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Biomarkers of Nutrition for Development—Folate Review[1–5]

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Abstract

The Biomarkers of Nutrition for Development (BOND) project is designed to provide evidence-based advice to anyone with an interest in the role of nutrition in health. Specifically, the BOND program provides state-of-the-art information and service with regard to selection, use, and interpretation of biomarkers of nutrient exposure, status, function, and effect. To accomplish this objective, expert panels are recruited to evaluate the literature and to draft comprehensive reports on the current state of the art with regard to specific nutrient biology and available biomarkers for assessing nutrients in body tissues at the individual and population level. Phase I of the BOND project includes the evaluation of biomarkers for 6 nutrients: iodine, iron, zinc, folate, vitamin A, and vitamin B-12. This review represents the second in the series of reviews and covers all relevant aspects of folate biology and biomarkers. The article is organized to provide the reader with a full appreciation of folate's history as a public health issue, its biology, and an overview of available biomarkers (serum folate, RBC folate, and plasma homocysteine concentrations) and their interpretation across a range of clinical and population-based uses. The article also includes a list of priority research needs for advancing the area of folate biomarkers related to nutritional health status and development. *J Nutr* 2015;145:1636S–80S.

Keywords: BOND, folate biomarkers, serum folate, RBC folate, homocysteine

Introduction

Folate's key role in ensuring normal development, growth, and maintenance of optimal health is the focus of the background section of this article, which begins with historical highlights and continues with capstones of clinical, chronic disease, and developmental disorder considerations. Public health applications follow with global intake recommendations coupled with

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status estimates and the impact of fortification with a focus on neural tube defect (NTD)[18] risk reduction. An overview of folate's chemistry, metabolism, and critically important role in one-carbon metabolism precedes the biomarker-specific sections of the review. After the identification of "priority" biomarkers for folate status assessment, the characteristics of pertinent analytical methods and technical considerations of each are presented (in general in the body of the article and in detail in **Supplemental Table 1**). Assay-specific issues are addressed for each priority biomarker and advantages and disadvantages summarized. Guidance to the most appropriate choice of method for the purpose and setting including laboratory infrastructure is provided in conjunction with how best to optimize specific analytical methods. In addition to analytical considerations, the review addresses the important issues on interpretation of folate status assessment data with the use of defined cutoff values determined by specific methods. After the method-specific sections, new directions and technologies, including the use of technology as research tools, are addressed. Concluding the review is a summary of research gaps and needs that present challenges and opportunities for research scientists with the interest and expertise to advance the field related to folate biomarkers for nutrition and development.

Background

Historical overview

Beginning with the observations by Lucy Wills in 1931 (1) of a factor in marmite that produces a cure of macrocytic anemia in pregnant women, a number of events have occurred to reinforce the role of folate as a key nutrient for human health (**Table 1**). This review will cover what we have learned over the years about this role and how best to assess folate status of humans across a range of developmental and environmental circumstances. The recent reviews by Shane (8) and Pfeiffer et al. (9) provide a perspective of the evolution of methods used to assess folate in both physiologic fluids and foods.

Clinical considerations and the role of folate in health and disease

Clinical stages of folate insufficiency. Because the Biomarkers of Nutrition for Development (BOND) project is intended to serve the breadth of users involved in the nutrition enterprise, including clinicians, it is useful to appreciate the clinical stages of folate insufficiency in order to inform that community. Inadequate intake is a leading cause of folate deficiency. Other major causes include increased requirements due to pregnancy or neoplastic diseases, malabsorptive conditions, and antifolate drugs or other metabolic inhibitors (10, 11). In Western societies, alcoholism (which affects both folate intake and absorption) is a common cause of low folate status, whereas in developing countries malabsorptive conditions such as tropical sprue are more common causative factors (10). Body stores of folate generally represent a 2- to 3-mo supply, and folate deficiency can develop in persons of any age with an inadequate intake and/or increased requirement for the vitamin (11). The clinical presentation of folate deficiency covers a wide range of symptoms, the basic progression of which is highlighted in **Table 2**.

Increased folate requirements for maternal health and fetal development. Although essential throughout life, folate is particularly critical during early stages of human development. Since the first report of amelioration of macrocytic anemia by exposure to folate-rich food sources (1), pregnancy has been recognized as a time when folate requirements are increased to sustain the demand for rapid cell replication and growth of fetal, placental, and maternal tissue, relating to the critical role it plays in DNA, RNA, and protein synthesis. Maintaining an adequate folate status throughout pregnancy is important not only for the mother's health but also for the developing infant because folate inadequacy in pregnancy has been associated with a number of adverse outcomes (21). These include folate-responsive NTDs and neural crest disorders (e.g., congenital heart defects), fetal growth retardation, low birth weight, preterm delivery, and neonatal folate deficiency. It is also notable that folate requirements are increased during lactation in order to meet both maternal and neonatal needs (21).

Folate and disease. Increased folate requirements in clinical settings have been linked with some anemias, malignancy, and in patients undergoing renal dialysis (22). In addition, folate status may become an issue in the context of therapeutic drug use including the following:

- anticonvulsant drugs (phenytoin, primidone);
- sulfasalazine (used in the treatment of inflammatory bowel disease);
- triamterene (a diuretic); and
- metformin (used in type 2 diabetes).

Some malabsorptive conditions can lead to folate deficiency (22). These include extensive inflammatory bowel disease (Crohn disease and ulcerative colitis), tropical sprue, and celiac disease, a genetically determined chronic inflammatory intestinal condition involving gluten-sensitive enteropathy and associated deficiency of iron, folate and other vitamins due to impaired absorption. In this condition, megaloblastic anemia is commonly encountered at the time of diagnosis, and subclinical deficiency is found in patients reported to have persistent mucosal damage (23).

Chronic alcoholism is associated with severe folate deficiency linked to poor dietary intake, intestinal malabsorption, impaired hepatic uptake with reduced storage of endogenous folates, and increased renal excretion (24). Hepatic methionine metabolism is also impaired in chronic alcoholism (24).

[5] Supplemental Tables 1 and 2 are available from the "Online Supporting Material" link in the online posting of the article and from the same link in the online table of contents at http://jn.nutrition.org.

[18] Abbreviations used: apABG, para-acetaminobenzoylglutamate; BOND, Biomarkers of Nutrition for Development; DBS, dried blood spot; DFE, dietary folate equivalent; DHF, dihydrofolate; dTMP, deoxythymidine monophosphate (deoxythymidylate); dTTP, deoxythymidine triphosphate; dUMP, deoxyuridine monophosphate (deoxyuridylate); dUTP, deoxyuridine triphosphate; FBP, folate-binding protein; GC-MS; gas chromatography–mass spectrometry; GCPII, glutamate carboxypeptidase II; hmTHF, 4α-hydroxy-5-methyltetrahydrofolate; IOM, Institute of Medicine; LC-MS/MS, liquid chromatography–tandem mass spectrometry; LINE-1, long interspersed nucelotide elements-1; LSD1, lysine-specific demethylase 1; MBA, microbiological assay; MeFox, pyrazino-s-triazine derivative of hmTHF; MTHFD, methylenetetrahydrofolate dehydrogenase; MTHFR, methylenetetrahydrofolate reductase; MTR, methionine synthase; MVM, multivitamin/mineral; NIST, National Institute of Standards and Technology; NTD, neural tube defect; pABG, para-aminobenzoylglutamate; PBA, protein-binding assay; PCFT, proton coupled folate transporter; QA, quality assessment; QC, quality control; RFC, reduced folate carrier; SAH, *S*-adenosylhomocysteine; SAM, *S*-adenosylmethionine; SPE, solid-phase extraction; SRM, standard reference material; THF, tetrahydrofolate; TYMS, thymidylate synthase; UDG, uracil glycosylase; USPHS, US Public Health Service; 5-formyl-THF, 5-formyltetrahydrofolate (folinic acid); 5-methyl-5,6-DHF, 5-methyl-5,6-dihydrofolate; 5-methyl-THF, 5-methyltetrahydrofolate; 5,10-methylene-THF, 5,10-methylenetetrahydrofolate; 5,10-methenyl-THF, 5,10-methenyltetrahydrofolate; 10-formyl-DHF, 10-formyldihydrofolate; 10-formyl-THF, 10-formyltetrahydrofolate.

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TABLE 1 Landmark events in folate history and critical points in assessment

Landmark events

- 1931, Lucy Wills, working in India, published an article on ``The treatment of ``pernicious anemia of pregnancy´´ and ``tropical anemia´´´´ in which she reported curing tropical macrocytic anemia with marmite. She was not able to identify the specific factor that produced the cure, noting that ``at present it is only possible to state that in marmite, and probably in other yeast extracts, there appears to be a curative agent for this dread disease....´´ (1)
- 1941 Mitchell et al. (2) described ``folic acid´´ extracted from 4 tons of spinach. The name was derived from the Latin word *folium* for leaf. They also noted that ``folic acid´´ stimulated the growth of *Lactobacillus casei*.
- 1945 Spies (3) demonstrated that folic acid produced a prompt hematologic response in macrocytic anemia; among the patients he treated were some patients with pernicious anemia, providing one of the first reports of masking of pernicious anemia by folic acid.

Critical points in folate assessment (4)

- Assessing folate status is complicated by the large number of folate forms that may be readily interconverted.
- Microbiological assays have been used for many decades due to the ability of some bacteria to grow in the presence of many different forms of folate (e.g., *L. casei*, now known as *Lactobacillus rhamnosus*, responds to all active monoglutamate forms). Microbiological assays are viewed by many researchers as the ``gold standard.´´
- Subsequently, assays using competitive protein binding became common because of their simplicity (4).
- In recent years the use of LC-MS/MS[1] has been recommended to quantitate individual folate forms which may be useful to:
  - Characterize metabolic alterations due to the common polymorphism in methylenetetrahydrofolate reductase (677 C>T) capable of reducing blood folate (5) and causing alterations in the proportion of folate forms in RBCs (6).
  - Identify blood folic acid resulting from food fortification and use of folic acid containing supplements.

[1] LC-MS/MS, liquid chromatography–tandem mass spectrometry.

Role of folate in chronic disease risk. Metabolic changes associated with impaired folate status have been linked to increased risk of chronic diseases including cancer and cardiovascular disease and cognitive dysfunction. As described in detail later in the review, folate is required for remethylation of homocysteine to methionine and DNA synthesis and cell proliferation in addition to methylation reactions that affect critical processes such as methylation of cytosine in DNA for control of gene expression and neurotransmitter synthesis. The types of chronic diseases linked to folate status and folate-related metabolic abnormalities are summarized below.

Cancer. Several cancers such as leukemia, lymphoma, and colorectal, breast, and prostate cancer have been associated epidemiologically with low folate status (25–28). Carcinogenesis has been linked with poor folate status because of the induction of DNA breaks by uracil incorporation in DNA (29). These deleterious genomic events lead to chromosome fragmentation and rearrangements, causing the generation of cells with aberrant karyotypes and altered gene dosage from which cancers have a higher probability of evolving (30). Hypomethylation of DNA associated with poor folate status may lead to inappropriate expression of genes that potentially predispose to cancer (e.g., uncontrolled expression of proto-oncogenes) (31). Although the prevention of folate deficiency has been linked to diminished cancer initiation, it has been hypothesized that excessive folate intake may fuel the growth of initiated cancers (32, 33). A recent meta-analysis with data on 50,000 individuals concluded that folic acid supplementation does not significantly increase or decrease site-specific cancer during the first 5 y of treatment (34).

Cardiovascular disease. As outlined later, one of the key folate-dependent pathways is associated with the generation of homocysteine. Hyperhomocysteinemia, which may result from poor folate intake or impaired folate metabolism as well as vitamin B-12 deficiency, has been associated with an increased risk of hypertension, cardiovascular disease, and cerebrovascular disease in epidemiologic studies (35–38). Although these observational findings do not support a causal effect, several mechanisms have been proposed by which hyperhomocysteinemia may mediate the risk of these diseases (38, 39).

Results of placebo-controlled intervention studies of folic acid supplementation, with or without vitamin B-12, have not yielded evidence of a strong protective effect against the incidence or progression of cardiovascular disease or cerebrovascular disease in the short term (<2 y). The evidence from these intervention studies does not support the previously hypothesized causal relation between homocysteine and cardiovascular disease that was based on observational studies.

Neurological conditions. Recent trials with longer intervention time frames using multiple B vitamins including folic acid suggest potential benefits against stroke, brain shrinkage, cognitive decline, and depression, particularly in those with above-average concentrations of homocysteine within the cohorts studied (40–43). In contrast, a recent meta-analysis of 11 trials on the effects of homocysteine lowering with B vitamins found no significant effect on individual cognitive domains or global cognitive function or on cognitive aging (44). However, meta-analyses on the effect of B vitamins on cognition (44, 45) included trials in which any effect would be difficult to detect because of the low sample size, short trial duration, or inclusion of healthy subjects not experiencing cognitive decline, subjects with already excellent B vitamin status, or severely demented patients in whom the treatment comes too late for any meaningful effect.

TABLE 2 Stages of folate insufficiency

- Events in the blood (12)
  - a decrease in plasma folate concentration followed by
  - an increase in plasma homocysteine concentration and
  - a reduction in RBC folate
- At the morphologic level, megaloblastic changes in the bone marrow and other rapidly dividing tissues becomes evident (13)
- Megaloblastic cells are abnormally large cells with large nuclei (characterized by a finely stippled lacy pattern of nuclear chromatin) and apparently normal cytoplasm, which give rise to the classic morphologic finding of nuclear-cytoplasmic dissociation
- Other abnormal nuclear changes may also occur: these include hypersegmentation of nuclei in neutrophils or generation of micronuclei in lymphocytes, which are a biomarker of chromosome breakage or loss; when found in RBCs, these are also known as Howell-Jolly bodies (14–18)
- The incapacitation of erythroblast replication results in reduced and abnormal erythrocyte production leading to anemia and reduced oxygen-carrying capacity of the blood, which may lead to symptoms of weakness, fatigue, irritability, and shortness of breath (19, 20)

Genetic considerations. An emerging body of evidence and research effort point to the potential for genetics to significantly affect folate metabolism and disease risk. Common polymorphisms in genes that code for proteins/enzymes required for folate uptake [e.g., glutamate carboxypeptidase II (*GCPII*; 1561 C>T), reduced folate carrier (*RFC*; 80 G>A)] and metabolism [e.g., methylenetetrahydrofolate reductase (*MTHFR*; 677C>T), methionine synthase (*MTR*; 2756 A>G)] have been shown to alter the catalytic activity or expression of these proteins, which can have a substantial influence on developmental or degenerative disease risk, providing further support for the central role of this key vitamin in health maintenance (46). Because some of these enzymes require other dietary cofactors for their function (e.g., vitamin B-12 and riboflavin as cofactors for MTR and MTHFR, respectively), it is important to take into account not only nutrient-gene interactions but also interactions of folate with other nutrients on health effects.

Role of folate in birth defects. A major driver of much of the recent public health attention to the importance of folate has been its link to a specific set of serious development disorders associated with defects in the closure of the neural tube. These disorders lead to an extremely serious set of disorders called "neural tube defects." Although considerable epidemiologic and experimental evidence links folate status to NTD risk, occurrence, and recurrence (47), the metabolic mechanisms by which folate promotes neural tube closure and reduces NTD risk are yet to be delineated. Because folate functions as an essential cofactor for the de novo synthesis of purine and thymidine nucleotides and for the remethylation of homocysteine to methionine, it has been suggested that folate can influence NTD risk by impairing nucleotide biosynthesis and cell division, elevating homocysteine, or altering the cellular methylation potential and gene expression (48). However, it is unlikely that one mechanism will suffice to explain the link between folate status and NTD risk. It is more likely to be an outcome of the complex interactions between folate nutriture, genetic, and environmental factors (49, 50).

Despite our lack of clarity with regard to the etiology of NTDs, a series of reports culminating in 2 landmark clinical trials that showed that folic acid taken periconceptionally could dramatically reduce the risk of NTDs (51, 52) led the US Public Health Service (USPHS) to recommend that all women of childbearing age capable of becoming pregnant take 400 μg folic acid daily (53). The approach and impact of this decision will be covered in greater detail in the section below.

Public health approaches

Low dietary intake remains the most common cause of folate inadequacy, both in developed and developing countries, and generally those of lower socioeconomic status do not consume high-folate-content foods. Furthermore, although the diets of many people worldwide may be adequate in preventing clinical deficiency (i.e., megaloblastic anemia), they may be insufficient to achieve a biomarker status of folate that is associated with optimal health and fetal development (i.e., NTD risk reduction). Accordingly, folate intakes of such diets would be considered suboptimal. This widespread underprovision of folate is generally attributed to the poor stability and incomplete bioavailability of natural food folates when compared with the synthetic vitamin folic acid (54). As a consequence, a large public health effort has gone into addressing the folate needs of the global population. The following is coverage of the key elements of those efforts.

Folate intake recommendations

Table 3 provides select examples of the folate intake recommendations across the world, including the US Institute of Medicine's (IOM's) DRIs. The FAO/WHO Expert Consultation adopted the RDAs set by the IOM (55) as the basis for the Recommended Nutrient Intakes (56). Other countries/regions with specific guidance include the following: Australia and New Zealand (57); the United Kingdom (58); Ireland (59); Germany, Austria, and Switzerland (60); The Netherlands (61); Denmark, Sweden, Norway, Iceland, and Finland (62); and Southeast Asia, encompassing Indonesia, Malaysia, The Philippines, Singapore, Thailand, and Vietnam (63). The basis of these recommendations and how they compare with the IOM DRIs have been reviewed (65).

The IOM recommendations consist of several categories (55). Of most relevance to folate are the following:

- Estimated Average Requirement: the median usual intake of the nutrient that meets the requirements of 50% of the population
- RDA: based on the Estimated Average Requirement, corrected for population variance, and represents the average daily dietary intake level sufficient to meet the nutrient requirement of ~98% of the population
- Adequate Intake: the quantity of a nutrient consumed by a group with no evidence of inadequacy
- Tolerable Upper Intake Level: defined as the "maximum daily intake levels at which no risk of adverse health effects is expected for almost all individuals in the general population, including sensitive individuals, when the nutrient is consumed over long periods of time" (66)

Table 4 provides some key points with regard to the derivation of DRIs for folate.

Folic acid intake recommendation for NTD risk reduction. For NTD risk reduction, the IOM (55) recommends that all women capable of becoming pregnant consume 400 μg folic acid/d from supplements or fortified foods in addition to folate from a varied diet. This recommended intake for NTD risk reduction is consistent with that of the USPHS (53) and is not the same as the RDA (400 μg dietary folate equivalents (DFEs), equivalent to 235 μg folic acid), a common misconception. The implications and impact of this policy are discussed in detail in the section below entitled "A case study in public health intervention: folic acid and NTDs."

Folate/folic acid intake and adequacy in the United States. The USDA Food and Nutrient Database for Dietary Studies (69) can be used to estimate dietary folate intake (μg/d) in specific categories including the following:

- naturally occurring food folate;
- folic acid, including that in enriched cereal-grain products (140 μg/100 g flour) and in folic acid–fortified ready-to-eat cereals, including those with ~100 to 400 μg/serving (70);
- total folate in μg/d; and
- total folate in μg/d DFEs.

Yang et al. (71) estimated folic acid intake provided by different food intake categories for the nonpregnant adult US population aged ≥19 y from NHANES 2003–2004 and 2005–2006. These results confirmed those of other studies that consumption of ready-to-eat cereals and/or supplements contributes significantly to intakes of folic acid. **Table 5** provides some additional detail with regard to folic acid exposure in the United States.

TABLE 3 Selected examples of folate intake recommendations worldwide[1]

	United States and Canada (55), μg/d DFE	FAO/WHO (56), μg/d DFE	Australia,New Zealand (57), μg/d DFE	United Kingdom (58), μg/d folate	Ireland (59), μg/d folate	Germany, Switzerland, Austria (60), μg/d DFE	The Netherlands (61), μg/d DFE	Nordic countries (62), μg/d folate	Southeast Asia (63), μg/d folate
Category	RDA/AI	RNI	RDI/AI	RNI	RDA	RI	RDA/AI	RI	RDA
Infants									
0–6 mo	65[2]	80	65[2]	50	50	(<4 mo) 60	(0–5 mo) 50[2]	None set	(0–5 mo) 80
7–12 mo	80[2]	80	80[2]	50	50	(4–11 mo) 80	(6–11 mo) 60[2]	50	(6–11 mo) 80
Children									
1–3 y	150	160	150	70	100	(1–4 y) 200	85[2]	(12–23 mo) 60	160
4–8 y	200	(4–6 y) 200; (7–9 y) 300	200	(4–6 y) 100; (7–10 y) 150	(4–10 y) 200	(4–9 y) 300	150[2]	(2–5 y) 80; (6–9 y) 130	(4–6 y) 200; (7–9 y) 300
Males									
9–13 y	300	(≥10 y) 400	300	(≥11 y) 200	(≥11 y) 300	(≥10 y) 400	225[2]	(10–13 y) 200	(≥10 y) 400
≥14 y	400		400				(14–18 y) 300[2]; (≥19 y) 300	(≥14 y) 300	
Females									
9–13 y	300	(≥10 y) 400	300	(≥11 y) 200	(≥11 y) 300	(≥10 y) 400	225[2]	(10–13 y) 200	(≥10 y) 400
≥14 y	400		400				(14–18 y) 300[2]; (≥19 y) 300	(14–17 y) 300; (18–30 y) 400; (≥30 y) 300	
Pregnancy (all ages)	600	600	600	300	(Second half) 500	600	400[2]	500	600
Lactation (all ages)	500	500	500	260	(First 6 mo) 500	600	400[2]	500	(First 6 mo) 500; (second 6 mo) 500

[1] Note that units used to express intake recommendations differ among countries. AI, Adequate Intake; DFE, dietary folate equivalent; RI, recommended intake; RNI, recommended nutrition intake. Adapted from reference 64 with permission.
[2] Indicates AI, which is believed to cover the needs of all individuals in the group, but lack of data or uncertainty in the data prevents being able to specify with confidence the percentage of individuals covered by this intake.

TABLE 4 Key points with regard to IOM folate recommendations[1]

- Primary biomarker used for DRI was RBC folate concentration, an index of tissue stores and long-term status.
- Ancillary biomarkers included serum folate and Hcy concentrations.
- The EAR for adults was based primarily on data from controlled metabolic studies in which folate response to defined diets was determined.
- Additional supporting evidence included data from epidemiologic studies in which folate intake was estimated in conjunction with status indicators.
- The DRIs are expressed in DFEs, defined as micrograms of naturally occurring food folate plus 1.7 times the micrograms of synthetic folic acid. The use of DFEs is intended to
  - account for differences in bioavailability between synthetic folic acid in fortified foods and naturally occurring dietary folate and
  - establish equivalency of all forms of folate, including folic acid in fortified foods.

  The 1.7 multiplier for converting micrograms of folic acid to DFEs was based on the assumption that added folic acid (consumed with a meal) is ~85% available (67) and food folate is ~50% available (68); thus, the ratio 85:50 yielded the multiplier of 1.7 in the DFE calculation.
- UL for adults (≥19 y) for folic acid is 1000 μg/d. There is no UL for naturally occurring food folate.
- UL for folic acid is based on case reports in patients treated for vitamin B-12 deficiency treated with high doses of folic acid (≥5 mg/d in most cases) and the observation that hematologic but not neurological symptoms were reversed in the majority of cases with the folic acid treatment.
- UL for children
  - No direct data available for children aged 1–18 y
  - Used adult UL and adjusted by weight: 300–800 μg/d, depending on the age group
  - No UL established for infants

[1] DFE, dietary folate equivalent; EAR, Estimated Average Requirement; Hcy, total homocysteine; IOM, Institute of Medicine; UL, Tolerable Upper Intake Level. Adapted from reference 55 with permission.

Folate status estimates

Folate status in the United States based on specified biomarkers and the effect of fortification.

Serum and RBC folate. Serum and RBC folate have been assessed for the US population as part of the NHANES, first (1988–2006) with the Bio-Rad radioassay, and later (2007–2010) with the microbiological assay (MBA) (4). Population-based reference data for serum and RBC folate concentrations from the 2003–2006 NHANES were recently summarized (72) (**Figures 1–3**, **Table 6**). In the most recent NHANES folate status report of the 2007–2010 time period, previously published data generated with the Bio-Rad assay (1988–2006) were adjusted by using statistical models to a microbiologic equivalent value to bridge known assay differences, thus enabling time trend evaluations (73) (Table 6).

Homocysteine. Because of its reputed impact on health, as discussed above, plasma homocysteine was assessed as a nonspecific "functional" indicator of folate status in NHANES (72). Elevated plasma homocysteine concentrations (>13 μmol/L) were found in ~8% of the population aged ≥20 y and in 19% of persons aged ≥60 y participating in NHANES 2003–2006 (72). Pfeiffer et al. (74) estimated a 10% decrease in plasma homocysteine when comparing prefortification (1991–1994) to postfortification (1999–2004) concentrations in a national sample of the US population.

Folate status in countries other than the United States based on specified biomarkers and guidelines in current use. Because folic acid in foods is more stable and bioavailable than naturally occurring food folates, the biomarker status of folate tends to be highest in countries with mandatory folic acid fortification, followed by those countries with voluntary fortification. Voluntary fortification of a wide variety of foods is practiced in many countries worldwide (75, 76). Because of the variability in the number of foods fortified and subsequently consumed, folate status (and related health outcomes) in countries with voluntary fortification is more disparate than in countries with mandatory fortification (77). Mandatory fortification may not reach all women of reproductive age adequately; however, the lowest folate status in population groups is found in those countries without access to folic acid–fortified foods, even on a voluntary basis (78). As of November 2012, 75 countries have passed regulations for mandatory fortification of staple foods with folic acid and iron (79). Features of the mandatory fortification can be found in **Table 7**. **Figure 4** shows a map of the global efforts to actualize folic acid fortification of foods.

Folate status—a global perspective. The ability to develop global, regional, or national consensus on folate status of populations at risk is contingent on having reliable and comparable data. Challenges to this goal include the use of different analytical methods and/or different biomarker cutoff points applied to define the severity of deficiency in different countries. Moreover, meeting a particular criterion or prevalence estimate of folate deficiency in a given population (e.g., >5% with a folate biomarker value falling outside a cutoff point indicative of deficiency) does not mean that folate status is optimal in the "nondeficient" portion of the population. In fact, in many settings, although folate deficiency may be relatively rare, suboptimal folate status with accompanying implications for health may be common.

TABLE 5 Folic acid intake in the United States[1]

- Based on NHANES data (2003–2004 and 2005–2006), the estimated usual median folic acid (μg/d) intakes provided by different food intake categories were as follows:
  - 138 from ECGPs only,
  - 274 from ECGPs plus RTECs,
  - 479 from ECGPs plus supplements, and
  - 635 from ECGPs + RTECs + supplements (71).
- An estimated 60% of US adults consumed folic acid from RTECs and/or supplements and 15% from both (71).
- Regular consumption of RTECs with folic acid was associated with an ~100% higher usual intake.
- Use of folic acid–containing supplements was associated with >200% higher intake compared with consumption of ECGPs only (71).
- Folic acid intake exceeding the UL was reported in NHANES (71).
  - Overall, <3% of US adults exceeded the UL.
  - Among the 60% of adults who did not take supplements postfortification (NHANES 2003–2004 and 2005–2006), 0% exceeded the UL for folic acid.
  - Among the 34% and 6% of adults who consumed supplements with an average of ≤400 or >400 μg/d folic acid, <1% and 47.8% (95% CI: 39.6%, 56.0%) consumed more than the UL, respectively.

[1] ECGP, enriched cereal grain product; RTEC, ready-to-eat cereal; UL, Tolerable Upper Intake Level.

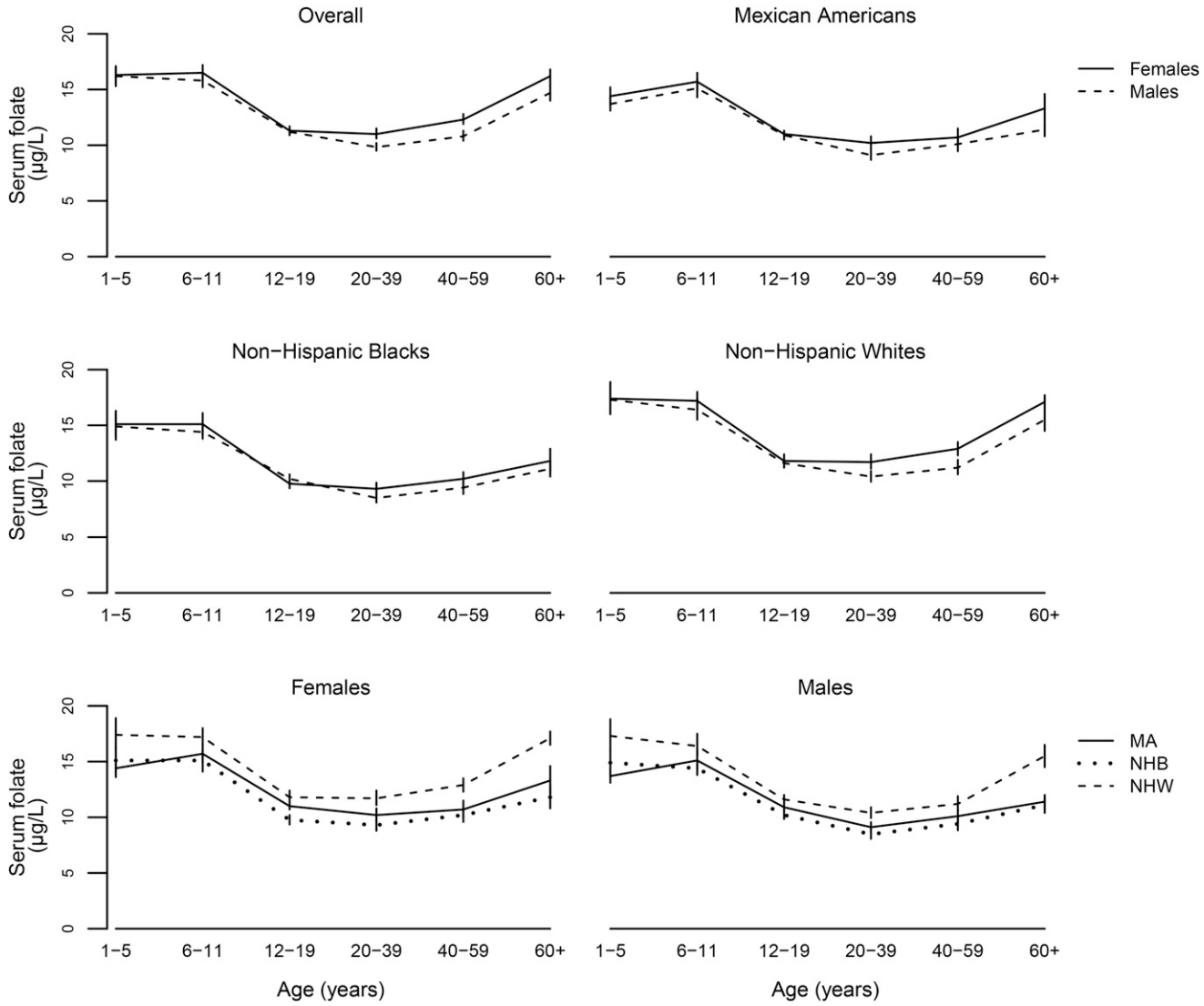

FIGURE 1 Serum folate concentrations by age group in the US population aged ≥1 y stratified by sex and race/ethnicity: NHANES 2003–2006. Values are geometric means; error bars represent 95% CIs. To convert μg/L to nmol/L, multiply by 2.266. MA, Mexican American; NHB, Non-Hispanic Black; NHW, Non-Hispanic White. Adapted from reference 72 with permission.

A recent review of folate deficiency worldwide compared surveys of folate status published between 1995 and 2005 (84) and highlighted many of the challenges confronting the global community (**Table 8**). The overall conclusion of the report was that to gain a better understanding of the magnitude of folate deficiency worldwide, there was a need for more population-based studies specifically designed to assess folate status, consensus on the best indicators for assessing folate status, and agreement on the appropriate biomarker cutoff point to define the severity of deficiency (84).

The European Community has been addressing this issue in a variety of ways and several points can be made about these efforts:

- Although nationally representative dietary surveys are available for several European countries (85), such surveys are often conducted without the inclusion of corresponding blood samples for determination of biomarker status, thereby preventing biomarker concentrations to be examined in relation to population intakes of folate.
- Observed variability in folate status among European countries is primarily due to differences in exposure to folic acid–fortified foods:
  - o National fortification policy varies considerably throughout the European Union.
  - o Many European countries (e.g., the United Kingdom and Ireland) permit the addition of folic acid and other nutrients to foods on a voluntary basis (i.e., at the manufacturer's discretion); others (e.g., Denmark) prohibit fortification of any kind or specifically limit fortification with folic acid (e.g., The Netherlands).
  - o The United Kingdom and Ireland have voluntary, relatively liberal, fortification policies that permit folic acid and other micronutrients to be added to various foods (e.g., breakfast cereals), thus allowing the consumer to have ready access to fortified foods. Under these conditions, studies show that typically ~75% of the population will consume fortified foods on a regular basis.
- The impact of voluntary fortification was examined in a convenience sample of 441 healthy adults aged 18–92 y who were not taking folic acid supplements in Northern Ireland (86):
  - o Fortified foods were associated with significantly higher total folate and folic acid intakes.

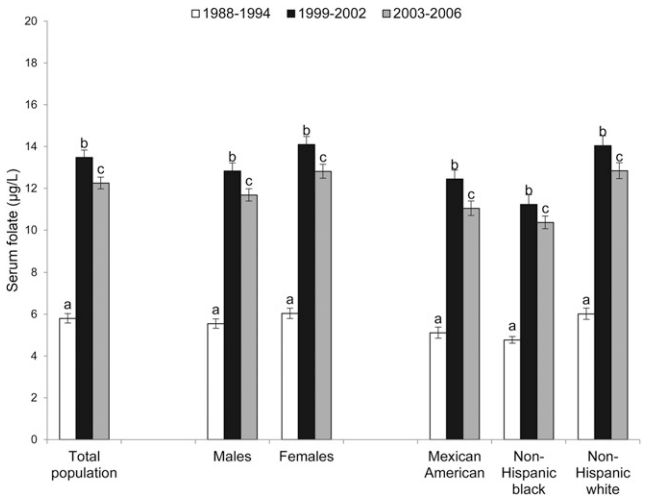

FIGURE 2 Serum folate concentrations in the US population aged ≥4 y stratified by sex or race/ethnicity: NHANES 1988–2006. Values are age-adjusted geometric means; error bars represent 95% CIs. Within a demographic group, bars not sharing a common letter differ ($P < 0.05$). To convert μg/L to nmol/L, multiply by 2.266. Adapted from reference 72 with permission.

- RBC folate concentrations were 387 nmol/L higher, and plasma homocysteine concentrations were 2 μmol/L lower, in the group in the highest tertile of fortified food intake (median intake of 208 μg/d folic acid) compared with nonconsumers of fortified foods (0 μg/d folic acid).

Although a comprehensive review of efforts to address folate status globally is beyond the scope of this article, a comparison of folate intake recommendations by different countries provides insight into global efforts to establish folate intake recommendations to maintain adequate folate status. Table 3 presents a comparison of these country- or region-specific folate intake recommendations, which have been previously reviewed (65).

Folate status in children. As will be discussed in further detail below, folate biology and subsequent requirements are developmentally sensitive. As noted, the bulk of surveillance has focused on the folate status of adults. However, some effort has gone into the assessment of status of children. Two prime examples are published data from population-based surveys conducted in the United Kingdom (87) (**Figure 5**) and the United States (88). Both reports describe a decline in folate status biomarkers with age from childhood to adolescence; these observations in British and American children are consistent with findings from Belgian, Dutch, and Greek children (albeit not population-based cohorts but convenience samples) (89–91), also showing age-related decreases in folate concentrations. Of particular note, where dietary intakes were also measured by using validated methodologies (87, 88, 91), the data showed that dietary folate intakes in general compared favorably with dietary reference values across all age groups and were not lower in the older children after adjustment for total energy. The mechanism for the decline in folate status biomarkers with age in children and adolescents, despite no corresponding decline in dietary folate intakes, is not clear but may be an indication that folate requirements of older children are increased due to higher metabolic demands for growth from childhood to adolescence (**Table 9**).

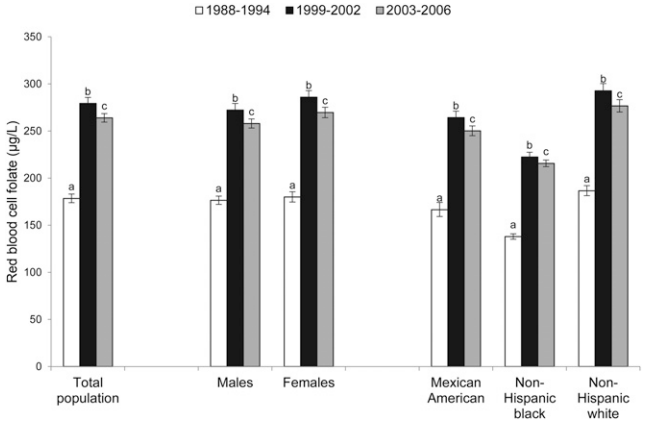

FIGURE 3 RBC folate in the US population aged ≥4 y stratified by sex or race/ethnicity: NHANES 1988–2006. Values are age-adjusted geometric means; error bars represent 95% CIs. Within a demographic group, bars not sharing a common letter differ ($P < 0.05$). To convert μg/L to nmol/L, multiply by 2.266. Adapted from reference 72 with permission.

Major causes of folate inadequacy in developed and underdeveloped countries. Folate inadequacy is not uncommon, even in otherwise well-nourished populations. As with any nutrient, a low or deficient status of folate can arise in any situation in which requirements are increased or availability is decreased or both, with the clinical manifestation of folate deficiency (i.e., megaloblastic anemia) more likely to be present when both occur simultaneously. The major causes of folate inadequacy are shown in Table 9. The biology of folate, including increased demands from a life stage and clinical perspective, will be covered in the subsequent sections. The assessment of these factors linked to folate inadequacy requires accurate and reliable measures of folate exposure. The following section addresses our current tools to evaluate that need.

Determining adequacy of folate intake/status. As discussed in greater detail below, RBC folate, widely considered the most robust biomarker of long-term status, is found to be moderately correlated with habitual folate intake when the latter is expressed as DFEs (as is done in the United States), thus accounting for the greater bioavailability of folic acid compared with naturally occurring food folates (86) (**Figure 6**). This conversion factor is not applied in most European countries, where folate intakes are expressed as total folate in micrograms per day (Table 3). As a result, the relation between folate

TABLE 6 Summary of key findings of folate status in the US population after initiation of mandatory fortification in 1998

- Serum and RBC folate concentrations followed a U-shaped age pattern, with the lowest concentrations seen in adolescents and young adults, respectively (Figure 1) (72).
- Serum folate concentrations more than doubled and RBC folate concentrations increased by ~50% (Figures 2 and 3) (72).
- Small decreases (<10%) in serum and RBC folate concentrations were observed from the earlier (1999–2002) to the later (2003–2006) postfortification period (Figures 2 and 3) (72).
- Based on microbiologically equivalent blood folate data for 6 prefortification (1988–1994) and 12 postfortification (1999–2010) years, the prevalence of low serum (<10 nmol/L) or RBC (<340 nmol/L) folate concentrations was ≤1% postfortification, regardless of demographic subgroup, compared with 24% for serum folate and 3.5% for RBC folate prefortification (72).

TABLE 7 Features of mandatory fortification

- Mandatory fortification of ≥1 food products is carried out under specific regulations or laws that are country-specific (80).
- Mandatory fortification can include many food products or be limited to only 1 staple food product.
- The level of folic acid fortification should be determined by the average daily consumption of the chosen food product and the mean target intake of folic acid desired in the target population (81).
- In the United States and Canada, all cereal-grain food products labeled as enriched are fortified through a standard of identity regulation at 1.4 mg/kg flour or cereal-grain product (70).
- Case study: Chile
  - Only bread is fortified with folic acid based on the goal of providing an average of 400 μg/d folic acid to women 15–44 y of age.
  - Food consumption patterns were used to support the decision to fortify bread flour with 220 μg folic acid per 100 g of bread flour (82).

biomarkers and dietary intake is found to be weaker, i.e., for RBC vs. natural food folates $r = 0.290$ ($P < 0.001$) and for RBC vs. food folic acid $r = 0.416$ ($P < 0.001$) (86). The contrasting approaches to expressing dietary folate intakes make any evaluation of adequacy of dietary folate intake in relation to biomarker status, or comparison of folate recommendations between countries, inherently complicated.

Folic acid in circulation: what does it mean? Concerns have been raised about the low concentrations of fasting unmetabolized circulating folic acid (0–2 nmol/L) found in subjects consuming fortified foods and/or folic acid–containing supplements (92). The concern about folic acid is primarily because folic acid is not a naturally occurring form of the vitamin. The "absence" of unmetabolized folic acid in plasma at lower folic acid doses in some studies most likely reflects limitations of the assay methods used to detect very low concentrations of folic acid.

A review of the extant literature revealed a single human study in postmenopausal women that reported what the authors concluded was a potential adverse effect of folic acid (93). Unmetabolized folic acid, but not total folate, in plasma was related to a decrease in NK cell cytotoxicity. Many of the elderly subjects in the study were obese, and it is possible they had conditions that influenced the concentration of soluble folate-binding protein (FBP) in plasma. If so, plasma folic acid concentrations would be merely reporting on the concentrations of soluble FBP (folic acid has a very high affinity for FBP), which, in turn, would be reporting on an underlying condition, and neither would be causative for the decrease in NK cell cytotoxicity. Other studies showed no effect of folic acid on NK cell cytotoxicity (94).

The effect of folic acid supplements on human-milk folate content was assessed in women who had received a folic acid prenatal supplement (750 μg/d) and who consumed 400 μg/d dietary folate during the third trimester of pregnancy (95). In this study, the prenatal folic acid dose did not increase human-milk total folate, but the proportion of folic acid increased to 40% of total breast-milk folate. A possible concern was raised about the effect of prenatal folic acid supplements on breast-milk folic acid concentrations; however, folic acid has been added to infant formula for many years without any apparent adverse effect. Aside from the results reported above, no confirmed metabolic effects have been found for plasma folic acid that would not be mimicked by the much higher concentrations of reduced folate in plasma. Thus, it is difficult to envisage a scenario in which the very low concentrations of circulating folic acid could have a specific adverse effect.

A case study in public health intervention: folic acid and NTDs. Two landmark clinical trials (51, 52) showed that folic acid, taken periconceptionally, could reduce the risk of both recurrent (51) and the first occurrence of (52) NTDs dramatically and led the USPHS to recommend that all women of childbearing age who are capable of becoming pregnant take 400 μg folic acid daily. Only a minority of women followed this recommendation, prompting the FDA to mandate that enriched cereal grain products (e.g., bread, pasta, rice) be fortified with 140 μg folic acid per 100 g grain in order to reach the population at risk.

The impact of this effort has not been easy to measure. In the United States, most NTDs are diagnosed prenatally and pregnancies may be terminated without being identified in vital records. Thus, determining the prevalence of NTDs reported on birth certificates results in many missed cases (78). Fortunately, in Canada, which has a very similar fortification program, more information is available on prenatally diagnosed cases (96). Comparing the impact of fortification between US and Canadian studies showed that the decrease in prevalences was greater in areas where ascertainment was more complete (97). However, the percentage reduction in prevalence was greater in Canada because the baseline prevalence was higher than in the United States, whereas the NTD prevalence postfortification was lower in the United States than in Canada. Because percentage reduction is a function of the baseline prevalence (98) it should only be used to monitor progress within a population and not be used to compare the impact of folic acid intervention on NTDs among countries.

The lowest achievable NTD prevalence in response to folic acid intervention is estimated to be ~5–6 per 10,000 pregnancies and consists of largely folate-insensitive NTDs. Other causes of NTDs are not preventable by folic acid (e.g., drug exposure, other genetic conditions) and these are thought to contribute to ~5 NTDs per 10,000 births because folic acid fortification results in reductions to approximately this rate in multiple population settings (78).

Can folate status biomarkers be used to assess NTD risk? The number of NTDs that could be prevented in a population has been shown to be dependent on folate status; specifically,

FIGURE 4 All countries shown in black fortify flour with at least iron and folic acid, except for Australia, which does not include iron, and Venezuela, the United Kingdom, the Philippines, and Trinidad and Tobago, which do not include folic acid. Reproduced from reference 83 with permission.

TABLE 8 Key points regarding the extant global folate survey data[1]

- Folate status was most frequently assessed in
  - women of reproductive age (34 countries) and
  - in adults generally (27 countries).
- Surveys of preschool-aged children and pregnant women, those likely to be at greatest risk of deficiency, are greatly lacking.
- The majority of the 145 studies examined (78%) assessed folate status by serum (or plasma) folate; far fewer reported RBC folate (45%).
- Few nationally representative studies were available for investigation; but, on the basis of evidence from countries with such survey data, deficient folate status (i.e., >5% of the population with a serum folate value below the normal range) was identified in specific age groups in 6 of 8 countries, most notably in preschool-aged children in Venezuela, pregnant women in Costa Rica (before mandatory fortification) and Venezuela, and the elderly in the United Kingdom.

[1] Data from reference 84.

RBC folate concentration has been shown to be a reasonable biomarker of NTD risk (99, 100). Daly et al. (100) found that the prevalence of NTD in an Irish population was <8 per 10,000 when RBC folate concentrations were ≥906 nmol/L. The dose-response between RBC folate concentrations and NTD risk in the Irish study (100) agreed with data modeled from Chinese folic acid intervention studies by Crider et al. (99). Crider et al. (99) reported that the NTD risk was substantially attenuated at RBC folate concentrations >1000 nmol/L. Their results indicated that an RBC folate concentration of ~1000–1300 nmol/L may achieve optimal prevention of folate-sensitive NTDs, with a resulting overall risk of NTDs of ~6 per 10,000 births. On the basis of distributions of RBC folate concentration (88, 99), the US population level of NTDs prefortification was estimated to be 10.1–16.4 per 10,000 births and postfortification prevalence to be in the range of 4.2–7.7 per 10,000 births (99). The WHO has developed new guidelines for optimal RBC folate concentrations associated with NTD risk reduction on the basis of published findings (101).

Has food fortification with folic acid prevented all folate-related NTDs in the United States? In 2 studies (102, 103), investigators collected data on folate/folic acid exposure in women who had NTD pregnancies and women who had unaffected pregnancies. They found that women who had an affected child were not significantly less likely to have used folic acid supplements. The data from these 2 epidemiologic studies suggest that because folic acid supplement use was not determined to significantly affect NTD risk, the amount of folic acid in fortified food alone in the United States may be sufficient to prevent a large percentage of folate-related NTDs. Conclusions from these studies are not definitive because the numbers of affected cases may have been insufficient to detect a true difference. The small sample sizes prevented subgroup analysis, which may have revealed racial/ethnic differences because a smaller decline in NTDs was detected in Hispanics during the postfortification period relative to non-Hispanic whites. In addition, recall bias cannot be ruled out because of the long period of time between interview and neural tube formation (up to 3 y).

A subgroup of US women who are non–supplement users may still be at increased risk of folic acid–preventable NTDs. This conclusion is based on the following facts: *1*) folic acid supplements are taken infrequently by women of childbearing age in the United States (~30%) (104), *2*) there is a high rate of unplanned pregnancies in the United States (~50%) (105), and *3*) the neural tube closes by day 28 of gestation, before most women know they are pregnant (106).

Because NTDs are known to have a strong genetic component, there has been an extensive search for folate gene variants that increase the risk of NTDs. The results have been mixed, which may relate to the mitigating effect that folic acid intake has on genetic risk. Thus, although many genetic variants in folate enzyme genes have been identified, a relatively small number have been shown to influence folate or homocysteine concentrations (107). Nonetheless, *MTHFR* 677 C>T has been shown to be a risk factor for NTDs in most studies and in a large meta-analysis (108).

The question of whether birth abnormalities other than NTDs are significantly affected by maternal folic acid intake has been investigated (109–113). There is, however, a lack of consistent evidence that the risk of birth defects other than NTDs is positively affected by folic acid in the amount consumed in supplements and/or fortified foods (109–113).

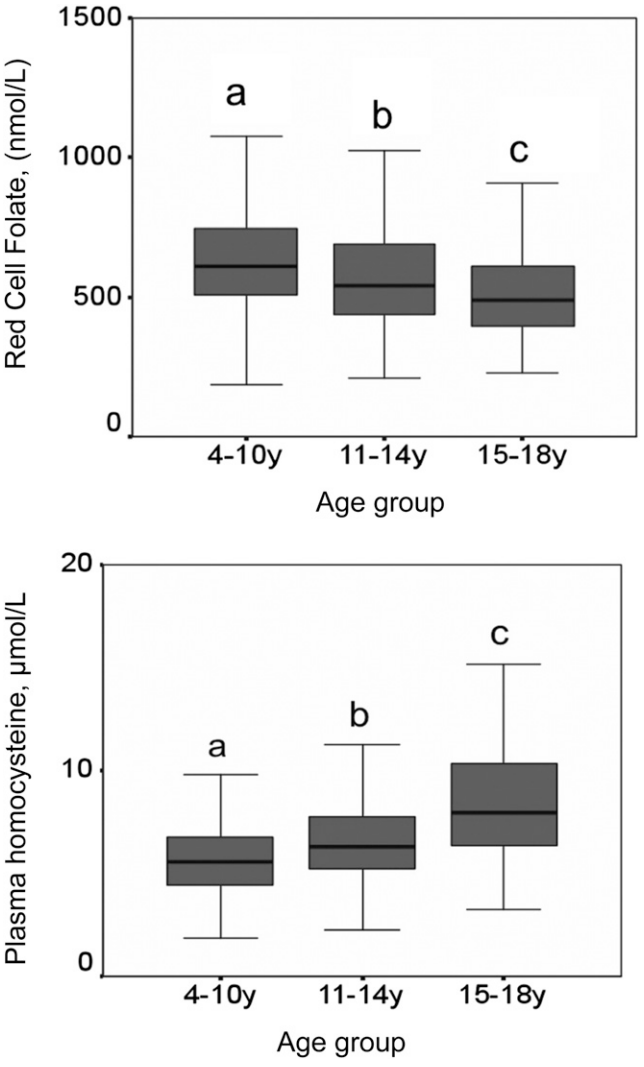

FIGURE 5 RBC folate and plasma homocysteine concentrations in a representative sample of British children aged 4–18 y. Differences between groups were assessed by using 1-factor ANOVA (with Tukey's post hoc test), adjusted for sex, smoking, breakfast cereal consumption, and supplement use (ANCOVA: $P < 0.05$). Bars not sharing a common letter differ, $P < 0.05$. Adapted from reference 87 with permission.

TABLE 9 Primary causes of folate inadequacy

- Reduced dietary intake (e.g., low intake of folic acid–fortified foods, dark-green leafy vegetables, legumes, select fruit such as orange juice)
- Increased requirement
  - Increased physiologic requirement (e.g., pregnancy, lactation, rapid growth in adolescence; see section on folate status in children)
  - Pathological conditions (e.g., malignancy, inflammatory conditions, certain anemias)
  - Drugs (e.g., anticonvulsants, methotrexate, sulfasalazine)
- Decreased availability
  - Impaired folate absorption (e.g., celiac disease, use of drugs such as sulfasalazine)

Folate Biology and Homeostasis

The ability to discover and develop biomarkers, particularly those reflecting nutrient function or effect, is contingent on an appreciation of the role of nutrients within relevant biological systems. To provide some perspective on the folate expert panel recommendations, the following sections describe the specific roles and interrelations of the folates.

Current understanding of the biology: dependent systems

Structure, function, and absorption. The tetrahydrofolates (THFs), a family of structurally related, water-soluble vitamins composed of a fully reduced pterin ring, a *p*-aminobenzoyl group, and a polyglutamate peptide containing up to 9 glutamate residues linked by unusual γ-peptide linkages, have been well described (114) and are represented in **Figure 7**. The key elements of folate metabolism are highlighted in **Table 10**.

Overview of folate-mediated one-carbon metabolism. The intracellular functions of folate are interconnected through competition for a limiting pool of folate cofactors within the network, because the concentration of folate enzymes exceeds intracellular folate concentrations (114). More recent studies indicate that the activity of these pathways is also regulated by dynamic physical compartmentation and formation of multienzyme complexes that are required for pathway function. The dynamic assembly of metabolic complexes adds additional dimensions and complexity to the regulation of these pathways, including the necessity for regulated trafficking of folate cofactors among compartmentalized pathways (115). **Figure 8** summarizes the essential components of THF-related pathways. The key elements of those pathways are as follows:

- In the mammalian cell, one-carbon metabolism occurs in the cytosol, mitochondria, and nucleus.
- THF polyglutamates are found in the lysosome where they are converted to THF monoglutamates through the activity of γ-glutamyl hydrolase (121).
- Folate-mediated one-carbon metabolism in the cytosol is a network of 3 interdependent biosynthetic pathways that catalyze the de novo synthesis of purine nucleotides, deoxythymidylate (dTMP), and remethylation of homocysteine to methionine.
- In the nucleus, THF is required for the synthesis of thymidylate at the replication fork (122) and may function in histone demethylation catalyzed by lysine-specific demethylase 1 (LSD1) (123).
- Formate is the primary source of one-carbon units for nuclear and cytosolic one-carbon metabolism and is generated through mitochondrial one-carbon metabolism (120), although one-carbons carried by THF can be derived directly in the cytosol from the catabolism of histidine, purines, and serine (114).

Methionine. The importance of these folate-dependent pathways is exemplified by the role of methionine in numerous pathways including serving as a precursor for protein biosynthesis. Methionine can be converted to *S*-adenosylmethionine (SAM), which, in the decarboxylated form, participates in polyamine synthesis and can serve as a cofactor and methyl group donor for numerous methylation reactions including the methylation of chromatin (CpG islands in DNA) and histone proteins, RNA, and numerous proteins and synthesis of neurotransmitters, phosphatidylcholine, and other small molecules. SAM-dependent methylation regulates fundamental biological processes including nuclear transcription, mRNA translation, cell signaling (124), protein localization (125), and the degradation of small molecules (126). The essential elements of the 3 primary folate-dependent pathways are shown in Figure 8 and **Figure 9** and are described in **Table 11**.

Each of these pathways highlights the important role of folates in critical metabolic systems. Although the purine pathway has no folate relevant biomarkers, the other 2 offer implications for potential biomarkers to assess folate. In the context of thymidylate biosynthesis, uracil accumulation in DNA is a biomarker of impaired de novo thymidylate biosynthesis (131). Uracil has been suggested to be a biomarker of folate (132) and vitamin B-12 status (133), but not all studies agree (134). In mice, distinct tissues have different levels of uracil incorporation into DNA (135).

Implications of homocysteine remethylation for folate assessment/biomarkers include the following:

- Biomarkers of impaired homocysteine remethylation include depressed SAM concentrations and elevations in plasma homocysteine and *S*-adenosylhomocysteine (SAH) concentrations (136, 137), leading to hypomethylated DNA

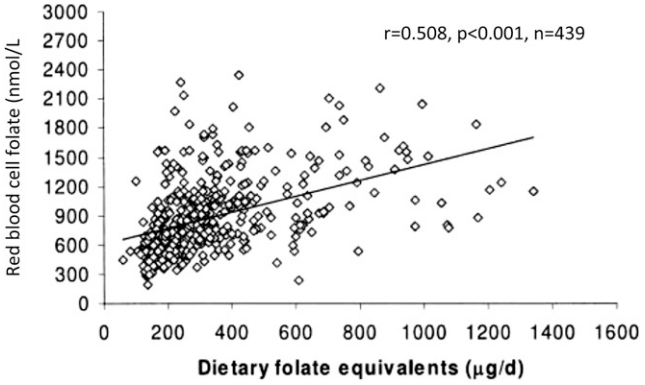

FIGURE 6 Relation between dietary intake and biomarker status of folate. Correlations were carried out on log-transformed data and were calculated by using Pearson correlation coefficients (r). Correlations for which $P < 0.05$ were considered significant. Total folate intake was expressed as DFEs, which were introduced in the United States to account for the higher bioavailability of synthetic folic acid added to food compared with natural food folate. DFEs were calculated as micrograms of natural folate plus 1.7 × μg added folic acid. DFE, dietary folate equivalent. Adapted from reference 86 with permission.

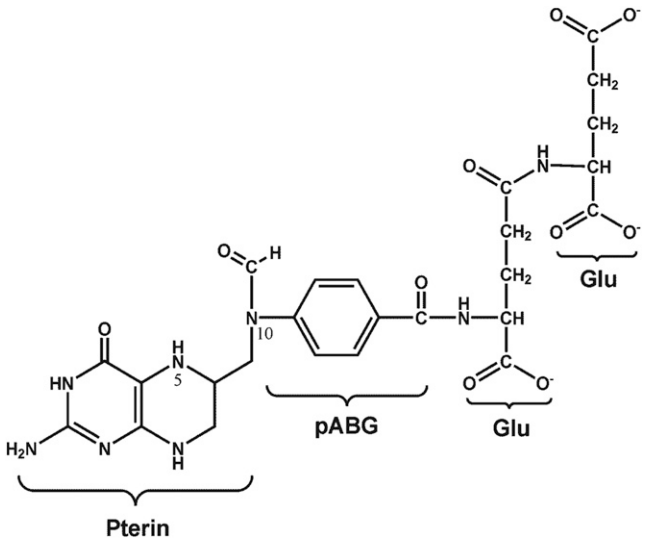

FIGURE 7 Structure of 10-formyltetrahydrofolate diglutamate. pABG, para-aminobenzoylglutamate. Reproduced from reference 115 with permission.

and protein (including histones), which affect gene expression and DNA stability (138–141).

- Folate-dependent homocysteine remethylation and plasma homocysteine concentrations can be affected by both genetic variation as well as other B-vitamin nutrient deficiencies including vitamin B-12.
- Because the MTHFR-catalyzed generation of 5-methyltetrahydrofolate (5-methyl-THF) is irreversible in vivo, 5-methyl-THF accumulates in the cell and cannot be utilized during severe vitamin B-12 deficiency due to lack of MTR activity, resulting in a folate "methyl trap," which can impair purine and thymidylate de novo biosynthesis (142).

One-carbon metabolism in mitochondria. THF monoglutamates are transported into mitochondria by the mitochondrial folate transporter (143), where they constitute as much as 40% of total cellular folate (144, 145). Folate monoglutamates must be converted to THF polyglutamates to be retained in mitochondria and become a distinct cofactor pool that is not in equilibrium with THF polyglutamates in the cytosol (145). One-carbon metabolism in mitochondria is essential for glycine synthesis from serine, N-formylmethionine–transfer RNA synthesis for mitochondrial protein synthesis initiation and the generation of formate from the amino acids serine, glycine, dimethylglycine, and sarcosine for cytosolic one-carbon metabolism (120) (Figure 9). Mitochondria-derived formate traverses to the cytosol where it is a major source of one-carbon units for cytosolic one-carbon metabolism. More recently, mitochondria have been shown to synthesize dTMP for mitochondrial DNA replication (146). Whereas synthesis of dTMP for nuclear DNA replication is cell cycle regulated, mitochondrial DNA replication is not linked to cell cycle or nuclear DNA replication and occurs in proliferating and nonproliferating cells (147).

All mammalian cells, with the exception of RBCs, can convert serine to glycine and formate, whereas the generation of formate from glycine, sarcosine, and dimethylglycine is restricted to liver, kidney, and stem cells and other undifferentiated cell types. The disruption of glycine cleavage to formate, as well as the generation of formate from 10-formyltetrahydrofolate (10-formyl-THF) catalyzed by methylene-THF dehydrogenase (MTHFD) 1L, in mouse models and human subjects is associated with increased risk of NTD-affected pregnancies (148–150). The THF-dependent catabolism of amino acids generates 5,10-methylenetetrahydrofolate (5,10-methylene-THF), which is oxidized to 10-formyl-THF by the bifunctional enzymes MTHFD2 (151, 152) and MTHFD2L (153). MTHFD1L hydrolyzes 10-formyl-THF to formate in an ATP-generating reaction (154, 155). Formate traverses to the cytosol for cytosolic and nuclear one-carbon metabolism (120). There are no established biomarkers for impaired mitochondrial one-carbon metabolism.

Homeostatic control of metabolism/nutrient-nutrient interactions

Overview—homeostatic controls of one-carbon metabolism. Research over the past ~10 y has shown that strong homeostatic controls exist to reduce fluctuation in folate-dependent metabolic processes (8, 115). Were it not for homeostatic controls, the rates of many of these biochemical reactions would be very sensitive to fluctuation in dietary intakes of folate, vitamins B-6 and B-12, and choline and to postprandial fluctuations in cellular concentrations of macronutrient substrates such as serine, glycine, and methionine. Elements of folate homeostasis are described in Table 11. These regulatory processes have been investigated by mathematical modeling studies (127, 156–160) and by many biochemical and genetic approaches. Controlled nutritional studies that use stable-isotopic tracers also have provided evidence of strong homeostatic regulation (161–167). Severe deficiency of folate and vitamin B-12 as well as genetic disorders clearly lead to impaired homeostasis of one-carbon metabolism with reduced thymidylate and purine synthesis, impaired homocysteine remethylation, and decreased SAM:SAH ratio associated with reduced methylation capacity.

TABLE 10 Essentials of folate absorption and metabolism[1]

- THF polyglutamates are the form of the vitamin present in cells and in food from natural sources.
- THF polyglutamates must be hydrolyzed to THF monoglutamates in the gastrointestinal tract before absorption across the intestinal epithelium of the duodenum by the PCFT (116).
- THF monoglutamates circulate in serum and are transported into cells through either the reduced folate carrier or through receptor-mediated endocytosis of the folate receptors (117, 118).
- Intracellular THF monoglutamates are processed into functional metabolic cofactors through the re-establishment of the polyglutamate peptide (119).
- The glutamate polypeptide is essential to retain the vitamin within cells and to increase its affinity for folate-dependent enzymes (114).
- Folic acid, a chemically stable and fully oxidized pro-vitamin that is found in fortified foods and vitamin supplements
  - is readily transported across the intestinal epithelium by PCFT,
  - must be reduced to THF by the enzyme dihydrofolate reductase, and
  - converted to a polyglutamate form to function as a metabolic cofactor.
- There are 5 one-carbon substituted forms of THF cofactors in cells. The one-carbon moiety is covalently attached at the N5 and/or N10 position of the cofactor at the oxidation level of formate (e.g., 10-formyl-THF), formaldehyde (5,10-methylene-THF) or methanol (5-methyl-THF) (Figure 7).
- Intracellular THF cofactors function as a family of metabolic cofactors that chemically activate and either accept or donate single carbons for a network of interconnected metabolic pathways referred to as one-carbon metabolism (114, 120) (Figure 8).

[1] PCFT, proton coupled folate transporter; THF, tetrahydrofolate.

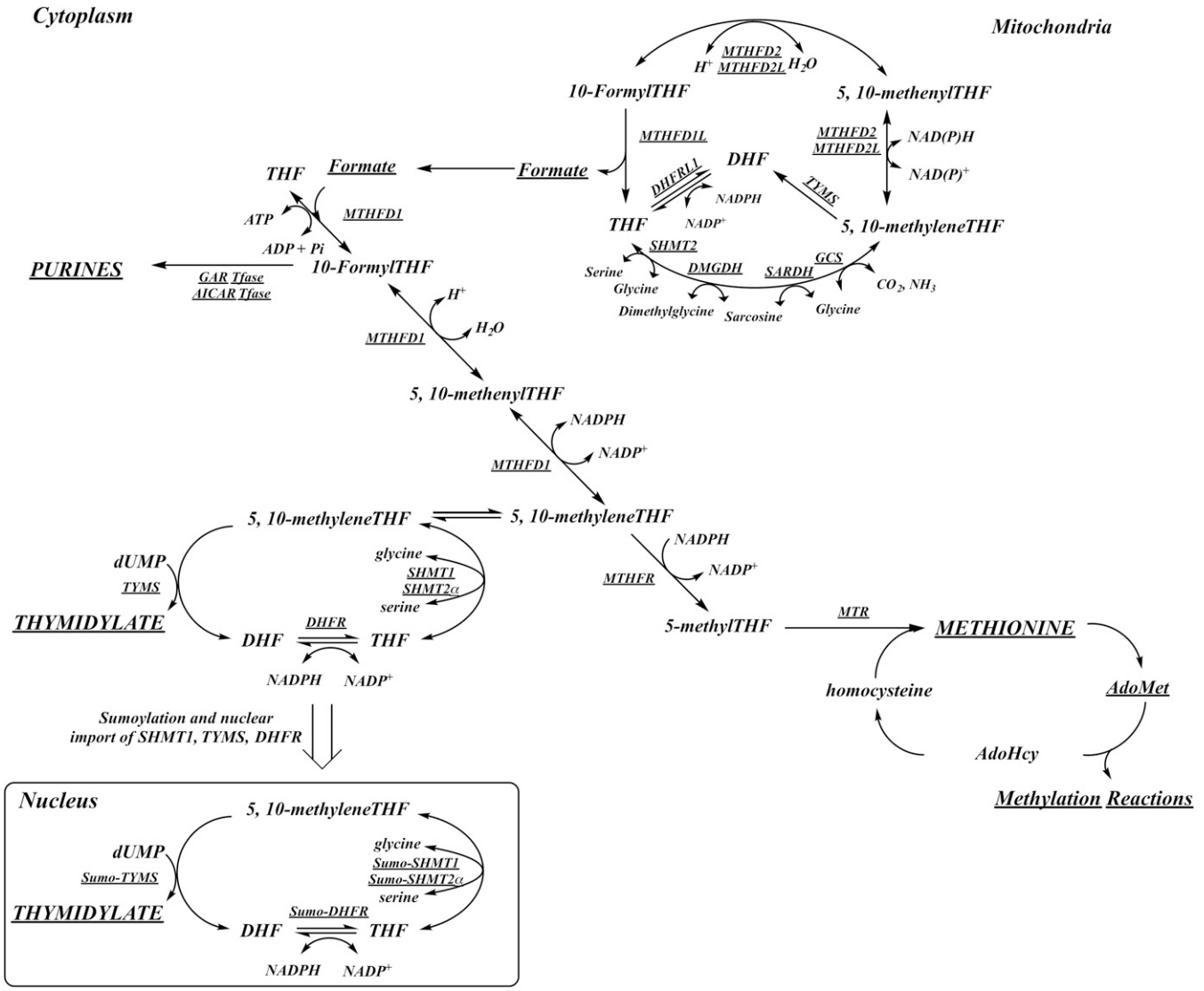

FIGURE 8 Folate- and vitamin B-12–mediated one-carbon metabolism. One-carbon metabolism is required for the synthesis of purines, thymidylate (dTMP), and methionine. The hydroxymethyl group of serine is a major source of one-carbon units, which are generated in the mitochondria in the form of formate via SHMT2 or in the cytosol through the activity of SHMT1 or SHMT2α. Mitochondria-derived formate can enter the cytoplasm and function as a one-carbon unit for folate metabolism. The synthesis of dTMP occurs in the nucleus and mitochondria. At the S phase, the enzymes of the thymidylate (dTMP) synthesis pathway undergo SUMO-dependent translocation to the nucleus. The remethylation of homocysteine to methionine by MTR requires vitamin B-12. The one-carbon is labeled in bold type. The inset shows the thymidylate synthesis cycle, which involves the enzymes SHMT1, SHMT2α, TYMS, and DHFR. AdoHcy, S-adenosylhomocysteine; AdoMet, S-adenosylmethionine; AICAR Tfase, glycinamide ribonucleotide transformylase and aminoimidazolecarboxamide ribonucleotide transformylase; DHF, dihydrofolate; DHFR, dihydrofolate reductase; dTMP, deoxythymidine monophosphate; dUMP, deoxyuridine monophosphate; GAR Tfase, glycinamide ribonucleotide transformylase; GCS, glycine cleavage system; MTHFD1, methylenetetrahydrofolate dehydrogenase 1; MTHFR, methylenetetrahydrofolate reductase; MTR, methionine synthase; SARDH, sarcosine dehydrogenase; SHMT, serine hydroxymethyltransferase; Sumo, small ubiquitin-like molecule; THF, tetrahydrofolate; TYMS, thymidylate synthase. Adapted from reference 115 with permission.

Other nutrient linkages with folate. As outlined above, folate's role in a myriad of critical biochemical pathways occurs in close association with other essential nutrient cofactors. The potential for interactions among these nutrients demands an appreciation of these interrelations and their implications. **Table 12** summarizes the nature of these interactions.

Specific considerations about absorption and metabolism of folic acid. As discussed earlier (Table 10), folic acid is not naturally found in food but is the more stable form of the vitamin used in food fortification and supplements. In light of the extent of its use, a brief review of essential components of folic acid absorption and metabolism is warranted, particularly in light of unknown implications of circulating free folic acid. Most of the folic acid absorbed in the gut is rapidly converted to reduced folates, primarily 5-methyl-THF during its passage across the intestinal mucosa and during first-pass metabolism in the liver. Some folic acid may appear in the peripheral circulation, the amount depending on the dose. Folate retention by tissues requires their conversion to long-chain-length polyglutamate forms. Folic acid and 5-methyl-THF are poor substrates for conversion to retainable polyglutamates. Incomplete conversion to polyglutamates by tissues such as intestinal mucosa and liver leads to the release of folate, mainly as methylfolate, into plasma. The utilization of folic acid differs from that of dietary folates primarily in that it has to be reduced

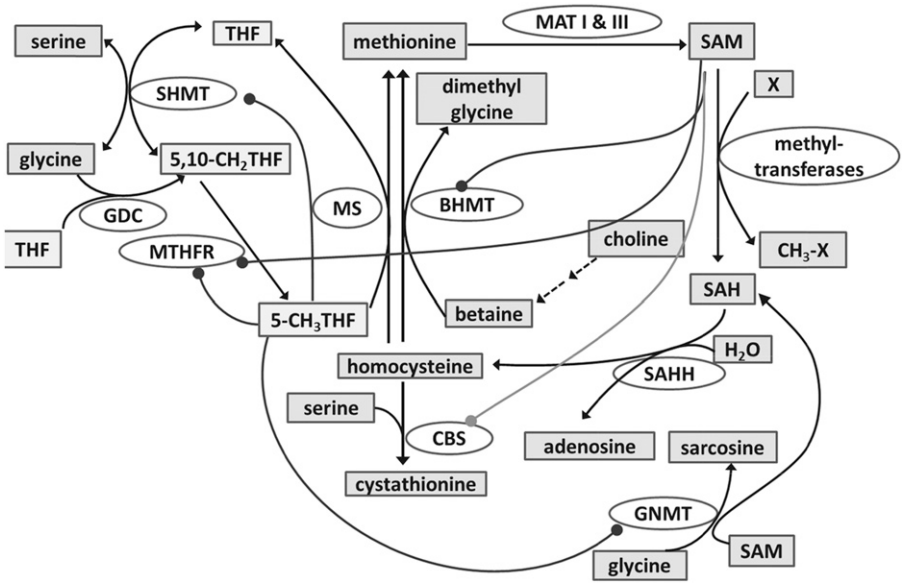

FIGURE 9 Schematic representation of one-carbon metabolic pathways and their homeostatic regulation. Black lines designate enzymatic reactions. Light lines with rounded ends designate stimulation, and dark lines with rounded ends indicate inhibition. BHMT, betaine:homosysteine methyltransferase; CBS, cystatione-β-synthase; GDC, glycine decarboxylase complex; GNMT, glycine N-methyltransferase; MAT, methionine adenosyltransferase; MS, methionine synthase; MTHFR, methylenetetrahydrofolate reductase; SAH, *S*-adenosylhomocysteine; SAHH, S-adenosylhomocysteine hydrolase; SAM, *S*-adenosylmethionine; SHMT, serine hydroxymethyltransferase; THF, tetrahydrofolate; X, indicates DNA, protein, or other compound involved in methylation reaction. Adapted from reference 127 with permission.

to THF via dihydrofolate (DHF) by DHF reductase (DHFR) (**Figure 10**). The first step, the reduction of folic acid to DHF, is quite slow and may be influenced by individual variations in DHFR activity (221). Folic acid would be poorly transported by many peripheral tissues. The limited peripheral tissue uptake may be explained by folic acid's very poor affinity for the RFC transporter, so transport would primarily be into tissues that express the proton coupled folate transporter (PCFT) and the limited number of tissues that express folate receptors (membrane-associated FBPs). The clearance of folate from plasma is very fast. After a single oral dose of folic acid, plasma folate (which would be mainly 5-methyl-THF) peaks after ~2 h and then falls, with a mean residence time of ~10 min (222). Plasma folate concentrations decrease with a similar half-life after an intravenous injection of folic acid (223). This rapid clearance indicates that fasting plasma folate concentrations primarily reflect reduced folates released by tissues. Why unmetabolized folic acid should still be present in plasma after fasting is less clear.

The concentrations of unmetabolized folic acid in fasting plasma are very low, representing at most a small percentage of total folate, and, somewhat unexpectedly, are poorly correlated with total folate (7, 92). These folic acid concentrations are very similar to those reported for soluble FBP in serum (224, 225). Folic acid has a very high affinity for FBP and binding to this protein would explain the persistence of low concentrations of folic acid in plasma in the fasted state. Human serum contains primarily FBP-γ, derived from neutrophil granulocytes, and some FBP-α, with total concentrations ranging from 0.5 to 1.5 nmol/L (225). Early studies, before the identification of FBP, indicated a high-affinity folic acid binder in serum that was increased in folate deficiency (up to 1 nmol/L), pregnancy, and in some cancers (226). These early studies probably measured unligated FBP rather than total binding capacity. There is little known about conditions that influence the concentrations of soluble plasma FBP.

As stated above, the Folate Expert Panel is not aware of any toxic or abnormal effects of circulating folic acid. Folic acid has been added to supplements for many years and in larger amounts than are obtained by food fortification in the United States without any apparent adverse effects.

Clearly, the family of folates is intimately and inextricably involved in numerous biological systems with significant implication for health and disease. As we learn more about these interrelations the need for better tools to assess folate status assumes even greater importance. The following section on folate biomarker overview summarizes the Folate Expert Panel's evaluation of the currently available biomarkers of folate covering a range of uses.

Folate Biomarker Overview

The usefulness, advantages, disadvantages, and analytical considerations for the folate priority biomarkers (serum, RBC folate, and homocysteine) have been summarized for all users by the Folate Expert Panel (**Table 13**). Later sections and Supplemental Table 1 include specific details for the priority biomarkers identified by the Folate Expert Panel as being most useful for the range of uses covered by the BOND community.

Biomarker-Specific Issues

This section is an overview of the conclusions of the Folate Expert Panel with regard to those biomarkers that were deemed

TABLE 11 Homeostatic controls of folate metabolism[1]

- SAM, an allosteric activator of cystathionine β-synthase and an allosteric inhibitor of MTHFR.
- 5-Methyl-THF (in polyglutamyl form), which inhibits GNMT and the use of SAM and glycine to produce sarcosine (N-methylglycine) and SAH.
- Homeostatic regulation also occurs under conditions of high folate intake and the accompanying high intracellular folate concentration.
- Many of the folate-dependent enzymes also exhibit inhibition by nonsubstrate polyglutamyl forms of THFs such as THF, 5-methyl-THF, and 5-formyl-THF (128–130).
- Shifts in the concentration of certain metabolites tend to maintain flux through metabolic pathways. These concurrent effects strongly support homeostasis (Figure 9).

[1] GNMT, glycine N-methyltransferase; MTHFR, methylenetetrahydrofolate reductase; SAH, *S*-adenosylhomocysteine; SAM, *S*-adenosylmethionine; THF, tetrahydrofolate.

TABLE 12 Summary of folate: nutrient interactions[1]

Nutrient	Implicated enzymes/metabolic pathways	Key features of interactions	Effect of nutrient deficiency on folate biomarkers
Vitamin B-12 (cobalamin)	• Vitamin B-12 (as methylcobalamin) serves as a coenzyme in the methionine synthase reaction. • Thus, vitamin B-12 is necessary for the methionine synthase–based conversion of 5-methyl-THF to THF and subsequent entry into other phases of one-carbon metabolism. • Another proposed aspect of vitamin B-12–dependent homeostasis involves translational regulation of methionine synthase expression (168). • Vitamin B-12 serves in the form of adenosylcobalamin as a coenzyme for methylmalonyl-CoA mutase for the conversion of methylmalonyl-CoA to succinyl-CoA. The methylmalonyl-CoA pathway is involved in the catabolism of branched-chain amino acids and odd-chain FAs and supports the TCA cycle by supplying succinyl-CoA.	• Via its role in methionine synthase, vitamin B-12 is intimately associated with the function of folate-dependent one-carbon metabolism needed for homocysteine regulation, cellular methylation reactions, and nucleotide synthesis. • Insufficiency of vitamin B-12 leads to an accumulation of methylmalonyl-CoA, which undergoes hydrolysis to MMA, which provides a functional biomarker of vitamin B-12 deficiency (169). • Despite the elevation of MMA and homocysteine due to vitamin B-12 deficiency, little is known about the functional impact of the changes in flux. • Severe vitamin B-12 deficiency, as occurs in untreated pernicious anemia, perturbs homoeostasis of one-carbon metabolism through reduced flux of the MS reaction. • Because the only metabolic function of 5-methyl-THF is as a substrate in the MS reaction for homocysteine remethylation, reduced MS activity causes 5-methyl-THF to accumulate in the ``methyl trap´´ (170, 171). • Because 5-methyl-THF monoglutamate is a poor substrate for elongation by folate polyglutamate synthetase, the accumulated 5-methyl-THF readily diffuses from the cell, leading to a progressive reduction in in vivo folate pool size and thus indirect induction of intracellular folate deficiency.	• Because vitamin B-12 is required for folate retention in developing RBCs, RBC folate concentrations are dependent on vitamin B-12 as well as folate availability, and low concentrations may reflect vitamin B-12 deficiency as well as folate deficiency. • A case study of a patient with vitamin B-12 deficiency and the *MTHFR* C677T SNP indicated higher Hcy, lower RBCs, and a higher percentage of 5-methyl-THF in RBCs before as compared with after vitamin B-12 supplementation, providing evidence of ``methyl trapping´´ in human vitamin B-12 deficiency (172) • In infants, vitamin B-12 rather than folate status predicts Hcy (173, 174), and high serum folate is attributed to methyl folate trapping as demonstrated by reduction in both Hcy and serum folate after vitamin B-12 supplementation (173). • Likewise, adults with pure vitamin B-12 deficiency (pernicious anemia) also have falsely increased serum folate concentrations, which return to baseline only after vitamin B-12 replacement (175–178). • Insufficiency of vitamin B-12, folate, or both leads to an accumulation of homocysteine. In population groups who consume folic acid–fortified foods or folic acid supplements, Hcy is considered a more reliable biomarker of vitamin B-12 status than of folate status (179).
Iron	• Both nutrients are intimately involved in the erythropoiesis process. • Heme carrier protein 1, also called the PCFT, constitutes a physiologic intersection of iron and folate because PCFT serves as a major intestinal folate absorption mechanism while also serving as a heme transporter (115). • Ferritin has been reported to catalyze a single-turnover cleavage of 5-formyl-THF to a pterin and para-aminobenzoylglutamate; this has been proposed as a mechanism contributing to folate turnover (180, 181). • Heavy-chain ferritin also has been shown to increase SHMT expression and favor thymidylate synthesis in cultured cells (181), an effect shown to be regulated by a ferritin-responsive internal ribosome entry site that is not dependent on iron availability (182).	• Iron is not directly involved in folate metabolism or metabolic functioning associated with one-carbon metabolism. However, metabolic and physiologic interactions exist between iron and folate. • Insufficient iron impairs heme production, whereas folate and/or vitamin B-12 deficiency leads to impaired nucleotide synthesis needed for the maturation of the RBCs, which leads to macrocytic anemia. • The observed greater incidence of neutrophil hypersegmentation in iron deficiency anemia concurrent with folate and/or vitamin B-12 deficiency implies interactive effects on the erythropoietic process (183, 184). • PCFT expression analysis suggests that the PCFT also serves along with other folate transporters in delivering folate to tissues such as the placenta and brain (185). • Overexpression of ferritin in cultured cells lowered the intracellular folate concentration, although alternative mechanisms of folate breakdown and turnover do exist (180, 186). • Oxidative stress associated with iron overload may also be proven to contribute to nonspecific oxidative turnover of folates.	• Iron and folate deficiencies often occur simultaneously, with the prevalence most common among pregnant women and individuals suffering from gastrointestinal disorders and intestinal parasites (187). • Some investigators suggest that iron deficiency can alter folate metabolism, inducing a functional deficiency, whereas others propose that the 2 conditions develop independently (187, 188). • The impact of iron status on folate biomarkers may depend on contributing physiologic influences and severity of iron and folate deficiencies. • Patients suffering from iron deficiency due to intestinal parasites and gastrointestinal disorders often have no evidence of impaired folate intake or absorption but present with megaloblastic dysplasia and other signs of compromised folate status, including low serum folate but normal or elevated RBC folate (187, 188). These symptoms, caused by impaired DNA synthesis, can be reversed by iron repletion alone (188). • In contrast, treatment of uncomplicated iron deficiency anemia with iron alone may ``unmask´´ folate deficiency, manifesting morphologic and hematologic changes, despite normal serum and RBC folate concentrations (187). • In rodent models, it is well established that maternal iron deficiency decreases milk folate content and retards neonatal growth, independent of maternal folate status (188). Although the impact of iron deficiency on folate status during lactation has not been well studied in humans, a study from Mexico indicated that milk folate secretions were not impaired during maternal iron deficiency associated with altered neonatal growth (189).

(Continued)

TABLE 12 *Continued*

Nutrient	Implicated enzymes/metabolic pathways	Key features of interactions	Effect of nutrient deficiency on folate biomarkers
Choline	• Does not directly participate in folate metabolism, but interactions exist which affect folate metabolism and homeostatis. • FAD-dependent choline dehydrogenase catalyzes oxidation of choline to betaine aldehyde, which is further oxidized by NAD-dependent betaine aldehyde dehydrogenase to form betaine (trimethylglycine). • Betaine acts as a methyl donor for BHMT, which catalyzes a folate-independent alternative remethylation mechanism to form methionine and dimethylglycine. • In contrast to widely distributed methionine synthase, BHMT exists only in the liver, kidney, and the lens of the eye. • Dimethylglycine is further oxidized with concurrent transfer of the methyl groups to the folate cycle by the sequential action of NAD-dependent dimethylglycine dehydrogenase forming N-methylglycine (sarcosine) and sarcosine dehydrogenase forming glycine. Both of these utilize THF as the one-carbon acceptor to form 5,10-methylene-THF.	• Although the oxidation pathway is intimately linked to one-carbon metabolism, quantitative aspects of such interactions with folate homeostasis remain unclear. • Because adequate choline intake provides a supply of betaine to allow non–folate-dependent homocysteine remethylation, choline can partially compensate in maintaining methylation despite dietary folate insufficiency (190). • Folate, but not choline, modified NTD risk in an *Shmt* knockout mouse model (33), whereas dietary choline intake and variants in choline metabolizing genes associate with NTDs in human populations (191–193) and the amount of folate intake has been shown to modulate choline metabolism in mice (194). • Choline intake appears to be an ameliorative factor affecting human NTD risk, but the literature is not consistent (191, 192, 195). • Common genetic polymorphisms of one-carbon metabolism also influence the susceptibility to effects of low choline intake (196) and men with the *MTHFR* 677TT (vs. 677CC) genotype use more choline as a methyl donor (197). The effect of the *MTHFD1* polymorphism is stronger than that of *MTHFR* and other SNPs in the folate pathway (196). • In controlled studies, healthy young adults exhibited no evidence of impaired folate-dependent remethylation or total remethylation during either mild folate restriction (164, 198) or vitamin B-6 restriction (162), and folate-dependent remethylation (i.e., via methionine synthase) accounted for nearly all of the whole-body remethylation flux in both studies. • In contrast, population studies indicated that betaine may be a significant determinant of plasma homocysteine (199).	• Dietary choline inadequacy may also affect biomarkers of folate status (200). • Short-term (<3 wk) administration of a choline-deficient diet to rats depleted hepatic folate content, decreased tissue concentrations of SAM, diminished global DNA methylation, and/or increased homocysteine (201–205). • In chronically (12 mo) choline-deficient rats, hepatic total folate concentration was not altered; however, polyglutamation was elevated, possibly due to increased conservation of the folate coenzymes (206). • In humans, dietary choline inadequacy elevated plasma homocysteine (after a methionine load) but did not influence circulating concentrations of folate (203).
Vitamin B-6 (pyridoxine)	PLP, the coenzyme form of vitamin B-6, functions as a cofactor in: • the glycine cleavage system and SHMT, both of which mediate the supply of 5,10-methylene-THF • the transsulfuration pathway enzymes cystathionine β-synthase and cystathionine γ-lyase, which mediate the disposal of homocysteine and synthesis of cysteine.	• Regulatory mechanisms exist to buffer the components of one-carbon metabolism against the effects of marginal vitamin B-6 deficiency, primarily via increases in substrate concentrations for key PLP-dependent enzymes as a result of reduced activity of the glycine cleavage system, SHMT, and cystathionine γ-lyase associated with reduced intracellular PLP availability (163, 167, 207). • Consequently, the supply of 5,10-methylene-THF, 10-formyl-THF, and 5-methyl-THF remain adequate to support the demands for nucleotide synthesis and methylation over a wide range of vitamin B-6 intakes, whereas cellular and plasma homocysteine are only weakly affected by vitamin B-6 insufficiency (208, 209). • Homocysteine and glycine are relatively weak biomarkers of vitamin B-6 status, and cystathionine is a more sensitive functional indicator of low vitamin B-6 status (162, 165, 210).	• Vitamin B-6 deficiency weakly affects plasma homocysteine concentration (208). • Cell culture studies and mathematical modeling suggest that the proportions of formyl-, methyl-, methylene-, and unsubstituted THFs can be altered by vitamin B-6 deficiency (211).

(Continued)

TABLE 12 *Continued*

Nutrient	Implicated enzymes/metabolic pathways	Key features of interactions	Effect of nutrient deficiency on folate biomarkers
Riboflavin (vitamin B-2)	FAD as a coenzyme for MTHFR	• Insufficient riboflavin can impair one-carbon metabolism, as indicated by elevated plasma homocysteine, particularly under conditions of low folate status. • The effects of low riboflavin status on one-carbon metabolic function are most prominent in individuals having the *MTHFR* 677TT genotype (212). • Molecular studies show that decreased activity of the variant enzyme is caused by reduced affinity for its FAD cofactor (213) • The molecular basis of this interaction involves greater susceptibility of the variant form of *MTHFR* to loss of activity due to the facile dissociation of FAD under conditions of low folate status (214). • Functionally, this interaction can influence blood pressure, although the precise mechanism remains to be established (215). • Hypertensive patients with the *MTHFR* 677TT genotype appear to be highly responsive to systolic and diastolic blood pressure lowering with riboflavin intervention at doses of just 1.6 mg/d, an effect that is independent of the number and type of antihypertensive drugs being taken (216, 217).	• When folate or riboflavin status is low, individuals with the homozygous mutant TT genotype typically present with low serum and RBC folate (5, 218), along with elevated homocysteine concentrations (219). • Riboflavin supplementation of individuals with the TT genotype appears to stabilize the variant enzyme in vivo, restoring 5-methyl-THF and thus lowering homocysteine concentrations (220).

[1] BHMT, betaine-homocysteine methyltransferase; MMA, methylmalonic acid; MS, methionine synthase; MTHFD1, methylenetetrahydrofolate dehydrogenase 1; MTHFR, methylenetetrahydrofolate reductase; NTD, neural tube defect; PCFT, proton coupled folate transporter; PLP, pyridoxal-*P*; SAM, *S*-adenosylmethionine; SHMT, serine hydroxymethyltransferase; SNP, single nucleotide polymorphism; TCA, tricarboxylic acid; THF, tetrahydrofolate.

to be best suited for wide application across the uses targeted by the BOND program. The material presented in this and the subsequent section on assay-specific queries for each of the priority biomarkers selected represents responses to a specific set of issues derived from an outline developed by the BOND Secretariat (**Supplemental Table 2** and on Tier 1 of the BOND website). This approach was used by each of the nutrient-specific BOND expert panels. The issues outlined were intended to *1*) provide a common format for the work of all BOND nutrient expert panels and *2*) address core issues that might be important to the range of user groups to be served by the BOND. On the basis of the quality and strength of the extant evidence, the Folate Expert Panel selected the following priority biomarkers for expanded coverage:

- serum folate concentration;
- RBC folate concentration; and
- plasma homocysteine concentration.

The characteristics and technical considerations for each of these biomarkers are listed in Supplemental Table 1. The following sections provide some general information about these biomarkers. In addition to the priority biomarkers, the Folate Expert Panel has included a summary of issues pertaining to dietary/supplemental folate/folic acid exposure methodology as an essential component of a comprehensive coverage of folate assessment.

Dietary folate/folic acid intake assessment

As highlighted in earlier sections, folate (THF polyglutamate) is the predominant form of the vitamin naturally found in food, whereas folic acid is the form of the vitamin used in food fortification/enrichment and dietary supplements. Total folate encompasses all dietary and supplemental exposure to folate and folic acid. Both dietary folate and total folate should be calculated by using DFEs to account for the differential bioavailablity of the natural and synthetic forms (*55*). The following sections cover the core elements of current approaches to dietary folate assessment.

Dietary/supplement intake assessment

Assessment of the diet can be done on a short- or long-term basis. Short-term instruments aim to capture data on recent or current diet, whereas long-term instruments aim to capture dietary data over a longer period of time. Methods used to assess dietary folate intake rely on the same assessment approaches typically used to assess overall nutrient intake, including food records, 24-h recall, and FFQs (227–231).

Dietary supplement use can be measured by using the same techniques as dietary assessment of foods: a record or diary, 24-h recalls, or a frequency-based instrument. No validation studies exist to compare the different methods for the assessment of supplement use. However, because dietary supplement use can be habitual (daily) or episodic (contextual), it may be ideal to use a frequency-based questionnaire to obtain a longer time period. Data from the NHANES 2003–2006 estimated that 43% of adults ($\geq$19 y) and 28% of children (1–18 y) use a dietary supplement product that contains folic acid, most often a multivitamin/mineral (MVM) product (232, 233). In adults, but not in children, those who use a dietary supplement tend to have significantly higher intakes of folate from food sources alone (234, 235).

The Dietary Supplement Ingredient Database is a federally funded program to determine the analytically derived content of dietary supplements relative to the labeled amount. The Dietary

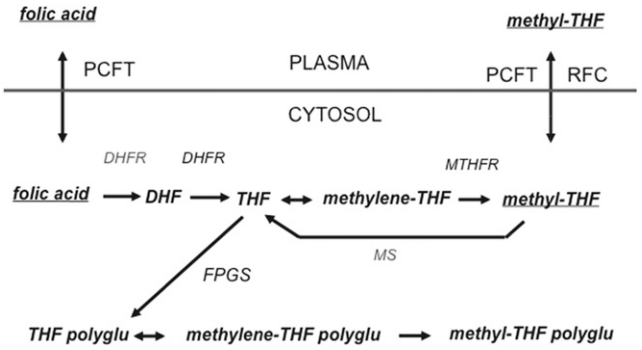

FIGURE 10 Transport of folic acid and 5-methyl-THF into tissues and their metabolism to retainable polyglutamate forms. DHF, dihydrofolate; DHFR, dihydrofolate reductase; FPGS, folylpolyglutamate synthase; MS, methionine synthase; MTHFR, methylenetetrahydrofolate reductase; PCFT, proton coupled folate transporter; polyglu, polyglutamate; RFC, reduced folate carrier; THF, tetrahydrofolate.

Supplement Ingredient Database uses a complex sampling program to ensure that products represent the US market for products. The folic acid content in children's and adults' MVM products was determined by using an MBA with the National Institute of Standards and Technology (NIST) standard reference material (SRM) 3280 to evaluate product content for folic acid. For both children (≥4 y) and adults, the most common label dose of folic acid in an MVM product is 400 μg, and the measured content was ~13% higher in the supplement than on the label. For products intended for children aged 1 to <4 y, the most common labeled dose of folic acid was 200 μg and the actual content was ~16% higher in the supplement than on the label.

Usual dietary and total intakes. Dietary recommendations are intended to be met over time (236). Many procedures have been described to adjust the dietary estimates from 24-h recalls to reduce within-person variation to produce usual intakes (237–241). To produce total usual intakes, it is recommended to first adjust the dietary folate estimates by using one of these methods and then add the daily estimate of folic acid from dietary supplements to the usual dietary intakes (242).

Food-composition tables. The most current national database for the folate/folic acid content of foods is the USDA National Nutrient Database for Standard Reference, release 25. The Standard Reference values are the basis for USDA Food and Nutrient Database for Dietary Studies values. The National Food and Nutrient Analysis Program is a federally funded research program to enhance the analytical estimates of the nutrient content of foods and dietary supplements. The folate content of foods is determined by a tri-enzyme microbiological procedure (243), which is appropriate for the estimation of total folate content, not the specific forms. To determine the amount of folic acid added to foods, an additional microbiological procedure without enzymes is used to estimate the amount of added folic acid; food folate then is assumed to be the remainder after the folic acid contribution is subtracted from the total (243). The DFE is then calculated by multiplying the amount of folic acid by 1.7 and adding that to the value for food folate. There are 7042 foods with values for total folate and 6381 foods with values for DFEs present in the Standard Release 25.

Measuring the folate/folic acid content in foods. Estimates of dietary intakes rely on the accuracy of the food-composition databases. Estimates of the folate and folic acid content of foods can be determined by MBAs and by LC . The MBA with enzyme extraction is unable to differentiate among the various forms of folate in foods. The LC techniques can differentiate among the folate forms but have been reported to have difficulties measuring all folates [summarized in (244)]. The variability in results obtained for folate/folic acid in foods can be attributed to agricultural variability, heat processing, pH, food matrix, extraction procedure, failure to account for all or different folate vitamers, incubation time, and number of enzyme extractions (244, 245).

Priority folate biomarkers

Serum folate concentration. The measurement of serum folate is the earliest indicator of altered folate exposure and reflects recent dietary intake (i.e., short-term status) (9). However, recurrent measures of serum folate in the same individual over time can reveal chronic folate deficiency. Serum folate is highly responsive to intervention with folic acid, with natural food folates typically resulting in a poorer serum folate response. Likewise, population data show that irrespective of whether they are from regions with mandatory or voluntary fortification, serum folate concentrations are highly reflective of exposure to folic acid, with the highest concentrations observed in persons who consume folic acid in both supplements and fortified foods (71, 246, 247).

RBC concentration. RBC folate is a sensitive indicator of long-term folate status, which represents the amount of folate that accumulates in RBCs during erythropoiesis, thereby reflecting folate status during the preceding 120 d (i.e., the half-life of RBCs) (248, 249). Moreover, RBC folate parallels liver concentrations (accounting for ~50% of total body folate) and is thus considered to reflect tissue folate stores (250). Similar to serum folate, RBC folate is highly responsive to intervention with folic acid, with natural food folates typically resulting in a poorer RBC folate response than folic acid at similar intervention amounts. Likewise, population data show that RBC folate concentrations are highly reflective of exposure to folic acid, with the highest concentrations observed in persons who consume folic acid in both supplements and fortified foods, in regions with mandatory or voluntary-only fortification (71, 99, 247).

Plasma homocysteine concentration. The measurement of plasma homocysteine provides a sensitive functional biomarker of folate status. As described in detail in the section on Folate Biology and Homeostasis, folate-mediated one-carbon metabolism is a network of 3 interdependent biosynthetic pathways including the remethylation of homocysteine to methionine. When the status of folate is low or deficient, plasma homocysteine is invariably found to be elevated. However, the folate-dependent remethylation of homocysteine to methionine is catalyzed by the vitamin B-12–dependent enzyme MTR and involves a number of other nutritional cofactors. Thus, plasma homocysteine is not a specific marker of folate status, because it will also be elevated with other B-vitamin deficiencies, lifestyle factors, renal insufficiency, and drug treatments (179, 251). In population groups who consume folic acid–fortified foods or folic acid supplements, homocysteine is considered to be a more reliable biomarker of vitamin B-12 than folate status (179).

Plasma homocysteine is inversely related to folate status (whether measured as serum or RBC folate). Plasma homocysteine is also highly responsive to intervention with folate, alone or in combination with the other methyl donors involved in

TABLE 13 Relative strengths and weaknesses of folate biomarkers[1]

Biomarker name	Population groups	Usefulness for purpose	Advantages	Disadvantages	Analytical considerations
Serum folate	All individuals	Measurement of serum folate provides information on the short-term folate status of the individual. Population data indicate that serum folate concentrations are highly reflective of exposure to folic acid, with the highest concentrations observed in persons who consume folic acid in both supplements and fortified foods.	Serum folate is the earliest indicator of altered folate exposure, will reflect recent dietary intake, and is highly responsive to intervention. Serum folate requires less processing at the time of blood collection vs. RBC folate.	Natural food folates typically result in a poorer serum folate response than does folic acid at similar intervention levels. The inconsistent use of cutoff values over time to assess the proportion of the population with low serum folate concentrations has led to confusion. Contrasting approaches to expressing dietary folate intakes makes any evaluation of adequacy of dietary folate intake in relation to biomarker status, or comparison of folate recommendations between countries, inherently complicated. The measurement of folate forms circulating in serum may further elucidate the role of folate vitamers relative to various health outcomes; however, to date, no cutoff values for either low or high concentrations or desirable ranges have been identified. Fasting blood samples are recommended when using serum folate to assess folate status.	Measurement of serum folate cannot be done in the field and requires at minimum a midlevel laboratory infrastructure with uninterrupted electrical power. Methods to assess serum folate have not yet reached the point where they produce sufficiently comparable results across methods or laboratories. Folate is the least stable of the B vitamins; careful sample handling and use of antioxidants are required to maintain sample integrity. Because RBCs contain much higher folate concentrations than serum, the presence of hemolysis will inflate serum folate values, regardless of technique used for measurement.
RBC folate	All individuals	RBC folate is a sensitive indicator of long-term folate status. RBC folate represents the amount of folate that accumulates in blood cells during erythropoiesis and reflects folate status during the preceding 120 d, i.e., the half-life of RBCs. RBC folate parallels liver concentrations (accounting for ~50% of total body folate) and is thus considered to reflect tissue folate stores. Population data indicate that RBC folate concentrations are highly reflective of exposure to folic acid, with the highest concentrations in persons who consume folic acid in both supplements and fortified foods.	RBC folate is highly correlated with habitual folate intake when the latter is expressed as DFEs.	Natural food folates typically result in a poorer RBC folate response than does folic acid at similar intervention levels. If the DFE conversion is not made, the relation between folate intake and status is weak. Contrasting approaches to expressing dietary folate intakes makes any evaluation of adequacy of dietary folate intake in relation to biomarker status, or comparison of folate recommendations between countries, inherently complicated. The measurement of folate forms present in RBCs may further elucidate the role of folate vitamers relative to various health outcomes; however, to date, no cutoff values for either low or high concentrations or desirable ranges have been identified.	Measurement of RBC folate cannot be done in the field and requires at minimum a midlevel laboratory infrastructure with uninterrupted electrical power. As for serum folate, methods to assess RBC folate have not yet reached the point where they produce sufficiently comparable results across methods or laboratories. The comparability of RBC folate methods is worse than that of serum folate methods. The measurement of RBC folate is even more complex than that of serum folate, because of the need to convert polyglutamates to monoglutamates before analysis.

(Continued)

TABLE 13 *Continued*

Biomarker name	Population groups	Usefulness for purpose	Advantages	Disadvantages	Analytical considerations
Hcy	All individuals	Measurement of plasma Hcy provides a sensitive functional biomarker of folate status.	When folate status is low or deficient, plasma Hcy is invariably found to be elevated. Plasma Hcy is highly responsive to intervention with folate, alone or in contribution with the other methyl donors involved in one-carbon metabolism: vitamin B-12, vitamin B-6, vitamin B-2, and betaine (or choline). Plasma Hcy responds within 3–4 wk of folate depletion (increases) and subsequent repletion (declines) in healthy subjects.	The remethylation of Hcy to methionine is catalyzed by the vitamin B-12–dependent enzyme MTR and involves a number of other nutritional cofactors. Thus, plasma Hcy is not a specific marker of folate status. It will also be elevated with other B-vitamin deficiencies, lifestyle factors, renal insufficiency, and drug treatments. In population groups who consume folic acid–fortified foods or folic acid supplements, Hcy is considered a more reliable biomarker of vitamin B-12 status than folate status.	Requires specialized laboratory equipment and trained technicians. Measurement of Hcy produces fairly comparable results across different method types Plasma Hcy is a very stable analyte as long as the plasma is separated from the RBCs within 1 h of blood collection (or within <8 h if the whole blood is kept on ice) (179).

[1] DFE, dietary folate equivalent; Hcy, total homocysteine; MTR, methionine synthase.

one-carbon metabolism: vitamins B-12, B-6, and B-2 (riboflavin) and betaine (or choline). Plasma homocysteine responds within 3–4 wk of folate depletion (increases) and subsequent repletion (declines) in healthy subjects (251). The fast response probably reflects that methyl groups for homocysteine remethylation are dependent on "small" folate pool(s) with a fast turnover rate (186).

Other biomarkers—general descriptions

Although the focus of this section is on a detailed coverage of the key issues specific to the priority biomarkers selected, the Folate Expert Panel concluded that it would be useful to provide brief summaries of those biomarkers that, although not chosen as priorities for immediate widespread use, nevertheless offer either *1*) some utility under defined circumstances or *2*) potential for eventual widespread application.

Serum folic acid. As discussed in earlier sections, unmetabolized folic acid in serum or plasma may be considered as a biomarker of exposure, status, function, and/or effect. The appearance and quantity of unmetabolized folic acid in circulation have been associated with folic acid exposure via fortified foods, dietary supplements, and a combination of both (7, 252, 253). Greater concentrations of unmetabolized folic acid are associated with higher serum folate concentrations (7, 254), suggesting that the amount of folic acid in blood is related to whole-body folate status. However, there is large variation in folic acid concentrations and the dose response relation between folic acid exposure and unmetabolized folic acid in circulation is not entirely clear. Any effect of unmetabolized folic acid in blood on cellular function and/or health remains to be elucidated.

Urinary folate/folic acid. Although not commonly used, in research settings 24-h urinary folate excretion in combination with serum or RBC folate can provide unique information about folate status and metabolism. Twenty-four-hour urinary folate excretion captures the rise and fall of circulating folate concentrations in response to feeding and fasting and thus may be considered an indicator of "average" folate exposure and status over that 24-h period (95). Although responsive to folate intake (255, 256), urinary folate excretion exhibits a large degree of inter- and intraindividual variability (257). Because of this variability and because little intact folate is excreted at doses up to the RDA, urinary folate would not be a sensitive biomarker for folate intake or status.

Urinary and serum para-aminobenzoylglutamate and para-acetamidobenzoylglutamate. The oxidative folate catabolites para-aminobenzoylglutamate (pABG) and para-acetamidobenzoylglutamate (apABG) are biomarkers of folate status and turnover. Although pABG and apABG are found in blood, urinary pABG and apABG are studied most often. Urinary pABG and apABG reflect turnover in endogenous folate pools (258). Total urinary catabolite excretion is positively correlated with serum total folate, RBC folate, and folate intake (259); however, it is not as sensitive to folate intake as urinary folate excretion (260), serum folate (261), and plasma homocysteine (261).

Genomic biomarkers of folate status. The following genomic biomarkers for folate status are used exclusively in research settings and are associated with problems due to lack of specificity, equivocal results, and methodologic challenges.

DNA cytosine methylation. Folate plays an important role in DNA metabolism because it is required as a methyl donor for

the synthesis of methionine and SAM and for the synthesis of phosphorylated nucleotides such as deoxythymidine triphosphate (dTTP) (262). SAM is required as a methyl donor for the maintenance or induction of cytosine methylation, which is essential for silencing of genes or structural integrity of specific regions of the chromosomes (131, 263). When SAM is depleted and/or the enzyme DNA methyltransferase is defective, it becomes increasingly probable that the maintenance of DNA methylation is compromised, leading to hypomethylation of cytosine and structural changes in chromatin (264). Some studies suggest that global DNA methylation status is reduced when folate is deficient and plasma homocysteine is elevated (265–272). However, folate depletion-repletion studies have produced differing results with regard to DNA methylation (273–276).

Uracil misincorporation into DNA. Uracil content in DNA has been explored as a biomarker of folate deficiency. dTTP synthesis requires adequate amounts of 5,10-methylene-THF to donate methyl groups to deoxyuridine monophosphate (deoxyuridylate; dUMP). If 5,10-methylene-THF is limiting as a substrate of thymidylate synthase (TYMS) and/or TYMS is defective, dUMP accumulates, the deoxyuridine triphosphate (dUTP):dTTP ratio increases, and it becomes more probable that uracil is incorporated into DNA instead of thymidine during DNA synthesis (131, 262). The conversion of dUMP to dTTP is entirely dependent on one-carbon donation by 5,10-methylene-THF. In contrast, methylation of CpG is dependent on the supply of SAM and its precursor methionine, the synthesis of which is influenced not only by folate but also by the availability of choline and methionine (131, 262). Therefore, uracil accumulation into DNA may be more specific than DNA cytosine methylation as a potential genomic biomarker of folate status.

Micronuclei. Excessive uracil incorporation into DNA and hypomethylation of pericentromeric DNA can lead to lagging chromosomes or chromosome fragments that form micronuclei (14, 131, 132, 277). Micronuclei have the same morphologic features as normal nuclei with the exception that they are much smaller, usually one-third to one-sixteenth in diameter. Cross-sectional studies have shown that micronuclei in lymphocytes or in erythrocytes are inversely associated with dietary folate intake and/or RBC folate and positively with plasma homocysteine (132, 278–285). Four intervention studies (284–287) reported on the effect of folic acid supplementation on micronuclei in lymphocytes, 3 of which showed a significant reduction in micronuclei frequency.

Because micronuclei frequency is also associated with intakes of other vitamins and minerals (279, 282) and exposure to lifestyle or environmental genotoxins (288, 289), it is not specific to folate status and therefore cannot be used on its own as an indicator of folate deficiency. It is, however, very sensitive to folate deficiency within the physiologic range and in combination with uracil and DNA methylation measurements has the potential to provide a reliable assessment of genome pathology resulting from inadequate folate. It is also important to note that it is now possible to score micronuclei automatically and reliably by using a wide range of image cytometry platforms (290, 291), making this technique amenable to mass screening.

Assay-Specific Queries

The previous section covered the physiologic aspects of each biomarker reviewed by the Folate Expert Panel. This section is focused principally on the priority biomarkers and in particular includes the following:

- an overview of the analytical methods;
- tools used to ensure the quality of the biomarker measurement; and
- a coverage of preanalytical considerations relevant to sample collection, processing, and storage.

Because the technical issues for serum and RBC folate are generally similar, these 2 biomarkers are presented together, pointing out differences where appropriate. Information on plasma homocysteine is presented separately.

Measurement of serum and RBC folate

Introduction. The essentials of folate as pertains to its measurement in serum/RBCs are outlined in **Table 14**. Folates are susceptible to interconversions and oxidative degradation (**Figure 11**) (295, 296), and their oxidation and breakdown products can also be found in serum. Some of these products no longer exhibit folate activity, yet one needs to ensure that they do not interfere with the measurement of folate vitamers. Understanding folate interconversions and degradations helps to better understand what different methods measure.

- 5-Methyl-THF is relatively stable at different pH values, with and without heat treatment, but it can undergo mild reversible oxidation to 5-methyl-5,6-dihydrofolate (5-methyl-5,6-DHF). This compound undergoes spontaneous cleavage of the C9-N10 bond in acidic solution (297). Under prolonged or severe oxidative conditions, 5-methyl-THF or 5-methyl-5,6-DHF can convert to 4α-hydroxy-5-methyl-THF, an intermediate product also called 4α-hydroxy-5-methyltetrahydrofolate (hmTHF) (298). In the absence of a reducing agent, hmTHF undergoes structural rearrangement to form a pyrazino-s-triazine derivative, which is no longer biologically active (299). This stable oxidation product of 5-methyl-THF is also known as pyrazino-s-triazine derivative of hmTHF (MeFox) (293, 300).
- Under heat and/or low pH conditions, THF can oxidize to folic acid via the highly unstable DHF intermediate. Assays measuring unmetabolized folic acid in serum should therefore verify that this compound is not an artifactual result of THF oxidation due to analytical steps. THF and DHF can also degrade to the biologically inactive catabolite pABG (301).
- There is a pH-driven equilibrium between 3 major formyl-folate vitamers: 10-formyl-THF is present at neutral and alkaline pH, folinic acid (5-formyl-THF) at slightly acidic pH, and 5,10-methenyl-THF at acidic pH. Heat accelerates the conversion of 10-formyl-THF to 5-formyl-THF at slightly acidic pH. Both 5-formyl-THF and 10-formyl-THF cyclize to 5,10-methenyl-THF at acidic pH. If the pH changes from acidic to slightly acidic or neutral, the equilibrium is pushed toward 10-formyl-THF (302).
- 10-Formyl-THF is readily oxidized to 10-formyl-folic acid (via 10-formyl-DHF), a stable form of the vitamin that was shown to exhibit folate activity (303).
- 5,10-methylene-THF is only stable at pH 10 and dissociates to formaldehyde and THF at physiologic pH values. On the other hand, heating samples in ascorbate-containing buffers in the absence of other reducing agent can also cause THF conversion to 5,10-methylene-THF via formaldehyde generated by the breakdown of ascorbate (295).

Analytical methods. Over the past 50 y, methods to assess serum and RBC folate concentrations have been continuously improved; however, they have not yet reached the point where they produce sufficiently comparable results across methods or laboratories. A comprehensive review article of analytical approaches and related issues (9) and 2 articles discussing issues pertaining to folate measurements in NHANES (4, 304) provide a wealth of information on this topic. **Table 15** is a summary of the 3 main method types and their advantages and disadvantages.

MBAs. Historically considered the "gold standard" measurement procedure for serum and RBC total folate because it "fully measures the multiple forms of folate species that exhibit folate vitamin activity and does not measure folate species that lack vitamin activity" (304), the underlying principle of the MBA is that a folate-dependent microorganism, namely *Lactobacillus rhamnosus* (formerly called *Lactobacillus casei*), grows proportionally to the amount of folate present in serum or whole blood and that the folate concentration can be quantified by measuring the turbidity of the inoculated medium after a nearly 2-d incubation at 37°C. Some of the key advantages and disadvantages are itemized in Table 15. However, a few points are worth highlighting. The MBA has received renewed interest during the past decade because of improvements in efficiency and robustness, so that the assay can be reliably used in a high-throughput routine setting such as NHANES (305, 306). Important improvements introduced in the 1970s and 1980s included the development of a chloramphenicol-resistant strain of *L. rhamnosus* (307), the ability to cryopreserve the inoculum (308), and the introduction of automated microtiter plate technology (309).

As a result, the need for sterilization or aseptic addition was eliminated, growth curves could be reproduced with much higher precision for hundreds of assays, and the assay was miniaturized by using disposable labware such as 96-well plates. Since then, 2 "variants" of the MBA have been adopted: *1*) continued use of the wild-type organism (American Type Culture Collection 7469), but incorporation of the 96-well plate technology and cryopreservation, and *2*) use of the chloramphenicol-resistant organism [American Type Culture Collection 27773 or National Collection of Industrial and Marine Bacteria (NCIMB) 10463] as well as incorporation of the 96-well plate technology and cryopreservation (310–312). A recent method comparison showed less than optimum correlation between these 2 "variants" (serum: $r \sim 0.8$; whole blood: $r \sim 0.7$) but very good correlation between 2 laboratories using the chloramphenicol-resistant organism (serum and whole blood: $r > 0.9$) (305).

The sensitivity of the MBA is a particular advantage when only small-sample volume is available, such as for samples collected from a fingerstick or as a dried blood spot (DBS). To date, no other folate assessment method has been applied successfully to DBS. The MBA method for DBS developed by O'Broin et al. (313, 314) and implemented at the CDC (315) is a suitable tool to assess folate status in a population when no venous sample can be collected (9). Despite the limitations outlined in Table 15, based on data from thousands of samples from NHANES, CDC analysts reported <1% of samples exhibited a pattern of interference due to the potential presence of antibiotics or antifolates (9). Some of the key factors that help to successfully perform the MBA are outlined in **Table 16**.

Protein-binding assays. Protein-binding assays (PBAs) were developed with the clinical laboratory in mind, to enable the diagnosis of folate deficiency. These assays use the highly specific FBP (mainly from milk or milk fractions, sometimes from porcine plasma or kidney) to "extract" folate from the sample. The strengths and weaknesses of PBAs are outlined in Table 15 and were recently reviewed by Pfeiffer et al. (9). A few key issues are highlighted below.

- PBA specificity comes with its own problems (9, 316), including that the various folate forms have different affinities to FBP (317) and slight deviations from the optimal pH of 9.3 (where folic acid and 5-methyl-THF show equivalent binding affinity to FBP) or in the protein content of the sample can lead to inaccurate measurements.
- PBAs are not sensitive to antibiotics, but they are influenced by certain antifolates such as methotrexate.
- If a person has consumed a diastereoisomeric supplement [e.g., (6R,S)-5-methyl-THF], the 6R form will readily bind to the FBP and the assay will give a spurious result (as will chromatographic assays because they also cannot distinguish between diastereoisomers).
- Folate polyglutamates generally exhibit a higher response than do monoglutamates, requiring a complete deconjugation of RBC folates (317). The conditions for the deconjugation step vary across assays and the preparation of whole-blood hemolysates needs to be performed off-line, potentially contributing to the larger assay differences noted for RBC folate than for serum folate (9).
- Additional shortcomings of PBAs are related to their limited dynamic range:
  - Potential problems with inaccurate dilution linearity, particularly if dilutions are not conducted in sample matrix.
  - Need to dilute and reanalyze a high percentage of samples from populations who are exposed to folic acid fortification, if the purpose of the analysis is to describe the distribution of folate concentrations.
- Other characteristics of PBAs include the following:
  - Different types of analyte binding: competitive (i.e., the sample and the labeled folate conjugate are mixed before the introduction of a limited amount of FBP, ensuring competition for the binding sites) or noncompetitive (i.e., an excess capacity of FBP is incubated with the sample before the addition of the labeled folate conjugate).

TABLE 14 Folate essentials[1]

- Serum folate represents the sum of several folate vitamers circulating in the blood stream, often referred to as ``total folate.´´
  - The analysis of these compounds is complicated by their appearance as pteroylmonoglutamates with variations in the oxidation state of the pteridine moiety and in the one-carbon substituent group at the N-5 and/or N-10 positions.
  - The main circulating folate vitamer is 5-methyl-THF (292),
  - Unmetabolized folic acid can be present in varying concentrations (7).
  - Reduced folate vitamers such as THF and formyl-folates (5- or 10-formyl-THF, sometimes 5,10-methenyl-THF) may also be present, albeit in very small concentrations (293, 294).
- RBC folate
  - RBCs contain much higher folate concentrations than serum.
  - 5-Methyl-THF polyglutamates are the main folate forms (292).
  - In persons with the *MTHFR* 677 C>T polymorphism, a portion of the 5-methyl-THF polyglutamates is replaced by formyl-folates (6).
  - The measurement of RBC folate is even more complex than that of serum folate, because of the need to convert polyglutamates to monoglutamates before analysis.

[1] MTHFR, methylenetetrahydrofolate reductase; THF, tetrahydrofolate.

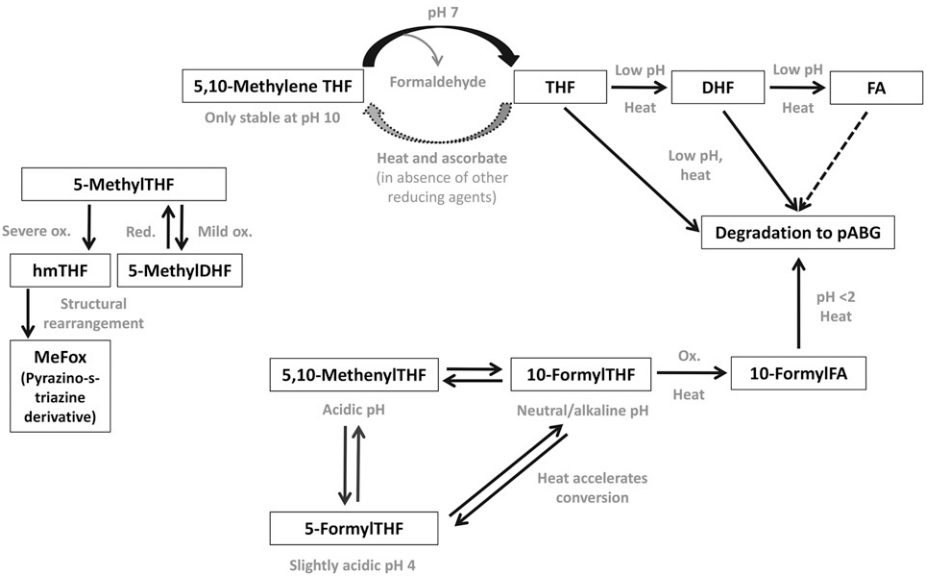

FIGURE 11 Folate interconversions and degradation. DHF, dihydrofolate; FA, folic acid; hmTHF, 4α-hydroxy-5-methyltetrahydrofolate; MeFox, pyrazino-s-triazine derivative of hmTHF; pABG, para-aminobenzoylglutamate; THF, tetrahydrofolate. Adapted from reference 295 with permission.

- Different types of sample preparation: heterogeneous (i.e., excess unbound analyte or excess reaction components have to be removed, requiring that either the FBP or the folate conjugate be immobilized to a solid support) or homogeneous (i.e., no separation step is required).

Most current PBAs are competitive and heterogeneous. Generally, a pretreatment step on the instrument with alkaline reagent and antioxidant ensures the release of folate from endogenous FBP and its stabilization to prevent oxidation.

Chromatography-based assays. Chromatography-based methods provide information on individual folate forms and are based on either measurement of intact folates via HPLC or measurement of folate breakdown products as an indicator for total folate via GC. General characteristics of these 2 approaches are listed in **Table 17**.

Over the past decade, tandem mass spectrometers have become more affordable and user-friendly and smaller in footprint, and their performance in terms of sensitivity and linear dynamic range has greatly improved. They are now considered to be the preferred detectors for HPLC-based methods, at least for specialized laboratories. General characteristics of LC-tandem mass spectrometry (LC-MS/MS) are outlined in **Table 18**.

Assay calibration for chromatographic assays. Ensuring accurate calibration is a big task for chromatography-based methods, partially due to the number of folate forms, but also because of their instability. General recommendations and detailed procedures for the preparation and handling of folate standards are described elsewhere (9, 254). Fazili and Pfeiffer (293) recently reported that high-concentration stock solutions of 5-methyl-THF (100 mg/L) were stable for up to 9 y if stored at –70°C in the presence of 1% ascorbic acid. However, mixed working calibrators have to be prepared freshly from frozen individual folate stock solutions for each analytical run and discarded after use.

Key principles in sample extraction and preparation for chromotographic assays. Chromatography-based analytical methods require sample extraction and cleanup, and some methods also use a sample concentration step to enable detection of minor folate forms. Pfeiffer et al. (9) provided a detailed table that summarizes features of LC-MS/MS methods used to measure serum and RBC folates. Most newer methods perform sample cleanup by use of solid-phase extraction (SPE) as an efficient way that can be easily automated for high throughput. Newer generation 96-probe SPE instruments allow fast sample cleanup (<1 h) with excellent folate recovery (300). Generally, reversed-phase cartridges are used in the SPE step, but strong anion-exchange cartridges have also been used. Because the latter require elution buffers with high salt content that can cause ion suppression and negatively influence analyte sensitivity, they are less favored by MS-based methods. Solid-phase affinity chromatography using FBP has also been used for sample cleanup by some methods. It has been shown that as long as the pH of the sample is low enough (<3.5) to prevent rebinding of 5-methyl-THF to the native proteins in the sample, the SPE step is sufficient, alleviating the need for a manual protein precipitation step (321). Yet, some investigators prefer to use heat, acid, or an organic solvent to precipitate proteins from either serum or whole blood. Regardless, internal standards should be added at the earliest possible time to account for procedural losses, and antioxidants have to be added to extraction buffers and reagents to protect labile reduced folates.

The extraction of folate polyglutamates from whole blood is complex and requires the hemolysis of erythrocytes (typically by diluting whole blood with ascorbic acid) as well as the deconjugation of polyglutamates to monoglutamates (typically through the action of the endogenous plasma conjugase at a slightly acidic pH and incubation at 37°C). Research is still ongoing to optimize conditions such that the LC-MS/MS method generates whole-blood total folate concentrations comparable to the MBA or to explain why the MBA measures ~20% higher (4). An alternative approach to the extraction of whole-blood folates at an acidic pH is to inactivate the endogenous plasma conjugase by heat in a high-pH borate buffer and to conduct in-line FBP affinity chromatography and HPLC-electrochemical detection analysis at a neutral pH (320). Although this preserves the folate polyglutamate pattern and prevents folate interconversions, it requires the quantitation of a large number of folate compounds, some of which have no commercially available calibrator materials. Furthermore, there are no appropriate internal standards available for this procedure and it is presently not amenable to LC-MS/MS.

TABLE 15 Main analytical method types used for the measurement of serum and RBC folate[1]

Method	Advantages	Disadvantages
MBA for total folate	• Great sensitivity; small sample volume needed (~50 μL) • Measures all biologically active forms approximately equally • Inexpensive, simple instrumentation, suited for low-resource settings • In-house control of performance • Can be used with dried blood spot samples	• Manual assay; relatively laborious unless automated liquid handling is introduced • Lengthy assay; limited throughput (can only be performed 3 d/wk) • Precision is relatively low (need for replicates) • Limited linear range (need multiple dilutions) • Not standardized (use of different calibrators, microorganisms) • Prone to contamination issues • Inhibited by presence of antibiotics or antifolates
PBA for total folate	• High sample throughput • Quick turnaround time to first result • Available in commercial kit form • Minimum operator involvement • Good precision for some assays • Relatively low reagent cost	• Folate forms have different affinities to the folate binding protein • Questionable accuracy when mixtures of folate are present • Limited linear range • Matrix effects when sample is diluted • No control over lot-to-lot variability or assay recalibration/reformulation
Chromatography-based MS/MS assay for folate vitamers	• Provides information on folate vitamers • Use of stable-isotope-labeled internal standards compensates for procedural losses • Highly selective and specific • Good sensitivity • Good precision • In-house control of performance	• Requires expensive instrumentation, experienced operator, frequent technical service • Manual assay; relatively laborious unless automated liquid handling is introduced • Complex sample extraction/cleanup • Interconversions of folate forms need to be considered in the interpretation of data • Conversion of erythrocyte polyglutamates to monoglutamates needed for RBC folate • Summation of folate forms to total folate

[1] MBA, microbiological assay; MS/MS, tandem mass spectrometry; PBA, protein-binding assay.

As mentioned earlier, folates are susceptible to oxidative degradation during preanalytical sample handling and storage processes. Two recent LC-MS/MS methods showed that an oxidation product of 5-methyl-THF known as MeFox is present in serum at low concentrations and can interfere with the quantitation of 5-formyl-THF because it is an isobaric compound of 5-formyl-THF (293, 322). This shows that even the highly specific LC-MS/MS methods are not immune from interference problems.

Choice of method. The choice of method is contingent on the purpose and setting. For example, the aim of clinical laboratories is to determine whether a patient is folate deficient. As a result, clinical laboratories require inexpensive, automated, and high-throughput assays to be able to report results within a day or less of receiving a sample. They prefer ready-to-use reagents and supplies and generally do not have the technical staff to develop, validate, improve, or troubleshoot assays. Therefore, for clinical settings, PBAs meet these criteria. However, as exemplified in the report by Raiten and Fisher (323), kit assays can change over time as a result of manufacturer recalibration or reformulation and may not be a good choice for a public health laboratory that needs to monitor trends in folate concentration distributions in a population over time.

Public health laboratories require stable assays, preferably where the method performance can be verified and controlled in-house. Either the MBA or LC-MS/MS may be suitable methods depending on the availability of resources (financial as well as staffing and instrumentation) and sample volume and whether total folate only or information on individual folate forms is of interest. By contrast, advantages of the MBA assay for low-resource settings include its relative low cost, its calibration and long-term performance can be controlled in-house, and it generates results that are generally in good agreement with higher-order LC-MS/MS methods. For the research setting, chromatographic assays that allow the determination of individual folate forms may be of greatest interest, but depending on the research question, other analytical approaches may also be appropriate.

Serum/RBC cutoff values and interpretation of data. The measurement of total folate provides information on the folate status of the individual, either short-term through serum folate or long-term through RBC folate. The inconsistent use of cutoff values over time to assess the proportion of the population with low blood folate concentrations has led to confusion. Some historical perspectives on the use of folate cutoffs are presented in **Table 19**. What unifies the various folate cutoff values is the fact that most were derived from data generated with the MBA. What is less clear is how the older MBA methods compare to today's MBA.

Crider et al. (99) estimated that a reasonable cutoff for optimal prevention of NTDs would be population RBC folate concentrations of ~1000 nmol/L, which is in agreement with the only prospective study that has been conducted to date (100), which found that the prevalence of NTD in an Irish population was lowest when RBC folate concentrations were ≥906 nmol/L (400 μg/L) (100). The WHO has estimated that the RBC folate concentration should be >400 μg/L (906 nmol/L) in women of reproductive age to achieve the greatest NTD risk reduction (101).

The presence of unmetabolized folic acid in serum is a result of folic acid intake from supplements or fortified foods in excess of ~200 μg/meal, which exceeds the capacity of the DHFR enzyme to reduce folic acid during intestinal absorption to the bioactive folate vitamer THF (328). The measurement of folate

TABLE 16 Keys to optimizing performance of MBAs[1]

Suggestion	Rationale	Comment
Use of a liquid handler to dilute and dispense samples and reagents	Improves assay precision. Reduces manual labor and increases efficiency.	With manual pipetting four 96-well plates can be handled by an experienced analyst in an analytical run and the pipetting steps take ~3 h. If a liquid handler is used, the analytical run can be increased to seven 96-well plates/d and the pipetting steps only take half the time. This is important because the MBA can only be set up 3 d/wk to avoid reading the plates on the weekend.
Select an appropriate calibrator	Because 5-methyl-THF constitutes the largest portion of total folate in both serum and whole-blood samples, it was recommended to use this folate form as a calibrator (305).	Traditionally, most MBAs have used folic acid as a calibrator for convenience because of the greater stability of this folate form compared to reduced folates.
	The response of the chloramphenicol-resistant *L. rhamnosus* has recently been shown to be slightly greater for 5-methyl-THF than for folic acid, resulting in lower calculated concentrations by ~20% (9, 305). Because 5-methyl-THF constitutes the largest portion of total folate in both serum and whole-blood samples, it was recommended to use this folate form as a calibrator (305).	Folic acid is probably preferable for a novice laboratory until it establishes good proficiency with the assay. Most of the synthetic 5-methyl-THF forms commercially available are 6-R,S diastereoisomeric mixtures. Because in vivo vitamin activity of THFs and in vitro coenzymatic activity are only observed with the (6S)-THF isomers, the MBA will show 50% activity.
Handle calibrator with great care	5-Methyl-THF is sensitive to oxidation; therefore, stock solutions should be prepared with great care (use of antioxidants, purging solutions with nitrogen).	The concentration of the primary stock solution should be determined spectrophotometrically, and secondary stock solutions should be stored in small single-use aliquots at −70°C (9).
Prepare ready-to-use reagents	To reduce daily workload (e.g., preparation of additional reagents to be added to the medium). To reduce the chance of contamination.	Intermediate ready-to-use reagents can be prepared and stored at −70°C for up to 6 mo and easily added to the medium at the time of preparation.
Generate a ``sensitive'' *Lactobacillus rhamnosus* inoculum	To ensure good assay sensitivity (growth response per unit of folate).	Measure the turbidity of the culture regularly to identify the log phase growth (usually at ~20 h), stop the growth at that point, and freeze many inoculum aliquots at −70°C for subsequent sample analysis. Create each new inoculum from the original culture (freeze-dried organism); however, avoid generating more than 2 subsequent inoculi to avoid potential changes in the organism. Test the new inoculum before using it for sample analysis. Use a negative control (medium without the addition of folate) when preparing the inoculum to ensure that no organism is growing in the absence of folate.
Use appropriate sample dilution	The required sample dilution factor varies with the folate concentrations found in serum and whole-blood samples.	Samples from a population exposed to folic acid fortification require higher dilution (e.g., 1/100 for serum, 1/140 for hemolysate) than samples from a folate-deficient population (e.g., 1/20 for serum, 1/40 for hemolysate). A dilution linearity test over a relatively wide range (including lower and higher dilution than typically needed) should be carried out periodically to verify that the assay recovery (100 ± 15%) and precision (CV ≤15%) are adequate.
Prevent contamination	To ensure accurate results and minimize time to troubleshoot assay problems.	Use separate areas for the preparation of calibrator stock solutions and for the analysis of samples or preparation of reagents to avoid cross-contamination. Use disposable supplies when possible. Thoroughly clean durable supplies and dedicate their use to the same purpose to avoid cross-contamination.
Follow pre-established sample and run QC criteria	To ensure objective data review. To allow for automated (rule-based) result acceptance, rejection, and repeat analysis determination.	If an assay calls for 4 replicates (from 2 different dilutions) to calculate the final folate concentration for each sample: accept results if $n = 4$ and CV ≤15% or if $n = 3$ and CV ≤10%; reject a result if $n \leq 2$ and repeat sample. If the result is outside the calibration range, repeat sample at higher or lower than regular dilutions. If run QC is outside of pre-established limits, repeat entire run.
Conduct regular calibration verification and instrument checks	Verify calibration accuracy of pipettes and automatic liquid handler. Ensure proper wavelength calibration of the microplate reader. Monitor temperature of the incubator, refrigerator, and freezer.	

[1] MBA, microbiological assay; QC, quality control; THF, tetrahydrofolate

TABLE 17 General characteristics of chromatography-based assays

HPLC-based methods
- Older methods were HPLC-based and used fairly inexpensive detectors, such as UV, diode array, fluorescence, or electrochemical (295).
- Folates were separated mainly on RP stationary materials.
- To facilitate retention of negatively charged folates on RP columns, it was common to use low pH (<4.0) mobile phases (phosphate buffer) with organic modifiers (typically acetonitrile), so-called ion suppression chromatography (318).
- Due to the acidic mobile phase, this approach led to some folate interconversion of formyl forms.
- Alternatively, folate forms were separated at a neutral pH after forming ion pairs with cationic surfactants (e.g., tetrabutylammonium phosphate), so-called ion-pair chromatography (319).
- However, ion-pairing reagents were generally not used with electrochemical detection (320) and may negatively affect the robustness of the chromatography.
- Both isocratic (typically shorter run times) and gradient (more flexible in resolving coeluting folate forms) elution methods were used.
- Aside from having limited specificity, these methods also suffered from the lack of appropriate internal standards to correct for procedural losses (9).

GC-based methods
- Developed for whole-blood samples before the availability of HPLC coupled to tandem MS (LC-MS/MS)
- Used acid hydrolysis to break folate to pABA and used stable-isotope-labeled pABA as an internal standard for the MS detection.
- Required lengthy multistep preparation and were not widely used.

[1] LC-MS/MS, liquid chromatography–tandem mass spectrometry; pABA, para-aminobenzoic acid; RP, reversed-phase.

forms circulating in serum or present in RBCs may further elucidate the functional role of folate vitamers relative to various health outcomes; however, cutoff values for low or high concentrations or desirable ranges have yet to be identified. In conclusion, irrespective of what cutoff values are used, an earlier expert panel tasked to assess the folate methodology used in the NHANES III came to the still valid conclusion that "there is an inherent inadequacy in the reliance of single indices as sole determinants of inadequate folate nutriture" (323).

Laboratory infrastructure. Because of the technical and capacity requirements, measurements of serum and RBC folate cannot be conducted in the field. **Table 20** outlines specific needs for each of the methods described above for assessment of serum/RBC folate.

Measurement of plasma total homocysteine

Introduction. Relevant information regarding homocysteine is itemized in **Table 21.**

Analytical methods. As described in detail by Ducros et al. (332) and Refsum et al. (179), various method types are available for homocysteine determination, from fully automated commercial kits (immunoassay or enzymatic method) to chromatographic assays with MS detection, overall providing comparable results and good assay performance. **Table 22** presents a summary of the main method types and their advantages and disadvantages.

All methods require the reduction of the disulfide bonds to allow measurement of homocysteine (sum of the various disulfide forms and the reduced homocysteine form). The general characteristics and some specific features of commonly available methodologies for homocysteine assessment are outlined in **Table 23.**

Choice of method. Because the measurement of homocysteine produces fairly comparable results across different method types, the choice of method is mainly dependent on the availability of instrumentation and technical expertise. The following may help to inform this decision:

- The use of commercial kits (either immunoassay or enzymatic assay) on a fully automated clinical analyzer will provide the highest throughput and quickest turnaround time with the least effort; however, the relatively high reagent costs can make the measurements quite expensive, particularly for a large number of samples.
- If an HPLC system with fluorescence detector is available, setting up a manual assay may be the least expensive approach, particularly in settings with low labor costs.
- The disadvantage of the manual HPLC assay is the number of samples that can be run, likely limited to 50–70 unknown samples per analytical run.
- If a laboratory has access to an LC-MS/MS system and the required technical know-how, homocysteine can be measured in a high-throughput semiautomated manner, with quite low reagent costs.
- Because of the high initial cost, an LC-MS/MS system is economical only if a large number of samples are measured regularly.
- For the research setting, where other thiols besides homocysteine may be of interest, chromatographic assays are the method of choice.
- Gas chromotography–mass spectrometry (GC-MS) or GC-tandem MS (GC-MS/MS) have also been used in high-throughput research settings, particularly when the determination of both methylmalonic acid and methionine is of interest (343).

TABLE 18 General characteristics of LC-MS/MS[1]

- Aside from offering great sensitivity, selectivity, and specificity, LC-MS/MS can also offer high throughput for routine measurements as long as the chromatographic separation is rapid and the sample preparation is mostly automated.
- Sample preparation and automation can be facilitated by using UPLC, a technique that is becoming more established, but requires specific instrumentation and columns to withstand the higher pressure.
- UPLC columns are less rugged than ordinary HPLC columns, requiring more frequent replacement.
- An important advantage of LC-MS/MS compared with HPLC with conventional detection is that it can use stable-isotope-labeled internal standards that improve the accuracy and precision of the method by
  - behaving almost identically to the analyte of interest and
  - correcting for procedural losses, sample-to-sample ionization variations, and ion suppression issues (9).
- Isotope dilution LC-MS/MS is now considered a state-of-the-art technology and is used by higher-order reference methods (4).
- Most recent LC-MS/MS methods have used ESI (a softer ionization form preferred for folate analysis) and positive ion mode.
- To enhance sample evaporation and ionization, MS-based methods require a mobile phase with high organic (acetonitrile or methanol) and low salt content.
- Acid modifiers (acetic or formic acid) are most often used for positive ion mode, whereas volatile buffers (ammonium acetate or formate) are used for negative ion mode.

[1] ESI, electrospray ionization; LC-MS/MS, liquid chromatography–tandem mass spectrometry; UPLC, ultra-high pressure liquid chromatography.

TABLE 19 Historical perspectives on folate cutoffs

- During the late 1960s, cutoff values for sequential stages of folate deficiency were established through depletion/repletion experiments.
- A serum folate concentration <7 nmol/L (3 μg/L) indicated negative folate balance at the time the blood sample was drawn (324).
- For RBC folate, concentrations of
  - <363 nmol/L (160 μg/L) indicated the onset of folate depletion,
  - <272 nmol/L (120 μg/L) marked the beginning of folate-deficient erythropoiesis, and
  - <227 nmol/L (100 μg/L) marked folate-deficient anemia (324).
- More commonly, investigators used a single cutoff value of <317 nmol/L (140 μg/L) for RBC folate to designate deficiency (325).
- More recently, cutoff values for folate deficiency (serum folate <10 nmol/L and RBC folate <340 nmol/L) were defined on the basis of a metabolic indicator (increased plasma total homocysteine) (326). These cutoff values were derived from NHANES III data generated with the Bio-Rad radioassay and have been recommended by the 2005 WHO Technical Consultation on folate and vitamin B-12 deficiencies for the assessment of folate status of populations (327).

- GC-based methods provide better precision, higher resolution, and longer column life than do LC-based methods and are not subject to ion suppression issues, which can be a problem in LC-MS/MS methods.

Interpretation of data. As discussed above, abnormal plasma homocysteine concentrations are not specific for folate deficiency. They are found in persons whose folate and vitamin B-12, B-6, and or B-2 status is suboptimal (208, 344) and in persons with impaired renal function regardless of their B-vitamin status (345). Although elevated plasma homocysteine concentrations are associated with an increased risk of cardiovascular disease, clinical trials have shown that reducing homocysteine is not associated with a decrease in recurrence of cardiovascular disease (346). Furthermore, a recent meta-analysis on overall results from large unpublished data sets showed that lifelong moderate homocysteine elevation had little or no effect on coronary heart disease and that results from previously published studies may reflect publication bias or methodologic problems (347). The 2009 US National Academy of Clinical Biochemistry Laboratory Medicine Practice Guidelines on "Emerging Biomarkers of Cardiovascular Disease and Stroke" categorized homocysteine concentrations (μmol/L) derived from standardized assays as follows: desirable, ≤10; intermediate (low to high), >10 to <15; high, ≥15 to <30; and very high, ≥30 (348).

Laboratory infrastructure. Measurements of homocysteine have to be conducted in a laboratory with uninterrupted electrical power supply for analytical instrumentation, freezers, refrigerators, and a water purification system for deionized water. Issues mentioned for folate PBAs, such as the need for a certified service engineer to handle repairs and more complex maintenance, analyzers often operating on a closed-channel basis, and calibrators and reagents typically being purchased in a ready-to-use form, are also true for homocysteine immunoassays or enzymatic assays. In general, immunoanalyzers are more expensive than basic clinical chemistry analyzers that can conduct colorimetric tests.

Most research laboratories have access to an HPLC system and although a fluorescence detector is more expensive than a standard UVA detector, the cost is still much lower than for any mass spectrometer. An autosampler (preferably with a thermostat), column oven, computer, and software are part of standard HPLC packages. The protein precipitation step requires a centrifuge, whereas the derivatization reaction may require a heating block. Regardless of the method specifics, the laboratory needs to have access to a number of basic instruments, such as a precision balance, pH meter, various adjustable air displacement pipettes, vortex mixer, stirring plate, and ideally a barcode scanner. The use of a liquid handler to automate the pipetting steps can greatly increase the throughput of the method; however, some steps still have to be handled manually by the operator (e.g., centrifugation step, derivatization step). The laboratory has to purchase chemicals and prepare calibrators, buffers, mobile phase, and other reagents on a regular basis. Most of this is true for LC-MS/MS and GC-MS/MS assays, with the additional need for staff with relevant technical expertise, a good understanding of instrument software and hardware, and much more frequent access to a service engineer for repairs and maintenance than for HPLC and GC instrumentation.

General principles in quality assessment

Quality assessment (QA) ensures that the laboratory results are accurate and of highest quality and is designed to address the following:

- avoidance of mistakes;
- consistency of performance;
- data integrity; and
- full staff participation in opportunities for training, which is needed to achieve high-quality results.

The basic components of a QA system are listed in **Table 24**.

Before the quality and consistency of a laboratory method can be monitored, prospect methods must be validated (for accuracy, precision, sensitivity, and ruggedness) and verified periodically (verification of assay calibration, verification of accuracy of pipettes, instruments). For a more detailed description of each QA system component, an example of a minimum QA system for a low-resource setting, and instructions on how to prepare, characterize, and use quality-control (QC) materials, the reader is referred to the Survey Toolkit for Nutritional Assessment, Laboratory and Field section, Quality Control and Quality Assurance subsection, developed by the CDC and hosted by the Micronutrient Initiative (349).

For convenience, some users prefer to purchase commercial QC materials. Unless one obtains the same batch of material, this practice may not be advisable for use over longer periods of time because frequent lot changes may prevent an assessment of assay shifts. In-house preparation of large batches of QC pools has the advantages of being more cost-efficient and being able to closely monitor assay performance. It is advisable to prepare 2 (normal and abnormal) or 3 (low, medium, and high) levels of QC pools, characterize them over the course of 20 individual analytical runs to establish target values and assay-associated variability, and then include them in every analytical run together with the unknown samples to judge whether the run is in control.

Although participation in proficiency testing programs is required to comply with certain laboratory certifications as well as recommended for good laboratory practice to allow external verification of results, the limitations of proficiency testing programs should be recognized and include the following:

- Most proficiency testing programs use method means (so-called peer-group means) to evaluate laboratories, making it difficult to identify methods with unsatisfactory performance or even monitor method shifts over time due to the lack of a stable reference point.

TABLE 20 Requirements for laboratory analysis of serum/RBC folate[1]

Method	Requirements
Serum or RBC folate (general)	• A midlevel laboratory infrastructure that guarantees uninterrupted electrical power supply for freezers, refrigerators, and the operation of analytical instrumentation. • A water purification system that provides deionized water. • Protection of samples and particularly folate calibrators from direct sunlight and artificial light is highly recommended.
PBA methods	• A suitable immunoanalyzer to measure folate with commercial kit assays; however, the cost for such an instrument is relatively expensive and most analyzers operate on a closed-channel basis allowing only reagents from 1 particular manufacturer to be used. • Calibrators and reagents are typically purchased from the manufacturer in a ready-to-use form or they require minimal handling.
MBA	• Several, albeit comparatively less expensive, pieces of instrumentation, including the following: o microplate reader; o 37°C incubator; o stirring hotplate; o vortex mixer; balance accurate to at least 2, preferably 3, decimal points (0.001 g); o UV/Vis spectrophotometer to determine the concentration of the folate calibrator; and o various adjustable air displacement pipettes including an 8-channel pipettor and a repeater pipettor. • Specific chemicals, calibrator preparation, and reagents such as the growth medium. • A plate rotor can help to carefully mix the whole-blood hemolysates without causing foaming of the sample. • Throughput of this method can be increased by use of a liquid handler to automate the various pipetting steps including the dilution of serum and whole-blood hemolysates, which is fairly laborious and can take several hours if conducted manually. • A computer-based sample tracking system can speed up sample log-in and avoid transcription errors by using a barcode scanner.
Chromatography-based	• Highly specialized instrumentation. • Staff with technical expertise in analytical chemistry and a good understanding of instrument software and hardware, regular access to a service engineer who performs repairs and more complex maintenance, generally as part of an annual service agreement. • LC-MS/MS instrumentation is very expensive and considerably more maintenance-intensive than HPLC coupled to other forms of detection such as UV, fluorescence, or electrochemical. • Because most methods rely on some form of solid-phase extraction for sample extraction and cleanup, at least a manual vacuum manifold is needed. • Automated solid-phase extraction and automated sample pipetting using a liquid handler are required to achieve high throughput/volume. • Depending on the method, other instrumentation may be needed, such as a sample evaporator (either through centrifugation in the presence of vacuum or through application of heat and/or the flow of an inert gas). • Regardless of the method specifics, the laboratory requires access to a number of basic instruments such as precision balance, UV/Vis spectrophotometer, pH meter, various adjustable air displacement pipettes, vortex mixer, stirring plate, and ideally a barcode scanner. • Chromatography-based assays produce a large amount of data that cannot be easily handled without an appropriate laboratory information management system; consideration has to be given to the time needed for data review and how the data will be stored so that it can be retrieved later; good access to IT services is essential. • The laboratory has to purchase numerous chemicals and prepare calibrators, buffers, mobile phase, and other reagents on a regular basis. • LC-MS/MS methods require stable-isotope-labeled internal standards, which are comparatively more expensive than unlabeled folate calibrators and only available from limited commercial sources (9); although certain folate polyglutamates (folic acid, 5-methyl-THF, and 5-formyl-THF) are commercially available, other reduced folate polyglutamates need to be custom synthesized (9).

[1] IT, information technology; LC-MS/MS, liquid chromatography–tandem mass spectrometry; MBA, microbiolgical assay; PBA, protein-binding assay; THF, tetrahydrofolate; UV/Vis, ultraviolet/visible.

- Proficiency testing samples are often modified (e.g., adding preservatives or other additives, supplementing materials with nonnative forms of analyte, using animal plasma or outdated human plasma from blood banks), potentially changing their behavior in the assay compared with fresh-frozen samples. This may lead to commutability problems with proficiency testing materials (350).
- As a result, information gained from the proficiency testing program may not be used to adjust assays.

Serum and RBC folate. To date, no formal standardization program exists for folate measurements, which may explain why considerable differences exist among laboratories and methods, particularly for RBC folate (9). A 2010 expert roundtable advising on folate biomarkers and methods for future NHANES surveys came to the conclusion that the close agreement obtained for serum folate results between the MBA and LC-MS/MS supported the introduction of the LC-MS/MS procedure for future NHANES (4). This allows for the measurement of individual folate vitamers, including unmetabolized folic acid, and calculation of total folate by summation of the individual vitamers. However, because the MBA gave ~25% higher concentrations than the LC-MS/MS procedure for RBC folate, NHANES retained the MBA for the measurement of RBC folate (4).

NIST has developed higher-order reference measurement procedures mainly for the measurement of serum 5-methyl-THF; these methods compared well with the CDC's LC-MS/MS method (351). Serum-based international reference materials produced by NIST (351) and the UK National Institute for Biological Standards and Control (352) have been available for several years; unfortunately, certified concentrations are provided for 5-methyl-THF only. No whole-blood reference materials are currently available with certified concentrations for any folate form or total folate. **Table 25** provides additional information on currently available reference materials and gives a selected list of proficiency testing programs for folate. Although there are no accuracy-based proficiency testing

TABLE 21 Homocysteine essentials

- Homocysteine is a thiol-containing amino acid found in normal human plasma.
- Only a very small portion (1–2%) occurs as the thiol, whereas the remaining amount is in the form of various disulfides, such as homocystine and homocysteine-cysteine disulfide, and minor amounts of other mixed disulfides (329, 330).
- The sum of these forms is called homocysteine.
- The majority of the total (~75%) is bound to protein (mainly albumin), whereas the remainder occurs in nonprotein-bound ``free´´ forms.
- In patients with abnormally elevated homocysteine concentrations, the relative contribution of the thiol homocysteine to the total increases to 10–25% (331).

programs available for folate, the UK National External Quality Assessment Service Haematinics Survey recently showed that the all-laboratory consensus mean (instead of the method mean) proved to be sufficiently accurate (compared with LC-MS/MS assigned values) and stable to be used as the target for monitoring laboratory performance for serum folate (355).

Plasma total homocysteine. Although no formal standardization program exists for plasma homocysteine measurements, results obtained with common methods generally compare well (356–358) and the assays display good performance, such as accuracy, precision, and linearity (356). Several proficiency testing programs are available for homocysteine and NIST has developed higher-order reference measurement procedures for the measurement of homocysteine (359, 360) and assigned certified concentrations for homocysteine to 2 serum-based international SRMs: SRM 1955 (351) and SRM 1950 (Table 25). NIST has also conducted a commutability study for SRM 1955, which showed that this material was commutable for the majority of immunoassays available at the time (361). As part of the 2009 National Academy of Clinical Biochemistry Laboratory Medicine Practice Guidelines on "Emerging Biomarkers of Cardiovascular Disease and Stroke," analytical performance goals for the clinical usefulness of homocysteine measurements have been set to <10% for bias, <5% for precision, and <18% for total error (348). The guidelines also recommended that manufacturers of diagnostic assays for homocysteine should follow approved value transfer protocols to ensure that standardized assays are used for vascular risk assessment.

Preanalytical factors

Serum and RBC folate. Folate is the least stable of the B vitamins; careful sample handling and use of antioxidants are required to maintain sample integrity. **Table 26** provides an overview of sample collection and processing requirements, storage, and freeze/thaw stability. Most of the information for serum and RBC folate is described in greater detail in a comprehensive review article on analytical approaches and related issues by Pfeiffer et al. (9); however, newly available information has been added as appropriate.

- *Variables related to the subject.* Data from several thousand US adults participating in NHANES 2003–2006 showed that samples from fasted (≥8 h, no dietary supplement consumed during the fast) participants had, on average, significantly lower serum (10%) and RBC folate (5%) concentrations than samples from nonfasted (<3 h) participants, but the difference was relatively small, indicating that fasting may not be essential when assessing the folate status of populations (362). However, in the individual, serum folate concentrations can increase drastically as a result of folate intake (either with food or as a dietary supplement), reaching a peak concentration ~1–2 h after the dose and depending on the size of the dose, the baseline

TABLE 22 Main analytical method types used for the measurement of plasma total homocysteine[1]

Method	Advantages	Disadvantages
Immunoassay (FPIA, ICL, EIA)	• High sample throughput • Quick turnaround time to first result • Available in commercial kit form • Minimum operator involvement • Good precision • Small sample volume needed (≤50 μL)	• Limited linear range (up to 50 μmol/L) • Relatively high reagent cost • No control over lot-to-lot variability or assay recalibration/reformulation • Requires patented antibody
Enzymatic assay	• Very small sample volume needed (≤20 μL) • Can be performed on basic clinical chemistry analyzer or manually on a microplate reader	• Manual pretreatment of samples needed • Background determination of D-amino acids needed
Chromatography-based assay (HPLC-FD, LC-MS/MS, GC-MS)	• Can provide information on other thiols • GC-MS methods can simultaneously determine methylmalonic acid, methionine, and other compounds of the transsulfuration pathway • HPLC-FD instrumentation commonly available • Use of internal standard (stable-isotope labeled for MS-based methods; other compounds for non-MS methods) compensates for procedural losses • Highly selective and specific • Good precision, in particular GC-MS • In-house control of performance	• GC-MS and LC-MS/MS methods require expensive instrumentation, experienced operator, and frequent technical service and are therefore limited to specialized laboratories • Manual assay; several pipetting steps unless automated liquid handling is introduced • Complex sample extraction for most GC-MS methods because they require ion exchange chromatography step to separate amino acids • Derivatization is needed for all methods but LC-MS/MS, making full automation difficult
Capillary electrophoresis (with laser-induced fluorescence)	• Good peak resolution • Short analysis time • No use of organic solvents • Ease of automation	• Requires specialized expensive instrumentation not commonly available and experienced operator • Manual assay, complex sample extraction, derivatization needed

[1] EIA, enzyme immunoassay; FPIA, fluorescence polarization immunoassay; GC-MS, gas chromatography–mass spectrometry; HPLC-FD, HPLC with fluorescence detection; ICL, immunochemiluminescence; LC-MS/MS, liquid chromatography–tandem mass spectrometry.

TABLE 23 Characteristics of methodologies used for homocysteine assessment[1]

Method	Characteristics
Immunoassays	• Rapid, fully automated immunoassay methods providing high sample throughput were developed in the 1990s due to the increasing clinical interest in Hcy as a potential risk factor for cardiovascular disease. • They were adapted for various clinical analyzer platforms and the majority of clinical laboratories still use this approach. The common principle of all assays is the enzymatic conversion of free Hcy to *S*-adenosyl-L-homocysteine by the action of *S*-adenosyl-L-homocysteine hydrolase (333). • The distinguishing feature of the assays is the detection mode, mainly including FPIA, ICL, and EIA. • The FPIA assay has been used in the continuous NHANES survey from 1999 to 2006 (334) and compares well with a rapid and simple HPLC assay with fluorescence detection (335). • The EIA assay, carried out on microtiter plates with a microplate reader, o has the potential for high throughput without the need for an expensive clinical analyzer; o however, the imprecision of this assay is somewhat higher than that of fully automated immunoassays (243) and o is not commonly used by laboratories participating currently in proficiency testing programs.
Enzymatic assays	• Enzymatic colorimetric assays do not require an immunoanalyzer; they can be performed on simpler clinical chemistry analyzers or manually using a microplate reader to record the colorimetric reaction. • Various commercial assays have been developed: o the A/C Diagnostics (San Diego, California) single-enzyme assay using homocysteine α,γ-lyase (336), which can now also be performed on a small portable fluorescence reader (337); o the Catch (Bio-Pacific Diagnostics, Inc; Bellevue, Washington) homogeneous enzymic assay based on pyruvate detection (338); and o the Diazyme (Diazyme Laboratories, Poway, California) enzymic cycling assay based on ammonia detection (339).
Capillary electrophoresis assays	• This analytical approach seemed once an interesting alternative to HPLC methods; however, the emergence of simpler and more powerful LC-MS/MS methods has made this approach less attractive.
Chromatography-based assays	• This method type comprises a wide spectrum from the less expensive HPLC assays using mostly fluorescence detection (some methods use electrochemical or photometric detection) to the more cumbersome and less used GC-MS methods and to the newer and simpler LC-MS/MS methods that no longer require derivatization but rely on expensive tandem mass spectrometer instrumentation. • Main advantages are as follows: o they allow simultaneous measurement of other thiols in the same sample and o the laboratory has in-house control of the assay performance and is not faced with unpredictable assay recalibration or reformulation by the manufacturer. • All of these methods require the same first 2 steps: o the conversion of the disulfide forms to reduced Hcy and o the precipitation of proteins. • A number of reducing reagents have been used (332): sulfhydryl agents such as dithiothreitol, dithioerythreitol, or 2-mercaptoethanol; sodium or potassium borohydride; phosphine agents such as TBP and TCEP. o TCEP has emerged as the reducing reagent with most advantages: ■ it is nonvolatile, ■ stable, and ■ soluble in aqueous solutions (335).
Subtype HPLC	• Less expensive. • Uses mostly fluorescence detection (some methods use electrochemical or photometric detection). • HPLC methods with fluorescence detection require a derivatization step; a number of reagents have been used: monobromobimane; halogenoslfonylbenzofurazans (SBD-F and ABD-F; o-phthaldialdehyde) (332). o SBD-F and ABD-F seem to have some important practical advantages: ■ they are not fluorescent; ■ their thiol adducts are stable (no fluorescent degradation products), allowing the application of a simple isocratic chromatography (with shorter retention times for SBD-F compared with ABD-F); ■ they are thiol-specific; ■ however, the derivatization needs to be performed at elevated temperature.
Subtype GC-MS	• More cumbersome and less commonly used than other methods in this class. Generally better precision than LC-MS/MS.
Subtype LC-MS/MS	• Superior analytical specificity and sensitivity. • Expensive tandem mass spectrometer instrumentation. • No longer require a derivatization step. • Has also been adapted for dried blood spots (340, 341). • A commercial kit specifically designed for LC-MS/MS has also been recently reported (342). • Can incorporate the inexpensive deuterium-labeled Hcy as an internal standard to control for procedural losses and are considered higher-order reference methods. • If only Hcy is of interest, the chromatographic run can be very short ($\leq$2 min) and the data processing can be quick and easy.

[1] ABD-F, 4-(Aminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole; EIA, enzyme immunoassay; FPIA, fluorescence polarization immunoassay; GC-MS, gas chromatography–mass spectrometry; Hcy, homocysteine; ICL, immunochemiluminescence; LC-MS/MS, liquid chromatocraphy–tandem mass spectrometry; SBD-F, 7-Fluorobenzofurazan sulfonic acid; TBP, tri-n-butylphosphine; TCEP, tris(2-caroboxyl-ethyl) phosphine.

TABLE 24 Basic components of QA[1]

- Internal QC through the use of bench and blind QC samples
- External QA via participation in proficiency testing programs
- Equipment monitoring and maintenance
- Documentation of policies and procedures
- Proper staff training
- Laboratory audits

[1] QA, quality assessment; QC, quality control.

folate status, and the vehicle in which folate was administered. The within-person variability for serum folate [CV (CV_w) = 21.5%] is about twice that for RBC folate (9.1%) (370). It has been recommended that the method imprecision should be less than one-half of the CV_w. Although this can be achieved with most current serum folate methods, RBC folate methods are nowhere near to achieving the much tighter requirement.

- *Variables related to the sample collection.* Although most laboratories prefer serum over plasma, both matrices generally produce comparable results for serum total folate (300, 363, 364), as long as the sample processing is not delayed (367).
- *Variables related to the sample processing.* Blood should be processed and frozen promptly. If delays in processing are unavoidable, the sample should be protected from light and kept cool; it should be processed no later than a few days after collection. Intact whole blood shows better folate stability than hemolysate (313, 364).
- *Variables related to the sample storage.* Generally, the lower the storage temperature, the better the folate stability. Folate in serum and hemolysates (but not in whole blood) can withstand a few short freeze/thawing cycles, particularly if the vials are closed most of the time to minimize the exposure of the sample to air (313, 366, 369). Folate in serum/plasma degrades rapidly at room temperature, particularly in the presence of EDTA (300, 367, 371). When Hannisdal et al. (367) kept matched serum and plasma samples at room temperature in the dark for up to 192 h, they did not find sample type differences at baseline (blood processed within <1 h and frozen immediately at −80°C) and 5-methyl-THF concentrations were essentially stable for 48 h in serum. However, in EDTA plasma, 5-methyl-THF decreased and MeFox (tentatively termed hmTHF in earlier publications) increased at a rate of 1.92%/h and 25.7%/h, respectively, during the first phase of rapid change. In serum, the reduction in 5-methyl-THF was totally recovered as MeFox after 96 h, whereas in EDTA plasma a smaller percentage of 5-methyl-THF was recovered as MeFox. Therefore, moderately degraded folate can be quantitatively recovered as MeFox. However, in serum samples stored for decades in biobanks, folate is degraded beyond MeFox but can be recovered as pABG equivalents after oxidation and mild acid hydrolysis of the folate species (372). These recovery strategies are useful for the assessment of folate status in epidemiologic studies, in which serum/plasma was processed, transported, and stored in biobanks under conditions that did not stabilize folate (i.e., in the absence of ascorbic acid).

Plasma total homocysteine. Plasma homocysteine is a very stable analyte as long as the plasma is separated from the RBCs within 1 h of blood collection (or within <8 h if the whole blood is kept on ice) (179) (Table 26). The within-person variability CV_w for plasma homocysteine is 12.2% (370). Most current homocysteine methods have an imprecision of between 5% and 10% and therefore could likely achieve a method imprecision of less than one-half of the CV_w.

Other biomarker methods.

Urinary folate/folic acid. Although urine contains some folate derivatives, the bulk of the excretion products in humans are folate cleavage products. A small and variable amount of a folate dose is recovered as intact folate derivatives in urine in the first 24 after the dose. Folate is freely filtered at the glomerulus and is reabsorbed in the proximal renal tubule. The net effect is that most of the secreted folate is reabsorbed (292). When large folate doses are given, more of the folate is excreted in the urine (373, 374) and the amount excreted depends on the type of

TABLE 25 Available reference materials and proficiency testing programs for folate status biomarkers[1]

Biomarker	Reference materials	Selected list of proficiency testing programs
Serum folate	NIST SRM 1955 (human serum, frozen; 3 levels; certified values for 5-methyl-THF, reference values for FA, information values for total folate and 5-formyl-THF) (353) NIST SRM 1950 (human plasma, frozen; 1 level; certified value for 5-methyl-THF, reference value for FA) (353) NIBSC RM 03/178 (human serum, freeze-dried; 1 level; LC-MS/MS values for 5-methyl-THF, FA, 5-formyl-THF, and total folate) 354	CAP Ligand Assay General Survey CAP Cal V/L Survey UK NEQAS Haematinics Survey
Whole-blood folate	NIBSC RM 95/528 (human whole-blood hemolysate, freeze-dried; 1 level; consensus value) (354)	CAP Ligand Assay General Survey CAP Cal V/L Survey UK NEQAS Haematinics Survey
Plasma Hcy	NIST SRM 1955 (human serum, frozen; 3 levels; certified values) (353) NIST SRM 1950 (human plasma, frozen; 1 level; certified value) (353)	CAP Homocysteine Survey CAP Cal V/L Survey NY State Department of Health Wadsworth Center General Clinical Chemistry DEKS

[1] Cal V/L, Calibration Verification and Linearity Survey; CAP, College of American Pathologists; DEKS, Danish External Quality Assessment Program; FA, folic acid; Hcy, total homocysteine; NEQAS, National External Quality Assessment Scheme; NIBSC, National Institute for Biological Standards and Control; NIST, National Institute of Standards and Technology; SRM, standard reference material; THF, tetrahydrofolate; UK, United Kingdom.

folate given as well as the dose. Humans have limited ability to reduce large doses of folic acid (221, 375, 376). More folic acid is excreted than 5-methyl-THF when large doses are given. Urinary folates can be measured by chromatography-based methods, which were discussed previously in the section on analytical methods under measurement of serum and RBC folate. Because folates occur as monoglutamates in urine, no deconjugation is necessary and the sample can be cleaned up by affinity chromatography, which requires no additional extraction step (377).

Serum folic acid. The main circulating folate vitamer is 5-methyl-THF (292), but unmetabolized folic acid can be present in varying concentrations (7, 294). This vitamer is measured by chromatography-based methods that provide information on individual folate forms and that were discussed previously in the section on analytical methods under measurement of serum and RBC folate. More recent methods have used tandem MS as the detector (300), but electrochemical detection (378) and MBA of folic acid–containing HPLC fractions (379, 380) have been used as well. It is important to note that under heat and/or low-pH conditions, THF can oxidize to folic acid via the highly unstable DHF intermediate. Assays measuring unmetabolized folic acid in serum should therefore verify that this compound is not an artifactual result of THF oxidation due to analytical steps.

Urinary and serum pABG and apABG. The oxidative folate catabolites pABG and apABG, biomarkers of folate status and turnover, are found in both urine and blood. Urinary pABG and apABG, which reflect turnover in endogenous folate pools, are frequently measured by chromatography-based methods (HPLC, GC-MS) after preanalytical passage through immobilized FBP affinity columns to remove potential interference from intact folate (258, 381). More recent studies reported the development of faster and highly sensitive LC-MS/MS procedures for the quantification of pABG and apABG in urine (382, 383) and the use of this procedure for quantifying these catabolites in fasted spot urine as a noninvasive alternative to 24-h urine analysis (384). pABG and apABG in serum can also be measured by LC-MS/MS by using procedures developed for the selective and simultaneous quantification of pABG and apABG and other folate species (5-methyl-THF, 5-formyl-THF, hmTHF, folic acid) (367). In serum samples stored for decades in biobanks, degraded folate can be recovered as pABG equivalents after oxidation and mild acid hydrolysis of the folate species (372). As indicated earlier, this recovery strategy can be useful for the assessment of folate status in epidemiologic studies in which serum/plasma was processed, transported, and stored in biobanks under conditions that did not stabilize folate (i.e., in the absence of ascorbic acid).

Genetic markers.

DNA methylation. One can examine cytosine methylation across all cytosines in the genome or in specific regions of the genome such as long interspersed nucleotide elements-1 (LINE-1) repetitive sequences or promoter sequences of single genes. The most commonly used methods require that DNA is isolated from cells to perform DNA methylation analysis (264, 385); however, it is possible to visualize methylated cytosine in the nuclei or chromosomes by using labeled antibodies (386). DNA methylation in human epidemiologic studies is usually measured in DNA from isolated lymphocytes or in DNA from whole blood. The preferred approach is to use DNA from isolated lymphocytes because DNA methylation status may vary between leukocyte subsets (387, 388) and leukocyte subset ratios may differ significantly between individuals depending on their age, sex, health, level of physical exercise, and lifestyle or nutritional status (389–391), which may then make interpretation of data difficult.

One of the earlier most utilized techniques in folate-related DNA methylation research is the CpG methyl transferase Sss1 (from *Spiroplasma* sp. strain) methyl acceptance assay in which DNA is incubated with the Sss1 enzyme with SAM containing a tritiated methyl group (392). The capacity of the DNA to accept tritiated methyl groups from SAM is then calculated from the degree of its radioactivity after isolation from the reaction mixture. A higher degree of radioactivity reflects a higher capacity to accept methyl groups and therefore indicates the extent to which the DNA's cytosines at CpG sites were initially hypomethylated. Another method that has been used successfully is based on the initial digestion of isolated DNA to single bases and subsequent measurement of the abundance of cytosine and 5-methylcytosine by LC in combination with UVA detection (393) or MS (139). The 5-methylcytosine:(cytosine + 5-methylcytosine) ratio provides a measure of global cytosine methylation.

The LINE-1 retrotransposon is a mobile parasitic genetic element that is abundant in the human genome, representing ~17% of the total human DNA (394). Methylation of LINE-1 sequences is essential for suppressing its expression, which if left unchecked, can multiply rapidly and insert itself randomly into the human genome, disrupting normal gene expression and causing chromosomal instability (264, 394). Several investigators are now measuring LINE-1 methylation in relation to folate status and folate metabolism gene polymorphisms by pyrosequencing of bisulfited DNA (395, 396). The DNA of interest is extracted and treated with bisulfite to convert unmethylated cytosines into uracils. After PCR amplification, by which all uracils result in thymidine, the sample is denatured to form single-stranded DNA. The single-stranded DNA is then sequenced and the degree of methylation at each CpG position in a sequence is determined from the ratio of T and C at that position.

Uracil misincorporation. Initially indirect methods were used to measure folate status of cells by determining either their resistance to incorporate tritiated thymidine when supplied with deoxyuridine (deoxyuridine suppression test) (397) or their uptake and metabolism of tritiated deoxyuridine by measuring radiolabeled uracil or thymidine in DNA, which reflected the cells' capacity to synthesize dTTP (398).

Other indirect methods involve the treatment of either nuclei or isolated DNA with uracil glycosylase (UDG), which converts uracil in DNA into an abasic site. In the former case, it is possible to use the comet assay under alkaline conditions (pH 13) to measure double-strand breaks in nuclei resulting from abasic sites that are either occurring spontaneously or are induced by UDG (399). The difference between the 2 measures then provides an estimate of the presence of uracil in DNA. In the case of isolated DNA, one can measure spontaneous and UDG-induced abasic sites by using labeled aldehyde reactive probe and ELISA detection (400); similarly, the difference between these 2 parameters provides a measure of uracil in DNA. More direct and quantitative measurement of uracil in DNA can be achieved by HPLC (401) or GC-MS analysis (402) of abundance of each base in hydrolyzed DNA. An alternative approach is to specifically release the uracil in DNA by UDG digestion and then analyze uracil extracted from the reaction mixture by GC-MS (403, 404).

Micronuclei. Micronuclei are best scored in once-divided cells because only cells that complete nuclear division can efficiently express micronuclei. Usually, micronuclei in humans are measured in mitogen-stimulated lymphocytes cultured ex

TABLE 26 Preanalytical factors influencing biochemical indicators of folate status[1]

Variables	Serum folate	RBC folate	Plasma Hcy
Subject			
Fasting	Essential for individual but probably not for population (~10% average difference between overnight fasted and <3 h fasted) (362)	Not required (362)	Generally not required (179); variations in Hcy in response to a high-protein meal (332)
Biological variation,[2] %			
Within-person	21.5	9.1	12.2
Between-person	48.7	35.8	37.1
Sample collection			
Venous vs. capillary blood	No data	Nonvolumetrically prepared capillary blood samples (finger-stick) compared well to venous blood samples if folate concentration was normalized to hemoglobin (313)	No data
Influence of anticoagulants	Serum preferred over plasma (might contain fibrinogen clots); generally both matrices provide similar results (300, 363, 364)	EDTA whole blood is used; other anticoagulants are not customary	EDTA plasma preferred over serum because the evacuated tubes can be immediately centrifuged.
Sample processing			
General requirements	Protect evacuated tubes with whole blood from light and keep cool (avoid freezing to keep RBCs intact); prompt processing and freezing of serum recommended	Protect evacuated tubes with anticoagulated EDTA whole blood from light and keep cool (avoid freezing to keep RBC intact); prompt processing and freezing of hemolysate recommended; Measure hematocrit to correct for packed cells; prepare hemolysate with ascorbic acid (1% wt:vol) by using accurate pipetting; use of serum folate concentration in calculation of RBC folate concentration preferred	It has been recommended that Hcy be measured in plasma because the sample can be processed immediately; to obtain serum, on the other hand, a blood sample has to be left at room temperature for 30–60 min to allow coagulation, which leads to an artificial increase in Hcy due to an ongoing release of Hcy from RBCs. Serum concentrations will therefore be ~5–10% higher than those obtained in optimally prepared plasma. Separate plasma from RBC within 1 h of collection to avoid artificial increase in Hcy [ongoing release of Hcy from RBCs; ~1 μmol /(L × h) at room temperature] (179).
Delayed processing	Prepare serum within 1 d, but no later than within 2–3 d of blood collection	Prepare hemolysate with ascorbic acid (1% wt:vol) within 1 d, but no later than within 4 d of blood collection (folate recovery is >90% at 4°C (313, 364)]	Alternatively, keep evacuated tubes with anticoagulated EDTA whole blood on ice for <8 h before preparation of plasma (179). Adenosine analogs (e.g., 3-deazaadenosine) prevent formation or release of Hcy from RBCs but are not compatible with immunoassays based on *S*-adenosylhomocysteine hydrolase (179).
	Storage of unprocessed whole blood at elevated temperature is unacceptable, particularly if it exceeds a few hours [10–20% loss after 6–12 h at 37°C (365); 30% loss after 1 d at 37°C (366)]	Storage of unprocessed whole blood at elevated temperature is unacceptable (~10% loss after 1 d at 22°C (267); 20–30% loss after 1 d at 37°C (313, 366)]	
Sample storage			
Storage stability	Stable for 1–2 d at room temperature in serum, but not in EDTA plasma; EDTA accelerates folate degradation [2%/h (367)] Stable for 1 wk refrigerated (<10% loss) (366, 368) Stable for a few years at −70°C (2) Ascorbic acid can be added (0.5% wt:vol) before storage to improve stability	Hemolysate with ascorbic acid (1% wt:vol) stable for several weeks at −20°C (313) and a few years at −70°C (9) Moderate folate losses can occur if whole blood is stored frozen [<20% loss after 2 y at −70°C (369)]	Stable for days at RT; stable for weeks refrigerated; stable for years frozen (179, 332)
Freeze/thaw stability	Little deterioration for at least 3 cycles (366)	Little deterioration in hemolysates for at least 3 cycles (369); significant folate loss in whole blood already at 2 cycles (369) Significant folate losses can occur if frozen whole blood is subjected to prolonged thawing times (313, 369)	Excellent stability; however, thorough mixing of samples required after thawing (179)

[1] Hcy, homocysteine; RT, room temperature.
[2] Data from reference 263.

vivo and blocked at the binucleate stage of mitosis by using cytochalasin B, a cytokinesis inhibitor, to identify once-divided cells (405). Micronuclei may also be measured in erythrocytes due to micronuclei formation in vivo in bone marrow normoblasts from which erythrocytes are derived after enucleation. Preferably, micronuclei are measured in very young erythrocytes known as reticulocytes, which can be identified by their larger size, higher RNA content, and by being positive for the transferrin receptor (131, 132, 406).

New Directions and Technologies

Omics (genome, epigenome, transcriptome, proteome, metabolome)

A biomarker may not necessarily refer to a single metabolic or genomic indicator. Rather, it could represent a panel of markers (407, 408) that reflect physiologic patterns or disease states (407, 409). Genome-related markers have the potential to serve as functional biomarkers of folate-dependent nucleotide biosynthesis and/or homocysteine remethylation, including the cellular methylation potential. Potential genomic biomarkers of folate status and function include alterations in chromatin methylation (410, 411), gene expression (412, 413), nuclear genome stability, and uracil content in nuclear DNA (29, 131, 414). Uracil misincorporation into nuclear DNA increases during folate deficiency, leading to increased levels of uracil content in DNA and DNA instability, but uracil accumulation in DNA is not specific to folate deficiency because vitamin B-12 and B-6 deficiency can impair folate utilization, leading to elevated uracil in DNA. Uracil in DNA also exhibits cell and tissue-type variations and has not been shown to be dose responsive to folate supplementation, limiting its role as a biomarker. Disruption of de novo dTMP synthesis in mitochondria results in elevated uracil levels in mitochondrial DNA (146), but the utility of uracil in mitochondrial DNA as a robust biomarker of folate nutritional status or de novo dTMP synthesis has yet to be established. Functional variation in folate-dependent homocysteine remethylation, which generates the cofactor SAM, includes CpG DNA methylation levels and protein methylation (including histones), which affect gene expression and DNA stability (138–141). However, methylation patterns are affected by not only folate but also by vitamin B-12, choline, threonine, and other one-carbon donors (415, 416). Changes in the DNA and histone methylome can be either global or specific to one or more genetic loci.

New technologies as research tools

High-throughput omics platforms for data collection. Biomarker discovery and validation, and determination of interindividual variation in biomarker responses to dietary exposures, increasingly require profiling of the metabolome, transcriptome, genetic variation, and the epigenome, including for quantitative profiling of biomarkers of one-carbon metabolism (417, 418). Rates and costs of data collection are limiting factors in biomarker discovery, validation, and use. Untargeted metabolomic approaches have the potential to reveal unexpected and new associations among metabolites and disease risks but are limited by the ability to detect low-abundance compounds beyond what current MS technologies can provide (419, 420). This approach is vulnerable to false discovery, as shown by the reported association between prostate cancer and urinary sarcosine (421), which could not be confirmed, probably because of interference from alanine (422). The risk of false discovery can be addressed by large sample size, data splitting, and validation cohorts (423). Therefore, candidate biomarkers identified through untargeted approaches must be validated and their biological plausibility established to become true targets.

Targeted metabolic profiling may be hypothesis driven, and it may be difficult to formulate adequate hypotheses within a system composed of complex interactive metabolic networks. However, methods can be developed and optimized to quantitatively determine a panel of low- and high-abundance metabolites within a defined metabolic pathway and the statistical problem of false discovery rate is mitigated. Last, the development and implementation of appropriate QC systems is currently a limiting factor in all "omics" research (424). Looking forward, the greatest need and opportunity in biomarker research and development is to exploit new or existing technologies to serve as platforms for simultaneous measurement of multiple biomarkers, particularly in field settings. Such methods are under development and offer the potential to assess comprehensively the nutritional status of individuals and populations.

Mathematical models to predict, identify, and integrate biomarkers of impaired folate metabolism. Deterministic mathematical models of one-carbon metabolism have been reported that describe the network including the effect of genetic variation and nutritional status on network outputs (158, 425). These deterministic modeling approaches use ordinary differential equations and represent metabolites as continuous variables (426) and serve to model system behavior from reaction velocities, usually described in terms of Michaelis-Menten kinetic parameters. Deterministic models have several limitations, the most important being that the combinatorial complexity of the system, when including all reactions, interactions, and gene expression data, can become a limiting factor (427). Thus, other approaches are needed to model folate metabolism and all of its interactions and regulation in the context of whole-body physiology.

Computable models can be generated for integrating and interpreting large-scale omics data (428), including transcriptomic (429–431), proteomic, and metabolomic data (432, 433). These reconstructions can be incorporated into in silico models for mathematical modeling to study network properties, structure, and dynamics. Mathematical modeling enables simulations and predictions of network responses to genetic and environmental perturbations, including drug treatments and nutritional interventions, and can assist in the discovery and validation of biomarkers that inform preventative or management treatments (159, 425). They have the potential to inform DRIs for essential nutrients (434). As an example, Recon 1, a global reconstruction of the human metabolic network (430), was built from a human genome sequence and the accumulated knowledge of human metabolism, encompassing 1496 genes, 2004 proteins, 2712 metabolites, and 3311 metabolic reactions. Although still in development, Recon 1 has been used to stimulate hypothesis-driven studies of human metabolism (435, 436), for computational simulation to identify biomarkers for disease management strategies (428, 430), and to investigate the effect of dietary interventions on transcriptome profiles at different stages of the intervention (429), which revealed interactions between metabolic and inflammatory pathways on insulin sensitivity. Computer simulations have also led to the systematic effort to predict biomarkers for >300 metabolic disorders (437), which were 10 times higher than random chance (438). The prediction of extracellular biomarkers in folate metabolism will benefit from a whole-body network model that incorporates different cells and tissues (438).

Research Gaps and Needs

Lacking are functional biomarkers that report on individual pathways within the folate-mediated one-carbon metabolic network as well as validated biomarkers of risk of folate-associated pathologies including developmental anomalies, neurodegeneration, cancers, and other diseases of aging. There are no established blood metabolite biomarkers that are specific to impaired de novo purine or thymidylate biosynthesis. There are several robust biomarkers of homocysteine remethylation function, including plasma concentratons of SAM, homocysteine, and SAH. However, apart from folate, these biomarkers are sensitive to choline and vitamin B-12 status, as well as genetic variation. Blood biomarkers that report on the activity of mitochondrial one-carbon metabolism are also lacking. Plasma formate concentrations may report on mitochondrial formate production (439) but its use as a functional biomarker has not been extensively investigated. Nonketotic hyperglycinemia, an inborn error of metabolism resulting in compromised folate-dependent glycine cleavage in mitochondria, results in elevated glycine concentrations in cerebral spinal fluid, but this biomarker is not relevant for healthy populations (440).

Recent evidence suggests that alterations in gene expression specific to folate deficiency (441, 442) and proteomic changes in response to folic acid supplementation (443) can be identified, which potentially could be used to supplement interpretation of established biomarkers. Furthermore, gene expression network analysis was used to identify gene expression patterns associated with micronuclei formation under different environmental exposure conditions such as acrylamide or nitrosamine exposure (444, 445). A similar approach could be developed to identify gene expression networks that relate to folate deficiency–induced uracil misincorporation, DNA hypomethylation, and micronuclei and possibly also include folate deficiency–specific microRNAs (e.g., miR-222) in the diagnostics (446).

Future research can address all of the above but should also seek to consider the following:

- studies in better defined leukocyte subpopulations rather than total leukocytes;
- studies in easily accessible epithelial tissues, such as buccal cells and skin;
- larger studies to be able to determine the impact of genotype;
- replication studies within the same population and across populations;
- harmonization of assay protocols across laboratories;
- continuing and sustained efforts to improve assay robustness, cost-effectiveness, and transportability to low-resource settings;
- simultaneous measurement of different but complementary biomarkers (i.e., DNA methylation, uracil in DNA, and DNA or chromosomal breaks; gene expression patterns) associated with folate deficiency or excess;
- harmonization of robust study designs to determine the genomic effects of depletion and repletion of folate; and
- inclusion of gene expression network analysis to verify mechanisms underlying observed genomic effects.

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References

1. Wills L. Treatment of "pernicious anaemia of pregnancy" and "tropical anaemia" with special reference to yeast extract as a curative agent. BMJ 1931;1:1059–64.
2. Mitchell HK, Snell EE, Williams RJ. Journal of the American Chemical Society, vol. 63, 1941: the concentration of "folic acid" by Herschel K. Mitchell, Esmond E. Snell, and Roger J. Williams. Nutr Rev 1988;46:324–5.
3. Spies TD. Treatment of macrocytic anaemia with folic acid. Lancet 1946;250: 225–8.
4. Yetley EA, Pfeiffer CM, Phinney KW, Fazili Z, Lacher DA, Bailey RL, Blackmore S, Bock JL, Brody LC, Carmel R, et al. Biomarkers of folate status in NHANES: a roundtable summary. Am J Clin Nutr 2011;94 (Suppl):303S–12S.
5. Molloy AM, Daly S, Mills JL, Kirke PN, Whitehead AS, Ramsbottom D, Conley MR, Weir DG, Scott JM. Thermolabile variant of 5,10-methylenetetrahydrofolate reductase associated with low red-cell folates: implications for folate intake recommendations. Lancet 1997;349:1591–3.
6. Bagley PJ, Selhub J. A common mutation in the methylenetetrahydrofolate reductase gene is associated with an accumulation of formylated tetrahydrofolates in red blood cells. Proc Natl Acad Sci USA 1998;95:13217–20.
7. 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;92:383–9.
8. Shane B. Folate status assessment history: implications for measurement of biomarkers in NHANES. Am J Clin Nutr 2011;94(Suppl):337S–42S.
9. Pfeiffer CM, Fazili Z, Zhang M. Folate analytical methodology. In: Bailey LB, editor. Folate in health and disease. 2nd ed. Boca Raton (FL): CRC Press, Taylor & Francis Group; 2010. p. 517–74.
10. Hillman RS, Ault KA, editors. Macrocytic anemias. In: Hematology in clinical practice, 3rd ed. New York: McGraw-Hill; 2002. p. 91–100.
11. Horne III. Nutritional deficiences. In: Rodgers GP, Young NS, editors. In: Hematology in clinical practice. 3rd ed. Philadelphia: Lippincott, Williams and Wilkins; 2005. p. 11–8.
12. Wickramasinghe SN. Diagnosis of megaloblastic anaemias. Blood Rev 2006;20:299–318.
13. Aslinia F, Mazza JJ, Yale SH. Megaloblastic anemia and other causes of macrocytosis. Clin Med Res 2006;4:236–41.
14. Bull CF, Mayrhofer G, Zeegers D, Mun GLK, Hande MP, Fenech MF. Folate deficiency is associated with the formation of complex nuclear anomalies in the cytokinesis-block micronucleus cytome assay. Environ Mol Mutagen 2012;53:311–23.
15. Bills T, Spatz L. Neutrophilic hypersegmentation as an indicator of incipient folic-acid deficiency. Am J Clin Pathol 1977;68:263–7.
16. Howell WH. The life-history of the formed elements of the blood, especially the red blood corpuscles. J Morphol 1890;4:57–116.
17. Jolly JM. Sur la formation des globules rouges des mammifères. [On the formation of red blood cells in mammals.] Comptes rendus de la Societe de Biologie 1905;58:528–31 (in French).
18. Lal A, Ames BN. Association of chromosome damage detected as micronuclei with hematological diseases and micronutrient status. Mutagenesis 2011;26:57–62.
19. Higgins C. Deficiency testing for iron, vitamin B12 and folate. Nurs Times 1995;91:38–9.
20. Thuesen BH, Husemoen LLN, Ovesen L, Jorgensen T, Fenger M, Gilderson G, Linneberg A. Atopy, asthma, and lung function in relation to folate and vitamin B-12 in adults. Allergy 2010;65:1446–54.

21. Tamura T, Picciano MF, McGuire MK. Folate in pregnancy and lactation. In: Bailey LB, editor. Folate in health and disease, 2nd ed. Boca Raton (FL): CRC Press, Taylor and Francis Group; 2010. p. 111–31.
22. Stabler SP. Clinical folate deficiency. In: Bailey LB, editor. Folate in health and disease. 2nd ed. Boca Raton (FL): CRC Press, Taylor and Francis Group; 2010. p. 409–28.
23. Dickey W, Ward M, Whittle CR, Kelly MT, Pentieva K, Horigan G, Patton S, McNulty H. Homocysteine and related B-vitamin status in coeliac disease: effects of gluten exclusion and histological recovery. Scand J Gastroenterol 2008;43:682–8.
24. Halsted CH, Medici V, Esfandiari F. Influence of alcohol on folate status and methionine metabolism in relation to alcoholic liver disease. In: Bailey LB, editor. Folate in health and disease. 2nd ed. Boca Raton (FL): CRC Press, Taylor and Francis Group; 2010. p. 429–48.
25. Tower RL, Spector LG. The epidemiology of childhood leukemia with a focus on birth weight and diet. Crit Rev Clin Lab Sci 2007;44:203–42.
26. Xu X, Chen J. One-carbon metabolism and breast cancer: an epidemiological perspective. J Genet Genomics 2009;36:203–14.
27. Kennedy DA, Stern SJ, Moretti M, Matok I, Sarkar M, Nickel C, Koren G. Folate intake and the risk of colorectal cancer: a systematic review and meta-analysis. Cancer Epidemiol 2011;35:2–10.
28. Collin SM, Metcalfe C, Refsum H, Lewis SJ, Zuccolo L, Smith GD, Chen L, Harris R, Davis M, Marsden G, et al. Circulating folate, vitamin B12, homocysteine, vitamin B12 transport proteins, and risk of prostate cancer: a case-control study, systematic review, and meta-analysis. Cancer Epidemiol Biomarkers Prev 2010;19:1632–42.
29. Berger SH, Pittman DL, Wyatt MD. Uracil in DNA: consequences for carcinogenesis and chemotherapy. Biochem Pharmacol 2008;76: 697–706.
30. Duthie SJ. Folate and cancer: how DNA damage, repair and methylation impact on colon carcinogenesis. J Inherit Metab Dis 2011;34:101–9.
31. Duthie SJ. Epigenetic modifications and human pathologies: cancer and CVD. Proc Nutr Soc 2011;70:47–56.
32. Mason JB. Folate, cancer risk, and the Greek god, Proteus: a tale of two chameleons. Nutr Rev 2009;67:206–12.
33. Miller DR. A tribute to Sidney Farber—the father of modern chemotherapy. Br J Haematol 2006;134:20–6.
34. 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;381:1029–36.
35. Blom HJ, Smulders Y. Overview of homocysteine and folate metabolism: with special references to cardiovascular disease and neural tube defects. J Inherit Metab Dis 2011;34:75–81.
36. Jacka FN, Maes M, Pasco JA, Williams LJ, Berk M. Nutrient intakes and the common mental disorders in women. J Affect Disord 2012; 141:79–85.
37. Kim JM, Stewart R, Kim SW, Yang SJ, Shin IS, Yoon JS. Predictive value of folate, vitamin B12 and homocysteine levels in late-life depression. Br J Psychiatry 2008;192:268–74.
38. McCully KS. Chemical pathology of homocysteine. IV. Excitotoxicity, oxidative stress, endothelial dysfunction, and inflammation. Ann Clin Lab Sci 2009;39:219–32.
39. Chou YF, Huang RF. Mitochondrial DNA deletions of blood lymphocytes as genetic markers of low folate-related mitochondrial genotoxicity in peripheral tissues. Eur J Nutr 2009;48:429–36.
40. Malouf R, Grimley Evans J. Folic acid with or without vitamin B12 for the prevention and treatment of healthy elderly and demented people. Cochrane Database Syst Rev 2008;4:CD004514.
41. Wang X, Qin X, Demirtas H, Li J, Mao G, Huo Y, Sun N, Liu L, Xu X. Efficacy of folic acid supplementation in stroke prevention: a meta-analysis. Lancet 2007;369:1876–82.
42. Smith AD, Smith SM, de Jager CA, Whitbread P, Johnston C, Agacinski G, Oulhaj A, Bradley KM, Jacoby R, Refsum H. Homocysteine-lowering by B vitamins slows the rate of accelerated brain atrophy in mild cognitive impairment: a randomized controlled trial. PLoS ONE 2010;5:e12244.
43. de Jager CA, Oulhaj A, Jacoby R, Refsum H, Smith AD. Cognitive and clinical outcomes of homocysteine-lowering B-vitamin treatment in mild cognitive impairment: a randomized controlled trial. Int J Geriatr Psychiatry 2012;27:592–600.
44. Clarke R, Bennett D, Parish S, Lewington S, Skeaff M, Eussen SJ, Lewerin C, Stott DJ, Armitage J, Hankey GJ, et al. Effects of homocysteine lowering with B vitamins on cognitive aging: meta-analysis of 11 trials with cognitive data on 22,000 individuals. Am J Clin Nutr 2014;100:657–66.
45. Ford AH, Almeida OP. Effect of homocysteine lowering treatment on cognitive function: a systematic review and meta-analysis of randomized controlled trials. J Alzheimers Dis 2012;29:133–49.
46. Stover PJ. Polymorphisms in 1-carbon metabolism, epigenetics and folate-related pathologies. J Nutrigenet Nutrigenomics 2011;4: 293–305.
47. Beaudin AE, Stover PJ. Insights into metabolic mechanisms underlying folate-responsive neural tube defects: a minireview. Birth Defects Res A Clin Mol Teratol 2009;85:274–84.
48. Beaudin AE, Abarinov EV, Malysheva O, Perry CA, Caudill M, Stover PJ. Dietary folate, but not choline, modifies neural tube defect risk in Shmt1 knockout mice. Am J Clin Nutr 2012;95:109–14.
49. Beaudin AE, Stover PJ. Folate-mediated one-carbon metabolism and neural tube defects: balancing genome synthesis and gene expression. Birth Defects Res C Embryo Today 2007;81:183–203.
50. Copp AJ, Stanier P, Greene ND. Neural tube defects: recent advances, unsolved questions, and controversies. Lancet Neurol 2013;12:799–810.
51. MRC Vitamin Research Study Group. Prevention of neural-tube defects—results of the Medical-Research-Council Vitamin Study. Lancet 1991;338:131–7.
52. Czeizel AE, Dudas I. Prevention of the first occurrence of neural-tube defects by periconceptional vitamin supplementation. N Engl J Med 1992;327:1832–5.
53. Centers for Disease Control and Prevention, Recommendations for the use of folic acid to reduce the number of cases of spina bifida and other neural tube defects, Morbidity and Mortality Weekly Report (MMWR) 1992;41(No. RR-14):1–7.
54. McNulty HPK. Folate bioavailability. In: Bailey LB, editor. Folate in health and disease. 2nd ed. Boca Raton (FL): CRC Press, Taylor and Francis Group; 2010. p. 25–47.
55. Institute of Medicine. Dietary Reference Intakes for thiamin, riboflavin, niacin, vitamin B6, folate, vitamin B12, pantothenic acid, biotin, and choline. Washington (DC): National Academies Press; 1998.
56. Food and Agriculture Organization; World Health Organization. Report of a joint FAO/WHO Expert Consultation. Folate and folic acid: human vitamin and mineral requirements. Rome (Italy): Food and Agriculture Organization, World Health Organization, United Nations; 2004.
57. Australian Government Department of Health and Aging, National Health and Medical Research Council. Nutrient reference values for Australia and New Zealand including recommended dietary intakes. Canberra (Australia): National Health and Medical Research Council; 2006.
58. Department of Health. Dietary reference values for food energy and nutrients for the United Kingdom. Report of the Panel on Dietary Reference Values of the Committee on Medical Aspects of Food Policy. London: HMSO; 1991. Report on Health and Social Subjects No. 41.
59. Food Safety Authority of Ireland; RDA Working Group. Recommended dietary allowances for Ireland. Dublin (Ireland): Food Safety Authority of Ireland; 1999.
60. German Nutrition Society; Austrian Nutrition Society; Swiss Society for Nutrition Research; Swiss Nutrition Association. Reference values for nutrient intake. 1st English ed. Frankfort/Main (Germany): Umschau Braus GmbH, German Nutrition Society; 2002.
61. Health Council of The Netherlands. Towards an optimal use of folic acid. The Hague (The Netherlands): Health Council of The Netherlands; 2008. Publication No. 2008/02E.
62. Nordic Council of Ministers. Nordic nutrition recommendations 2004: integrating nutrition and physical activity. 4th ed. Copenhagen (Denmark); 2004.
63. Barba CV, Cabrera MI. Recommended dietary allowances harmonization in Southeast Asia. Asia Pac J Clin Nutr 2008;17(Suppl 2):405–8.
64. Bailey LB, editor. Folate in health and disease. 2nd ed. Boca Raton (FL): CRC Press, Taylor and Francis Group; 2010. p. 473–4.
65. Kauwell GPA, Diaz ML, Yang Q, Bailey LB. Folate: recommended intakes, consumption, and status. In: Bailey LB, editor. Folate in health and disease. 2nd ed. Boca Raton (FL): CRC Press, Taylor and Frances Group; 2010. p. 467–90.

66. Institute of Medicine. Using the Tolerable Upper Intake level for nutrient assessment of groups. In: Dietary Reference Intakes: applications in dietary assessment. Washington (DC): National Academies Press; 2000. p. 113–26.
67. Pfeiffer CM, Rogers LM, Bailey LB, Gregory JF III. Absorption of folate from fortified cereal-grain products and of supplemental folate consumed with or without food determined by using a dual-label stable-isotope protocol. Am J Clin Nutr 1997;66:1388–97.
68. Sauberlich HE, Kretsch MJ, Skala JH, Johnson HL, Taylor PC. Folate requirement and metabolism in nonpregnant women. Am J Clin Nutr 1987;46:1016–28.
69. USDA. USDA Food and Nutrient Database for Dietary Studies. Version 1.0 [database on the Internet]. [cited 2013 Feb 25]. Available from: www.ars.usda.gov/ba/bhnrc/fsrg. US Department of Agriculture, Agricultural Research Service; 2004.
70. FDA. Food Standards: Amendment of Standards of Identity for Enriched Grain Products to Require Addition of Folic Acid, Final Rule, 21 CFR Parts 136, 137, and 139 (1996).
71. Yang Q, Cogswell ME, Hamner HC, Carriquiry A, Bailey LB, Pfeiffer CM, Berry RJ. Folic acid source, usual intake, and folate and vitamin B-12 status in US adults: National Health and Nutrition Examination Survey (NHANES) 2003–2006. Am J Clin Nutr 2010;91:64–72. Erratum in: Am J Clin Nutr 2010;92(4):1001.
72. Centers for Disease Control and Prevention. Second National Report on Biochemical Indicators of Diet and Nutrition in the U.S. Population. Atlanta (GA): CDC; 2012.
73. Pfeiffer CM, Hughes JP, Durazo-Arvizu RA, Lacher DA, Sempos CT, Zhang M, Yetley EA, Johnson CL. Changes in measurement procedure from a radioassay to a microbiologic assay necessitate adjustment of serum and RBC folate concentrations in the U.S. population from the NHANES 1988–2010. J Nutr 2012;142:894–900.
74. Pfeiffer CM, Osterloh JD, Kennedy-Stephenson J, Picciano MF, Yetley EA, Rader JI, Johnson CL. Trends in circulating concentrations of total homocysteine among US adolescents and adults: findings from the 1991–1994 and 1999–2004 National Health and Nutrition Examination Surveys. Clin Chem 2008;54:801–13.
75. Chen LT, Rivera MA. The Costa Rican experience: reduction of neural tube defects following food fortification programs. Nutr Rev 2004;62: S40–3.
76. Food Safety Authority of Ireland. Currently no need for mandatory fortification—increased folate status negates mandatory folic acid fortification at this time. March 2009. [cited 2012 Nov 20]. Available from: https://www.fsai.ie/details.aspx?id=7706.
77. Bower C, Miller M, Payne J, Serna P. Promotion of folate for the prevention of neural tube defects: who benefits? Paediatr Perinat Epidemiol 2005;19:435–44.
78. Crider KS, Bailey LB, Berry RJ. Folic acid food fortification-its history, effect, concerns, and future directions. Nutrients 2011;3:370–84.
79. Food Fortification Initiative. Global progress. 2012. [cited 2012 Nov 20]. Available from: http://www.ffinetwork.org/global_progress/.
80. Berry R, Mullinare J, Hamner HC. Folic acid fortification: neural tube defect risk reduction—a global perspective. In: Bailey LB, editor. Folate in health and disease. 2nd ed. Boca Raton (FL): CRC Press, Taylor and Frances Group; 2010, p. 179–204.
81. World Health Organization; Food and Agriculture Organization. Guidelines on food fortification with micronutrients, Annex 7. Geneva (Switzerland): World Health Organization, Food and Agriculture Organization of the United Nations; 2006.
82. Hertrampf E, Cortes F. Folic acid fortification of wheat flour: Chile. Nutr Rev 2004;62(6 Pt 2):S44–8; discussion S49.
83. Food Fortification Initiative. Homepage [cited 2014 Aug 29]. Available from: http://ffinetwork.org/.
84. McLean E, de Benoist B, Allen LH. Review of the magnitude of folate and vitamin B12 deficiencies worldwide. Food Nutr Bull 2008;29(2, Suppl):S38–51.
85. Flynn A, Hirvonen T, Mensink GB, Ocke MC, Serra-Majem L, Stos K, Szponar L, Tetens I, Turrini A, Fletcher R, et al. Intake of selected nutrients from foods, from fortification and from supplements in various European countries. Food Nutr Res 2009;53 (DOI: 10.3402/fnr.v53i0.2038).
86. Hoey L, McNulty H, Askin N, Dunne A, Ward M, Pentieva K, Strain J, Molloy AM, Flynn CA, Scott JM. Effect of a voluntary food fortification policy on folate, related B vitamin status, and homocysteine in healthy adults. Am J Clin Nutr 2007;86:1405–13.
87. Kerr MA, Livingstone B, Bates CJ, Bradbury I, Scott JM, Ward M, Pentieva K, Mansoor MA, McNulty H. Folate, related B vitamins, and homocysteine in childhood and adolescence: potential implications for disease risk in later life. Pediatrics 2009;123:627–35.
88. Pfeiffer CM, Hughes JP, Lacher DA, Bailey RL, Berry RJ, Zhang M, Yetley EA, Rader JI, Sempos CT, Johnson CL. Estimation of trends in serum and RBC folate in the U.S. population from pre- to postfortification using assay-adjusted data from the NHANES 1988–2010. J Nutr 2012;142:886–93.
89. De Laet C, Wautrecht JC, Brasseur D, Dramaix M, Boeynaems JM, Decuyper J, Kahn A. Plasma homocysteine concentration in a Belgian school-age population. Am J Clin Nutr 1999;69:968–72.
90. van Beynum IM, den Heijer M, Thomas CM, Afman L, Oppenraay-van Emmerzaal D, Blom HJ. Total homocysteine and its predictors in Dutch children. Am J Clin Nutr 2005;81:1110–6.
91. Papandreou D, Mavromichalis I, Makedou A, Rousso I, Arvanitidou M. Reference range of total serum homocysteine level and dietary indexes in healthy Greek schoolchildren aged 6–15 years. Br J Nutr 2006;96:719–24.
92. Sweeney MR, Staines A, Daly L, Traynor A, Daly S, Bailey SW, Alverson PB, Ayling JE, Scott JM. Persistent circulating unmetabolised folic acid in a setting of liberal voluntary folic acid fortification: implications for further mandatory fortification? BMC Public Health 2009;9:295.
93. 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;136:189–94.
94. Hirsch S, Miranda D, Munoz 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;29:772–6.
95. West AA, Yan J, Perry CA, Jiang X, Malysheva OV, Caudill MA. Folate-status response to a controlled folate intake in nonpregnant, pregnant, and lactating women. Am J Clin Nutr 2012;96:789–800.
96. Berry RJ, Bailey L, Mulinare J, Bower C, Grp FAW. Fortification of flour with folic acid. Food Nutr Bull 2010;31:S22–35.
97. Mills JL, Signore C. Neural tube defect rates before and after food fortification with folic acid. Birth Defects Res A Clin Mol Teratol 2004;70(11):844–5.
98. Berry RJ, Li Z, Erickson JD, Li S, Moore CA, Wang H, Mulinare J, Zhao P, Wong LY, Gindler J, et al. Prevention of neural-tube defects with folic acid in China. China-U.S. Collaborative Project for Neural Tube Defect Prevention. N Engl J Med 1999;341:1485–90.
99. Crider KS, Devine O, Hao L, Dowling NF, Li S, Molloy AM, Li Z, Zhu J, Berry RJ. Population red blood cell folate concentrations for prevention of neural tube defects: Bayesian model. BMJ 2014;349: g4554.
100. Daly LE, Kirke PN, Molloy A, Weir DG, Scott JM. Folate levels and neural tube defects: implications for prevention. JAMA 1995;274: 1698–702.
101. World Health Organization. Guidelines for optimal serum and red blood cell folate concentrations in women of reproductive age for prevention of neural tube defects. Geneva (Switzerland): World Health Organization; 2015.
102. Mosley BS, Cleves MA, Siega-Riz AM, Shaw GM, Canfield MA, Waller DK, Werler MM, Hobbs CA, Stud NBDP. Neural tube defects and maternal folate intake among pregnancies conceived after folic acid fortification in the United States. Am J Epidemiol 2009;169:9–17.
103. Ahrens K, Yazdy MM, Mitchell AA, Werler MM. Folic acid intake and spina bifida in the era of dietary folic acid fortification. Epidemiology 2011;22:731–7.
104. Tinker SC, Cogswell ME, Devine O, Berry RJ. Folic acid intake among U.S. women aged 15–44 years, National Health and Nutrition Examination Survey, 2003–2006. Am J Prev Med 2010;38:534–42.
105. Finer LB, Zolna MR. Unintended pregnancy in the United States: incidence and disparities, 2006. Contraception 2011;84:478–85.
106. Botto LD, Moore CA, Khoury MJ, Erickson JD. Neural-tube defects. N Engl J Med 1999;341:1509–19.
107. West AA, Caudill MA. Genetic variation: impact on folate (and choline) bioefficacy. Int J Vitam Nutr Res 2010;80:319–29.
108. Botto LD, Yang QH. 5,10-Methylenetetrahydrofolate reductase gene variants and congenital anomalies: a HuGE review. Am J Epidemiol 2000;151:862–77.

109. López-Camelo JS, Castilla EE, Orioli IM, Instituto Nacional de Genetica Medica Populacional, Estudio Colaborative Latino Americano de Malformaciones Congenitas. Folic acid flour fortification: impact on the frequencies of 52 congenital anomaly types in three South American countries. Am J Med Genet A 2010;152A: 2444–58.
110. Canfield MA, Collins JS, Botto LD, Williams LJ, Mai CT, Kirby RS, Pearson K, Devine O, Mulinare J, National Birth Prevention Network. Changes in the birth prevalence of selected birth defects after grain fortification with folic acid in the United States: findings from a multistate population-based study. Birth Defects Res A Clin Mol Teratol 2005;73(10):679–89.
111. Botto LD, Lisi A, Bower C, Canfield MA, Dattani N, De Vigan C, De Walle H, Erickson DJ, Halliday J, Irgens LM, et al. Trends of selected malformations in relation to folic acid recommendations and fortification: an international assessment. Birth Defects Res A Clin Mol Teratol 2006;76(10):693–705.
112. Ionescu-Ittu R, Marelli AJ, Mackie AS, Pilote L. Prevalence of severe congenital heart disease after folic acid fortification of grain products: time trend analysis in Quebec, Canada. BMJ 2009;338:b1673.
113. Godwin KA, Sibbald B, Bedard T, Kuzejevic B, Lowry RB, Arbour L. Changes in frequencies of select congenital anomalies since the onset of folic acid fortification in a Canadian birth defect registry. Can J Public Health 2008;99:271–5.
114. Fox JT, Stover PJ. Folate-mediated one-carbon metabolism. Vitam Horm 2008;79:1–44.
115. Stover PJ, Field MS. Trafficking of intracellular folates. Adv Nutr 2011;2:325–31.
116. Qiu A, Jansen M, Sakaris A, Min SH, Chattopadhyay S, Tsai E, Sandoval C, Zhao R, Akabas MH, Goldman ID. Identification of an intestinal folate transporter and the molecular basis for hereditary folate malabsorption. Cell 2006;127:917–28.
117. Zhao R, Matherly LH, Goldman ID. Membrane transporters and folate homeostasis: intestinal absorption and transport into systemic compartments and tissues. Expert Rev Mol Med 2009;11:e4.
118. Low PS, Kularatne SA. Folate-targeted therapeutic and imaging agents for cancer. Curr Opin Chem Biol 2009;13:256–62.
119. Shane B, Garrow T, Brenner A, Chen L, Choi YJ, Hsu JC, Stover P. Folylpoly-gamma-glutamate synthetase. Adv Exp Med Biol 1993;338: 629–34.
120. Tibbetts AS, Appling DR. Compartmentalization of mammalian folate-mediated one-carbon metabolism. Annu Rev Nutr 2010;30:57–81.
121. Panetta JC, Wall A, Pui CH, Relling MV, Evans WE. Methotrexate intracellular disposition in acute lymphoblastic leukemia: a mathematical model of gamma-glutamyl hydrolase activity. Clin Cancer Res 2002;8:2423–9.
122. Anderson DD, Woeller CF, Chiang EP, Shane B, Stover PJ. Serine hydroxymethyltransferase anchors de novo thymidylate synthesis pathway to nuclear lamina for DNA synthesis. J Biol Chem 2012; 287:7051–62.
123. Luka Z, Moss F, Loukachevitch LV, Bornhop DJ, Wagner C. Histone demethylase LSD1 is a folate-binding protein. Biochemistry 2011;50: 4750–6.
124. Miranda TB, Jones PA. DNA methylation: the nuts and bolts of repression. J Cell Physiol 2007;213:384–90.
125. Winter-Vann AM, Kamen BA, Bergo MO, Young SG, Melnyk S, James SJ, Casey PJ. Targeting Ras signaling through inhibition of carboxyl methylation: an unexpected property of methotrexate. Proc Natl Acad Sci USA 2003;100:6529–34.
126. Stead LM, Jacobs RL, Brosnan ME, Brosnan JT. Methylation demand and homocysteine metabolism. Adv Enzyme Regul 2004;44:321–33.
127. Nijhout HF, Reed MC, Anderson DF, Mattingly JC, James SJ, Ulrich CM. Long-range allosteric interactions between the folate and methionine cycles stabilize DNA methylation reaction rate. Epigenetics 2006;1:81–7.
128. Wagner C. Symposium on the subcellular compartmentation of folate metabolism. J Nutr 1996;126(4, Suppl):1228S–34S.
129. Min H, Shane B, Stokstad EL. Identification of 10-formyltetrahydrofolate dehydrogenase-hydrolase as a major folate binding protein in liver cytosol. Biochim Biophys Acta 1988;967:348–53.
130. Stover P, Schirch V. 5-Formyltetrahydrofolate polyglutamates are slow tight binding inhibitors of serine hydroxymethyltransferase. J Biol Chem 1991;266:1543–50.
131. Fenech M. Folate (vitamin B9) and vitamin B12 and their function in the maintenance of nuclear and mitochondrial genome integrity. Mutat Res 2012;733:21–33.
132. Blount BC, Mack MM, Wehr CM, MacGregor JT, Hiatt RA, Wang G, Wickramasinghe SN, Everson RB, Ames BN. Folate deficiency causes uracil misincorporation into human DNA and chromosome breakage: implications for cancer and neuronal damage. Proc Natl Acad Sci USA 1997;94:3290–5.
133. Kapiszewska M, Kalemba M, Wojciech U, Milewicz T. Uracil misincorporation into DNA of leukocytes of young women with positive folate balance depends on plasma vitamin B12 concentrations and methylenetetrahydrofolate reductase polymorphisms: a pilot study. J Nutr Biochem 2005;16:467–78.
134. van den Donk M, Pellis L, Crott JW, van Engeland M, Friederich P, Nagengast FM, van Bergeijk JD, de Boer SY, Mason JB, Kok FJ, et al. Folic acid and vitamin B-12 supplementation does not favorably influence uracil incorporation and promoter methylation in rectal mucosa DNA of subjects with previous colorectal adenomas. J Nutr 2007;137:2114–20.
135. Nilsen H, Stamp G, Andersen S, Hrivnak G, Krokan HE, Lindahl T, Barnes DE. Gene-targeted mice lacking the Ung uracil-DNA glycosylase develop B-cell lymphomas. Oncogene 2003;22:5381–6.
136. Clarke S, Banfield K. S-adenosylmethionine-dependent methyltransferases. In: Carmel R, Jacobson DW, editors. Homocysteine in health and disease. Cambridge (United Kingdom): Cambridge Press; 2001. p. 63–78.
137. Herbig K, Chiang EP, Lee LR, Hills J, Shane B, Stover PJ. Cytoplasmic serine hydroxymethyltransferase mediates competition between folate-dependent deoxyribonucleotide and S-adenosylmethionine biosyntheses. J Biol Chem 2002;277:38381–9.
138. Friso S, Choi SW, Girelli D, Mason JB, Dolnikowski GG, Bagley PJ, Olivieri O, Jacques PF, Rosenberg IH, Corrocher R, et al. A common mutation in the 5,10-methylenetetrahydrofolate reductase gene affects genomic DNA methylation through an interaction with folate status. Proc Natl Acad Sci USA 2002;99:5606–11.
139. Friso S, Choi SW, Dolnikowski GG, Selhub J. A method to assess genomic DNA methylation using high-performance liquid chromatography/electrospray ionization mass spectrometry. Anal Chem 2002;74:4526–31.
140. Jaenisch R, Bird A. Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. Nat Genet 2003;33(Suppl):245–54.
141. Huang C, Sloan EA, Boerkoel CF. Chromatin remodeling and human disease. Curr Opin Genet Dev 2003;13:246–52.
142. Hoffbrand AV, Herbert V. Nutritional anemias. Semin Hematol 1999;36(4, Suppl 7):13–23.
143. McCarthy EA, Titus SA, Taylor SM, Jackson-Cook C, Moran RG. A mutation inactivating the mitochondrial inner membrane folate transporter creates a glycine requirement for survival of chinese hamster cells. J Biol Chem 2004;279:33829–36.
144. Shin YS, Chan C, Vidal AJ, Brody T, Stokstad EL. Subcellular localization of gamma-glutamyl carboxypeptidase and of folates. Biochim Biophys Acta 1976;444:794–801.
145. Lin BF, Huang RF, Shane B. Regulation of folate and one-carbon metabolism in mammalian cells. III. Role of mitochondrial folylpoly-gamma-glutamate synthetase. J Biol Chem 1993;268:21674–9.
146. Anderson DD, Quintero CM, Stover PJ. Identification of a de novo thymidylate biosynthesis pathway in mammalian mitochondria. Proc Natl Acad Sci USA 2011;108:15163–8.
147. Bogenhagen D, Clayton DA. Mouse L cell mitochondrial DNA molecules are selected randomly for replication throughout the cell cycle. Cell 1977;11:719–27.
148. Momb J, Lewandowski JP, Bryant JD, Fitch R, Surman DR, Vokes SA, Appling DR. Deletion of Mthfd1l causes embryonic lethality and neural tube and craniofacial defects in mice. Proc Natl Acad Sci USA 2013;110:549–54.
149. Parle-McDermott A, Pangilinan F, O'Brien KK, Mills JL, Magee AM, Troendle J, Sutton M, Scott JM, Kirke PN, Molloy AM, et al. A common variant in MTHFD1L is associated with neural tube defects and mRNA splicing efficiency. Hum Mutat 2009;30:1650–6.
150. Narisawa A, Komatsuzaki S, Kikuchi A, Niihori T, Aoki Y, Fujiwara K, Tanemura M, Hata A, Suzuki Y, Relton CL, et al. Mutations in genes encoding the glycine cleavage system predispose to neural tube defects in mice and humans. Hum Mol Genet 2012;21:1496–503.

151. Di Pietro E, Sirois J, Tremblay ML, MacKenzie RE. Mitochondrial NAD-dependent methylenetetrahydrofolate dehydrogenase-methenyltetrahydrofolate cyclohydrolase is essential for embryonic development. Mol Cell Biol 2002;22:4158–66.
152. Di Pietro E, Wang XL, MacKenzie RE. The expression of mitochondrial methylenetetrahydrofolate dehydrogenase-cyclohydrolase supports a role in rapid cell growth. Biochim Biophys Acta 2004;1674:78–84.
153. Bolusani S, Young BA, Cole NA, Tibbetts AS, Momb J, Bryant JD, Solmonson A, Appling DR. Mammalian MTHFD2L encodes a mitochondrial methylenetetrahydrofolate dehydrogenase isozyme expressed in adult tissues. J Biol Chem 2011;286:5166–74.
154. Pike ST, Rajendra R, Artzt K, Appling DR. Mitochondrial C1-tetrahydrofolate synthase (MTHFD1L) supports the flow of mitochondrial one-carbon units into the methyl cycle in embryos. J Biol Chem 2010;285:4612–20.
155. Christensen KE, MacKenzie RE. Mitochondrial one-carbon metabolism is adapted to the specific needs of yeast, plants and mammals. BioEssays 2006;28:595–605.
156. Reed MC, Nijhout HF, Neuhouser ML, Gregory JF III, Shane B, James SJ, Boynton A, Ulrich CM. A mathematical model gives insights into nutritional and genetic aspects of folate-mediated one-carbon metabolism. J Nutr 2006;136:2653–61.
157. Nijhout HF, Reed MC, Lam SL, Shane B, Gregory JF III, Ulrich CM. In silico experimentation with a model of hepatic mitochondrial folate metabolism. Theor Biol Med Model 2006;3:40.
158. Nijhout HF, Reed MC, Budu P, Ulrich CM. A mathematical model of the folate cycle: new insights into folate homeostasis. J Biol Chem 2004;279:55008–16.
159. Nijhout HF, Gregory JF, Fitzpatrick C, Cho E, Lamers KY, Ulrich CM, Reed MC. A mathematical model gives insights into the effects of vitamin B-6 deficiency on 1-carbon and glutathione metabolism. J Nutr 2009;139:784–91.
160. Martinov MV, Vitvitsky VM, Banerjee R, Ataullakhanov FI. The logic of the hepatic methionine metabolic cycle. Biochim Biophys Acta 2010;1804:89–96.
161. Cuskelly GJ, Stacpoole PW, Williamson J, Baumgartner TG, Gregory JF III. Deficiencies of folate and vitamin B(6) exert distinct effects on homocysteine, serine, and methionine kinetics. Am J Physiol Endocrinol Metab 2001;281:E1182–90.
162. Davis SR, Scheer JB, Quinlivan EP, Coats BS, Stacpoole PW, Gregory JF III. Dietary vitamin B-6 restriction does not alter rates of homocysteine remethylation or synthesis in healthy young women and men. Am J Clin Nutr 2005;81:648–55.
163. Lima CP, Davis SR, Mackey AD, Scheer JB, Williamson J, Gregory JF III. Vitamin B-6 deficiency suppresses the hepatic transsulfuration pathway but increases glutathione concentration in rats fed AIN-76A or AIN-93G diets. J Nutr 2006;136:2141–7.
164. Davis SR, Quinlivan EP, Shelnutt KP, Ghandour H, Capdevila A, Coats BS, Wagner C, Shane B, Selhub J, Bailey LB, et al. Homocysteine synthesis is elevated but total remethylation is unchanged by the methylenetetrahydrofolate reductase 677C->T polymorphism and by dietary folate restriction in young women. J Nutr 2005;135:1045–50.
165. Lamers Y, Coats B, Ralat M, Quinlivan EP, Stacpoole PW, Gregory JF III. Moderate vitamin B-6 restriction does not alter postprandial methionine cycle rates of remethylation, transmethylation, and total transsulfuration but increases the fractional synthesis rate of cystathionine in healthy young men and women. J Nutr 2011;141:835–42.
166. Lamers Y, Williamson J, Gilbert LR, Stacpoole PW, Gregory JF III. Glycine turnover and decarboxylation rate quantified in healthy men and women using primed, constant infusions of [1,2-(13)C2]glycine and [(2)H3]leucine. J Nutr 2007;137:2647–52.
167. Lamers Y, Williamson J, Ralat M, Quinlivan EP, Gilbert LR, Keeling C, Stevens RD, Newgard CB, Ueland PM, Meyer K, et al. Moderate dietary vitamin B-6 restriction raises plasma glycine and cystathionine concentrations while minimally affecting the rates of glycine turnover and glycine cleavage in healthy men and women. J Nutr 2009;139:452–60.
168. Oltean S, Banerjee R. Nutritional modulation of gene expression and homocysteine utilization by vitamin B12. J Biol Chem 2003;278:20778–84.
169. Savage DG, Lindenbaum J, Stabler SP, Allen RH. Sensitivity of serum methylmalonic acid and total homocysteine determinations for diagnosing cobalamin and folate deficiencies. Am J Med 1994;96:239–46.
170. Shane B, Stokstad EL. Vitamin B12-folate interrelationships. Annu Rev Nutr 1985;5:115–41.
171. Molloy AM. Folate-vitamin B12 interrelationships: links to disease risk. In: Bailey LB, editor. Folate in health and disease. 2nd ed. Boca Raton (FL): CRC Press, Taylor and Francis Group; 2010, p. 381–408.
172. Smulders YM, Smith DE, Kok RM, Teerlink T, Swinkels DW, Stehouwer CD, Jakobs C. Cellular folate vitamer distribution during and after correction of vitamin B12 deficiency: a case for the methylfolate trap. Br J Haematol 2006;132:623–9.
173. Bjørke-Monsen AL, Torsvik I, Saetran H, Markestad T, Ueland PM. Common metabolic profile in infants indicating impaired cobalamin status responds to cobalamin supplementation. Pediatrics 2008;122:83–91.
174. Hure AJ, Collins CE, Smith R. A longitudinal study of maternal folate and vitamin B12 status in pregnancy and postpartum, with the same infant markers at 6 months of age. Matern Child Health J 2012;16:792–801.
175. Herbert V, Zalusky R. Interrelations of vitamin B12 and folic acid metabolism: folic acid clearance studies. J Clin Invest 1962;41:1263–76.
176. Waters AH, Mollin DL. Observations on the metabolism of folic acid in pernicious anaemia. Br J Haematol 1963;9:319–27.
177. Cooper BA, Lowenstein L. Relative folate deficiency of erythrocytes in pernicious anemia and its correction with cyanocobalamin. Blood 1964;24:502–21.
178. Nixon PF, Bertino JR. Impaired utilization of serum folate in pernicious anemia. A study with radiolabeled 5-methyltetrahydrofolate. J Clin Invest 1972;51:1431–9.
179. Refsum H, Smith AD, Ueland PM, Nexo E, Clarke R, McPartlin J, Johnston C, Engbaek F, Schneede J, McPartlin C, et al. Facts and recommendations about total homocysteine determinations: an expert opinion. Clin Chem 2004;50:3–32.
180. Suh JR, Oppenheim EW, Girgis S, Stover PJ. Purification and properties of a folate-catabolizing enzyme. J Biol Chem 2000;275:35646–55.
181. Oppenheim EW, Adelman C, Liu X, Stover PJ. Heavy chain ferritin enhances serine hydroxymethyltransferase expression and de novo thymidine biosynthesis. J Biol Chem 2001;276:19855–61.
182. Woeller CF, Fox JT, Perry C, Stover PJ. A ferritin-responsive internal ribosome entry site regulates folate metabolism. J Biol Chem 2007;282:29927–35.
183. Westerman DA, Evans D, Metz J. Neutrophil hypersegmentation in iron deficiency anaemia: a case-control study. Br J Haematol 1999;107:512–5.
184. Metz J. A high prevalence of biochemical evidence of vitamin B12 or folate deficiency does not translate into a comparable prevalence of anemia. Food Nutr Bull 2008;29(2, Suppl):S74–85.
185. 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.
186. Gregory JF III, Quinlivan EP. In vivo kinetics of folate metabolism. Annu Rev Nutr 2002;22:199–220.
187. O'Connor DL. Interaction of iron and folate during reproduction. Prog Food Nutr Sci 1991;15:231–54.
188. Herbig AK, Stover P. Regulation of folate metabolism by iron. In: Massaro EJ, Rogers JM, editors. Folate and human development. Clinton (NJ): Humana Press; 2002. p. 241–62.
189. Khambalia A, Latulippe ME, Campos C, Merlos C, Villalpando S, Picciano MF, O'Connor DL. Milk folate secretion is not impaired during iron deficiency in humans. J Nutr 2006;136:2617–24.
190. Shin W, Yan J, Abratte CM, Vermeylen F, Caudill MA. Choline intake exceeding current dietary recommendations preserves markers of cellular methylation in a genetic subgroup of folate-compromised men. J Nutr 2010;140:975–80.
191. Shaw GM, Carmichael SL, Yang W, Selvin S, Schaffer DM. Periconceptional dietary intake of choline and betaine and neural tube defects in offspring. Am J Epidemiol 2004;160:102–9.
192. Shaw GM, Finnell RH, Blom HJ, Carmichael SL, Vollset SE, Yang W, Ueland PM. Choline and risk of neural tube defects in a folate-fortified population. Epidemiology 2009;20:714–9.
193. Enaw JO, Zhu H, Yang W, Lu W, Shaw GM, Lammer EJ, Finnell RH. CHKA and PCYT1A gene polymorphisms, choline intake and spina bifida risk in a California population. BMC Med 2006;4:36.

194. Chew TW, Jiang X, Yan J, Wang W, Lusa AL, Carrier BJ, West AA, Malysheva OV, Brenna JT, Gregory JF III, et al. Folate intake, MTHFR genotype, and sex modulate choline metabolism in mice. J Nutr 2011;141:1475–81.
195. Carmichael SL, Yang W, Shaw GM. Periconceptional nutrient intakes and risks of neural tube defects in California. Birth Defects Res A Clin Mol Teratol 2010;88:670–8.
196. Kohlmeier M, da Costa KA, Fischer LM, Zeisel SH. Genetic variation of folate-mediated one-carbon transfer pathway predicts susceptibility to choline deficiency in humans. Proc Natl Acad Sci USA 2005;102:16025–30.
197. Yan J, Wang W, Gregory JF III, Malysheva O, Brenna JT, Stabler SP, Allen RH, Caudill MA. MTHFR C677T genotype influences the isotopic enrichment of one-carbon metabolites in folate-compromised men consuming d9-choline. Am J Clin Nutr 2011;93:348–55.
198. Davis SR, Quinlivan EP, Shelnutt KP, Maneval DR, Ghandour H, Capdevila A, Coats BS, Wagner C, Selhub J, Bailey LB, et al. The methylenetetrahydrofolate reductase 677C->T polymorphism and dietary folate restriction affect plasma one-carbon metabolites and red blood cell folate concentrations and distribution in women. J Nutr 2005;135:1040–4.
199. Ueland PM, Holm PI, Hustad S. Betaine: a key modulator of one-carbon metabolism and homocysteine status. Clin Chem Lab Med 2005;43:1069–75.
200. Caudill MA. Folate and choline interrelationships: metabolic and potential health implications. In: Bailey LB, editor. Folate in health and disease. 2nd ed. Boca Raton (FL): CRC Press, Taylor and Francis Group; 2009. p. 449–65.
201. Selhub J, Seyoum E, Pomfret EA, Zeisel SH. Effects of choline deficiency and methotrexate treatment upon liver folate content and distribution. Cancer Res 1991;51:16–21.
202. Varela-Moreiras G, Ragel C, Perez de Miguelsanz J. Choline deficiency and methotrexate treatment induces marked but reversible changes in hepatic folate concentrations, serum homocysteine and DNA methylation rates in rats. J Am Coll Nutr 1995;14:480–5.
203. da Costa KA, Gaffney CE, Fischer LM, Zeisel SH. Choline deficiency in mice and humans is associated with increased plasma homocysteine concentration after a methionine load. Am J Clin Nutr 2005;81:440–4.
204. Svardal AM, Ueland PM, Berge RK, Aarsland A, Aarsaether N, Lonning PE, Refsum H. Effect of methotrexate on homocysteine and other sulfur compounds in tissues of rats fed a normal or a defined, choline-deficient diet. Cancer Chemother Pharmacol 1988;21:313–8.
205. Pomfret EA, daCosta KA, Zeisel SH. Effects of choline deficiency and methotrexate treatment upon rat liver. J Nutr Biochem 1990;1:533–41.
206. Varelamoreiras G, Selhub J, Dacosta KA, Zeisel SH. Effect of chronic choline deficiency in rats on liver folate content and distribution. J Nutr Biochem 1992;3:519–22.
207. Scheer JB, Mackey AD, Gregory JF III. Activities of hepatic cytosolic and mitochondrial forms of serine hydroxymethyltransferase and hepatic glycine concentration are affected by vitamin B-6 intake in rats. J Nutr 2005;135:233–8.
208. Selhub J, Jacques PF, Wilson PW, Rush D, Rosenberg IH. Vitamin status and intake as primary determinants of homocysteinemia in an elderly population. JAMA 1993;270:2693–8.
209. Miller JW, Ribaya-Mercado JD, Russell RM, Shepard DC, Morrow FD, Cochary EF, Sadowski JA, Gershoff SN, Selhub J. Effect of vitamin B-6 deficiency on fasting plasma homocysteine concentrations. Am J Clin Nutr 1992;55:1154–60.
210. Davis SR, Quinlivan EP, Stacpoole PW, Gregory JF III. Plasma glutathione and cystathionine concentrations are elevated but cysteine flux is unchanged by dietary vitamin B-6 restriction in young men and women. J Nutr 2006;136:373–8.
211. da Silva VR, Ralat MA, Quinlivan EP, DeRatt BN, Garrett TJ, Chi YY, Frederik Nijhout H, Reed MC, Gregory JF III. Targeted metabolomics and mathematical modeling demonstrate that vitamin B-6 restriction alters one-carbon metabolism in cultured HepG2 cells. Am J Physiol Endocrinol Metab 2014;307:E93–101.
212. Hustad S, Midttun O, Schneede J, Vollset SE, Grotmol T, Ueland PM. The methylenetetrahydrofolate reductase 677C→T polymorphism as a modulator of a B vitamin network with major effects on homocysteine metabolism. Am J Hum Genet 2007;80:846–55.
213. Yamada K, Chen Z, Rozen R, Matthews RG. Effects of common polymorphisms on the properties of recombinant human methylenetetrahydrofolate reductase. Proc Natl Acad Sci USA 2001;98:14853–8.
214. Pejchal R, Campbell E, Guenther BD, Lennon BW, Matthews RG, Ludwig ML. Structural perturbations in the Ala → Val polymorphism of methylenetetrahydrofolate reductase: how binding of folates may protect against inactivation. Biochemistry 2006;45:4808–18.
215. Ward M, Wilson CP, Strain JJ, Horigan G, Scott JM, McNulty H. B-vitamins, methylenetetrahydrofolate reductase (MTHFR) and hypertension. Int J Vitam Nutr Res 2011;81:240–4.
216. Horigan G, McNulty H, Ward M, Strain JJ, Purvis J, Scott JM. Riboflavin lowers blood pressure in cardiovascular disease patients homozygous for the 677C→T polymorphism in MTHFR. J Hypertens 2010;28:478–86.
217. Wilson CP, McNulty H, Ward M, Strain JJ, Trouton TG, Hoeft BA, Weber P, Roos FF, Horigan G, McAnena L, et al. Blood pressure in treated hypertensive individuals with the MTHFR 677TT genotype is responsive to intervention with riboflavin: findings of a targeted randomized trial. Hypertension 2013;61:1302–8.
218. Jacques PF, Bostom AG, Williams RR, Ellison RC, Eckfeldt JH, Rosenberg IH, Selhub J, Rozen R. Relation between folate status, a common mutation in methylenetetrahydrofolate reductase, and plasma homocysteine concentrations. Circulation 1996;93:7–9.
219. Frosst P, Blom HJ, Milos R, Goyette P, Sheppard CA, Matthews RG, Boers GJ, den Heijer M, Kluijtmans LA, van den Heuvel LP, et al. A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. Nat Genet 1995;10:111–3.
220. McNulty H, Dowey le RC, Strain JJ, Dunne A, Ward M, Molloy AM, McAnena LB, Hughes JP, Hannon-Fletcher M, Scott JM. Riboflavin lowers homocysteine in individuals homozygous for the MTHFR 677C->T polymorphism. Circulation 2006;113:74–80.
221. 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 USA 2009;106:15424–9.
222. 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;80:680–91.
223. Metz J, Stevens K, Krawitz S, Brandt V. The plasma clearance of injected doses of folic acid as an index of folic acid deficiency. J Clin Pathol 1961;14:622–5.
224. Zettner A, Duly PE. New evidence for a binding principle specific for folates as a normal constituent of human serum. Clin Chem 1974;20:1313–9.
225. Høier-Madsen M, Holm J, Hansen SI. alpha Isoforms of soluble and membrane-linked folate-binding protein in human blood. Biosci Rep 2008;28:153–60.
226. Waxman S, Schreiber C. Measurement of serum folate levels and serum folic acid-binding protein by 3H-PGA radioassay. Blood 1973;42:281–90.
227. Thompson FE, Byers T. Dietary assessment resource manual. J Nutr 1994;124(Suppl):2245S–317S.
228. Campbell VA, Dodds ML. Collecting dietary information from groups of older people. J Am Diet Assoc 1967;51:29–33.
229. Marr JW, Heady JA. Within- and between-person variation in dietary surveys: number of days needed to classify individuals. Hum Nutr Appl Nutr 1986;40:347–64.
230. Heady JA. Diets of bank clerks—development of a method of classifying the diets of individuals for use in epidemiological studies. J R Stat Soc Ser A Stat Soc 1961;124(3):336–61.
231. Subar AF, Kipnis V, Troiano RP, Midthune D, Schoeller DA, Bingham S, Sharbaugh CO, Trabulsi J, Runswick S, Ballard-Barbash R, et al. Using intake biomarkers to evaluate the extent of dietary misreporting in a large sample of adults: the OPEN study. Am J Epidemiol 2003;158:1–13.
232. 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;91:231–7.
233. 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;92:353–8.
234. Bailey RL, Fulgoni VL, Keast DR, Dwyer JT. Examination of vitamin intakes among US adults by dietary supplement use. J Acad Nutr Diet 2012;112:657–63.
235. Bailey RL, Fulgoni VL, Keast DR, Lentino CV, Dwyer JT. Do dietary supplements improve micronutrient sufficiency in children and adolescents? J Pediatr 2012;161:837.

236. National Cancer Institute. Measurement error webinar series [cited 2014 Aug 29]. Available from: http://appliedresearch.cancer.gov/measurementerror/.
237. Dodd KW, Guenther PM, Freedman LS, Subar AF, Kipnis V, Midthune D, Tooze JA, Krebs-Smith SM. Statistical methods for estimating usual intake of nutrients and foods: a review of the theory. J Am Diet Assoc 2006;106:1640–50.
238. National Research Council. Nutrient adequacy. Washington (DC): National Academies Press; 1986.
239. Nusser SM, Carriquiry AL, Dodd KW, Fuller WA. A semiparametric transformation approach to estimating usual daily intake distributions. J Am Stat Assoc 1996;91:1440–9.
240. Subar AF, Dodd KW, Guenther PM, Kipnis V, Midthune D, McDowell M, Tooze JA, Freedman LS, Krebs-Smith SM. The Food Propensity Questionnaire: concept, development, and validation for use as a covariate in a model to estimate usual food intake. J Am Diet Assoc 2006;106:1556–63.
241. Tooze JA, Midthune D, Dodd KW, Freedman LS, Krebs-Smith SM, Subar AF, Guenther PM, Carroll RJ, Kipnis V. A new statistical method for estimating the usual intake of episodically consumed foods with application to their distribution. J Am Diet Assoc 2006;106:1575–87.
242. Carriquiry AL. Estimation of usual intake distributions of nutrients and foods. J Nutr 2003;133(Suppl):601S–8S.
243. USDA. Composition of foods raw, processed, prepared. USDA National Nutrient Database for Standard Reference, release 25. Washington (DC): USDA; 2012.
244. Koontz JL, Phillips KM, Wunderiach KM, Exler J, Holden JM, Gebhardt SE, Haytowitz DB. Comparison of total folate concentrations in foods determined by microbiological assay at several experienced US commercial laboratories. J AOAC Int 2005;88:805–13.
245. Hyun TH, Tamura T. Trienzyme extraction in combination with microbiologic assay in food folate analysis: an updated review. Exp Biol Med (Maywood) 2005;230:444–54.
246. Yeung L, Yang Q, Berry RJ. Contributions of total daily intake of folic acid to serum folate concentrations. JAMA 2008;300:2486–7.
247. Hopkins SM, McNulty BA, Walton J, Flynn A, Molloy AM, Scott JM, McNulty H, Nugent AP, Gibney MJ. Impact of voluntary fortification and supplement use on dietary intakes of folate and status in an Irish adult population. Proc Nutr Soc 2012;71:E38.
248. Mason JB. Biomarkers of nutrient exposure and status in one-carbon (methyl) metabolism. J Nutr 2003;133(Suppl 3):941S–7S.
249. Clifford AJ, Noceti EM, Block-Joy A, Block T, Block G. Erythrocyte folate and its response to folic acid supplementation is assay dependent in women. J Nutr 2005;135:137–43.
250. Wu A, Chanarin I, Slavin G, Levi AJ. Folate deficiency in the alcoholic–its relationship to clinical and haematological abnormalities, liver disease and folate stores. Br J Haematol 1975;29:469–78.
251. Jacob RA, Wu MM, Henning SM, Swendseid ME. Homocysteine increases as folate decreases in plasma of healthy men during short-term dietary folate and methyl group restriction. J Nutr 1994;124:1072–80.
252. Kalmbach RD, Choumenkovitch SF, Troen AM, D'Agostino R, Jacques PF, Selhub J. Circulating folic acid in plasma: relation to folic acid fortification. Am J Clin Nutr 2008;88:763–8.
253. Obeid R, Kirsch SH, Kasoha M, Eckert R, Herrmann W. Concentrations of unmetabolized folic acid and primary folate forms in plasma after folic acid treatment in older adults. Metabolism 2011;60:673–80.
254. Pfeiffer CM, Fazili Z, McCoy L, Zhang M, Gunter EW. Determination of folate vitamers in human serum by stable-isotope-dilution tandem mass spectrometry and comparison with radioassay and microbiologic assay. Clin Chem 2004;50:423–32.
255. Guinotte CL, Burns MG, Axume JA, Hata H, Urrutia TF, Alamilla A, McCabe D, Singgih A, Cogger EA, Caudill MA. Methylenetetrahydrofolate reductase 677C→T variant modulates folate status response to controlled folate intakes in young women. J Nutr 2003;133:1272–80.
256. Fukuwatari T, Shibata K. Urinary water-soluble vitamins and their metabolite contents as nutritional markers for evaluating vitamin intakes in young Japanese women. J Nutr Sci Vitaminol (Tokyo) 2008;54:223–9.
257. Gregory JF III. Case study: folate bioavailability. J Nutr 2001;131(4, Suppl):1376S–82S.
258. Caudill MA, Bailey LB, Gregory JF III. Consumption of the folate breakdown product para-aminobenzoylglutamate contributes minimally to urinary folate catabolite excretion in humans: investigation using [(13)C(5)]para-aminobenzoylglutamate. J Nutr 2002;132:2613–6.
259. Wolfe JM, Bailey LB, Herrlinger-Garcia K, Theriaque DW, Gregory JF III, Kauwell GP. Folate catabolite excretion is responsive to changes in dietary folate intake in elderly women. Am J Clin Nutr 2003;77:919–23.
260. Kownacki-Brown PA, Wang C, Bailey LB, Toth JP, Gregory JF III. Urinary excretion of deuterium-labeled folate and the metabolite p-aminobenzoylglutamate in humans. J Nutr 1993;123:1101–8.
261. Gregory JF III, Swendseid ME, Jacob RA. Urinary excretion of folate catabolites responds to changes in folate intake more slowly than plasma folate and homocysteine concentrations and lymphocyte DNA methylation in postmenopausal women. J Nutr 2000;130:2949–52.
262. Stover PJ. One-carbon metabolism-genome interactions in folate-associated pathologies. J Nutr 2009;139:2402–5.
263. Yehezkel S, Shaked R, Sagie S, Berkovitz R, Shachar-Bener H, Segev Y, Selig S. Characterization and rescue of telomeric abnormalities in ICF syndrome type I fibroblasts. Front Oncol 2013;3:35.
264. Jones PA. Functions of DNA methylation: islands, start sites, gene bodies and beyond. Nat Rev Genet 2012;13:484–92.
265. Kim YI. Nutritional epigenetics: impact of folate deficiency on DNA methylation and colon cancer susceptibility. J Nutr 2005;135:2703–9.
266. McGlynn AP, Wasson GR, O'Reilly SL, McNulty H, Downes CS, Chang CK, Hoey L, Molloy AM, Ward M, Strain JJ, et al. Low colonocyte folate is associated with uracil misincorporation and global DNA hypomethylation in human colorectum. J Nutr 2013;143:27–33.
267. Liu J, Hesson LB, Meagher AP, Bourke MJ, Hawkins NJ, Rand KN, Molloy PL, Pimanda JE, Ward RL. Relative distribution of folate species is associated with global DNA methylation in human colorectal mucosa. Cancer Prev Res (Phila) 2012;5:921–9.
268. Crider KS, Yang TP, Berry RJ, Bailey LB. Folate and DNA methylation: a review of molecular mechanisms and the evidence of folate's role. Adv Nutr 2012;3:21–38.
269. Kraunz KS, Hsiung D, McClean MD, Liu M, Osanyingbemi J, Nelson HH, Kelsey KT. Dietary folate is associated with p16(INK4A) methylation in head and neck squamous cell carcinoma. Int J Cancer 2006;119:1553–7.
270. Duthie SJ, Narayanan S, Blum S, Pirie L, Brand GM. Folate deficiency in vitro induces uracil misincorporation and DNA hypomethylation and inhibits DNA excision repair in immortalized normal human colon epithelial cells. Nutr Cancer 2000;37:245–51.
271. Piyathilake CJ, Johanning GL, Macaluso M, Whiteside M, Oelschlager DK, Heimburger DC, Grizzle WE. Localized folate and vitamin B-12 deficiency in squamous cell lung cancer is associated with global DNA hypomethylation. Nutr Cancer 2000;37:99–107.
272. Stern LL, Mason JB, Selhub J, Choi SW. Genomic DNA hypomethylation, a characteristic of most cancers, is present in peripheral leukocytes of individuals who are homozygous for the C677T polymorphism in the methylenetetrahydrofolate reductase gene. Cancer Epidemiol Biomarkers Prev 2000;9:849–53.
273. Rampersaud GC, Kauwell GP, Hutson AD, Cerda JJ, Bailey LB. Genomic DNA methylation decreases in response to moderate folate depletion in elderly women. Am J Clin Nutr 2000;72:998–1003.
274. Jacob RA, Gretz DM, Taylor PC, James SJ, Pogribny IP, Miller BJ, Henning SM, Swendseid ME. Moderate folate depletion increases plasma homocysteine and decreases lymphocyte DNA methylation in postmenopausal women. J Nutr 1998;128:1204–12.
275. Axume J, Smith SS, Pogribny IP, Moriarty DJ, Caudill MA. Global leukocyte DNA methylation is similar in African American and Caucasian women under conditions of controlled folate intake. Epigenetics 2007;2:66–8.
276. Shelnutt KP, Kauwell GP, Gregory JF III, Maneval DR, Quinlivan EP, Theriaque DW, Henderson GN, Bailey LB. Methylenetetrahydrofolate reductase 677C→T polymorphism affects DNA methylation in response to controlled folate intake in young women. J Nutr Biochem 2004;15:554–60.
277. Fenech M, Crott JW. Micronuclei, nucleoplasmic bridges and nuclear buds induced in folic acid deficient human lymphocytes—evidence for breakage-fusion-bridge cycles in the cytokinesis-block micronucleus assay. Mutat Res 2002;504:131–6.

278. Fenech M, Rinaldi J. The relationship between micronuclei in human-lymphocytes and plasma-levels of vitamin-C, vitamin-E, vitamin-B-12 and folic-acid. Carcinogenesis 1994;15:1405–11.
279. Fenech M, Baghurst P, Luderer W, Turner J, Record S, Ceppi M, Bonassi S. Low intake of calcium, folate, nicotinic acid, vitamin E, retinol, beta-carotene and high intake of pantothenic acid, biotin and riboflavin are significantly associated with increased genome instability—results from a dietary intake and micronucleus index survey in South Australia. Carcinogenesis 2005;26:991–9.
280. Andreassi MG, Botto N, Cocci F, Battaglia D, Antonioli E, Masetti S, Manfredi S, Colombo MG, Biagini A, Clerico A. Methylenetetrahydrofolate reductase gene C677T polymorphism, homocysteine, vitamin B12, and DNA damage in coronary artery disease. Hum Genet 2003;112:171–7.
281. Fenech M. Micronucleus frequency in human lymphocytes is related to plasma vitamin B12 and homocysteine. Mutat Res 1999;428:299–304.
282. MacGregor JT, Wehr CM, Hiatt RA, Peters B, Tucker JD, Langlois RG, Jacob RA, Jensen RH, Yager JW, Shigenaga MK, et al. 'Spontaneous' genetic damage in man: evaluation of interindividual variability, relationship among markers of damage, and influence of nutritional status. Mutat Res 1997;377:125–35.
283. Kliemann M, Pra D, Muller LL, Hermes L, Horta JA, Reckziegel MB, Burgos MS, Maluf SW, Franke SIR, da Silva J. DNA damage in children and adolescents with cardiovascular disease risk factors. An Acad Bras Cienc 2012;84:833–40.
284. Fenech MF, Dreosti IE, Rinaldi JR. Folate, vitamin B12, homocysteine status and chromosome damage rate in lymphocytes of older men. Carcinogenesis 1997;18:1329–36.
285. Fenech M, Aitken C, Rinaldi J. Folate, vitamin B12, homocysteine status and DNA damage in young Australian adults. Carcinogenesis 1998;19:1163–71.
286. Titenko-Holland N, Jacob RA, Shang N, Balaraman A, Smith MT. Micronuclei in lymphocytes and exfoliated buccal cells of postmenopausal women with dietary changes in folate. Mutat Res 1998; 417:101–14.
287. Stopper H, Treutlein AT, Bahner U, Schupp N, Schmid U, Brink A, Perna A, Heidland A. Reduction of the genomic damage level in haemodialysis patients by folic acid and vitamin B12 supplementation. Nephrol Dial Transplant 2008;23:3272–9.
288. Fenech M, Bonassi S. The effect of age, gender, diet and lifestyle on DNA damage measured using micronucleus frequency in human peripheral blood lymphocytes. Mutagenesis 2011;26:43–9.
289. Fenech M, Holland N, Chang WP, Zeiger E, Bonassi S. The HUman MicroNucleus Project—an international collaborative study on the use of the micronucleus technique for measuring DNA damage in humans. Mutat Res 1999;428:271–83.
290. Rossnerova A, Spatova M, Schunck C, Sram RJ. Automated scoring of lymphocyte micronuclei by the MetaSystems Metafer image cytometry system and its application in studies of human mutagen sensitivity and biodosimetry of genotoxin exposure. Mutagenesis 2011;26:169–75.
291. Decordier I, Papine A, Vande Loock K, Plas G, Soussaline F, Kirsch-Volders M. Automated image analysis of micronuclei by IMSTAR for biomonitoring. Mutagenesis 2011;26:163–8.
292. Shane B. Folate chemistry and metabolism. In: Bailey LB, editor. Folate in health and disease. 2nd ed. Boca Raton (FL): CRC Press, Taylor and Francis Group; 2010. p. 1–24.
293. Fazili Z, Pfeiffer CM. Accounting for an isobaric interference allows correct determination of folate vitamers in serum by isotope dilution-liquid chromatography-tandem MS. J Nutr 2013;143:108–13.
294. Fazili Z, Pfeiffer CM, Zhang M. Comparison of serum folate species analyzed by LC-MS/MS with total folate measured by microbiologic assay and Bio-Rad radioassay. Clin Chem 2007;53:781–4.
295. De Brouwer V, Zhang GF, Storozhenko S, Straeten DV, Lambert WE. pH stability of individual folates during critical sample preparation steps in prevision of the analysis of plant folates. Phytochem Anal 2007;18:496–508.
296. Gregory JF III. Chemical and nutritional aspects of folate research: analytical procedures, methods of folate synthesis, stability, and bioavailability of dietary folates. Adv Food Nutr Res 1989;33:1–101.
297. Foo SK, Cichowicz DJ, Shane B. Cleavage of naturally-occurring folates to unsubstituted para-aminobenzoylpoly-gamma-glutamates. Anal Biochem 1980;107:109–15.
298. Gapski GR, Whiteley JM, Huennekens FM. Hydroxylated derivatives of 5-methyl-5,6,7,8-tetrahydrofolate. Biochemistry 1971;10:2930–4.
299. Jongejan JAMH, Berends W. Autoxidation of 5-alkyl-tetrahydropteridines the oxidation product of 5-methyl-THF. In: Proceedings of the Sixth International Symposium on the Chemistry and Biology of Pteridines; 1978 Sep 25–28; La Jolla, CA. New York: Elsevier/North-Holland; 1979.
300. Fazili Z, Whitehead RD Jr., Paladugula N, Pfeiffer CM. A high-throughput LC-MS/MS method suitable for population biomonitoring measures five serum folate vitamers and one oxidation product. Anal Bioanal Chem 2013;405:4549–60.
301. Reed LS, Archer MC. Oxidation of tetrahydrofolic acid by air. J Agric Food Chem 1980;28:801–5.
302. Robinson DR. The nonenzymatic hydrolysis of N5,N10-methenyltetrahydrofolic acid and related reactions. In: Chytil F, editor. Methods in enzymology. New York: Academic Press; 1971. p. 716–25.
303. Gregory JF, Ristow KA, Sartain DB, Damron BL. Biological-activity of the folacin oxidation-products 10-formylfolic acid and 5-methyl-5,6-dihydrofolic acid. J Agric Food Chem 1984;32:1337–42.
304. Yetley EA, Johnson CL. Folate and vitamin B-12 biomarkers in NHANES: history of their measurement and use. Am J Clin Nutr 2011;94(Suppl):322S–31S.
305. Pfeiffer CM, Zhang M, Lacher DA, Molloy AM, Tamura T, Yetley EA, Picciano MF, Johnson CL. Comparison of serum and red blood cell folate microbiologic assays for national population surveys. J Nutr 2011;141:1402–9.
306. Centers for Disease Control and Prevention; National Center for Health Statistics. Serum and red blood cell folate [cited 2014 Aug 29]. Available from: http://www.cdc.gov/nchs/nhanes/nhanes2009–2010/FOLATE_F.htm.
307. Davis RE, Nicol DJ, Kelly A. An automated method for measurement of folate activity. J Clin Pathol 1970;23:47–53.
308. Grossowicz N, Waxman S, Schreiber C. Cryoprotected lactobacillus-casei—an approach to standardization of microbiological assay of folic-acid in serum. Clin Chem 1981;27:745–7.
309. Newman EM, Tsai JF. Microbiological analysis of 5-formyltetrahydrofolic acid and other folates using an automatic 96-well plate reader. Anal Biochem 1986;154:509–15.
310. Horne DW. Microbiological assay of folates in 96-well microtiter plates. Vitamins and Coenzymes. Pt K 1997;281:38–43.
311. Molloy AM, Scott JM. Microbiological assay for serum, plasma, and red cell folate using cryopreserved, microtiter plate method. Methods Enzymol 1997;281:43–53.
312. O'Broin S, Kelleher B. Microbiological assay on microtitre plates of folate in serum and red-cells. J Clin Pathol 1992;45:344–7.
313. O'Broin SD, Kelleher BP, Davoren A, Gunter EW. Field-study screening of blood folate concentrations: specimen stability and finger-stick sampling. Am J Clin Nutr 1997;66:1398–405.
314. O'Broin SD, Gunter EW. Screening of folate status with use of dried blood spots on filter paper. Am J Clin Nutr 1999;70:359–67.
315. Rabinowitz DJ, Zhang M, Paladugula N, LaVoie DJ, Pfeiffer CM. A fresh look at the folate microbiological assay, including dried blood spots and preanalytical conditions for whole blood samples. Clin Chem 2009;55:A227–8.
316. Wilson DH, Williams G, Herrmann R, Wiesner D, Brookhart P. Issues in immunoassay standardization: the ARCHITECT folate model for intermethod harmonization. Clin Chem 2005;51:684–7.
317. Shane B, Tamura T, Stokstad ELR. Folate assay—comparison of radioassay and microbiological methods. Clin Chim Acta 1980;100: 13–9.
318. Gregory JF, Sartain DB, Day BPF. Fluorometric determination of folacin in biological materials using high-performance liquid chromatography. J Nutr 1984;114:341–53.
319. Bagley PJ, Selhub J. Analysis of folates using combined affinity and ion-pair chromatography. Methods Enzymol 1997;281:16–25.
320. Bagley PJ, Selhub J. Analysis of folate form distribution by affinity followed by reversed-phase chromatography with electrical detection. Clin Chem 2000;46:404–11.
321. Nelson BC, Pfeiffer CM, Margolis SA, Nelson CP. Solid-phase extraction-electrospray ionization mass spectrometry for the quantification of folate in human plasma or serum. Anal Biochem 2004; 325:41–51.

322. Hannisdal R, Ueland PM, Svardal A. Liquid chromatography-tandem mass spectrometry analysis of folate and folate catabolites in human serum. Clin Chem 2009;55:1147–54.
323. Raiten DJ, Fisher KD. Assessment of folate methodology used in the Third National Health and Nutrition Examination Survey (NHANES III, 1988–1994). J Nutr 1995;125:1371S–98S.
324. Herbert V. Making sense of laboratory tests of folate status: folate requirements to sustain normality. Am J Hematol 1987;26:199–207.
325. Gibson RS. Principles of nutritional assessment. 2nd ed. New York: Oxford Press; 2005.
326. Selhub J, Jacques PF, Dallal G, Choumenkovitch S, Rogers G. The use of blood concentrations of vitamins and their respective functional indicators to define folate and vitamin B12 status. Food Nutr Bull 2008;29(2, Suppl):S67–73.
327. de Benoist B. Conclusions of a WHO Technical Consultation on folate and vitamin B12 deficiencies. Food Nutr Bull 2008;29(2, Suppl):S238–44.
328. Kelly P, McPartlin J, Goggins M, Weir DG, Scott JM. Unmetabolized folic acid in serum: acute studies in subjects consuming fortified food and supplements. Am J Clin Nutr 1997;65:1790–5.
329. Mansoor MA, Svardal AM, Ueland PM. Determination of the in vivo redox status of cysteine, cysteinylglycine, homocysteine, and glutathione in human plasma. Anal Biochem 1992;200:218–29.
330. Mudd SH, Finkelstein JD, Refsum H, Ueland PM, Malinow MR, Lentz SR, Jacobsen DW, Brattstrom L, Wilcken B, Wilcken DE, et al. Homocysteine and its disulfide derivatives: a suggested consensus terminology. Arterioscler Thromb Vasc Biol 2000;20:1704–6.
331. Mansoor MA, Ueland PM, Aarsland A, Svardal AM. Redox status and protein binding of plasma homocysteine and other aminothiols in patients with homocystinuria. Metabolism 1993;42:1481–5.
332. Ducros V, Demuth K, Sauvant MP, Quillard M, Causse E, Candito M, Read MH, Drai J, Garcia I, Gerhardt MF, et al. Methods for homocysteine analysis and biological relevance of the results. J Chromatogr B Analyt Technol Biomed Life Sci 2002;781:207–26.
333. Shipchandler MT, Moore EG. Rapid, fully automated measurement of plasma homocyst(e)ine with the Abbott IMx analyzer. Clin Chem 1995;41:991–4.
334. Yetley EA, Pfeiffer CM, Phinney KW, Bailey RL, Blackmore S, Bock JL, Brody LC, Carmel R, Curtin LR, Durazo-Arvizu RA, et al. Biomarkers of vitamin B-12 status in NHANES: a roundtable summary. Am J Clin Nutr 2011;94(Suppl):313S–21S.
335. Pfeiffer CM, Huff DL, Gunter EW. Rapid and accurate HPLC assay for plasma total homocysteine and cysteine in a clinical laboratory setting. Clin Chem 1999;45:290–2.
336. Tan Y, Tang L, Sun X, Zhang N, Han Q, Xu M, Baranov E, Tan X, Tan X, Rashidi B, et al. Total-homocysteine enzymatic assay. Clin Chem 2000;46:1686–8.
337. Tan Y, Hoffman RM. A highly sensitive single-enzyme homocysteine assay. Nat Protoc 2008;3:1388–94.
338. Kellogg MD, Parker R, Ricupero A, Rifai N. Evaluation of an enzymatic homocysteine assay for the Hitachi series chemistry analyzer. Clin Chim Acta 2005;354:117–22.
339. Dou C, Xia D, Zhang L, Chen X, Flores P, Datta A, Yuan C. Development of a novel enzymatic cycling assay for total homocysteine. Clin Chem 2005;51:1987–9.
340. Gempel K, Gerbitz KD, Casetta B, Bauer MF. Rapid determination of total homocysteine in blood spots by liquid chromatography-electrospray ionization-tandem mass spectrometry. Clin Chem 2000;46:122–3.
341. Turgeon CT, Magera MJ, Cuthbert CD, Loken PR, Gavrilov DK, Tortorelli S, Raymond KM, Oglesbee D, Rinaldo P, Matern D. Determination of total homocysteine, methylmalonic acid, and 2-methylcitric acid in dried blood spots by tandem mass spectrometry. Clin Chem 2010;56:1686–95.
342. Casetta B. Determination of total homocysteine in plasma using liquid chromatography coupled to tandem mass spectrometry (LC/MS/MS). Methods Mol Biol 2010;603:253–60.
343. Windelberg A, Arseth O, Kvalheim G, Ueland PM. Automated assay for the determination of methylmalonic acid, total homocysteine, and related amino acids in human serum or plasma by means of methylchloroformate derivatization and gas chromatography-mass spectrometry. Clin Chem 2005;51:2103–9.
344. Hustad S, Ueland PM, Vollset SE, Zhang Y, Bjorke-Monsen AL, Schneede J. Riboflavin as a determinant of plasma total homocysteine: effect modification by the methylenetetrahydrofolate reductase C677T polymorphism. Clin Chem 2000;46:1065–71.
345. Wollesen F, Brattstrom L, Refsum H, Ueland PM, Berglund L, Berne C. Plasma total homocysteine and cysteine in relation to glomerular filtration rate in diabetes mellitus. Kidney Int 1999;55:1028–35.
346. Clarke R, Halsey J, Lewington S, Lonn E, Armitage J, Manson JE, Bonaa KH, Spence JD, Nygard O, Jamison R, et al. Effects of lowering homocysteine levels with B vitamins on cardiovascular disease, cancer, and cause-specific mortality: meta-analysis of 8 randomized trials involving 37 485 individuals. Arch Intern Med 2010;170:1622–31.
347. Clarke R, Bennett DA, Parish S, Verhoef P, Dotsch-Klerk M, Lathrop M, Xu P, Nordestgaard BG, Holm H, Hopewell JC, et al. Homocysteine and coronary heart disease: meta-analysis of MTHFR case-control studies, avoiding publication bias. PLoS Med 2012;9:e1001177.
348. Myers GL, Christenson RH, Cushman M, Ballantyne CM, Cooper GR, Pfeiffer CM, Grundy SM, Labarthe DR, Levy D, Rifai N, et al. National Academy of Clinical Biochemistry Laboratory Medicine Practice guidelines: emerging biomarkers for primary prevention of cardiovascular disease. Clin Chem 2009;55:378–84.
349. Centers for Disease Control and Prevention. Survey toolkit for nutritional assessment. Hosted by the Micronutrient Initiative. Available from: http://www.micronutrient.org/nutritiontoolkit/.
350. Bock JL, Endres DB, Elin RJ, Wang E, Rosenzweig B, Klee GG. Comparison of fresh frozen serum to traditional proficiency testing material in a College of American Pathologists survey for ferritin, folate, and vitamin B12. Arch Pathol Lab Med 2005;129:323–7.
351. Satterfield MB, Sniegoski LT, Sharpless KE, Welch MJ, Hornikova A, Zhang NF, Pfeiffer CM, Fazili Z, Zhang M, Nelson BC. Development of a new standard reference material: SRM 1955 (homocysteine and folate in human serum). Anal Bioanal Chem 2006;385:612–22.
352. Thorpe SJ, Heath A, Blackmore S, Lee A, Hamilton M, O'Broin S, Nelson BC, Pfeiffer C. International standard for serum vitamin B(12) and serum folate: international collaborative study to evaluate a batch of lyophilised serum for B(12) and folate content. Clin Chem Lab Med 2007;45:380–6.
353. US National Institute of Standards and Technology. Standard reference materials [cited 2013 Aug 23]. Available from: http://www.nist.gov/srm/index.cfm.
354. UK National Institute for Biological Standards and Control. Biological reference materials and reagents [cited 2013 Aug 23]. Available from: http://www.nibsc.org/products/biological_reference_materials.aspx.
355. Blackmore S, Pfeiffer CM, Lee A, Fazili Z, Hamilton MS. Isotope dilution-LC-MS/MS reference method assessment of serum folate assay accuracy and proficiency testing consensus mean. Clin Chem 2011;57:986–94.
356. La'ulu SL, Rawlins ML, Pfeiffer CM, Zhang M, Roberts WL. Performance characteristics of six homocysteine assays. Am J Clin Pathol 2008;130:969–75.
357. Hanson NQ, Eckfeldt JH, Schwichtenberg K, Aras O, Tsai MY. Interlaboratory variation of plasma total homocysteine measurements: results of three successive homocysteine proficiency testing surveys. Clin Chem 2002;48:1539–45.
358. Pfeiffer CM, Huff DL, Smith SJ, Miller DT, Gunter EW. Comparison of plasma total homocysteine measurements in 14 laboratories: an international study. Clin Chem 1999;45:1261–8.
359. Nelson BC, Pfeiffer CM, Sniegoski LT, Satterfield MB. Development and evaluation of an isotope dilution LC/MS method for the determination of total homocysteine in human plasma. Anal Chem 2003;75:775–84.
360. Nelson BC, Satterfield MB, Sniegoski LT, Welch MJ. Simultaneous quantification of homocysteine and folate in human serum or plasma using liquid chromatography/tandem mass spectrometry. Anal Chem 2005;77:3586–93.
361. Nelson BC, Pfeiffer CM, Zhang M, Duewer DL, Sharpless KE, Lippa KA. Commutability of NIST SRM 1955 Homocysteine and Folate in Frozen Human Serum with selected total homocysteine immunoassays and enzymatic assays. Clin Chim Acta 2008;395:99–105.
362. Haynes BM, Pfeiffer CM, Sternberg MR, Schleicher RL. Selected physiologic variables are weakly to moderately associated with 29 biomarkers of diet and nutrition, NHANES 2003–2006. J Nutr 2013;143(Suppl):1001S–10S.

363. Fazili Z, Pfeiffer CM. Measurement of folates in serum and conventionally prepared whole blood lysates: application of an automated 96-well plate isotope-dilution tandem mass spectrometry method. Clin Chem 2004;50:2378–81.
364. O'Broin JD, Temperley IJ, Scott JM. Erythrocyte, plasma, and serum folate: specimen stability before microbiological assay. Clin Chem 1980;26:522–4.
365. Zhang DJ, Elswick RK, Miller WG, Bailey JL. Effect of serum-clot contact time on clinical chemistry laboratory results. Clin Chem 1998;44:1325–33.
366. Drammeh BS, Schleicher RL, Pfeiffer CM, Jain RB, Zhang M, Nguyen PH. Effects of delayed sample processing and freezing on serum concentrations of selected nutritional indicators. Clin Chem 2008;54: 1883–91.
367. Hannisdal R, Ueland PM, Eussen SJ, Svardal A, Hustad S. Analytical recovery of folate degradation products formed in human serum and plasma at room temperature. J Nutr 2009;139:1415–8.
368. Clement NF, Kendall BS. Effect of light on vitamin B12 and folate. Labmedicine 2009;40:657–9.
369. Fazili Z, Sternberg MR, Pfeiffer CM. Assessing the influence of 5,10-methylenetetrahydrofolate reductase polymorphism on folate stability during long-term frozen storage, thawing, and repeated freeze/thawing of whole blood. Clin Chim Acta 2012;413:966–72.
370. Lacher DAHJ, Carroll MD. Biological variation of laboratory analytes based on the 1999–2002 National Health and Nutrition Examination Survey. Hyattsville (MD): National Center for Health Statistics; 2010. National Health Statistics Report No. 21.
371. Hustad S, Eussen S, Midttun O, Ulvik A, van de Kant PM, Morkrid L, Gislefoss R, Ueland PM. Kinetic modeling of storage effects on biomarkers related to B vitamin status and one-carbon metabolism. Clin Chem 2012;58:402–10.
372. Hannisdal R, Gislefoss RE, Grimsrud TK, Hustad S, Morkrid L, Ueland PM. Analytical recovery of folate and its degradation products in human serum stored at -25 degrees C for up to 29 years. J Nutr 2010;140:522–6.
373. Whitehead VM. Pharmacokinetics and physiological disposition of folate and its derivatives. In: Blakley RL, Whitehead VM, editors. Folates and pterins. New York: Wiley; 1986. p. 177–206.
374. Scott JM, Weir DG. Excretion of folates, pterins, and their metabolic products. In: Blakley RL, Whitehead VM, editors. Folates and pterins. New York: Wiley; 1986. p. 297–324.
375. Whitehead VM, Kamen BA, Beaulieu D. Levels of dihydrofolate reductase in livers of birds, animals, primates, and man. Cancer Drug Deliv 1987;4:185–9.
376. Lucock MD, Wild J, Smithells RW, Hartley R. In vivo characterization of the absorption and biotransformation of pteroylmonoglutamic acid in man: a model for future studies. Biochem Med Metab Biol 1989;42:30–42.
377. Gregory JF III, Toth JP. Chemical synthesis of deuterated folate monoglutamate and in vivo assessment of urinary excretion of deuterated folates in man. Anal Biochem 1988;170:94–104.
378. Kalmbach R, Paul L, Selhub J. Determination of unmetabolized folic acid in human plasma using affinity HPLC. Am J Clin Nutr 2011;94:343S–7S.
379. Kelly P, McPartlin J, Scott J. A combined high-performance liquid chromatographic-microbiological assay for serum folic acid. Anal Biochem 1996;238:179–83.
380. 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;788:187–91.
381. Caudill MA, Gregory JF, Hutson AD, Bailey LB. Folate catabolism in pregnant and nonpregnant women with controlled folate intakes. J Nutr 1998;128:204–8.
382. Sokoro AA, Etter ML, Lepage J, Weist B, Eichhorst J, Lehotay DC. Simple method for the quantitative analysis of endogenous folate catabolites p-aminobenzoylglutamate (pABG) and its acetamido (apABG) derivative in human serum and urine by liquid chromatography-tandem mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci 2006;832:9–16.
383. Niesser M, Harder U, Koletzko B, Peissner W. Quantification of urinary folate catabolites using liquid chromatography-tandem mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci 2013; 929:116–24.
384. 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:e56194.
385. Toraño EG, Petrus S, Fernandez AF, Fraga MF. Global DNA hypomethylation in cancer: review of validated methods and clinical significance. Clin Chem Lab Med 2012;50:1733–42.
386. Pfarr W, Webersinke G, Paar C, Wechselberger C. Immunodetection of 5 '-methylcytosine on Giemsa-stained chromosomes. Biotechniques 2005;38:527–8, 530.
387. Nakkuntod J, Avihingsanon Y, Mutirangura A, Hirankarn N. Hypomethylation of LINE-1 but not Alu in lymphocyte subsets of systemic lupus erythematosus patients. Clin Chim Acta 2011;412: 1457–61.
388. Li Y, Chen GB, Ma LN, Ohms SJ, Sun C, Shannon MF, Fan JY. Plasticity of DNA methylation in mouse T cell activation and differentiation. BMC Mol Biol 2012;13:16.
389. Kramer TR, Moore RJ, Shippee RL, Friedl KE, Martinez Lopez L, Chan MM, Askew EW. Effects of food restriction in military training on T-lymphocyte responses. Int J Sports Med 1997;18:S84–90.
390. García-Dabrio MC, Pujol-Moix N, Martinez-Perez A, Fontcuberta J, Souto JC, Soria JM, Nomdedeu JF. Influence of age, gender and lifestyle in lymphocyte subsets: report from the Spanish Gait-2 Study. Acta Haematol 2012;127:244–9.
391. Li Q, Morimoto K, Nakadai A, Qu TL, Matsushima H, Katsumata M, Shimizu T, Inagaki H, Hirata Y, Hirata K, et al. Healthy lifestyles are associated with higher levels of perforin, granulysin and granzymes A/B-expressing cells in peripheral blood lymphocytes. Prev Med 2007;44:117–23.
392. Balaghi M, Wagner C. DNA methylation in folate-deficiency—use of Cpg methylase. Biochem Biophys Res Commun 1993;193:1184–90.
393. Sandhu J, Kaur B, Armstrong C, Talbot CJ, Steward WP, Farmer PB, Singh R. Determination of 5-methyl-2 '-deoxycytidine in genomic DNA using high performance liquid chromatography-ultraviolet detection. J Chromatogr B Analyt Technol Biomed Life Sci 2009; 877:1957–61.
394. Beck CR, Garcia-Perez JL, Badge RM, Moran JV. LINE-1 elements in structural variation and disease. Annu Rev Genomics Hum 2011;12:187–215.
395. Yang AS, Estecio MRH, Doshi K, Kondo Y, Tajara EH, Issa JPJ. A simple method for estimating global DNA methylation using bisulfite PCR of repetitive DNA elements. Nucleic Acids Res 2004;32:e38.
396. Tost J, Gut IG. DNA methylation analysis by pyrosequencing. Nat Protoc 2007;2:2265–75.
397. Metz J. The deoxyuridine suppression test. Crit Rev Clin Lab Sci 1984;20:205–41.
398. Wickramasinghe SN, Fida S. Bone marrow cells from vitamin B12- and folate-deficient patients misincorporate uracil into DNA. Blood 1994;83:1656–61.
399. Duthie SJ, Hawdon A. DNA instability (strand breakage, uracil misincorporation, and defective repair) is increased by folic acid depletion in human lymphocytes in vitro. FASEB J 1998;12:1491–7.
400. Atamna H, Cheung I, Ames BN. A method for detecting abasic sites in living cells: age-dependent changes in base excision repair. Proc Natl Acad Sci USA 2000;97:686–91.
401. de Korte D, Haverkort WA, Vangennip AH, Roos D. Nucleotide profiles of normal human-blood cells determined by high-performance liquid-chromatography. Anal Biochem 1985;147:197–209.
402. Blount BC, Ames BN. Analysis of uracil in DNA by gas chromatography-mass spectrometry. Anal Biochem 1994;219: 195–200.
403. Mashiyama ST, Hansen CM, Roitman E, Sarmiento S, Leklem JE, Shultz TD, Ames BN. An assay for uracil in human DNA at baseline: effect of marginal vitamin B6 deficiency. Anal Biochem 2008;372: 21–31.
404. Crott JW, Mashiyama ST, Ames BN, Fenech M. The effect of folic acid deficiency and MTHFR C677T polymorphism on chromosome damage in human lymphocytes in vitro. Cancer Epidemiol Biomarkers Prev 2001;10:1089–96.
405. Fenech M. Cytokinesis-block micronucleus cytome assay. Nat Protoc 2007;2:1084–104.
406. Dawson DW, Bury HPR. Significance of Howell-Jolly bodies and giant metamyelocytes in marrow smears. J Clin Pathol 1961;14:374–80.

407. Kouskoumvekaki I, Panagiotou G. Navigating the human metabolome for biomarker identification and design of pharmaceutical molecules. J Biomed Biotechnol 2011;2011 pii 25497.
408. Weljie AM, Dowlatabadi R, Miller BJ, Vogel HJ, Jirik FR. An inflammatory arthritis-associated metabolite biomarker pattern revealed by H-1 NMR spectroscopy. J Proteome Res 2007;6:3456–64.
409. Mamas M, Dunn WB, Neyses L, Goodacre R. The role of metabolites and metabolomics in clinically applicable biomarkers of disease. Arch Toxicol 2011;85:5–17.
410. LaSalle JM. A genomic point-of-view on environmental factors influencing the human brain methylome. Epigenetics 2011;6:862–9.
411. Parle-McDermott A, Ozaki M. The impact of nutrition on differential methylated regions of the genome. Adv Nutr 2011;2:463–71.
412. Caldwell PT, Manziello A, Howard J, Palbykin B, Runyan RB, Selmin O. Gene expression profiling in the fetal cardiac tissue after folate and low-dose trichloroethylene exposure. Birth Defects Res A Clin Mol Teratol 2010;88(2):111–27.
413. van Oostrom O, de Kleijn DPV, Fledderus JO, Pescatori M, Stubbs A, Tuinenburg A, Lim SK, Verhaar MC. Folic acid supplementation normalizes the endothelial progenitor cell transcriptome of patients with type 1 diabetes: a case-control pilot study. Cardiovasc Diabetol 2009;8:47.
414. Dhillon VS, Thomas P, Iarmarcovai G, Kirsch-Volders M, Bonassi S, Fenech M. Genetic polymorphisms of genes involved in DNA repair and metabolism influence micronucleus frequencies in human peripheral blood lymphocytes. Mutagenesis 2011;26:33–42.
415. Blusztajn JK, Mellott TJ. Choline nutrition programs brain development via DNA and histone methylation. Cent Nerv Syst Agents Med Chem 2012;12:82–94.
416. Shyh-Chang N, Locasale JW, Lyssiotis CA, Zheng YX, Teo RY, Ratanasirintrawoot S, Zhang J, Onder T, Unternaehrer JJ, Zhu H, et al. Influence of threonine metabolism on S-adenosylmethionine and histone methylation. Science 2013;339:222–6.
417. Fredriksen A, Meyer K, Ueland PM, Vollset SE, Grotmol T, Schneede J. Large-scale population-based metabolic phenotyping of thirteen genetic polymorphisms related to one-carbon metabolism. Hum Mutat 2007;28:856–65.
418. Midttun Ø, Hustad S, Ueland PM. Quantitative profiling of biomarkers related to B-vitamin status, tryptophan metabolism and inflammation in human plasma by liquid chromatography/tandem mass spectrometry. Rapid Commun Mass Spectrom 2009;23:1371–9.
419. Gibney MJ, Walsh M, Brennan L, Roche HM, German B, van Ommen B. Metabolomics in human nutrition: opportunities and challenges. Am J Clin Nutr 2005;82:497–503.
420. Rezzi S, Ramadan Z, Fay LB, Kochhar S. Nutritional metabonomics: applications and perspectives. J Proteome Res 2007;6:513–25.
421. Sreekumar A, Poisson LM, Rajendiran TM, Khan AP, Cao Q, Yu JD, Laxman B, Mehra R, Lonigro RJ, Li Y, et al. Metabolomic profiles delineate potential role for sarcosine in prostate cancer progression. Nature 2009;457:910–4.
422. Meyer TE, Fox SD, Issaq HJ, Xu X, Chu LW, Veenstra TD, Hsing AW. A reproducible and high-throughput HPLC/MS method to separate sarcosine from alpha- and beta-alanine and to quantify sarcosine in human serum and urine. Anal Chem 2011;83:5735–40.
423. Dahl FA, Grotle M, Benth JS, Natvig B. Data splitting as a countermeasure against hypothesis fishing: with a case study of predictors for low back pain. Eur J Epidemiol 2008;23:237–42.
424. Gika HG, Theodoridis GA, Earll M, Wilson ID. A QC approach to the determination of day-to-day reproducibility and robustness of LC-MS methods for global metabolite profiling in metabonomics/metabolomics. Bioanalysis 2012;4:2239–47.
425. Reed MC, Thomas RL, Pavisic J, James SJ, Ulrich CM, Nijhout HF. A mathematical model of glutathione metabolism. Theor Biol Med Model 2008;5:8.
426. Ullah M, Schmidt H, Cho KH, Wolkenhauer O. Deterministic modelling and stochastic simulation of biochemical pathways using MATLAB. IEE Proc Syst Biol 2006;153:53–60.
427. Bachman JA, Sorger P. New approaches to modeling complex biochemistry. Nat Methods 2011;8:130–1.
428. Bordbar A, Palsson BO. Using the reconstructed genome-scale human metabolic network to study physiology and pathology. J Intern Med 2012;271:131–41.
429. Capel F, Klimcakova E, Viguerie N, Roussel B, Vitkova M, Kovacikova M, Polak J, Kovacova Z, Galitzky J, Maoret JJ, et al. Macrophages and adipocytes in human obesity: adipose tissue gene expression and insulin sensitivity during calorie restriction and weight stabilization. Diabetes 2009;58:1558–67.
430. Duarte NC, Becker SA, Jamshidi N, Thiele I, Mo ML, Vo TD, Srivas R, Palsson BO. Global reconstruction of the human metabolic network based on genomic and bibliomic data. Proc Natl Acad Sci USA 2007;104:1777–82.
431. Zelezniak A, Pers TH, Soares S, Patti ME, Patil KR. Metabolic network topology reveals transcriptional regulatory signatures of type 2 diabetes. PLOS Comput Biol 2010;6:e1000729.
432. Deo RC, Hunter L, Lewis GD, Pare G, Vasan RS, Chasman D, Wang TJ, Gerszten RE, Roth FP. Interpreting metabolomic profiles using unbiased pathway models. PLOS Comput Biol 2010;6:e1000692.
433. Vo TD, Paul Lee WN, Palsson BO. Systems analysis of energy metabolism elucidates the affected respiratory chain complex in Leigh's syndrome. Mol Genet Metab 2007;91:15–22.
434. Stover PJ. Nutritional genomics. Physiol Genomics 2004;16:161–5.
435. Heinemann M, Sauer U. Systems biology of microbial metabolism. Curr Opin Microbiol 2010;13:337–43.
436. Orth JD, Palsson BO. Systematizing the generation of missing metabolic knowledge. Biotechnol Bioeng 2010;107:403–12.
437. Shlomi T, Benyamini T, Gottlieb E, Sharan R, Ruppin E. Genome-scale metabolic modeling elucidates the role of proliferative adaptation in causing the Warburg effect. PLOS Comput Biol 2011;7:e1002018.
438. Shlomi T, Cabili MN, Ruppin E. Predicting metabolic biomarkers of human inborn errors of metabolism. Mol Syst Biol 2009;5:263.
439. Lamarre SG, Molloy AM, Reinke SN, Sykes BD, Brosnan ME, Brosnan JT. Formate can differentiate between hyperhomocysteinemia due to impaired remethylation and impaired transsulfuration. Am J Physiol Endocrinol Metab 2012;302:E61–7.
440. Van Hove JL, Lazeyras F, Zeisel SH, Bottiglieri T, Hyland K, Charles HC, Gray L, Jaeken J, Kahler SG. One-methyl group metabolism in non-ketotic hyperglycinaemia: mildly elevated cerebrospinal fluid homocysteine levels. J Inherit Metab Dis 1998;21:799–811.
441. Crott JW, Liu Z, Keyes MK, Choi SW, Jang H, Moyer MP, Mason JB. Moderate folate depletion modulates the expression of selected genes involved in cell cycle, intracellular signaling and folate uptake in human colonic epithelial cell lines. J Nutr Biochem 2008;19:328–35.
442. Chango A, Nour AA, Bousserouel S, Eveillard D, Anton PM, Gueant JL. Time course gene expression in the one-carbon metabolism network using HepG2 cell line grown in folate-deficient medium. J Nutr Biochem 2009;20:312–20.
443. Duthie SJ, Horgan G, de Roos B, Rucklidge G, Reid M, Duncan G, Pirie L, Basten GP, Powers HJ. Blood folate status and expression of proteins involved in immune function, inflammation, and coagulation: biochemical and proteomic changes in the plasma of humans in response to long-term synthetic folic acid supplementation. J Proteome Res 2010;9:1941–50.
444. Hebels DGAJ, Jennen DGJ, van Herwijnen MHM, Moonen EJC, Pedersen M, Knudsen LE, Kleinjans JCS, de Kok TMCM. Whole-genome gene expression modifications associated with nitrosamine exposure and micronucleus frequency in human blood cells. Mutagenesis 2011;26:753–61.
445. Hochstenbach K, van Leeuwen DM, Gmuender H, Gottschalk RW, Lovik M, Granum B, Nygaard U, Namork E, Kirsch-Volders M, Decordier I, et al. Global gene expression analysis in cord blood reveals gender-specific differences in response to carcinogenic exposure in utero. Cancer Epidemiol Biomarkers Prev 2012;21:1756–67.
446. Marsit CJ, Eddy K, Kelsey KT. MicroRNA responses to cellular stress. Cancer Res 2006;66:10843–8.