One study analyzed hospital discharges in Chile before and after the mandatory January 2000 folic acid fortification of 220 µg/100 g of wheat flour (72). The study compared the periods 1992–1996 and 2001–2004. After mandatory fortification, among adults 45–64 yr old, the rate of a hospital discharge diagnosis of colon cancer increased by 162%. The rate ratio between the 2 periods for colon cancer was 2.6 (99% CI, 2.93–2.58) in 45–64-yr-olds and 2.9 (99% CI, 3.25–2.86) in 65–79-yr-olds.

Two clinical trials that used folic acid to reduce the recurrence rate for colorectal adenomas produced conflicting results (73, 74). Although neither showed a decrease in recurrence in those who received folic acid, one showed an increase in multiple adenomas in the folic acid group and the other did not. Ebbing *et al.* (75) followed subjects who had participated in a trial of B vitamins to prevent cardiovascular disease. They found that the subjects who had been assigned to the folic acid and B₁₂ group had significantly higher rates of cancer detected, cancer death, and all cause mortality. These findings require further study.

In another study (76), pre- and postfortification data from the United States and Canada showed a decline of colorectal cancer in the early 1990s but, coinciding with the implementation of folic acid fortification, a sharp increase with an increased absolute incidence of colorectal cancer of 4–6 cases/100,000 individuals for several yr with a subsequent drop. Other studies, however, have shown protective effects of moderate folate supplementation on the development of cancer, mainly through the enhancement of DNA repair mechanisms (77).

Another concern is that folic acid fortification of food will reduce the efficacy of pharmaceuticals that target folate. Some studies have shown that patients

taking high levels of folate supplementation require increased doses of methotrexate to treat rheumatoid arthritis (78). One study of women receiving methotrexate for ectopic pregnancies showed that those whose blood folate levels were in the upper half of the study group distribution were significantly less likely to respond to the initial methotrexate intervention than those whose blood folate levels were in the lower half (79), suggesting that high folate levels may block methotrexate action.

Concerns have been raised that folic acid fortification will mask symptoms of vitamin B₁₂ deficiency, delaying the diagnosis until after neurological injury has occurred, particularly in the elderly. Older literature demonstrated that even relatively low doses of folic acid could cause masking but that, conversely, very high doses often did not. One study showed that the proportion of elderly patients with newly diagnosed vitamin B₁₂ deficiency who did not have anemia did not increase after food fortification was instituted in the United States (80). Vitamin B₁₂ deficiency has also been implicated in neural tube defect risk, although not to the same extent as folic acid deficiency. It may be that the appropriate solution to concerns about folic acid fortification masking B₁₂ deficiency would be to fortify foods with both folic acid and vitamin B₁₂; however, this proposal is controversial because no clinical trials have demonstrated that vitamin B₁₂ can prevent neural tube defects, and the effect on the general population is not clear.

A recent study has observed an increased risk of pediatric asthma associated with maternal use of folic acid supplementation during pregnancy (81). Another recent study has shown that children's blood folate levels were inversely related to risk of atopic disease and wheeze (82). This area requires additional investigation.

CONCLUSIONS

Experimental animal studies have demonstrated the importance of folate in embryo development, and work is continuing on the developmental role of specific enzymes in the folate pathway. The experimental animal work supports and has been supported by multiple epidemiology studies showing that the use of multivitamins with folic acid preconceptionally and in early pregnancy decreases the risk of certain birth defects in offspring. The association of folic acid supplementation with a reduced risk of neural tube and other birth defects has led many countries to recommend folic acid supplementation. Because such recommendations have not appeared to result in adequate folic acid intake during early pregnancy, many nations have instituted folic acid food fortification programs, which have been successful in decreasing the prevalence of neural tube defects. Although concerns remain to be resolved regarding possible adverse effects, folic acid fortification of food has been documented to be a public health success, with an important effect on the

reduction in the prevalence of serious disabling neural tube defects. EJ

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REFERENCES

- Christianson, A., Howson, C. P., and Modell, B. (2006) *March of Dimes Global Report on Birth Defects*. March of Dimes Research Foundation, White Plains, NY, USA
- Greene, N. D., and Copp, A. J. (2009) Development of the vertebrate central nervous system: formation of the neural tube. *Prenat. Diagn.* **29**, 303–311
- Gorman, E. B., Wallis, D., Ballard, J. L., Goin-Kochel, R. P., and Finnell, R. H. (2010) Folate transport and folate responsive developmental disorders. *Pteridines* **20**, 156–162
- Wallis, D., Ballard, J. L., Shaw, G. M., Lammer, E. J., and Finnell, R. H. (2009) Folate-related birth defects: embryonic consequences of abnormal folate transport and metabolism. In *Folate in Health and Disease*, 2nd ed. (Bailey, L., ed) pp. 156–178. Taylor and Francis, New York
- Salbaum, J. M., Finnell, R. H., and Kappen, C. (2009) Regulation of folate receptor 1 gene expression in the visceral endoderm. *Birth Defects Res. A Clin. Mol. Teratol.* **85**, 303–313
- Piedrahita, J. A., Oetama, B., Bennett, G. D., van Waes, J., Kamen, B. A., Richardson, J., Lacey, S. W., Anderson, R. G., and Finnell, R. H. (1999) Mice lacking the folic acid-binding protein Folbp1 are defective in early embryonic development. *Nat. Genet.* **23**, 228–232
- Spiegelstein, O., Mitchell, L. E., Merriweather, M. Y., Wicker, N. J., Zhang, Q., Lammer, E. J., and Finnell, R. H. (2004) Embryonic development of folate binding protein-1 (Folbp1) knockout mice: effects of the chemical form, dose, and timing of maternal folate supplementation. *Dev. Dyn.* **231**, 221–231
- Gelineau-van Waes, J., Aleman, F., Maddox, J., Bauer, L., Wilberding, J., Rosenquist, T., and Finnell, R. (2007) Folic acid prevents conotruncal malformations in *Folbp1* knockout mice: role of folate in cardiac neural crest cell survival and migration. In *Chemistry and Biology of Pteridines and Folates* (Jansen, G., and Peters, G., eds) pp. 483–502. Kluwer Academic Publishers, Heilbronn, Germany
- Zhu, H., Wlodarczyk, B. J., Scott, M., Yu, W., Merriweather, M., Gelineau-van Waes, J., Schwartz, R. J., and Finnell, R. H. (2007) Cardiovascular abnormalities in *Folr1* knockout mice and folate rescue. *Birth Defects Res. A Clin. Mol. Teratol.* **79**, 257–268
- Wlodarczyk, B., Spiegelstein, O., Gelineau-van Waes, J., Vorce, R. L., Lu, X., Le, C. X., and Finnell, R. H. (2001) Arsenic-induced congenital malformations in genetically susceptible folate binding protein-2 knockout mice. *Toxicol. Appl. Pharmacol.* **177**, 238–246
- Zhao, R., Russell, R. G., Wang, Y., Liu, L., Gao, F., Kneitz, B., Edelmann, W., and Goldman, I. D. (2001) Rescue of embryonic lethality in reduced folate carrier-deficient mice by maternal folic acid supplementation reveals early neonatal failure of hematopoietic organs. *J. Biol. Chem.* **276**, 10224–10228
- Gelineau-vanWaes, J., Heller, S., Bauer, L. K., Wilberding, J., Maddox, J. R., Aleman, F., Rosenquist, T. H., and Finnell, R. H. (2008) Embryonic development in the reduced folate carrier knockout mouse is modulated by maternal folate supplementation. *Birth Defects Res. A Clin. Mol. Teratol.* **82**, 494–507
- Chen, Z., Karaplis, A. C., Ackerman, S. L., Pogribny, I. P., Melnyk, S., Lussier-Cacan, S., Chen, M. F., Pai, A., John, S. W., Smith, R. S., Bottiglieri, T., Bagley, P., Selhub, J., Rudnicki, M. A., James, S. J., and Rozen, R. (2001) Mice deficient in methylenetetrahydrofolate reductase exhibit hyperhomocysteinemia and decreased methylation capacity, with neuropathology and aortic lipid deposition. *Hum. Mol. Genet.* **10**, 433–443
- Swanson, D. A., Liu, M. L., Baker, P. J., Garrett, L., Stitzel, M., Wu, J., Harris, M., Banerjee, R., Shane, B., and Brody, L. C.

- (2001) Targeted disruption of the methionine synthase gene in mice. *Mol. Cell. Biol.* **21**, 1058–1065
15. Elmore, C. L., Wu, X., Leclerc, D., Watson, E. D., Bottiglieri, T., Krupenko, N. I., Krupenko, S. A., Cross, J. C., Rozen, R., Gravel, R. A., and Matthews, R. G. (2007) Metabolic derangement of methionine and folate metabolism in mice deficient in methionine synthase reductase. *Mol. Genet. Metab.* **91**, 85–97
 16. Deng, L., Elmore, C. L., Lawrance, A. K., Matthews, R. G., and Rozen, R. (2008) Methionine synthase reductase deficiency results in adverse reproductive outcomes and congenital heart defects in mice. *Mol. Genet. Metab.* **94**, 336–342
 17. Stover, P. J., Chen, L. H., Suh, J. R., Stover, D. M., Keyomarsi, K., and Shane, B. (1997) Molecular cloning, characterization, and regulation of the human mitochondrial serine hydroxymethyltransferase gene. *J. Biol. Chem.* **272**, 1842–1848
 18. Herbig, K., Chiang, E. P., Lee, L. R., Hills, J., Shane, B., and Stover, P. J. (2002) Cytoplasmic serine hydroxymethyltransferase mediates competition between folate-dependent deoxyribonucleotide and S-adenosylmethionine biosyntheses. *J. Biol. Chem.* **277**, 38381–38389
 19. Macfarlane, A. J., Liu, X., Perry, C. A., Flodby, P., Allen, R. H., Stabler, S. P., and Stover, P. J. (2008) Cytoplasmic serine hydroxymethyltransferase regulates the metabolic partitioning of methylenetetrahydrofolate but is not essential in mice. *J. Biol. Chem.* **283**, 25846–25853
 20. Pike, S. T., Rajendra, R., Artzt, K., and Appling, D. R. (2010) Mitochondrial C1-tetrahydrofolate synthase (MTHFD1L) supports the flow of mitochondrial one-carbon units into the methyl cycle in embryos. *Biol. Chem.* **285**, 4612–4620
 21. Di Pietro, E., Sirois, J., Tremblay, M. L., and MacKenzie, R. E. (2002) Mitochondrial NAD-dependent methylenetetrahydrofolate dehydrogenase-methylenetetrahydrofolate cyclohydrolase is essential for embryonic development. *Mol. Cell. Biol.* **22**, 4158–4166
 22. Weisberg, I., Tran, P., Christensen, B., Sibani, S., and Rozen, R. (1998) A second genetic polymorphism in methylenetetrahydrofolate reductase (MTHFR) associated with decreased enzyme activity. *Mol. Genet. Metab.* **64**, 169–172
 23. Weisberg, I. S., Jacques, P. F., Selhub, J., Bostom, A. G., Chen, Z., Curtis Ellison, R., Eckfeldt, J. H., and Rozen, R. (2001) The 1298A→C polymorphism in methylenetetrahydrofolate reductase (MTHFR): in vitro expression and association with homocysteine. *Atherosclerosis* **156**, 409–415
 24. Botto, L. D., and Yang, Q. (2000) 5,10-Methylenetetrahydrofolate reductase gene variants and congenital anomalies: a HuGE review. *Am. J. Epidemiol.* **151**, 862–877
 25. Smithells, R. W., Sheppard, S., and Schorah, C. J. (1976) Vitamin deficiencies and neural tube defects. *Arch. Dis. Child.* **51**, 944–950
 26. Laurence, K. M., James, N., Miller, M. H., Tennant, G. B., and Campbell, H. (1981) Double-blind randomised controlled trial of folate treatment before conception to prevent recurrence of neural-tube defects. *Br. Med. J.* **282**, 1509–1511
 27. Smithells, R. W., Sheppard, S., Schorah, C. J., Seller, M. J., Nevin, N. C., Harris, R., Read, A. P., and Fielding, D. W. (1981) Apparent prevention of neural tube defects by periconceptional vitamin supplementation. *Arch. Dis. Child.* **56**, 911–918
 28. Smithells, R. W., Nevin, N. C., Seller, M. J., Sheppard, S., Harris, R., Read, A. P., Fielding, D. W., Walker, S., Schorah, C. J., and Wild, J. (1983) Further experience of vitamin supplementation for prevention of neural tube defect recurrences. *Lancet* **1**, 1027–1031
 29. Holmes-Siedle, M., Lindenbaum, R. H., Galliard, A., and Bobrow, M. (1982) Vitamin supplementation and neural tube defects. *Lancet* **1**, 276
 30. Seller, M. J., and Nevin, N. C. (1984) Periconceptional vitamin supplementation and the prevention of neural tube defects in south-east England and Northern Ireland. *J. Med. Genet.* **21**, 325–330
 31. Vergel, R. G., Sanchez, L. R., Heredero, B. L., Rodriguez, P. L., and Martinez, A. J. (1990) Primary prevention of neural tube defects with folic acid supplementation: Cuban experience. *Prenat. Diagn.* **10**, 149–152
 32. Medical Research Council Vitamin Study Research Group. (1991) Prevention of neural tube defects: results of the Medical Research Council vitamin study. *Lancet* **338**, 131–137
 33. Czeizel, A. E., and Dudás, I. (1992) Prevention of the first occurrence of neural-tube defects by periconceptional vitamin supplementation. *N. Engl. J. Med.* **327**, 1832–1835
 34. Mulinare, J., Cordero, J. F., Erickson, J. D., and Berry, R. J. (1988) Periconceptional use of multivitamins and the occurrence of neural tube defects. *JAMA* **260**, 3141–3145
 35. Bower, C., and Stanley, F. J. (1989) Dietary folate as a risk factor for neural-tube defects: evidence from a case-control study in Western Australia. *Med. J. Aust.* **150**, 613–619
 36. Milunsky, A., Jick, H., Jick, S. S., Bruell, C. L., MacLaughlin, D. S., Rothman, K. J., and Willett, W. (1989) Multivitamin/folic acid supplementation in early pregnancy reduces the prevalence of neural tube defects. *JAMA* **262**, 2847–2852
 37. Werler, M. M., Shapiro, S., and Mitchell, A. A. (1993) Periconceptional folic acid exposure and risk of occurrent neural tube defects. *JAMA* **269**, 1257–1261
 38. Shaw, G. M., Schaffer, D., Velie, E., Morland, K., and Harris, J. A. (1995) Periconceptional vitamin use, dietary folate, and the occurrence of neural tube defects. *Epidemiology* **6**, 219–226
 39. Berry, R. J., Li, Z., Erickson, J. D., Li, S., Moore, C. A., Wang, H., Mulinare, J., Zhao, P., Wong, L. Y., Gindler, J., Hong, S. X., and Correa, A. (1999) Prevention of neural-tube defects with folic acid in China. China-U.S. Collaborative Project for Neural Tube Defect Prevention. *N. Engl. J. Med.* **341**, 1485–1490
 40. Hernandez-Diaz, S., Werler, M. M., Walker, A. M., and Mitchell, A. A. (2000) Folic acid antagonists during pregnancy and the risk of birth defects. *N. Engl. J. Med.* **343**, 1608–1614
 41. Yates, J. R. W., Ferguson-Smith, M. A., Shenkin, A., Guzman-Rodriguez, R., White, M., and Clark, B. J. (1987) Is disordered folate metabolism the basis for the genetic predisposition to neural tube defects? *Clin. Genet.* **31**, 279–287
 42. Kirke, P. N., Molloy, A. M., and Daly, L. E. (1993) Maternal plasma folate and vitamin B12 are independent risk factors for neural tube defects. *Q. J. Med.* **86**, 703–708
 43. Van der Put, N. M., Thomas, C. M., Eskes, T. K., Trijbels, F. J., Steegers-Theunissen, R. P., Mariman, E. C., De Graaf-Hess, A., Smeitink, J. A., and Blom, H. J. (1997) Altered folate and vitamin B12 metabolism in families with spina bifida offspring. *Q. J. Med.* **90**, 505–510
 44. Gardiki-Kouidou, P., and Seller, M. J. (1988) Amniotic fluid folate vitamin B₁₂ and transcobalmins in neural tube defects. *Clin. Genet.* **33**, 441–448
 45. Weekes, E. W., Tamura, T., Davis, R. O., Birch, R., Vaughn, W. H., Franklin, J. C., Barganier, C., Cosper, P., Finley, S. C., and Finley, W. H. (1992) Nutrient levels in amniotic fluid from women with normal and neural tube defect pregnancies. *Biol. Neonate* **61**, 226–231
 46. Economides, D. L., Ferguson, J., Mackenzie, I. Z., Darley, J., Ware, I. L., and Holmes-Siedel, M. (1992) Folate and vitamin B₁₂ concentration in maternal and fetal blood, and amniotic fluid in second trimester pregnancies complicated by neural tube defects. *Br. J. Obstet. Gynaecol.* **99**, 23–25
 47. Wild, J., Schorah, C. J., Sheldon, T. A., and Smithells, R. W. (1993) Investigation of factors influencing folate status in women who have had a neural tube-affected infant. *Br. J. Obstet. Gynaecol.* **100**, 546–549
 48. Luckock, M. D., Wild, J., Schorah, C. J., Levene, M. I., and Hartley, R. (1994) The methylfolate axis in neural tube defects: in vitro characterization and clinical investigation. *Biochem. Med. Metab. Biol.* **52**, 101–114
 49. Botto, L. D., Lisi, A., Bower, C., Canfield, M. A., Dattani, N., De Vigan, C., De Walle, H., Erickson, D. J., Halliday, J., Irgens, L. M., Lowry, R. B., McDonnell, R., Metneki, J., Poetzsch, S., Ritvanen, A., Robert-Gnansia, E., Siffel, C., Stoll, C., and Mastroiacovo, P. (2006) Trends of selected malformations in relation to folic acid recommendations and fortification: an international assessment. *Birth Defects Res. A Clin. Mol. Teratol.* **76**, 693–705
 50. U.S. Food and Drug Administration. (1996) Food standards: amendment of standards of identity for enriched grain products to require addition of folic acid. *Federal Register* **61**, 44
 51. Centers for Disease Control and Prevention. (2000) Weekly folate status in women of childbearing age: United States, 1999. *MMWR Morb. Mortal. Wkly. Rep.* **49**, 962–965
 52. Honein, M. A., Paulozzi, L. J., Mathews, T. J., Erickson, J. D., and Wong, L. Y. (2001) Impact of folic acid fortification of the US food supply on the occurrence of neural tube defects. *JAMA* **285**, 2981–2986
 53. Williams, L. J., Mai, C. T., Edmonds, L. D., Shaw, G. M., Kirby, R. S., Hobbs, C. A., Sever, L. E., Miller, L. A., Meaney, F. J., and

- Levitt, M. (2002) Prevalence of spina bifida and anencephaly during the transition to mandatory folic acid fortification in the United States. *Teratology* **66**, 33–39
54. Canfield, M. A., Collins, J. S., Botto, L. D., Williams, L. J., Mai, C. T., Kirby, R. S., Pearson, K., Devine, O., and Mulinare, J. (2005) Changes in the birth prevalence of selected birth defects after grain fortification with folic acid in the United States findings from a multi-state population-based study. *Birth Defects Res. A Mol. Clin. Teratol.* **73**, 679–689
 55. Centers for Disease Control and Prevention. (2008) Trends in wheat-flour fortification with folic acid and iron: worldwide, 2004 and 2007. *MMWR Morb. Mortal. Wkly. Rep.* **57**, 8–10
 56. Blencowe, H., Cousens, S., Modell, B., and Lawn, J. (2010) Folic acid to reduce neonatal mortality from neural tube disorders. *Int. J. Epidemiol.* **39**(Suppl. 1), i110–i121
 57. Shaw, G. M., O'Malley, C. D., Wasserman, C. R., Tolarova, M. M., and Lammer, E. J. (1995) Maternal periconceptional use of multivitamins and reduced risk for conotruncal heart defects and limb deficiencies among offspring. *Am. J. Med. Genet.* **59**, 536–545
 58. Botto, L. D., Khoury, M. J., Mulinare, J., and Erickson, J. D. (1996) Periconceptional multivitamin use and the occurrence of conotruncal heart defects: results from a population-based, case-control study. *Pediatrics* **98**, 911–917
 59. Shaw, G. M., Lammer, E. J., Wasserman, C. R., O'Malley, C. D., and Tolarova, M. M. (1995) Risks of orofacial clefts in children born to women using multivitamins containing folic acid periconceptionally. *Lancet* **345**, 393–396
 60. Loffredo, L. C. M., Souza, J. M. P., Freitas, J. A. S., and Mossey, P. A. (2001) Oral clefts and vitamin supplementation. *Cleft Palate Craniofac. J.* **38**, 76–83
 61. Czeizel, A. E., Toth, M., and Rockenbauer, M. (1996) Population-based case control study of folic acid supplementation during pregnancy. *Teratology* **53**, 345–351
 62. Czeizel, A. E., and Hirschberg, J. (1997) Orofacial clefts in Hungary: epidemiological and genetic data, primary prevention. *Folia Phoniatr. Logop.* **49**, 111–116
 63. Werler, M. M., Hayes, C., Louik, C., Shapiro, S., and Mitchell, A. A. (1999) Multivitamin supplementation and risk of birth defects. *Am. J. Epidemiol.* **150**, 675–682
 64. Munger R. (2002) Maternal nutrition and oral clefts. In *Cleft Lip and Palate: From Origin to Treatment* (Wyzsynski, D., ed), pp. 170–192, Oxford University Press, New York
 65. Van Rooij I. A. L. M., Ocké M. C., Straatman, H., Zielhuis, G. A., Merkus, H. M., and Steegers-Theunissen, R. P. (2004) Periconceptional folate intake by supplement and food reduces the risk of nonsyndromic cleft lip with or without cleft palate. *Prev. Med.* **39**, 689–694
 66. Beaty, T. H., Wang, H., Hetmanski, J. B., Fan, Y. T., Zeiger, J. S., Liang, K. Y., Chiu, Y. F., Vanderkolk, C. A., Seifert, K. C., Wulfsberg, E. A., Raymond, G., Panny, S. R., and McIntosh, I. (2001) A case-control study on nonsyndromic oral clefts in Maryland. *Ann. Epidemiol.* **11**, 434–442
 67. Selhub, J., Jacques, P. F., Rosenberg, I. H., Rogers, G., Bowman, B. A., Gunter, E. W., Wright, J. D., and Johnson, C. L. (1999) Serum total homocysteine concentrations in the third National Health and Nutrition Examination Survey (1991–1994): population reference ranges and contribution of vitamin status to high serum concentrations. *Ann. Intern. Med.* **131**, 331–339
 68. McLean, E., de Benoist, B., and Allen, L. H. (2008) Review of the magnitude of folate and vitamin B₁₂ deficiencies worldwide. *Food Nutr. Bull.* **29**, S38–S51
 69. Bukowski, R., Malone, F. D., Porter, F. T., Nyberg, D. A., Comstock, C. H., Hankins, G. D., Eddleman, K., Gross, S. J., Dugoff, L., Craigo, S. D., Timor-Tritsch I. E., Carr S. R., Wolfe, H. M., and D'Alton, M. E. (2009) Preconceptional folate supplementation and the risk of spontaneous preterm birth: a cohort study. *PLoS Med.* **6**, e1000061
 70. Wen, S. W., Chen, X. K., Rodger, M., White, R. R., Yang, Q., Smith G. N., Sigal, R. J., Perkins, S. L., and Walker, M. C. (2008) Folic acid supplementation in early second trimester and the risk of preeclampsia. *Am. J. Obstet Gynecol.* **198**, 45.e1–45.e7
 71. Wen, S. W., Zhou, J., Yang, Q., Fraser, W., Olatunbosun, O., and Walker, M. (2008) Maternal exposure to folic acid antagonists and placenta-mediated adverse pregnancy outcomes. *CMAJ* **179**, 1263–1268
 72. Hirsch, S., Sanchez, H., Albala, C., de la Maza, M. P., Barrera, G., Leiva, L., and Bunout, D. (2009) Colon cancer in Chile before and after the start of the flour fortification program. *Eur. J. Gastroenterol. Hepatol.* **21**, 436–439
 73. Cole, B. F., Baron, J. A., Sandler, R. S., Haile, R. W., Ahnen, D. J., Bresalier, R. S., McKeown-Eyssen, G., Summers, R. W., Rothstein, R. I., Burke, C. A., Snover, D. C., Church, T. R., Allen, J. I., Robertson, D. J., Beck, G. J., Bond, J. H., Byers, T., Mandel, J. S., Mott, L. A., Pearson, L. H., Barry, E. L., Rees, J. R., Marcon, N., Saibil, F., Ueland, P. M., and Greenberg, E. R. (2007) Folic acid for the prevention of colorectal adenomas: a randomized clinical trial. *JAMA* **297**, 2351–2359
 74. Logan, R. F., Grainge, M. J., Shepherd, V. C., Armitage, N. C., and Muir, K. R. (2008) Aspirin and folic acid for the prevention of recurrent colorectal adenomas. *Gastroenterology* **134**, 29–38
 75. Ebbing, M., Bønaa, K. H., Nygård, O., Arnesen, E., Ueland, P. M., Nordrehaug, J. E., Rasmussen, K., Njølstad, I., Refsum, H., Nilsen, D. W., Tverdal, A., Meyer, K., and Vollset, S. E. (2009) Cancer incidence and mortality after treatment with folic acid and vitamin B12. *JAMA* **302**, 2119–2126
 76. Mason, J. B., Dickstein, A., Jacques, P. F., Haggarty, P., Selhub, J., Dallal, G., and Rosenberg, I. H. (2007) A temporal association between folic acid fortification and an increase in colorectal cancer rates may be illuminating important biological principles: a hypothesis. *Cancer Epidemiol. Biomark. Prev.* **16**, 1325–1329
 77. Giovannucci, E., Stampfer, M. J., Colditz, G. A., Hunter, D. J., Fuchs, C., Rosner, B. A., Speizer, F. E., and Willett, W. C. (1998) Multivitamin use, folate, and colon cancer in women in the Nurses' Health Study. *Ann. Intern. Med.* **129**, 517–524
 78. Arabelovic, S., Sam, G., Dallal, G. E., Jacques, P. F., Selhub, J., Rosenberg, I. H., and Roubenoff, R. (2007) Preliminary evidence shows that folic acid fortification of the food supply is associated with higher methotrexate dosing in patients with rheumatoid arthritis. *J. Am. Coll. Nutr.* **26**, 453–455
 79. Takacs, P., and Rodriguez, L. (2005) High folic acid levels and failure of single-dose methotrexate in ectopic pregnancy. *Int. J. Gynaecol. Obstet.* **89**, 301–302
 80. Mills, J. L., Von Kohorn, I., Conley, M. R., Zeller, J. A., Cox, C., Williamson, R. E., and Dufour, D. R. (2003) Low vitamin B₁₂ concentrations in patients without anemia: the effect of folic acid fortification of grain. *Am. J. Clin. Nutr.* **77**, 1474–1477
 81. Whitrow, M. J., Moore, V. M., Rumbold, A. R., and Davies, M. J. (2009) Effect of supplemental folic acid in pregnancy on childhood asthma: a prospective birth cohort study. *Am. J. Epidemiol.* **170**, 1486–1493
 82. Matsui, E. C., and Matsui, W. (2009) Higher serum folate levels are associated with a lower risk of atopy and wheeze. *J. Allergy Clin. Immunol.* **123**, 1253–1259
 83. De Marco, P., Calevo, M. G., Moroni, A., Merello, E., Raso, A., Finnell, R. H., Zhu, H., Andreussi, L., Cama, A., and Capra, V. (2003) Reduced folate carrier polymorphism (80A→G) and neural tube defects. *Eur. J. Hum. Genet.* **11**, 245–252

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