Perspective

Knowledge gaps in understanding the metabolic and clinical effects of excess folates/folic acid: a summary, and perspectives, from an NIH workshop

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ABSTRACT

Folate, an essential nutrient found naturally in foods in a reduced form, is present in dietary supplements and fortified foods in an oxidized synthetic form (folic acid). There is widespread agreement that maintaining adequate folate status is critical to prevent diseases due to folate inadequacy (e.g., anemia, birth defects, and cancer). However, there are concerns of potential adverse effects of excess folic acid intake and/or elevated folate status, with the original concern focused on exacerbation of clinical effects of vitamin B-12 deficiency and its role in neurocognitive health. More recently, animal and observational studies have suggested potential adverse effects on cancer risk, birth outcomes, and other diseases. Observations indicating adverse effects from excess folic acid intake, elevated folate status, and unmetabolized folic acid (UMFA) remain inconclusive; the data do not provide the evidence needed to affect public health recommendations. Moreover, strong biological and mechanistic premises connecting elevated folic acid intake, UMFA, and/or high folate status to adverse health outcomes are lacking. However, the body of evidence on potential adverse health outcomes indicates the need for comprehensive research to clarify these issues and bridge knowledge gaps. Three key research questions encompass the additional research needed to establish whether high folic acid or total folate intake contributes to disease risk. *1*) Does UMFA affect biological pathways leading to adverse health effects? *2*) Does elevated folate status resulting from any form of folate intake affect vitamin B-12 function and its roles in sustaining health? *3*) Does elevated folate intake, regardless of form, affect biological pathways leading to adverse health effects other than those linked to vitamin B-12 function? This article summarizes the proceedings of an August 2019 NIH expert workshop focused on addressing these research areas. *Am J Clin Nutr* 2020;112:1390–1403.

Keywords: folic acid, folate, vitamin B-12, upper limit, excess intake, unmetabolized folic acid, adverse outcomes

Introduction

Folate, an essential nutrient integral to the function of numerous critical cellular processes, functions as a family of metabolic cofactors that participate in 1-carbon transfer reactions, cellular methylation reactions, amino acid metabolism, and nucleotide biosynthesis. Folate is present naturally in foods in

a reduced form, whereas fortified foods and nearly all dietary supplements contain a synthetic, oxidized form of folate termed folic acid. Some groups within the population consume folic acid in excess of the current Tolerable Upper Intake Level (UL). Precisely what constitutes an "excessive intake" remains ill-defined at present because the adverse health effects ascribed to excessive intake have not been established, and dose-response data are lacking, leaving open the possibility that future scientific advances might dictate adjustments in the existing UL. For the purposes of this article, excessive folic acid intake constitutes exposure doses that exceed the UL of 1000 μg/d for adults set by the Institute of Medicine in 1998 (1). Approximately 5% of American men and women aged 51–70 y have folic acid intakes exceeding the UL, primarily because of dietary supplement intakes (2). Furthermore, depending on age group, 30% (9- to 13-y-olds) to 66% (64% among 1- to 3-y-olds and 66% among 4- to 8-y-olds) of children who take folic acid–containing supplements have intakes exceeding the age-specific UL (3), and nearly all children aged 1–8 y who consume ≥200 μg folic acid/d from dietary supplements have total intakes that exceed the UL (4). For children not using dietary supplements containing folic acid, some still exceed the UL (7% among 1- to 3-y-olds; 6% among 4- to 8-y-olds; and 2% among 9- to 13-y-olds). Further, one of the unanswered uncertainties is whether the ULs for infants and children are legitimate because they have been extrapolated from adult data.

The use of dietary supplements is the primary source of excessive folic acid intake: ∼35% of US adults and 28% of children aged 1–13 y regularly use supplements containing folic acid; however, fortified food sources alone enable many infants and children to exceed the UL (2). Discretionary fortification of certain foodstuffs by the food industry, most notably ready-to-eat cereals, makes a smaller contribution as noted in the *Federal Register* on Food Additives Permitted for Direct Addition to Food for Human Consumption; Folic Acid (**Supplemental Table 1**), and an even smaller contribution is from federally mandated fortification of enriched grain (5, 6). The latter was established in 1998 in the United States and Canada as a result of strong evidence that folic acid substantially reduces the incidence of a common type of birth defect known as neural tube defects (7, 8), and the resulting mandatory fortification programs, now in >80 countries, have been highly successful (9, 10).

The NHANES reports the nearly ubiquitous presence of the fully oxidized, unsubstituted form of folate used in fortification and in most supplements, unmetabolized folic acid (UMFA), in the serum samples of US participants of all age groups (11). Although the body possesses a mechanism for converting ingested folic acid into a reduced and natural form of the vitamin, primarily in the intestine and liver, the system is readily saturated: in older studies UMFA was detected in serum with ingested doses ≥200 μg (12–15), but newer, more sensitive HPLC–tandem MS methodology reveals detectable UMFA in >95% of NHANES sera, regardless of recorded intake (11). The health implications, if any, of elevated folate or UMFA exposures are unknown. Some studies, primarily preclinical and observational in nature, have identified possible adverse outcomes related to folic acid exposure above the UL and/or elevated folate status at levels not seen in the absence of very-high-dose folic-acid supplements, although other studies have not reproduced these findings. The presence of UMFA is generally regarded as a marker of dihydrofolate reductase (DHFR) saturation in its capacity to convert folic acid to tetrahydrofolate (THF), whereas potential adverse health effects of UMFA are expected to demonstrate a dose–response relation. In those studies where adverse effects have been reported, elevated folate status has been associated with increased risk of various disease conditions, including cancer, cardiovascular disease, diabetes and metabolic syndrome, insulin resistance and obesity in offspring, other adverse birth outcomes, and autism (16–33). Other studies have indicated a potential for an interaction between elevated folate status and vitamin B-12 metabolism, with adverse effects on biomarkers of vitamin B-12 deficiency and an enhanced risk of neurocognitive decline among the elderly (34–37). In contrast, other studies have reported no significant effects of high folic acid and/or total folate exposure on the aforementioned adverse health outcomes (38–42). Given the heterogeneity and inconsistency in the findings among these studies, the data do not rise to the level of evidence needed for either policy recommendations or modifications in the approaches to medical care. However, it is critical to continue to monitor and investigate potential adverse effects of excess folate/folic acid given the occurrence of some individuals and population groups exceeding the UL.

Over the course of the 20th century, essential nutrients for life were identified, and DRIs were established for each of these nutrients. The DRIs specify the daily intakes necessary to prevent dietary deficiency diseases, and the ULs are defined as the highest amount of daily nutrient intake that is likely to pose no risk of adverse health effects in almost all individuals across age groups. In 2017, recognizing the role of nutrients in chronic disease, a committee of The National Academies of Sciences, Engineering, and Medicine developed guiding principles for arriving at DRIs for nutrients that have been shown to affect risks of chronic diseases and suggested using ranges over which the risk of chronic disease is reduced for a given nutrient (43). The DRI chronic disease framework described the types of evidence needed to establish causal relations between nutrient intake and chronic disease outcomes that describe nutrient intake–response relations, as well as a methodology—GRADE (Grading of Recommendations, Assessment, Development, and Evaluation)—to evaluate the strengths of evidence (44). The committee emphasized that the DRIs should be developed

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The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the NIH, the US FDA, the CDC/the Agency for Toxic Substances and Disease Registry, and the USDA.

Supplemental Table 1 is available from the "Supplementary data" link in the online posting of the article and from the same link in the online table of contents at https://academic.oup.com/ajcn/.

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Abbreviations used: BOND, Biomarkers of Nutrition for Development; CDRR, chronic disease risk reduction; DHFR, dihydrofolate reductase; FOLR, folate receptor; MTHFR, methyltetrahydrofolate reductase; NTP, National Toxicology Program; THF, tetrahydrofolate; UL, Tolerable Upper Intake Level; UMFA, unmetabolized folic acid; 5-MTHF, 5-methyltetrahydrofolate.

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only when at least moderately strong evidence exists for these associations; an exception to this in the report was that when a food substance increases chronic disease risk, the level of certainty considered acceptable might be lower (e.g., sodium, saturated fat, sugar). These newer approaches are emerging as methods of choice to evaluate the strengths of evidence from systematic reviews and meta-analyses and to develop this evidence into clinical guideline and policy recommendations (45–48).

This DRI framework was applied for the first time in a recent review of the relations between sodium and potassium intakes and cardiovascular disease (49). The expert committee undertaking this task used the term chronic disease risk reduction (CDRR) to describe the lowest amount at which a risk reduction in chronic disease is achieved for sodium intake, as supported by the strength of the evidence available for cardiovascular disease. However, no CDRR was developed for potassium because of the lack of sufficient evidence. In keeping with the report recommendations, the term UL was not used for the chronic disease DRI but remains in place to cover the adverse effects that are not necessarily considered risk factors for chronic disease. If risks of chronic diseases were found to be causally related to folate/folic acid intakes, the DRI should be reconsidered and the development of CDRRs explored.

In 2015, the National Toxicology Program (NTP)—a division of the NIH's National Institute of Environmental Health Sciences—and the NIH Office of Dietary Supplements conducted systematic reviews to evaluate the literature on high folic acid intake/elevated folate status and adverse health outcomes (16). The Biomarkers of Nutrition for Development (BOND) project convened by the NIH's Eunice Kennedy Shriver National Institute of Child Health and Human Development focused on summarizing the information on assessment of biomarkers of folate status (50). Although the BOND project and the NTP review identified many of the existing knowledge gaps, they did not focus on exploring and defining the research strategies that would be required to address these knowledge gaps and thereby establish any outcomes that might be causally related to high folic acid intake and/or elevated folate status, with the goal of determining what constitutes an "excess" folic acid/folate intake level. This expert workshop convened by the NIH updated and extended prior meetings on this topic by identifying knowledge gaps, as well as developing research strategies to address current gaps in knowledge. Moreover, these prior activities were undertaken >4 y ago, and many new observations have been reported in the ensuing years. Therefore, to define the research necessary to determine whether there is an excess folate/folic acid intake level that is causally related with adverse health outcomes and to define its potential interaction with vitamin B-12 deficiency, NIH—partnering with the US FDA and USDA—convened a 1.5-d workshop during the summer of 2019.

Challenges

The workshop focused on 3 research topics informed by the existing literature related to the potential effects of *1*) unmetabolized folic acid on biological/physiological pathways leading to adverse health effects; *2*) elevated folate status, resulting from intake of any form of folate, on vitamin B-12 function and associated adverse health effects; and *3*) elevated folate status, resulting from intake of any form of folate, on biological pathways leading to adverse health effects other than those linked to vitamin B-12 function. The participants were charged with identifying specific research strategies that are needed to bridge the knowledge gaps that currently exist (i.e., to establish whether high folic acid or high total folate intake causes adverse health outcomes and, if so, to establish biomarkers for those effects and elucidate the mechanistic basis for those adverse effects). Integral to establishing causation and mechanism are the establishment of dose–response relations and the development of animal and in vitro preclinical models, as well as robust clinical study designs.

The formal presentations at the workshop reviewed the state of the current literature, including both clinical and preclinical research, and described potential associations between folic acid/folate intake and adverse health outcomes, including interactions with vitamin B-12 status. A panel session further delineated the gaps in knowledge that need to be addressed to establish mechanisms and causation. Three breakout sessions then explored the specific research approaches needed to bridge the knowledge gaps identified by the panel members. The ultimate goal of these research agendas is to advance scientific understanding and generate a comprehensive body of knowledge related to the metabolic and clinical effects of excess folic acid and elevated folate status to inform evidence-based clinical and public health recommendations. This report summarizes the salient information that emerged in the presentations and discussions from the workshop.

What are the biological effects of folic acid currently not ascribed to other physiological forms of folate?

Existing gaps in understanding the health effects of folic acid.

Folic acid is a previtamin and not thought to be a metabolically functional form of folate. Its only established biological activities include its transport into cells through either the folate receptor (FOLR) or the intestinal proton-coupled transporter, and its function as a reduced folate carrier during transmembrane transport (51, 52). Further, it is processed to reduced folate through the enzyme DHFR. Incremental increases in the oral intake of folic acid result in increasing urinary excretion of 2 of its major catabolic products, p-aminobenzoylglutamate and p-acetamidobenzoylglutamate, providing an avenue of egress from the body (53, 54). Lacking are established biological mechanisms of folic acid underlying potential cause-and-effect relations with adverse health outcomes.

UMFA refers to the folic acid that accumulates in biological fluids (55), presumably because the catalytic capacity of DHFR has been saturated. Older studies in adults found that 200 μg oral folic acid resulted in detectable concentrations of UMFA in the bloodstream (12, 14, 15), and that the daily ingestion of 400 μg produced a sustained appearance of circulating folic acid (13) but newer, more sensitive, methodologies have shown that it circulates at low concentrations [~0.8 nmol/L (10)] even among those who take no form of supplementation. After ingestion, not all folic acid is reduced to THF in intestine. Folic acid

that escapes the intestine enters the hepatic portal vein and is almost completely metabolized to THF in the liver (13, 56, 57). The activity of DHFR is variable in human liver and much lower than found in rodents (51). UMFA persistence in the body is reflected by observations that ^{14}C-labeled folic acid can be detected in plasma, urine, and feces for >40 d after ingestion (58), although whether its detection in this study in erythrocytes indicates it might also accumulate in some tissues is debatable because it may have merely been bound to the cell membranes.

The appearance of UMFA in plasma is not only related to the dose, but also to the timing, of ingested folic acid (i.e., smaller doses consumed more frequently result in higher UMFA concentrations than larger doses consumed less frequently) (14). It has been shown that supplement use is associated with higher UMFA concentrations, but supplement use alone does not fully determine UMFA concentrations (11, 59). Furthermore, the exposure to supplemental folic acid at recommended levels during pregnancy does not appear to increase UMFA concentrations in maternal or cord blood (60). Nevertheless, the likelihood of having detectable UMFA in plasma among US adults whose folic acid intakes exceed the UL is greater than among those whose intake is less than the UL (OR: 17.6; CI: 5.5, 56) (61). It is also noteworthy that plasma UMFA was detected in ∼95% of an older Irish population at a time when a mandatory fortification program was absent, but voluntary high-dosage fortification of foodstuffs was widespread (62). Furthermore, folic acid has been found to exist in plasma and breast milk, where it is tightly bound to a soluble form of the FOLR (16, 63, 64). There are gaps in understanding the factors that contribute to UMFA accumulation in plasma, in both its FOLR-bound and unbound states.

FOLRs are glycosylphosphatidylinositol-anchored cell surface proteins that transport folate into the cell through receptor-mediated endocytosis. Unlike transmembrane folate transporters, FOLRs have a limited expression range, but are present in the blood–brain barrier, kidney, placenta, neural epithelium of the developing embryo, and cancer cells (52, 65, 66). They also are present in soluble form in plasma and breast milk. Members of the FOLR family exhibit high binding affinities for folic acid ($K_D = 10^{-10}$ to 10^{-11} M) that are 6- to 10-fold higher than for 5-methyltetrahydrofolate (5-MTHF), the primary form of folate in circulation. Circulating UMFA is converted to reduced folate in the kidney through a mechanism that involves binding by FOLR-α at the apical membrane of proximal tubule cells, endocytosis, and acid-dependent dissociation from FOLR-α, followed by transport into the cytoplasm, where it is reduced and methylated before its transport into the bloodstream. Alternatively, the entire folate/FOLR-α complex may re-enter the bloodstream through exocytosis (67). This complex is reportedly reabsorbed by the proximal tubular cells by megalin-linked endocytosis (68). How the complex of FOLR-α/folic acid is handled by other tissues is not known. In the choroid plexus, FOLR-α facilitates folate uptake from the basal plasma membrane, its transport through the cell, and then across the apical membrane into brain parenchyma (69). FOLRs are also found in various cancer cells and have been used as targets (70). Little is known about the function or accumulation of folic acid at the cell surface and in cells with respect to adverse effects, including effects of its interaction with FOLRs. However, it has been observed that autoantibodies can block the binding of folic acid to FOLRs and impair its transport across the blood–brain and placental barriers (71, 72).

Research regarding the effects of excess folic acid intake and continued population monitoring of UMFA and folic acid intake are needed. Supplemental Table 1 tabulates median and 95th percentile age-specific daily folic acid intakes from the *Federal Register*, showing current folic acid intakes from food and supplements among American free-living people. These data show that the 95th percentile intakes are well below the respective ULs, except for children 1–13 y of age (73). Continued monitoring of the folate status of the US population, including UMFA, is essential to clarify any unintended effects at high amounts of intake (74, 75). Likewise, research is needed to determine whether UMFA in serum or tissues genuinely produces adverse effects and to examine the associated biological pathways.

Potential mechanisms for activity of UMFA.

Folic acid is not known to accumulate at high concentrations in tissues, as do natural folates, because it is not converted to the polyglutamyl forms of folate that are retained within the cell (76) and it binds weakly to folate enzymes. Therefore, any postulated direct effects of UMFA on biological processes are likely to be independent of folate-mediated 1-carbon metabolism and may involve noncanonical pathways. For example, FOLRs may serve as signaling proteins (77) through their interaction with folate derivatives, including folic acid (78). The addition of folic acid to cultured cells can activate signaling pathways in a FOLR-α-dependent manner, in shorter periods of time than could plausibly be attributed to changes in 1-carbon metabolism, including phospho-activation of gene coding for cellular Sarcoma(c-src) and extracellular signal-regulated kinases (ERK) kinases and the activation of phospho-STAT (79–81). Reports also indicate that in isolated mouse cranial neural crest cells, FOLR-α translocates to the nucleus in response to folic acid exposure, where it functions as a transcription factor, activates the gene coding octamer-binding transcription factor 4 *(Oct4),* gene coding for sex determining region Y *(Sox2)*, and gene coding for gut-enriched Krüppel-like factor-4 *(Klf4)* genes, and represses biogenesis of microRNAs that target these genes or their effector molecules (82, 83). Studies of the nematode *Caenorhabditis elegans* (84) have shown that worms respond to a particular folate from their diet (10-CHO-THF polyglutamates but no other folates) by increasing the rate of germ cell proliferation, through a mechanism that requires the folate receptor FOLR-1. These studies argue for the existence of noncanonical folate-signaling pathways that are not part of 1-carbon metabolism. In 1 study of adult female rats, dietary folic acid, but not reduced folate, was found to regulate different stages of neurogenesis in the ventral hippocampus (85). Folic acid was shown to modulate endothelial NO synthase activity, although it is unclear whether the effect was due to folic acid as opposed to enhancement of the total folate pool (86). Additional exploratory studies will be required to better understand the roles of UMFA in noncanonical pathways (those not normally known to be associated with folate) and cellular processes and their connections to both health and disease.

Are there plausible adverse effects of excessive exposure to folates, independent of the form ingested, separate from its effects on vitamin B-12 metabolism?

Existing gaps in knowledge.

Among the potential adverse health outcomes of high total folate or folic acid exposure unrelated to vitamin B-12 are *1*) an increased risk of certain cancers; *2*) short- and long-term effects on pregnancy outcomes and on the developing fetus; *3*) risk of allergies, asthma, and other hypersensitivity illnesses; and *4*) risk of thyroid and other endocrine disorders (16, 20, 32). The vast majority of the observations to date apply to the first 2 of these potential adverse health outcomes.

Low consumption of total folate is considered to be an independent risk factor for colorectal cancer and perhaps also for cancers of the breast, lung, pancreas, and others (87), because without adequate folate uracil misincorporation and DNA double strand breaks can occur (88–90). However, some animal studies (91, 92) as well as some secondary post hoc analyses of human trials (23, 24) suggest that there may be adverse effects at excessive doses. Some controlled studies in laboratory rodent models of colorectal cancer have observed an enhancement of carcinogenesis with dietary amounts of folic acid severalfold above the basal requirement (91, 92), although the increase in tumorigenesis has not uniformly been observed in all rodent models (93). Furthermore, it remains to be determined whether the putative enhancing effect of high folate intake results from the effects of folic acid intake on folate status or relates specifically to folic acid. Human studies that have explored this phenomenon have not shown consistency; therefore, its relevance to human cancer biology remains unclear. To date, 5 clinical trials have been conducted with participants who had previously resected precancerous adenomatous polyps, an intermediary biomarker of colorectal cancer risk, and who were then randomly assigned to receive daily supplements containing 0.5–5 mg folic acid or placebo. Although the largest randomized trial with the longest follow-up failed to prevent the development of new colorectal adenomas, the study observed as a secondary outcome a 1.67-fold increased risk of incident advanced adenomas ($P = 0.05$), presumably due to stimulation of growth of microscopic early lesions (21). None of the other 4 trials observed a similar increase in tumorigenesis (94–96). Other clinical study designs—including secondary analyses of clinical trials (23, 24), large prospective cohort studies (40, 97), and systematic reviews and meta-analyses (42)—have similarly failed to arrive at a consensus as to whether excess folic acid intake enhances the risk of colorectal cancer [or other common cancers such as prostate (24)] in humans.

Women frequently consume quantities of folic acid that exceed the UL during pregnancy (98, 99), and they often consume these quantities through the second and third trimesters, far beyond the time of neural tube closure. Because serum and RBC folate concentrations of cord blood correlate with maternal values (100), the developing fetus also is exposed to elevated amounts of the vitamin. In a Canadian cohort of healthy pregnant women, the mean serum folate concentration of cord blood was 64 nmol/L, and 93% of the samples had detectable concentrations of UMFA (101). Nevertheless, daily maternal supplementation of 400 μg/d does not appear to produce a significant elevation in the concentration of cord blood UMFA (59). Two observational studies conducted in the Indian subcontinent have raised concerns about an apparent association between high maternal folate status during pregnancy and a variety of metabolic abnormalities in offspring, including obesity, insulin resistance, and high blood glucose concentrations (27, 33), although these links were not substantiated by results from a clinical trial conducted in that region (41). Similarly, although some animal studies involving supplementation of mouse and rat dams at 2.5–20 times the basal requirement of folic acid have corroborated the development of these metabolic abnormalities in offspring exposed in utero (25, 26, 30), those effects have not been observed uniformly (38).

Potential mechanisms explaining adverse effects of elevated folates.

The causal mechanisms by which high folate status, or specifically folic acid, is postulated to promote carcinogenesis have not been established, although several plausible pathways have been proposed: enhancing hyperproliferation of neoplastic cells via facilitated DNA synthesis (102), impairing natural killer cell activity (103–105), promoting a proinflammatory transcriptomic milieu in the colon (90), and folate operating as a component in procarcinogenic cell signaling pathways (106). Most recently, supraphysiologic concentrations of folic acid ($\geq$100 μmol) have been observed in cancer cell organoids to rescue methionine dependency, which is a common feature of cancer cells (107). Some animal and human studies have found positive associations between elevated UMFA and total folate in the blood and reduced natural killer cell activity (103–105), although the apparent suppressive effect on natural killer cells was not observed in either an in vitro study or a cross-sectional study among older adults (108, 109). Although severe folate deficiency reduces natural killer cell–mediated cytotoxicity in rodent models (110), a biological pathway (or pathways) underlying the associations between high folate/UMFA and reduced natural killer cell activity is unknown.

The mechanisms by which high maternal folic acid intake during pregnancy might alter birth outcomes, whether that be detrimental or beneficial, are not well defined, although epigenetic stem cell programming has been suggested as a potential cause. During embryogenesis, the patterns of genome methylation undergo dynamic modifications that are influenced by 1-carbon metabolism. Folic acid supplementation during pregnancy has been shown to alter patterns of both genome-wide and gene-specific DNA methylation in an organ-specific manner in a rodent model (111). Also in rodent models, methylation and expression of imprinted genes, histone marks, and heterochromatin assembly have each been shown to be modified by maternal folic acid supplementation [reviewed in (112)]. In a Canadian cohort of pregnant women, maternal RBC folate in early pregnancy and cord plasma UMFA were inversely correlated with DNA hydroxymethylation (100), an intermediary in the demethylation of DNA. Among ~2000 European newborns, site-specific alterations in their epigenome were found to correlate with maternal concentrations of plasma folate (113). Complementing these observational studies and supporting a genuine causal role for maternal folic acid supplementation on epigenetic mechanisms is a controlled trial in pregnant women, which observed that continuing supplementation with

400 μg folic acid/d into the 2nd and 3rd trimesters induced alterations in DNA methylation of candidate genes related to brain development in cord blood (114, 115). Importantly, these altered epigenetic patterns may persist throughout life. In the Aberdeen Folic Acid Supplementation Trial, site-specific patterns of DNA methylation in 86 offspring, aged 46–48 y, correlated in a dose-responsive manner with the amount of folic acid supplementation that their mothers received during pregnancy (116). It is nevertheless important to note that none of these epigenetic changes have been causally linked to phenotypic characteristics and should therefore not be assumed to be deleterious; indeed, continuing 400 μg/d supplementation into the second and third trimesters has been observed to result in higher cognition scores among offspring at 3 and 7 y of age (117).

Thus, significant gaps exist in our present understanding of the relations between folic acid supplementation and/or elevated folate status and their effects on physiology, both beneficial and deleterious, unrelated to vitamin B-12 metabolism. Stronger and more consistent evidence is required to determine whether the aforementioned relations are genuinely causal in humans. In addition to proving causality, the following 3 knowledge gaps are among the most important to examine. *1*) What are the dose–response relations between folate/folic acid exposure, physiology, and these putative effects (or biomarkers of effects)? *2*) What are the underlying mechanisms of action? *3*) Are there "at-risk" groups particularly susceptible to these effects?

What are plausible effects of elevated folate status resulting from intake of any form of folate on vitamin B-12 function and associated adverse health effects?

Existing gaps.

Vitamin B-12 and folate interact within folate-mediated 1-carbon metabolism, in which vitamin B-12 deficiency causes an accumulation of cellular folate as 5-MTHF, leading to a functional folate deficiency and impaired nucleotide and DNA biosynthesis. This effect of vitamin B-12 deficiency on impairing folate metabolism and nucleotide synthesis is known as the "methyl trap," which results in megaloblastic anemia (118, 119). The effects of vitamin B-12 deficiency on DNA synthesis and the associated anemia can be partially rescued by higher folic acid intakes. In human cultured cells, both genetic and nutritional vitamin B-12 deficiency impairs folate-dependent de novo thymidylate synthesis and causes increased DNA damage, and both of these outcomes are rescued, not exacerbated, by high 5-formylTHF in the culture medium (102). Regarding dose-response, it is also noteworthy that >95% of the 155 cases in which folic acid supplementation was reported to precipitate neurologic manifestations occurred before 1963, during which time the FDA recommended dosage was 5–20 mg folic acid/d (120).

In contrast, an emerging body of observational data and secondary data analyses suggests possible adverse interactions between elevated folate status and vitamin B-12 deficiency, with respect to risk of functional neurological decline and pathology (17–19, 22, 29, 31). These were recently reviewed with respect to the safety of folic acid (121). The Institute of Medicine's 1998 DRI report for folate (1) cited early studies indicating that vitamin-B-12-deficient monkeys and fruit bats receiving supplemental folate developed signs of neuropathology earlier than did controls (122, 123). Those early studies first raised the question regarding adverse effects of elevated folate status exacerbating the clinical sequelae of vitamin B-12 deficiency and consisted of a relatively small number of animals, in which there was significant "unexplained death" across the intervention groups (122, 123). Replication of these early findings has not been reported, and current rodent models do not exhibit the neurological clinical sequelae of human vitamin B-12 deficiency, which has limited further investigation of these findings. Two more recent studies in rodents reported that although a high-folate, vitamin-B-12-deficient diet consumed by dams was associated with unique gene expression changes in offspring liver and pancreas, in addition to changes in fasting insulin, this exposure did not affect such physiological outcomes as weight gain or adiposity (38, 124). In female offspring consuming a "Western diet" (i.e., high-fat, low-calcium, and low-vitamin-D diet), the vitamin-B-12-deficient diet appeared protective against similar outcomes (124). Among humans, a single cohort study in obese subjects observed that high folate status in conjunction with low vitamin B-12 status was associated with insulin resistance (28). It is important to note that although there are several validated transgenic mouse models of vitamin B-12 deficiency (*Mmachc*$^{+/-}$, *Mtr*$^{+/}$, *Mtrr*$^{+/-}$, *CD320*$^{-/-}$), studies using "high folate" exposure have not been performed in these models to date.

A recent human intervention trial investigated the effects of 1 intramuscular injection of cyanocobalamin (10 mg), pyridoxine (100 mg), and thiamin (100 mg) in vitamin-B-12-deficient elderly Chileans (17) on measures related to peripheral neuropathy. The intervention improved sensory nerve conduction velocity in these individuals, and this outcome was not affected by baseline folate status (17). However, a secondary data analysis indicated that individuals with serum folate above the study median (i.e., 33.9 nmol/L), compared with those below the median, had a weaker response to treatment with respect to a computed index of vitamin B-12 status comprising total serum vitamin B-12, holo-transcobalamin, methylmalonic acid, and total homocysteine (17).

What mechanisms of action might explain a folate–vitamin B-12 interaction?.

The most ready explanation for individuals who jointly present with high folate and low vitamin B-12 status, which is the focus of reports from several observational studies (17–19, 22, 31), is that they likely have impaired vitamin B-12 absorption or have very low intakes of vitamin B-12, the latter of which may be the case among strict vegans not using dietary supplements. Because both folic acid and vitamin B-12 are present in adequate or sometimes more-than-adequate amounts in most dietary supplements, supplement users could be expected to attain the elevated folate status observed in these studies. It has been proposed that the associations between low vitamin B-12/high folate status and adverse outcomes in observational studies are driven by severe defects in vitamin B-12 absorption, where dietary supplement users with vitamin B-12 malabsorption would be expected to exhibit a low vitamin B-12/high folate status and would be susceptible to the symptoms of vitamin B-12 deficiency (121). Moreover, the lack of a standardized definition of what constitutes "high folate status" among studies has

TABLE 1 Requisite gaps to fill in our understanding of the biological, physiological, and health effects of excess folate/folic acid and their interactions with vitamin B-12

Indicators of nutrient status and function
Establish biomarkers of status and function that exhibit dose–response relations, define exposure to excess folic acid/folate, and are on the mechanistic pathway and then validate cutoffs for these biomarkers.
Establish universally accepted definitions for accurately classifying vitamin B-12 status and excess folate.
Establish omic profiles (metabolome, transcriptome, epigenome) of folate status and function, from deficiency to excess.
Purported clinical outcomes and underlying mechanisms of unmetabolized folic acid and elevated folate status
Clarify authenticity of developmental and intergenerational outcomes.
Clarify authenticity of effects on clinical sequelae of vitamin B-12 deficiency.
Clarify authenticity of effects on carcinogenesis and promotion of cancer risk.
Clarify authenticity of effects on natural killer cell function.
Identification and delineation of effect modifiers and covariates
Genetic variation, including polymorphisms in dihydrofolate reductase and methylenetetrahydrofolate reductase
Dietary factors, including other B-vitamins and 1-carbon nutrients and metabolites
Microbiome
Sex, age, pregnancy, lactation, obesity, underlying disease states

further complicated interstudy comparisons. Also, no established mechanisms account for the long-standing putative effects of elevated folate status exacerbating the clinical sequelae of vitamin B-12 deficiency. Progress has been hampered by the limited availability of relevant animal and preclinical models.

Knowledge gaps that need to be addressed to establish whether adverse health effects of excess folic acid and elevated folate status exist

Participants in the workshop identified several knowledge and evidence gaps that need to be addressed to evaluate the safety of high folic acid intake and elevated folate status from all dietary sources. **Table 1** summarizes these knowledge gaps and highlights the need for robust dose-response data with respect to important clinical outcomes. The following high-priority research areas were identified:

- Develop functional biomarkers of elevated folate status. The lack of valid biomarkers that establish a dose-dependent relation between elevated folate status and biochemical, molecular, and/or physiologic endpoints that are on a causal pathway leading to adverse health outcomes is greatly impeding research progress in this field. Although biomarkers of elevated folate status and function are needed, an ideal biomarker would be one that not only quantitatively reflects exposure to high folic acid or total folate, but also functionally links to—and thereby predicts—a downstream outcome of interest. Once these biomarkers and endpoints are in hand, a consensus on the definitions of what constitutes "high" or "excess" folate status can be established, including the appropriate cutoffs for biomarkers. The biomarker cutoffs used to define deficiency have been validated, but the biomarkers and related cutoffs to define high intake or status are lacking (11, 125). Reference ranges and cutoffs for biomarkers of excess folic acid intake and elevated folate status (assuming ≥1 are identified and informative) should be derived and validated for at-risk populations and across age groups and life stages. Consistent data-driven approaches are needed to characterize high folate status and associated cutoffs, including consensus on experimental methods, measures, modifiers, and identification of relevant population subgroups (126). For example, increased BMI has been associated with altered circulating folate concentrations (127); biomarkers and related cutoffs should therefore be validated across BMI categories. Defining and validating biomarkers of adverse effects related to excess folate is essential and should include studies that establish whether the biomarker in question *1*) precedes and predicts the clinical endpoint of interest and *2*) is an integral component of the causal pathway leading to the clinical endpoint of interest. Some potential biomarkers of contemporary interest include genomic markers related to mutation rates or epigenetic signatures and/or downstream proteome and/or metabolome profiles (80, 128), although it is important to remain receptive to the idea of developing novel biomarkers. Mendelian randomization, an epidemiological methodology that uses genetic variants to strengthen causal inference (129), should be used where possible. Investigators need to be fully circumspect about the strengths and limitations of the biomarkers that they utilize in their studies (130–133); no intermediary biomarker of disease perfectly predicts the occurrence of an adverse health outcome and intermediary biomarkers often will lack sufficient specificity to apply to other adverse health outcomes.

Once validated status and functional biomarkers of excess folic acid intake, high folate status, and excess UMFA are established, effect modifiers—including genetic variants [e.g., methylenetetrahydrofolate reductase (MTHFR) variants and the DHFR 19-base-pair-deletion polymorphism (134)], as well as relevant exogenous factors, such as dietary exposures to the other 1-carbon nutrients—need to be identified and characterized. Furthermore, the roles of the intestinal microbiome will be an important factor to examine because it may interact with changes in folate exposure in a number of ways. For example, an observational study reported that the intestinal enterotype is linked to the quantity of ingested folate and other 1-carbon nutrients (135). Alternatively, different microbial profiles in the gut may metabolize ingested folates in distinctive ways, thereby

modifying the quantity or quality of folates available in the lumen of the intestine.

- Conduct dose-response studies with metabolic tracers. Dose–response relations among folate intake, folate status, and metabolic outcomes can be determined from tracer studies. Stable isotope tracer studies in both animal models and humans have defined the qualitative and quantitative flux of compounds through 1-carbon metabolism and in other folate-dependent pathways (136). Although their application is somewhat more restricted, studies utilizing radiolabeled tracers also have been very informative in both animals and humans (137). Such studies are needed to determine the dose–response relation between folate intake from deficient to elevated levels and metabolic fluxes, including variability across individuals, and any impact on physiological and health outcomes.
- Strengthen the study design of preclinical and observational studies. Although randomized and controlled human intervention trials are the design of choice for testing causal relations between excess folate exposure and adverse health outcomes, preclinical models (i.e., cell culture and other in vitro constructs, and nonhuman animal studies) and observational studies are more common and can make important contributions to the totality of evidence. Some of the benefits offered by preclinical studies include *1*) delineating tissue-specific effects, *2*) clarifying dose–response relations, and *3*) generating and confirming mechanistic hypotheses.

Although human observational studies lack the ability to prove causality, they can identify associations and generate hypotheses, among other insights. For example, in large-scale prospective cohorts, individuals can be stratified by genetic risk and followed over time to identify factors that associate with outcomes and evaluate gene–environment interactions. Owing to the fact that people who choose to consume supplements differ from those who do not in several important demographic and health characteristics, it is critical to look for uncontrolled confounding, reverse causality, and artifacts as common issues. However, Mendelian randomization can bolster the ability of observational studies to infer causation. Because of various logistical constraints and the extraordinary costs of intervention trials, observational studies often allow monitoring responses over longer periods of time, with larger numbers of subjects. Moreover, although adverse outcomes are the primary focus of this research agenda, ethical constraints substantially limit the feasibility of many of the intervention trials that would help resolve the issues of high folate intake, alone or in combination with low vitamin B-12 status, and cancer risk, transgenerational effects, or other health outcomes.

- Make better use of biorepositories. Efforts should be made to mine biorepositories similar to the Finnish Maternal Serum Bank, which has archived biological samples with linkage to medical registries for outcomes and allows access to extramural researchers. A collaborative consortium of birth cohorts has been formed to evaluate rare outcomes, particularly childhood cancers (138). These data and biological samples may also be used for the evaluation of other outcomes. Comparisons between countries with, and those without, mandatory folic acid fortification may offer important insights although they will require rigorous control of confounders. Consistent findings across international studies or longitudinal studies conducted in different decades within the same country are less prone to the residual confounding issues likely to affect current longitudinal studies conducted in 1 country. For example, the NIH has consolidated selected birth cohorts as part of its Environmental influences on Child Health Outcomes (commonly known as ECHO) program and will be launching new cohorts in the next few years (139). The data are being harmonized, and serum/plasma samples may be available, as well as information from follow-up studies. The Norwegian Birth Cohort (140) and the Danish Birth Cohort (141) are especially valuable in supporting collection and storage of biological samples and dietary supplement data, offer medical registries with outcome information, and welcome collaborations.
- Enhance resource and data sharing. A systematic cross-cohort approach, in which existing cohorts are combined, would facilitate the testing of high folate exposures among individuals, as well as potential dose–response relations and intergenerational effects. One example of this approach is the transnational "EpiBrain" project that is examining gradients of folate/folic acid exposure during pregnancy among 3 countries with differing fortification and supplementation policies, as well as effects on cognitive outcomes in children (142).
- Differentiate direct effects of UMFA from those of high folate status. UMFA cannot be considered in isolation because folic acid intake is intrinsically linked to higher folate status; hence, both should be considered in the design of all studies of UMFA. Furthermore, studies of UMFA should consider all other folate forms, including reduced folates and other oxidized forms of folate, such as MeFox (an oxidation product of 5-MTHF that does not possess the traditional biologic activity of the vitamin) (143). This includes identification of the differences between folic acid and reduced folates in whole-body and tissue-specific accumulation, metabolism, presence in biological fluids (urine, breast milk, blood), and biological effects, including effects on the microbiome, epigenome, metabolome, signaling pathways, genome stability, and mutation rates in various physiological states—including development, growth, lactation, weaning, and aging—as well as other potential critical windows of development and sex-specific effects.
- Develop appropriate animal and other preclinical dietary exposure models. For each health outcome, strong consideration should be given to conducting preclinical animal and cell culture studies, using dietary exposures consistent with human exposures. Traditional cell culture media often contain supraphysiologic concentrations of 1-carbon nutrients, necessitating use of custom formulas. Critical factors include consideration of the specific timing, duration, and frequency of the exposure to elevated folate and/or folic acid. Critical windows of time in the gestational period, as well as other phases of the life span during which the organism may be especially susceptible to adverse health outcomes resulting from excess folate exposure, need to be considered (111, 144).

- Use of appropriate animal models. Development of animal models that better capture the diet–disease relation and that are maximally relevant to human disease is a high-priority need. For example, hepatic DHFR activity in the human is only 2% of that possessed by the rat (51), thereby prompting the question of under what circumstances the effects of folic acid on disease outcomes in rodent models are translatable to humans (145). Thus, it is important to develop and validate appropriate animal models that optimally reflect human 1-carbon metabolism. Confirming observations in multiple strains or species helps to ensure that the results can be generalized across the mammalian spectrum and therefore are more likely relevant to human health.
- Consider off-target effects. It is unknown whether the underlying mechanisms whereby folate status and/or UMFA purportedly produce adverse health outcomes operate through the well-described avenues of canonical folate metabolism or via unknown off-target effects. These metabolic effects may be immediate consequences of excess folate intake, such as the stages of intestinal uptake of folate, its initial distribution to tissues, or its excretion. Alternatively, less immediate effects might be exerted through its roles in 1-carbon metabolism, or in other as-yet-unidentified biochemical pathways, via changes in the transcriptome (146) or epigenome (113, 147), or its role as a signaling molecule (84, 148). Cellular folate is partitioned in cytosolic, mitochondrial, and nuclear compartments and, therefore, adverse effects of excess folate may be a function of aberrant compartmentalization (149). Moreover, both the quantitative amount of folate and the distribution of its vitameric forms are highly tissue-specific (150), underscoring the necessity of demonstrating effects on the target tissue of interest and not inferring from effects observed in other tissues. Further complicating the issue of tissue specificity is that it can be influenced by the underlying genotype (or strain in animal models) (151).
- Precise classification of vitamin B-12 status. It is important to gain consensus on how to accurately classify vitamin B-12 status (152) because the current measures lack specificity. The biomarker methylmalonic acid is very sensitive to vitamin B-12 status and responds to vitamin B-12 supplementation, but its elevation does not always indicate clinical signs of vitamin B-12 deficiency. Caution is needed when multiple variables are combined into a computed variable (153), because vital information may be obscured. Newer biomarkers, such as 2-methyl citric acid and holo-transcobalamin, have not yet offered much in the way of additional advantages. Functional and clinical biomarkers should be developed that accurately predict the effects of variable folate status on vitamin B-12 deficiency. It will be critical to delineate the effects of a range of folate status on vitamin B-12 metabolism in the whole body and in other tissues (with particular focus on the central nervous system) in relevant preclinical and animal models. The impact of genetic variations, including the MTHFR variants and the DHFR 19-base-pair-deletion polymorphism, also should be considered (134).
- Seek strong experimental evidence through the systematic compilation of observations. Scientific knowledge generally moves ahead by consensus, so rigorous systematic reviews are needed to evaluate the totality and strength of evidence regarding the health effects of elevated folate status and to guide future studies, keeping in mind that such analyses can sometimes obscure effects that occur only in at-risk subgroups (154). Presently, few updated systematic reviews specific to this research agenda are available to inform such efforts.

At-risk groups for elevated folate status

At least 3 large segments of the US population have been proposed to be at elevated risk of any potential adverse effects of elevated folate status. Pregnant women and their offspring are exposed to higher amounts of folic acid as a result of supplement use during pregnancy and lactation. Currently 33% of pregnant US women exceed the UL for folic acid; among supplement users that number increases to 47% (155). Notably, all US women of reproductive age capable of getting pregnant are recommended to supplement with 400–800 μg folic acid/d (156), but most prenatal supplements contain 800–1000 μg. However, without the use of dietary supplements, almost 40% of US women of reproductive age do not meet their requirement for folate intake (155). Thus, pregnant women are at risk of both inadequacy and potential excess. Virtually nothing is known about excess folic acid exposures during lactation, despite many lactating women exceeding the UL for folic acid from dietary supplements alone (63). It should be noted, however, that 100% of the folate in most commercial infant formulas is in the form of folic acid and these are generally higher in folic acid content than human breast milk.

Another group vulnerable to the postulated adverse effects of excess folate is children. Although no US adults exceed the folic acid UL from foods alone (2), some children do, and among those who regularly use vitamin supplements more than half exceed their UL (98). At the time the UL values were devised, insufficient data existed to confidently establish a UL based on pediatric observations, so the values for infants and children were extrapolated from adult levels. It could be argued that the UL for folic acid in children is too low.

Finally, largely as a result of various types of vitamin B-12 malabsorption, adults older than the age of 60 y are at increased risk of vitamin B-12 deficiency and are concurrently more likely to have high folate status largely because of the prevalent use of supplements. Estimates vary according to the criteria used, but in the United States it is generally thought that 10%–15% of elderly have subclinical vitamin B-12 deficiency (i.e., biomarkers indicative of deficiency in the absence of overt clinical manifestations) (152). Susceptibility of the elderly to vitamin B-12 deficiency is compounded by the prevalent use of gastric acid suppressant medications in this segment of the population; a nationally representative survey indicated that 33% of all ambulatory medical visits in 2008–2009 among those aged 65–79 y involved individuals taking a proton pump inhibitor (157, 158).

Summary and Conclusions

At present, there is an insufficient body of evidence to support human adverse health outcomes that are a result of high amounts of folate or folic acid intake. However, owing to a provocative

body of observations and the potential public health ramifications of these observations, a comprehensive and rigorous body of future investigations is warranted to determine if there is a causal relation. Credible evidence of causality, delineation of the underlying mechanisms, and dose–response relations are each important pieces of the puzzle that figure prominently in establishing the totality of evidence required to determine the safety of excess folic acid intake and elevated folate status.

There is a pressing need for valid status and functional biomarkers or sets of biomarkers that *1*) reflect high folic intake and/or elevated folate status, *2*) act through a mechanistic pathway, and *3*) predict ≥1 of the adverse health outcomes in question. Moreover, better consensus is needed regarding the most accurate means of assessing vitamin B-12 status. The absence of such tools constitutes a major impediment to progress. Although randomized controlled trials provide the most definitive evidence for assessing causality, the workshop participants acknowledged that the limited availability, high costs, and in some instances the ethical barriers of controlled trials underscore the value of preclinical and observational studies to inform this research agenda. Workshop members also emphasized the value of mining new insights from existing human databases and utilizing data from multiple populations across the life span that are subject to different dietary and fortification policies and that have different genetic backgrounds and gene variant enrichments. There is the potential for meaningful public health ramifications if the evidence for purported adverse effects of elevated folate status and/or UMFA is shown to be causal. In this era of widespread use of dietary supplements, and discretionary fortification by the food industry (and to a far lesser degree mandatory federal fortification), some segments of the population are exceeding recommended guidelines on the upper level of folic acid intake. For this reason, it is critical for the scientific community to remain vigilant in its research pursuits and address directly the evidence and knowledge gaps related to the health effects of excess folate and/or folic acid intake.

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References

1. Institute of Medicine (US) Food and Nutrition Board. Dietary Reference Intakes: a risk assessment model for establishing upper intake levels for nutrients. Washington (DC): National Academies Press (US); 1998.
2. Bailey RL, Dodd KW, Gahche JJ, Dwyer JT, McDowell MA, Yetley EA, Sempos CA, Burt VL, Radimer KL, Picciano MF. Total folate and folic acid intake from foods and dietary supplements in the United States: 2003–2006. Am J Clin Nutr 2010;91(1):231–7.
3. Bailey RL, McDowell MA, Dodd KW, Gahche JJ, Dwyer JT, Picciano MF. Total folate and folic acid intakes from foods and dietary supplements of US children aged 1–13 y. Am J Clin Nutr 2010;92(2):353–8.
4. Yeung LF, Cogswell ME, Carriquiry AL, Bailey LB, Pfeiffer CM, Berry RJ. Contributions of enriched cereal-grain products, ready-to-eat cereals, and supplements to folic acid and vitamin B-12 usual intake and folate and vitamin B-12 status in US children: National Health and Nutrition Examination Survey (NHANES), 2003–2006. Am J Clin Nutr 2011;93(1):172–85.
5. Tinker SC, Cogswell ME, Hamner HC, Berry RJ. Usual folic acid intakes: a modelling exercise assessing changes in the amount of folic acid in foods and supplements, National Health and Nutrition Examination Survey, 2003–2008. Public Health Nutr 2012;15(7):1216–27.
6. Yeung L, Yang Q, Berry RJ. Contributions of total daily intake of folic acid to serum folate concentrations. JAMA 2008;300(21):2486–7.
7. CDC. Recommendations for the use of folic acid to reduce the number of cases of spina bifida and other neural tube defects. MMWR Recomm Rep 1992;41(RR-14):1–7.
8. US Preventive Services Task Force; Bibbins-Domingo K, Grossman DC, Curry SJ, Davidson KW, Epling JW Jr, Garcia FA, Kemper AR, Krist AH, Kurth AE, et al. Folic acid supplementation for the prevention of neural tube defects: US Preventive Services Task Force Recommendation Statement. JAMA 2017;317(2):183–9.
9. De Wals P, Tairou F, Van Allen MI, Uh SH, Lowry RB, Sibbald B, Evans JA, Van den Hof MC, Zimmer P, Crowley M, et al. Reduction in neural-tube defects after folic acid fortification in Canada. N Engl J Med 2007;357(2):135–42.
10. Wald NJ, Morris JK, Blakemore C. Public health failure in the prevention of neural tube defects: time to abandon the tolerable upper intake level of folate. Public Health Rev 2018;39:2.
11. Pfeiffer CM, Sternberg MR, Fazili Z, Yetley EA, Lacher DA, Bailey RL, Johnson CL. Unmetabolized folic acid is detected in nearly all serum samples from US children, adolescents, and adults. J Nutr 2015;145(3):520–31.
12. Kelly P, McPartlin J, Scott J. A combined high-performance liquid chromatographic–microbiological assay for serum folic acid. Anal Biochem 1996;238(2):179–83.
13. Sweeney MR, McPartlin J, Scott J. Folic acid fortification and public health: report on threshold doses above which unmetabolised folic acid appear in serum. BMC Public Health 2007;7:41.
14. Sweeney MR, McPartlin J, Weir DG, Daly L, Scott JM. Postprandial serum folic acid response to multiple doses of folic acid in fortified bread. Br J Nutr 2006;95(1):145–51.
15. Sweeney MR, McPartlin J, Weir DG, Scott JM. Measurements of sub-nanomolar concentrations of unmetabolised folic acid in serum. J Chromatogr B Analyt Technol Biomed Life Sci 2003;788(1):187–91.
16. National Toxicology Program. NTP Monograph: identifying research needs for assessing safe use of high intakes of folic acid. Research Triangle Park, NC: National Toxicology Program; 2015.
17. Brito A, Verdugo R, Hertrampf E, Miller JW, Green R, Fedosov SN, Shahab-Ferdows S, Sanchez H, Albala C, Castillo JL, et al. Vitamin B-12 treatment of asymptomatic, deficient, elderly Chileans improves conductivity in myelinated peripheral nerves, but high serum folate impairs vitamin B-12 status response assessed by the combined indicator of vitamin B-12 status. Am J Clin Nutr 2016;103(1):250–7.
18. Castillo-Lancellotti C, Margozzini P, Valdivia G, Padilla O, Uauy R, Rozowski J, Tur JA. Serum folate, vitamin B_{12} and cognitive impairment in Chilean older adults. Public Health Nutr 2015;18(14):2600–8.
19. Chanarin I, Deacon R, Lumb M, Muir M, Perry J. Cobalamin-folate interrelations: a critical review. Blood 1985;66(3):479–89.
20. Colapinto CK, O'Connor DL, Sampson M, Williams B, Tremblay MS. Systematic review of adverse health outcomes associated with high serum or red blood cell folate concentrations. J Public Health (Oxf) 2016;38(2):e84–97.

21. Cole BF, Baron JA, Sandler RS, Haile RW, Ahnen DJ, Bresalier RS, McKeown-Eyssen G, Summers RW, Rothstein RI, Burke CA, et al. Folic acid for the prevention of colorectal adenomas: a randomized clinical trial. JAMA 2007;297(21):2351–9.
22. Deng Y, Wang D, Wang K, Kwok T. High serum folate is associated with brain atrophy in older diabetic people with vitamin B12 deficiency. J Nutr Health Aging 2017;21(9):1065–71.
23. Ebbing M, Bønaa KH, Nygård O, Arnesen E, Ueland PM, Nordrehaug JE, Rasmussen K, Njølstad I, Refsum H, Nilsen DW, et al. Cancer incidence and mortality after treatment with folic acid and vitamin B_{12}. JAMA 2009;302(19):2119–26.
24. Figueiredo JC, Grau MV, Haile RW, Sandler RS, Summers RW, Bresalier RS, Burke CA, McKeown-Eyssen GE, Baron JA. Folic acid and risk of prostate cancer: results from a randomized clinical trial. J Natl Cancer Inst 2009;101(6):432–5.
25. Huang Y, He Y, Sun X, He Y, Li Y, Sun C. Maternal high folic acid supplement promotes glucose intolerance and insulin resistance in male mouse offspring fed a high-fat diet. Int J Mol Sci 2014;15(4):6298–313.
26. Keating E, Correia-Branco A, Araújo JR, Meireles M, Fernandes R, Guardão L, Guimarães JT, Martel F, Calhau C. Excess perigestational folic acid exposure induces metabolic dysfunction in post-natal life. J Endocrinol 2015;224(3):245–59.
27. Krishnaveni GV, Veena SR, Karat SC, Yajnik CS, Fall CH. Association between maternal folate concentrations during pregnancy and insulin resistance in Indian children. Diabetologia 2014;57(1):110–21.
28. Li Z, Gueant-Rodriguez RM, Quilliot D, Sirveaux MA, Meyre D, Gueant JL, Brunaud L. Folate and vitamin B12 status is associated with insulin resistance and metabolic syndrome in morbid obesity. Clin Nutr 2018;37(5):1700–6.
29. McNulty H, Ward M, Hoey L, Hughes CF, Pentieva K. Addressing optimal folate and related B-vitamin status through the lifecycle: health impacts and challenges. Proc Nutr Soc 2019;78(3):449–62.
30. Morakinyo AO, Samuel TA, Awobajo FO, Oludare GO, Mofolorunso A. High-dose perinatal folic-acid supplementation alters insulin sensitivity in Sprague-Dawley rats and diminishes the expression of adiponectin. J Diet Suppl 2019;16(1):14–26.
31. Paul L, Selhub J. Interaction between excess folate and low vitamin B12 status. Mol Aspects Med 2017;53:43–7.
32. Selhub J, Rosenberg IH. Excessive folic acid intake and relation to adverse health outcome. Biochimie 2016;126:71–8.
33. Yajnik CS, Deshpande SS, Jackson AA, Refsum H, Rao S, Fisher DJ, Bhat DS, Naik SS, Coyaji KJ, Joglekar CV, et al. Vitamin B_{12} and folate concentrations during pregnancy and insulin resistance in the offspring: the Pune Maternal Nutrition Study. Diabetologia 2008;51(1):29–38.
34. Miller JW, Garrod MG, Allen LH, Haan MN, Green R. Metabolic evidence of vitamin B-12 deficiency, including high homocysteine and methylmalonic acid and low holotranscobalamin, is more pronounced in older adults with elevated plasma folate. Am J Clin Nutr 2009;90(6):1586–92.
35. Morris MS, Jacques PF, Rosenberg IH, Selhub J. Folate and vitamin B-12 status in relation to anemia, macrocytosis, and cognitive impairment in older Americans in the age of folic acid fortification. Am J Clin Nutr 2007;85(1):193–200.
36. Morris MS, Jacques PF, Rosenberg IH, Selhub J. Circulating unmetabolized folic acid and 5-methyltetrahydrofolate in relation to anemia, macrocytosis, and cognitive test performance in American seniors. Am J Clin Nutr 2010;91(6):1733–44.
37. Selhub J, Morris MS, Jacques PF. In vitamin B_{12} deficiency, higher serum folate is associated with increased total homocysteine and methylmalonic acid concentrations. Proc Natl Acad Sci U S A 2007;104(50):19995–20000.
38. Aleliunas RE, Aljaadi AM, Laher I, Glier MB, Green TJ, Murphy M, Miller JW, Devlin AM. Folic acid supplementation of female mice, with or without vitamin B-12, before and during pregnancy and lactation programs adiposity and vascular health in adult male offspring. J Nutr 2016;146(4):688–96.
39. Clarke R, Sherliker P, Hin H, Molloy AM, Nexo E, Ueland PM, Emmens K, Scott JM, Evans JG. Folate and vitamin B_{12} status in relation to cognitive impairment and anaemia in the setting of voluntary fortification in the UK. Br J Nutr 2008;100(5):1054–9.
40. Stevens VL, McCullough ML, Sun J, Jacobs EJ, Campbell PT, Gapstur SM. High levels of folate from supplements and fortification are not associated with increased risk of colorectal cancer. Gastroenterology 2011;141(1):98–105.e1.
41. Stewart CP, Christian P, Schulze KJ, Arguello M, LeClerq SC, Khatry SK, West KP Jr. Low maternal vitamin B-12 status is associated with offspring insulin resistance regardless of antenatal micronutrient supplementation in rural Nepal. J Nutr 2011;141(10):1912–17.
42. Vollset SE, Clarke R, Lewington S, Ebbing M, Halsey J, Lonn E, Armitage J, Manson JE, Hankey GJ, Spence JD, et al. Effects of folic acid supplementation on overall and site-specific cancer incidence during the randomised trials: meta-analyses of data on 50,000 individuals. Lancet 2013;381(9871):1029–36.
43. Kumanyika S, Oria MP, editors. Guiding principles for developing Dietary Reference Intakes based on chronic disease: consensus study report. Washington (DC): National Academy of Sciences; 2017.
44. Yetley EA, MacFarlane AJ, Greene-Finestone LS, Garza C, Ard JD, Atkinson SA, Bier DM, Carriquiry AL, Harlan WR, Hattis D, et al. Options for basing Dietary Reference Intakes (DRIs) on chronic disease endpoints: report from a joint US-/Canadian-sponsored working group. Am J Clin Nutr 2017;105(1):249S–85S.
45. Guyatt GH, Oxman AD, Schünemann HJ, Tugwell P, Knottnerus A. GRADE guidelines: a new series of articles in the *Journal of Clinical Epidemiology*. J Clin Epidemiol 2011;64(4):380–2.
46. Guyatt GH, Oxman AD, Vist GE, Kunz R, Falck-Ytter Y, Alonso-Coello P, Schünemann HJ, GRADE Working Group. GRADE: an emerging consensus on rating quality of evidence and strength of recommendations. BMJ 2008;336(7650):924–6.
47. Johnston BC, Alonso-Coello P, Bala MM, Zeraatkar D, Rabassa M, Valli C, Marshall C, El Dib R, Vernooij RWM, Vandvik PO, et al. Methods for trustworthy nutritional recommendations NutriRECS (Nutritional Recommendations and accessible Evidence summaries Composed of Systematic reviews): a protocol. BMC Med Res Methodol 2018;18(1):162.
48. Zeraatkar D, Johnston BC, Guyatt G. Evidence collection and evaluation for the development of dietary guidelines and public policy on nutrition. Annu Rev Nutr 2019;39:227–47.
49. Oria M, Harrison M, Stallings VA, editors. Dietary Reference Intakes for sodium and potassium: consensus study report. Washington (DC): National Academies of Sciences, Engineering, and Medicine; 2019.
50. Bailey LB, Stover PJ, McNulty H, Fenech MF, Gregory JF 3rd, Mills JL, Pfeiffer CM, Fazili Z, Zhang M, Ueland PM, et al. Biomarkers of Nutrition for Development—folate review. J Nutr 2015;145(7):1636S–80S.
51. Bailey SW, Ayling JE. The extremely slow and variable activity of dihydrofolate reductase in human liver and its implications for high folic acid intake. Proc Natl Acad Sci U S A 2009;106(36):15424–9.
52. Zhao R, Diop-Bove N, Visentin M, Goldman ID. Mechanisms of membrane transport of folates into cells and across epithelia. Annu Rev Nutr 2011;31:177–201.
53. Niesser M, Demmelmair H, Weith T, Moretti D, Rauh-Pfeiffer A, van Lipzig M, Vaes W, Koletzko B, Peissner W. Folate catabolites in spot urine as non-invasive biomarkers of folate status during habitual intake and folic acid supplementation. PLoS One 2013;8(2):e56194.
54. Wolfe JM, Bailey LB, Herrlinger-Garcia K, Theriaque DW, Gregory JF 3rd, Kauwell GP. Folate catabolite excretion is responsive to changes in dietary folate intake in elderly women. Am J Clin Nutr 2003;77(4):919–23.
55. Obeid R. Serum unmetabolized folic acid: the straw that broke dihydrofolate reductase's back? J Nutr 2015;145(3):387–90.
56. Wright AJ, Dainty JR, Finglas PM. Folic acid metabolism in human subjects revisited: potential implications for proposed mandatory folic acid fortification in the UK. Br J Nutr 2007;98(4):667–75.
57. Wright AJ, Finglas PM, Dainty JR, Wolfe CA, Hart DJ, Wright DM, Gregory JF. Differential kinetic behavior and distribution for pteroylglutamic acid and reduced folates: a revised hypothesis of the primary site of PteGlu metabolism in humans. J Nutr 2005;135(3):619–23.
58. Clifford AJ, Arjomand A, Dueker SR, Schneider PD, Buchholz BA, Vogel JS. The dynamics of folic acid metabolism in an adult given a small tracer dose of ^{14}C-folic acid. Adv Exp Med Biol 1998;445:239–51.
59. Bailey RL, Mills JL, Yetley EA, Gahche JJ, Pfeiffer CM, Dwyer JT, Dodd KW, Sempos CT, Betz JM, Picciano MF. Unmetabolized serum

folic acid and its relation to folic acid intake from diet and supplements in a nationally representative sample of adults aged ≥60 y in the United States. Am J Clin Nutr 2010;92(2):383–9.
60. Pentieva K, Selhub J, Paul L, Molloy AM, McNulty B, Ward M, Marshall B, Dornan J, Reilly R, Parle-McDermott A, et al. Evidence from a randomized trial that exposure to supplemental folic acid at recommended levels during pregnancy does not lead to increased unmetabolized folic acid concentrations in maternal or cord blood. J Nutr 2016;146(3):494–500.
61. Bailey RL, Fulgoni VL, Taylor CL, Pfeiffer CM, Thuppal SV, McCabe GP, Yetley EA. Correspondence of folate dietary intake and biomarker data. Am J Clin Nutr 2017;105(6):1336–43.
62. Boilson A, Staines A, Kelleher CC, Daly L, Shirley I, Shrivastava A, Bailey SW, Alverson PB, Ayling JE, McDermott AP, et al. Unmetabolized folic acid prevalence is widespread in the older Irish population despite the lack of a mandatory fortification program. Am J Clin Nutr 2012;96(3):613–21.
63. Jun S, Gahche JJ, Potischman N, Dwyer JT, Guenther PM, Sauder KA, Bailey RL. Dietary supplement use and its micronutrient contribution during pregnancy and lactation in the United States. Obstet Gynecol 2020;135(3):623–33.
64. Page R, Robichaud A, Arbuckle TE, Fraser WD, MacFarlane AJ. Total folate and unmetabolized folic acid in the breast milk of a cross-section of Canadian women. Am J Clin Nutr 2017;105(5):1101–9.
65. Avagliano L, Massa V, George TM, Qureshy S, Bulfamante GP, Finnell RH. Overview on neural tube defects: from development to physical characteristics. Birth Defects Res 2019;111(19):1455–67.
66. Wang S, Low PS. Folate-mediated targeting of antineoplastic drugs, imaging agents, and nucleic acids to cancer cells. J Control Release 1998;53(1–3):39–48.
67. Birn H, Selhub J, Christensen EI. Internalization and intracellular transport of folate-binding protein in rat kidney proximal tubule. Am J Physiol 1993;264(2 Pt 1):C302–10.
68. Birn H, Zhai X, Holm J, Hansen SI, Jacobsen C, Christensen EI, Moestrup SK. Megalin binds and mediates cellular internalization of folate binding protein. FEBS J 2005;272(17):4423–30.
69. Grapp M, Wrede A, Schweizer M, Huwel S, Galla HJ, Snaidero N, Simons M, Buckers J, Low PS, Urlaub H, et al. Choroid plexus transcytosis and exosome shuttling deliver folate into brain parenchyma. Nat Commun 2013;4:2123.
70. Fernandez M, Javaid F, Chudasama V. Advances in targeting the folate receptor in the treatment/imaging of cancers. Chem Sci 2018;9(4):790–810.
71. Desai A, Sequeira JM, Quadros EV. The metabolic basis for developmental disorders due to defective folate transport. Biochimie 2016;126:31–42.
72. Rothenberg SP, da Costa MP, Sequeira JM, Cracco J, Roberts JL, Weedon J, Quadros EV. Autoantibodies against folate receptors in women with a pregnancy complicated by a neural-tube defect. N Engl J Med 2004;350(2):134–42.
73. US FDA. Food standards: amendment of standards of identity for enriched grain products to require addition of folic acid. Fed Regist 1996;61(44):8781–97.
74. Rader JI, Yetley EA. Nationwide folate fortification has complex ramifications and requires careful monitoring over time. Arch Intern Med 2002;162(5):608–9.
75. Yetley EA, Rader JI. Modeling the level of fortification and post-fortification assessments: U.S. experience. Nutr Rev 2004;62(6 Pt 2):S50–9; discussion S60–1.
76. Moran RG. Roles of folylpoly-gamma-glutamate synthetase in therapeutics with tetrahydrofolate antimetabolites: an overview. Semin Oncol 1999;26(2 Suppl 6):24–32.
77. Frigerio B, Bizzoni C, Jansen G, Leamon CP, Peters GJ, Low PS, Matherly LH, Figini M. Folate receptors and transporters: biological role and diagnostic/therapeutic targets in cancer and other diseases. J Exp Clin Cancer Res 2019;38(1):125.
78. Mohanty V, Siddiqui MR, Tomita T, Mayanil CS. Folate receptor alpha is more than just a folate transporter. Neurogenesis (Austin) 2017;4(1):e1263717.
79. Hansen MF, Greibe E, Skovbjerg S, Rohde S, Kristensen AC, Jensen TR, Stentoft C, Kjær KH, Kronborg CS, Martensen PM. Folic acid mediates activation of the pro-oncogene STAT3 via the Folate Receptor alpha. Cell Signal 2015;27(7):1356–68.
80. Kuo C-T, Chang C, Lee W-S. Folic acid inhibits COLO-205 colon cancer cell proliferation through activating the FRα/c-SRC/ERK1/2/NFκB/TP53 pathway: *in vitro* and *in vivo* studies. Sci Rep 2015;5:11187.
81. Zhang X-M, Huang G-W, Tian Z-H, Ren D-L, Wilson JX. Folate stimulates ERK1/2 phosphorylation and cell proliferation in fetal neural stem cells. Nutr Neurosci 2009;12(5):226–32.
82. Boshnjaku V, Shim KW, Tsurubuchi T, Ichi S, Szany EV, Xi G, Mania-Farnell B, McLone DG, Tomita T, Mayanil CS. Nuclear localization of folate receptor alpha: a new role as a transcription factor. Sci Rep 2012;2:980.
83. Mohanty V, Shah A, Allender E, Siddiqui MR, Monick S, Ichi S, Mania-Farnell B, McLone DG, Tomita T, Mayanil CS. Folate receptor alpha upregulates *Oct4*, *Sox2* and *Klf4* and downregulates miR-138 and miR-let-7 in cranial neural crest cells. Stem Cells 2016;34(11):2721–32.
84. Chaudhari SN, Mukherjee M, Vagasi AS, Bi G, Rahman MM, Nguyen CQ, Paul L, Selhub J, Kipreos ET. Bacterial folates provide an exogenous signal for *C. elegans* germline stem cell proliferation. Dev Cell 2016;38(1):33–46.
85. Qiu W, Gobinath AR, Wen Y, Austin J, Galea LAM. Folic acid, but not folate, regulates different stages of neurogenesis in the ventral hippocampus of adult female rats. J Neuroendocrinol 2019;31(10):e12787.
86. Taylor SY, Dixon HM, Yoganayagam S, Price N, Lang D. Folic acid modulates eNOS activity via effects on posttranslational modifications and protein–protein interactions. Eur J Pharmacol 2013;714(1–3):193–201.
87. Chen J, Xu X, Liu A, Ulrich C. Folate and cancer: epidemiological perspective. In: Bailey LB, editor. Folate in health and disease. Boca Raton, FL: Taylor & Francis LLC; 2009. p. 205–34.
88. Courtemanche C, Huang AC, Elson-Schwab I, Kerry N, Ng BY, Ames BN. Folate deficiency and ionizing radiation cause DNA breaks in primary human lymphocytes: a comparison. FASEB J 2004;18(1):209–11.
89. O'Reilly SL, McGlynn AP, McNulty H, Reynolds J, Wasson GR, Molloy AM, Strain JJ, Weir DG, Ward M, McKerr G, et al. Folic acid supplementation in postpolypectomy patients in a randomized controlled trial increases tissue folate concentrations and reduces aberrant DNA biomarkers in colonic tissues adjacent to the former polyp site. J Nutr 2016;146(5):933–9.
90. Protiva P, Mason JB, Liu Z, Hopkins ME, Nelson C, Marshall JR, Lambrecht RW, Pendyala S, Kopelovich L, Kim M, et al. Altered folate availability modifies the molecular environment of the human colorectum: implications for colorectal carcinogenesis. Cancer Prev Res (Phila) 2011;4(4):530–43.
91. Song J, Sohn KJ, Medline A, Ash C, Gallinger S, Kim YI. Chemopreventive effects of dietary folate on intestinal polyps in Apc+/−Msh2−/− mice. Cancer Res 2000;60(12):3191–9.
92. Wargovich MJ, Chen CD, Jimenez A, Steele VE, Velasco M, Stephens LC, Price R, Gray K, Kelloff GJ. Aberrant crypts as a biomarker for colon cancer: evaluation of potential chemopreventive agents in the rat. Cancer Epidemiol Biomarkers Prev 1996;5(5):355–60.
93. Kim YI, Salomon RN, Graeme-Cook F, Choi SW, Smith DE, Dallal GE, Mason JB. Dietary folate protects against the development of macroscopic colonic neoplasia in a dose responsive manner in rats. Gut 1996;39(5):732–40.
94. Jaszewski R, Misra S, Tobi M, Ullah N, Naumoff JA, Kucuk O, Levi E, Axelrod BN, Patel BB, Majumdar AP. Folic acid supplementation inhibits recurrence of colorectal adenomas: a randomized chemoprevention trial. World J Gastroenterol 2008;14(28):4492–8.
95. Logan RF, Grainge MJ, Shepherd VC, Armitage NC, Muir KR, ukCAP Trial Group. Aspirin and folic acid for the prevention of recurrent colorectal adenomas. Gastroenterology 2008;134(1):29–38.
96. Paspatis GA, Karamanolis DG. Folate supplementation and adenomatous colonic polyps. Dis Colon Rectum 1994;37(12):1340–1.
97. Gibson TM, Weinstein SJ, Pfeiffer RM, Hollenbeck AR, Subar AF, Schatzkin A, Mayne ST, Stolzenberg-Solomon R. Pre- and postfortification intake of folate and risk of colorectal cancer in a large prospective cohort study in the United States. Am J Clin Nutr 2011;94(4):1053–62.

98. Bailey RL, Fulgoni VL 3rd, Keast DR, Lentino CV, Dwyer JT. Do dietary supplements improve micronutrient sufficiency in children and adolescents? J Pediatr 2012;161(5):837–42.
99. Masih SP, Plumptre L, Ly A, Berger H, Lausman AY, Croxford R, Kim YI, O'Connor DL. Pregnant Canadian women achieve recommended intakes of one-carbon nutrients through prenatal supplementation but the supplement composition, including choline, requires reconsideration. J Nutr 2015;145(8):1824–34.
100. Plumptre L, Tammen SA, Sohn K-J, Masih SP, Visentin CE, Aufreiter S, Malysheva O, Schroder TH, Ly A, Berger H, et al. Maternal and cord blood folate concentrations are inversely associated with fetal DNA hydroxymethylation, but not DNA methylation, in a cohort of pregnant Canadian women. J Nutr 2020;150(2):202–11.
101. Plumptre L, Masih SP, Ly A, Aufreiter S, Sohn KJ, Croxford R, Lausman AY, Berger H, O'Connor DL, Kim Y-I. High concentrations of folate and unmetabolized folic acid in a cohort of pregnant Canadian women and umbilical cord blood. Am J Clin Nutr 2015;102(4): 848–57.
102. Palmer AM, Kamynina E, Field MS, Stover PJ. Folate rescues vitamin B_{12} depletion-induced inhibition of nuclear thymidylate biosynthesis and genome instability. Proc Natl Acad Sci U S A 2017;114(20):E4095–E102.
103. Paniz C, Bertinato JF, Lucena MR, De Carli E, Amorim P, Gomes GW, Palchetti CZ, Figueiredo MS, Pfeiffer CM, Fazili Z, et al. A daily dose of 5 mg folic acid for 90 days is associated with increased serum unmetabolized folic acid and reduced natural killer cell cytotoxicity in healthy Brazilian adults. J Nutr 2017;147(9):1677–85.
104. Sawaengsri H, Wang J, Reginaldo C, Steluti J, Wu D, Meydani SN, Selhub J, Paul L. High folic acid intake reduces natural killer cell cytotoxicity in aged mice. J Nutr Biochem 2016;30:102–7.
105. Troen AM, Mitchell B, Sorensen B, Wener MH, Johnston A, Wood B, Selhub J, McTiernan A, Yasui Y, Oral E, et al. Unmetabolized folic acid in plasma is associated with reduced natural killer cell cytotoxicity among postmenopausal women. J Nutr 2006;136(1):189–94.
106. Kaittanis C, Andreou C, Hieronymus H, Mao N, Foss CA, Eiber M, Weirich G, Panchal P, Gopalan A, Zurita J, et al. Prostate-specific membrane antigen cleavage of vitamin B9 stimulates oncogenic signaling through metabotropic glutamate receptors. J Exp Med 2018;215(1):159–75.
107. Zgheib R, Battaglia-Hsu S-F, Hergalant S, Quéré M, Alberto JM, Chéry C, Rouyer P, Gauchotte G, Guéant J-L, Namour F. Folate can promote the methionine-dependent reprogramming of glioblastoma cells towards pluripotency. Cell Death Dis 2019;10(8):596.
108. Hirsch S, Miranda D, Muñoz E, Montoya M, Ronco AM, de la Maza MP, Bunout D. Natural killer cell cytotoxicity is not regulated by folic acid in vitro. Nutrition 2013;29(5):772–6.
109. Ravaglia G, Forti P, Maioli F, Bastagli L, Facchini A, Mariani E, Savarino L, Sassi S, Cucinotta D, Lenaz G. Effect of micronutrient status on natural killer cell immune function in healthy free-living subjects aged ≥90 y. Am J Clin Nutr 2000;71(2):590–8.
110. Kim YI, Hayek M, Mason JB, Meydani SN. Severe folate deficiency impairs natural killer cell–mediated cytotoxicity in rats. J Nutr 2002;132(6):1361–7.
111. Ly A, Ishiguro L, Kim D, Im D, Kim S-E, Sohn K-J, Croxford R, Kim Y-I. Maternal folic acid supplementation modulates DNA methylation and gene expression in the rat offspring in a gestation period-dependent and organ-specific manner. J Nutr Biochem 2016;33: 103–10.
112. Guéant J-L, Namour F, Guéant-Rodriguez R-M, Daval J-L. Folate and fetal programming: a play in epigenomics? Trends Endocrinol Metab 2013;24(6):279–89.
113. Joubert BR, den Dekker HT, Felix JF, Bohlin J, Ligthart S, Beckett E, Tiemeier H, van Meurs JB, Uitterlinden AG, Hofman A, et al. Maternal plasma folate impacts differential DNA methylation in an epigenome-wide meta-analysis of newborns. Nat Commun 2016;7:10577.
114. Caffrey A, Irwin RE, McNulty H, Strain JJ, Lees-Murdock DJ, McNulty BA, Ward M, Walsh CP, Pentieva K. Gene-specific DNA methylation in newborns in response to folic acid supplementation during the second and third trimesters of pregnancy: epigenetic analysis from a randomized controlled trial. Am J Clin Nutr 2018;107(4):566–75.
115. Irwin RE, Thursby SJ, Ondicova M, Pentieva K, McNulty H, Richmond RC, Caffrey A, Lees-Murdock DJ, McLaughlin M, Cassidy T, et al. A randomized controlled trial of folic acid intervention in pregnancy highlights a putative methylation-regulated control element at ZFP57. Clin Epigenetics 2019;11(1):31.
116. Richmond RC, Sharp GC, Herbert G, Atkinson C, Taylor C, Bhattacharya S, Campbell D, Hall M, Kazmi N, Gaunt T, et al. The long-term impact of folic acid in pregnancy on offspring DNA methylation: follow-up of the Aberdeen Folic Acid Supplementation Trial (AFAST). Int J Epidemiol 2018;47(3):928–37.
117. McNulty H, Rollins M, Cassidy T, Caffrey A, Marshall B, Dornan J, McLaughlin M, McNulty BA, Ward M, Strain JJ, et al. Effect of continued folic acid supplementation beyond the first trimester of pregnancy on cognitive performance in the child: a follow-up study from a randomized controlled trial (FASSTT Offspring Trial). BMC Med 2019;17(1):196.
118. Herbert V, Zalusky R. Interrelations of vitamin B_{12} and folic acid metabolism: folic acid clearance studies. J Clin Invest 1962;41: 1263–76.
119. Shane B, Stokstad EL. Vitamin B_{12}-folate interrelationships. Annu Rev Nutr 1985;5:115–41.
120. Berry RJ. Lack of historical evidence to support folic acid exacerbation of the neuropathy caused by vitamin B12 deficiency. Am J Clin Nutr 2019;110(3):554–61.
121. Field MS, Kamynina E, Watkins D, Rosenblatt DS, Stover PJ. Human mutations in methylenetetrahydrofolate dehydrogenase 1 impair nuclear de novo thymidylate biosynthesis. Proc Natl Acad Sci U S A 2015;112(2):400–5.
122. Agamanolis DP, Chester EM, Victor M, Kark JA, Hines JD, Harris JW. Neuropathology of experimental vitamin B^{12} deficiency in monkeys. Neurology 1976;26(10):905–14.
123. van der Westhuyzen J, Fernandes-Costa F, Metz J. Cobalamin inactivation by nitrous oxide produces severe neurological impairment in fruit bats: protection by methionine and aggravation by folates. Life Sci 1982;31(18):2001–10.
124. Henderson AM, Tai DC, Aleliunas RE, Aljaadi AM, Glier MB, Xu EE, Miller JW, Verchere CB, Green TJ, Devlin AM. Maternal folic acid supplementation with vitamin B_{12} deficiency during pregnancy and lactation affects the metabolic health of adult female offspring but is dependent on offspring diet. FASEB J 2018;32(9):5039–50.
125. Pfeiffer CM, Sternberg MR, Hamner HC, Crider KS, Lacher DA, Rogers LM, Bailey RL, Yetley EA. Applying inappropriate cutoffs leads to misinterpretation of folate status in the US population. Am J Clin Nutr 2016;104(6):1607–15.
126. Raghavan R, Ashour FS, Bailey R. A review of cutoffs for nutritional biomarkers. Adv Nutr 2016;7(1):112–20.
127. Bird JK, Ronnenberg AG, Choi S-W, Du F, Mason JB, Liu Z. Obesity is associated with increased red blood cell folate despite lower dietary intakes and serum concentrations. J Nutr 2015;145(1):79–86.
128. Nutrition Research Division, Health Canada, MacFarlane AJ. 2019, Unpublished results, Ottawa, Canada.
129. Ebrahim S, Davey Smith G. Mendelian randomization: can genetic epidemiology help redress the failures of observational epidemiology? Hum Genet 2008;123(1):15–33.
130. Pepe MS, Etzioni R, Feng Z, Potter JD, Thompson ML, Thornquist M, Winget M, Yasui Y. Phases of biomarker development for early detection of cancer. J Natl Cancer Inst 2001;93(14):1054–61.
131. Pepe MS, Feng Z, Janes H, Bossuyt PM, Potter JD. Pivotal evaluation of the accuracy of a biomarker used for classification or prediction: standards for study design. J Natl Cancer Inst 2008;100(20): 1432–8.
132. Srivastava S, Kramer BS. Validation: a critical step in bringing biomarkers to clinical fruition. Ann Epidemiol 2018;28(2):135–8.
133. Whiting PF, Rutjes AW, Westwood ME, Mallett S, Deeks JJ, Reitsma JB, Leeflang MM, Sterne JA, Bossuyt PM, QUADAS-2 Group. QUADAS-2: a revised tool for the quality assessment of diagnostic accuracy studies. Ann Intern Med 2011;155(8):529–36.
134. Kalmbach RD, Choumenkovitch SF, Troen AP, Jacques PF, D'Agostino R, Selhub J. A 19-base pair deletion polymorphism in dihydrofolate reductase is associated with increased unmetabolized folic acid in plasma and decreased red blood cell folate. J Nutr 2008;138(12):2323–7.
135. Gurwara S, Ajami NJ, Jang A, Hessel FC, Chen L, Plew S, Wang Z, Graham DY, Hair C, White DL, et al. Dietary nutrients involved in one-carbon metabolism and colonic mucosa-associated gut microbiome in individuals with an endoscopically normal colon. Nutrients 2019;11(3):613.

136. Davis SR, Stacpoole PW, Williamson J, Kick LS, Quinlivan EP, Coats BS, Shane B, Bailey LB, Gregory JF 3rd. Tracer-derived total and folate-dependent homocysteine remethylation and synthesis rates in humans indicate that serine is the main one-carbon donor. Am J Physiol Endocrinol Metab 2004;286(2):E272–9.
137. Lin Y, Dueker SR, Follett JR, Fadel JG, Arjomand A, Schneider PD, Miller JW, Green R, Buchholz BA, Vogel JS, et al. Quantitation of in vivo human folate metabolism. Am J Clin Nutr 2004;80(3):680–91.
138. Murdoch Children's Research Institute. I4C Consortium, 2019 [Internet]. Parkville, Victoria: Murdoch Children's Research Institute; [cited November 7, 2019]. Available from: https://www.mcri.edu.au/research/projects/international-childhood-cancer-cohort-consortium-i4c/i4c-consortium.
139. NIH. Environmental influences on Child Health Outcomes (ECHO) Program [Internet]. Bethesda, MD: NIH; [cited November 7, 2019]. Available from: https://www.nih.gov/research-training/environmental-influences-child-health-outcomes-echo-program, 2019.
140. Norwegian Institute of Public Health. Norwegian Mother, Father and Child Cohort Study (MoBa), 2019 [Internet]. Oslo, Norway: Norwegian Institute of Public Health; [cited November 7, 2019]. Available from: https://www.fhi.no/en/studies/moba/.
141. Danish National Birth Cohort, 2019 [Internet]. Copenhagen, Denmark: Danish National Birth Cohort; [cited November 7, 2019]. Available from: https://www.dnbc.dk/.
142. The Joint Programming Initiative (JPI). EpiBrain, 2019 [Internet]. The Hague, Netherlands: JPI; [cited 24 September, 2019]. Available from: https://www.healthydietforhealthylife.eu/index.php/84-nutrition-the-epigenome/483-epibrain.
143. Pfeiffer CM, Sternberg MR, Fazili Z, Lacher DA, Zhang M, Johnson CL, Hamner HC, Bailey RL, Rader JI, Yamini S, et al. Folate status and concentrations of serum folate forms in the US population: National Health and Nutrition Examination Survey 2011–2. Br J Nutr 2015;113(12):1965–77.
144. Luebeck EG, Moolgavkar SH, Liu AY, Boynton A, Ulrich CM. Does folic acid supplementation prevent or promote colorectal cancer? Results from model-based predictions. Cancer Epidemiol Biomarkers Prev 2008;17(6):1360–7.
145. Peng L, Dreumont N, Coelho D, Guéant J-L, Arnold C. Genetic animal models to decipher the pathogenic effects of vitamin B_{12} and folate deficiency. Biochimie 2016;126:43–51.
146. Ouattara B, Bissonnette N, Duplessis M, Girard CL. Supplements of vitamins B9 and B12 affect hepatic and mammary gland gene expression profiles in lactating dairy cows. BMC Genomics 2016;17(1):640.
147. Tserga A, Binder AM, Michels KB. Impact of folic acid intake during pregnancy on genomic imprinting of IGF2/H19 and 1-carbon metabolism. FASEB J 2017;31(12):5149–58.
148. Balashova OA, Visina O, Borodinsky LN. Folate action in nervous system development and disease. Dev Neurobiol 2018;78(4):391–402.
149. Stover PJ, Field MS. Trafficking of intracellular folates. Adv Nutr 2011;2(4):325–31.
150. Varela-Moreiras G, Selhub J. Long-term folate deficiency alters folate content and distribution differentially in rat tissues. J Nutr 1992;122(4):986–91.
151. Ghandour H, Chen Z, Selhub J, Rozen R. Mice deficient in methylenetetrahydrofolate reductase exhibit tissue-specific distribution of folates. J Nutr 2004;134(11):2975–8.
152. Green R, Allen LH, Bjørke-Monsen A-L, Brito A, Guéant J-L, Miller JW, Molloy AM, Nexo E, Stabler S, Toh B-H, et al. Vitamin B_{12} deficiency. Nat Rev Dis Primers 2017;3:17040.
153. Fedosov SN, Brito A, Miller JW, Green R, Allen LH. Combined indicator of vitamin B_{12} status: modification for missing biomarkers and folate status and recommendations for revised cut-points. Clin Chem Lab Med 2015;53(8):1215–25.
154. Thompson SG, Higgins JP. Treating individuals 4: can meta-analysis help target interventions at individuals most likely to benefit? Lancet 2005;365(9456):341–6.
155. Bailey RL, Pac SG, Fulgoni VL 3rd, Reidy KC, Catalano PM. Estimation of total usual dietary intakes of pregnant women in the United States. JAMA Netw Open 2019;2(6):e195967.
156. CDC. Recommendations: women & folic acid [Internet]. Atlanta, GA: National Center on Birth Defects and Developmental Disabilities (NCBDDD); [accessed August 13, 2019]. 2019. Available from: https://www.cdc.gov/ncbddd/folicacid/recommendations.html.
157. Miller JW. Proton pump inhibitors, H_2-receptor antagonists, metformin, and vitamin B-12 deficiency: clinical implications. Adv Nutr 2018;9(4):511S–18S.
158. Rotman SR, Bishop TF. Proton pump inhibitor use in the U.S. ambulatory setting, 2002–2009. PLoS One 2013;8(2):e56060.