Supplemental Table 1 Biomarker Specific Characteristics

Serum Folate: General Characteristics

Humans versus animal models versus cell/molecular studies?	Model systems, including animals and cell cultures, are often used for preclinical and mechanistic studies of biological systems. For folate biomarker research, they can be used effectively to model gene-nutrient interactions, discover new folate-dependent enzymes and genes, understand metabolic pathways, investigate the responsiveness of biomarkers to dietary challenges and identify genes involved in folate-responsive NTDs (1). No model system faithfully recapitulates human physiology. Although the pathways in one-carbon metabolism are highly conserved between mice and humans, the regulation of the pathways can differ substantially (2). Serum/plasma folate concentrations are about 10-fold higher in mice than in humans (3). The limitations inherent with the model system must be understood and accounted for in the experimental design and interpretation of results.
Exposure (short-/long-term?)	The measurement of serum folate provides information on the short-term folate status of the individual. Serum folate is the earliest indicator of altered folate exposure and will reflect recent dietary intake (4). Repeated measures over time in the same individual may reflect chronic folate deficiency.
Status: are there validated norms to define deficiency/adequacy?	During the late 1960s, biological cut-off points for sequential stages of folate deficiency were established through depletion/repletion experiments. A serum folate concentration <7 nmol/L (3 ng/mL) indicated negative folate balance at the time the blood sample was drawn (5). More recently, cut-off points for folate deficiency (serum folate <10 nmol/L) were defined based on a metabolic indicator (increased plasma total homocysteine [Hcy]) (6). These cut-off points have been recommended by the 2005 WHO Technical Consultation on Folate and Vitamin B12 Deficiencies for the assessment of folate status of populations (7, 8). The measurement of serum folate may further elucidate the role of folate in relation to various health outcomes, however, no cut-off points indicative of low or high serum folate concentrations or desirable ranges have been identified in this context to date.

Function: does biomarker reflect direct function, (e.g, enzyme stimulation assays) or indirectly reflect function of biological systems, (e.g. growth)	Serum folate reflects recent folate intake and indicates short-term status, whereas RBC folate represents the amount of folate that accumulates in blood cells during erythropoiesis and is a long-term indicator of folate status. Recurrent measures of serum folate on the same individual overtime can reveal chronic folate deficiency. Thus serum folate reflects a different folate pool than RBC folate, and shows a moderate but variable correlation with RBC folate (r in the range 0.41 to 0.63) in different populations (9).
Effect: does the biomarker directly reflect a response to an intervention either positive or negative?	Serum folate is highly responsive to intervention with folic acid, with natural food folates typically resulting in a poorer serum folate response compared to folic acid at similar intervention levels. Likewise, population data show that serum folate concentrations are highly reflective of exposure to folic acid, with the highest concentrations observed in people who consume folic acid in both supplements and fortified foods, both in regions with mandatory fortification and voluntary-only fortification (10-12). Serum folate increased in a linear, dose-dependent manner in response to intervention with folic acid at doses of 200, 400 and 800 μg/d for 6 months (13).
User groups considerations for biomarker with regard to serum folate:	
Population (e.g. policy makers assessing status of population; agencies conducting national surveys; agencies responsible for development, implementation, and evaluation of food/nutrient based programs) Different environment and resources: • developed countries • developing-low resource countries	Although serum folate is a marker for short term folate exposure, it is the most practical marker for large scale studies in populations. As noted in section 5, stable assays which can be verified and controlled in-house are preferable for population assessments. Due to manufacture recalibration or reformulation, assay kit assays can change over time and thus 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 and compare folate status between population groups in different countries (14). For low-resource settings, the microbiologic assay (MBA) is the method of choice because it is the least expensive assay, 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. The CDC has developed a Nutrition Survey Toolkit that describes in detail how specimens can be collected, handled and assayed by the MBA (15). The use of blood spots collected on filter paper is being explored for use in developing countries where obtaining and managing serum samples may be difficult.

Research settings (e.g. researchers and educators involved in studies of nutrition/health including development and utilization of biomarkers and training students and research staff)	In research, serum folate is measured in human participants and animal models to investigate the effects of genetics on folate status and requirements, the effects of other nutrients and diets on folate status, and to study folate bioavailability. Serum folate reflects short-term status (*exposure*), is very responsive to folate intake (*effect*), and has wide utility in the research setting because there are established reference values and cut-offs (*status*) based on haematological indices (*function*) published in the folate DRI (16). Additional cut-offs indicating high or "supranutritional" status (17, 18). As it best reflects short-term status, serum folate is often considered in concert with RBC folate, a folate biomarker indicating long-term status. However, in populations with invariant folate intakes (19) and when measured repeatedly on the same individual over time, serum folate can also reflect long-term status. Serum folate (versus RBC folate) is the preferred status marker in dose-response studies where repeated measures of serum folate are obtained within the same individual to assess response to a known folate intake. It is important that interpretation of serum folate values in research settings fully consider the variety of biological and contextual factors (covered in detail below) that can impact circulating concentrations. Serum total folate is the most widely utilized folate biomarker; however, serum folate vitamers, including 5-methyltetrahydrofolate (20), formyl folate (20, 21), and folic acid (17, 22-25) are also important folate biomarkers in the research setting.
Clinical settings	Folate biomarkers used within the clinical context have more validity than when used in the absence of clinical information. Folate status is often altered during a number of pathological conditions, including cancer, psoriasis, inflammatory bowel disease, hemolytic anaemia, HIV infection and kidney failure (see below). The primary aim of folate testing in clinical laboratories is to determine whether a patient is folate deficient. Serum folate is a useful marker of folate status in the clinical setting, although it is somewhat labile with levels influenced by recent consumption of a high folate meal, folic acid supplement or alcohol. It has been shown that serum folate levels may be in the normal range in patients who have clear clinical evidence of folate deficiency and may be low in patients without clinical abnormalities. Repeated measures over time can make serum folate testing more informative. Confirmatory testing using a second marker such as Hcy is helpful.

	Clinical laboratories require inexpensive, automated, and high throughput assays to be able to report results within a day or less of receiving a sample. Protein-binding assays have been developed with the clinical laboratory in mind, to enable the diagnosis of folate deficiency. Most are fully automated for clinical analyzers to provide high throughput measurements with turnaround times of less than 1 h. Further, unlike the microbiologic assay they are not influenced by the presence of antibiotics or antifolates which may inhibit bacterial growth and could be an issue when working in the clinical setting.
Biological Factors; impact of the following on interpretation of each biomarker:	
Race/ethnicity	Folic acid fortification in the US, enacted in 1998, has markedly improved all markers of folate status across all ethnicities. Comparing data from NHANES III 1988—1994 with NHANES 1999-2000, NHANES 2001-2002 and NHANES 2003-2006, shows an approximate 130% and 60% increase in serum folate and RBC folate, respectively, and a moderate decline in plasma Hcy of 20% (26, 27). Notably, both before and after fortification, non-Hispanic whites (NHW) have higher serum and RBC folate than Mexican Americans (MA). Non-Hispanic blacks (NHB) have the lowest level of serum and RBC folate (26, 27). The differences in folate status according to ethnicity may be due to higher folate requirements in NHB (28) and/or higher intake of folate in NHW (11). In a recent analysis of NHANES 2003-2006 data, race-ethnic differentials in serum and RBC folate concentrations remained significant after adjustment for sociodemographic and lifestyle variables (29).
Age-life stage/ endocrinology (infants/children; adolescents; women of reproductive age; pregnancy; elderly)	*Age and gender*: Folate biomarkers and their interrelations change markedly from birth to senescence. Infants (< 1 year of age) have a biochemical profile characterized by relatively high serum folate (~ 30 nmol/L), relatively high Hcy (~ 7 µmol/L) and low serum cobalamin. RBC folate is high at birth (~ 550 nmol/L) but declines abruptly by nearly 50% within 6 weeks (30). No difference in folate biomarkers according to gender were reported in several studies (30, 31). In contrast, higher serum and RBC folate levels were recently reported for adult (aged ≥ 20 y) women as compared to men from NHANES 2003-2006 (29). This difference remained after adjustment for sociodemographic and lifestyle variables. After one year of age, serum folate declined markedly and RBC folate declined moderately. No differences in folate

	biomarkers were observed between girls and boys aged < 15 years (30-33), but in older age groups (32, 33) males attained lower serum folate and slightly lower RBC folate than females (26). The lowest values for serum folate (~10 nmol/L) and RBC folate (~230 nmol/L) were observed in age ranges of 10 - 40 (26, 30). Thereafter, all three biomarkers increased in both genders (26). Notably, in infants high serum folate is attributed to methyl folate trapping as demonstrated by reduction of both Hcy and serum folate following cobalamin supplementation (34). *Pregnancy*: There are substantial changes in folate biomarkers throughout pregnancy. Many studies do not allow quantification of such changes because of lack of preconception levels (35), and comparison of folate concentrations between studies is difficult due to different and non-standardized analytical methodology. A decrease in serum folate during pregnancy is a common observation whereas the change in RBC folate varies (36). In most (35-38) but not all (39) longitudinal studies, serum folate declines throughout pregnancy, in particular in women carrying the *MTHFR* 677-T allele (40, 41). RBC folate has been reported to show smaller changes with a moderate increase during mid pregnancy (38), or a decrease during the last 2 (37) or 5 months (35, 42). These changes in serum and RBC folate are modified by onset of folic acid intake (43). Plasma folate and RBC folate concentrations are correlated and both are inversely associated with Hcy in pregnant women (35, 42). In a recent analysis of NHANES 2003-2006 data, pregnant compared to non-pregnant women aged 20-49 y had 18% and 26% higher serum and RBC folate concentrations, respectively after adjusting for demographic variables, smoking, use of dietary supplements, fasting, inflammation, and renal function, but without adjusting for total folate intake from foods and supplements (44). Several mechanisms for the change in folate status during pregnancy have been proposed, including increased folate or methionine demand, increased folate catabolism, increased clearance and excretion of folate, decreased folate absorption, hormonal effects, hemodilution due to plasma volume expansion, increased renal Hcy clearance and decreased Hcy binding to albumin (36).

	Oral contraceptives: Early studies on the effect of oral contraceptives (OCs) suggest a negative effect on folate status (45). OCs with low estrogen content have no non-equivocal effect on serum or RBC folate in recent studies controlling for potential confounders, and no conclusion on the effect from OCs on folate status can be made (46). *Male sex hormones* affect the activities of folate metabolizing enzymes in the rat (47), but there is no data showing effects of male sex hormones on folate status in humans.
Genetics	The (single nucleotide) polymorphisms most commonly investigated in relation to folate status are located on genes encoding for enzymes involved in folate-dependent one-carbon metabolism and Hcy metabolism. These include methylenetetrahydrofolate reductase (*MTHFR*) c.665C>T (known as 677C>T; p.Ala222Val) and c.1286A4C (known as 1298A>C; p.Glu429Ala), methionine synthase (*MTR*) c.2756A>G (p.Asp919Gly), methionine synthase reductase (*MTRR*) c.66A>G(p.Ile22Met), methylenetetrahydrofolate dehydrogenase (*MTHFD1*) c.1958G>A (p.Arg653Gln), p.Arg239Gln), reduced folate carrier-1 (*SLC19A1*) c.80G>A (p.Arg27His) (48), cystathionine *beta*-synthase (*CBS*) c.844_845ins68 and *CBS* c.699C>T (p.Tyr233Tyr) (49). The strongest and most consistent effects have been observed for *MTHFR* 677C>T (50). The *MTHFR* 1298 A>C variant is also worth mentioning as it is in linkage disequilibrium with *MTHFR* 677C>T, which has caused considerable confusion in the literature. When the effects of the two variants have been dissected out, it has been shown that 1298 does not significantly affect folate levels. For the other polymorphisms, the associations with folate biomarkers are weak and somewhat inconsistent across different studies, some of which are small and lack power. There are consistent results demonstrating that Hcy increases and serum folate decreases in a dose-response manner according to the number of *MTHFR* 677-T alleles. These effects are most pronounced in subjects/populations with low folate status, and the inverse association between Hcy and serum folate is strongest in subjects with the TT genotype (50). The *MTHFR* 677 C->T polymorphism also seems to alter the distribution of RBC folates by decreasing the relative amount of methylated tetrahydrofolate in subjects with the variant TT genotype (51, 52), but the magnitude of the overall effect on RBC folate is related to the specificity of the applied folate assay (53). These effects on folate in serum and RBC are explained by reduced catalytic activity of MTHFR

	encoded by the 677 variant T-allele (so-called thermolabile enzyme), which has lower affinity for methylenetetrahydrofolate (and FAD), leading to impaired formation of 5-methyltetrahydrofolate and its polyglutamated derivatives (in RBC) (50). The *MTHFR* 677C->T distribution is different across geographic regions and ethnic groups. The T-allele frequency is 0.15 – 0.17 in south Asia, 0.30 – 0.35 in northern Europe, 0.39 or higher in Japan and 0.45 in Italy (54), 0.33 among US NHW, 0.11 among US NHB and 0.45 among US MA (55). The prevalences of the variant alleles of *MTHFR* 1298 A->C and *MTRR* 66A->G are also significantly higher among NHW than among NHB or MA (55). In the prefortification US population (NHANES III), persons with the *MTHFR* 677 TT genotype had a 22.1% (95% CI: 14.6%, 28.9%) lower serum folate and a 25.7% (95% CI: 18.6%, 33.2%) higher Hcy concentration than did persons with the CC genotype (55). The difference in serum folate becomes abolished in subjects taking > 400 µg/d of folic acid whereas the Hcy difference is only moderately reduced by supplementation or moderate intake of folic acid (56), but higher in Asia and Europe as compared to the US and ANZ (54). The serum folate response to intervention with folic acid (irrespective of dose) is however strongly affected by the common 677 C>T polymorphism in *MTHFR.* One large randomized controlled trial (RCT) in China showed that despite 6 months of supplementation with 4000 μg/d folic acid, women with the *MTHFR* 677TT genotype achieved lower serum folate and higher plasma Hcy concentrations than did those with the CC genotype (56). In the prefortification US population, differences in serum folate by *MTHFR* 677C->T genotype were noted for all race-ethnicity groups, with serum folate values lower among NHB (34 %), NHW (20%) and MA (21%) of the TT as compared with the CT genotype (55). See Table 12 for a description of the impact of poor riboflavin status on serum folate concentrations in individuals with the *MTHFR* 677 TT genotype.
Body mass index	There are several reports on an inverse relation between serum folate and BMI in both genders and in different age groups (29, 57-63). Studies on RBC folate and BMI are less consistent, demonstrating either a positive (29, 63) or no (58, 62, 64) association.

	The link between obesity and low folate status is potentially important in women of childbearing age, since both are risk factors for birth defects, including NTD (64). BMI was associated with low serum folate in US women aged 17 – 49 years both before (NHANES III) and after (NHANES 1999 - 2000) folic acid fortification, and the association was strongest in women aged > 20 years. Women with high BMI used less supplements and had lower folate intake from food, but the inverse association between BMI and serum folate persisted after adjustment for these factors and after adjustment for insulin levels and glucose. The latter observation indicates that insulin resistance does not fully explain the BMI-serum folate association (64). A recent study analyzing postfortification data from the NHANES 2003–2004, 2005–2006, and 2007–2008 cycles demonstrated that RBC folate increases with increasing BMI both in supplement users and non-users (63). This suggests the BMI affects cellular uptake and tissue distribution of folate. Furthermore, an inverse association between serum folate and BMI was only observed among supplement non-users suggesting that high intake of folic acid may compensate for altered folate distribution in obese women (63). Experimental evidence of differences in the pharmacokinetic response to the current recommended dose of folic acid between obese and normal weight women of childbearing age was provided by a recent study by da Silva et al. (65).
Endemic disease (e.g. malaria; HIV)	*Malaria:* There is some evidence that malarial infection may induce folate deficiency in adults (66). The causes include inadequate intake, malabsorption, hemolysis and antimalarial drugs. Notably, there are consistent reports of increased RBC folate in malarial infection (67, 68), which has been attributed to de novo parasite folate synthesis or predisposition of malaria in subjects with high RBC folate. Malaria may also induce increased hemolysis of folate-rich cells that can lead to an increase in serum folate. The levels of folate biomarkers in malaria seems to be influenced by the infection itself and their usefulness to assess folate status is uncertain. *HIV/AIDS:* Micronutrient deficiencies are common in subjects with HIV/AIDS (69). Low serum folate or RBC folate (70) has been reported in HIV-infected pregnant women (71), children (72) and adults (73) in developing countries, but folate status was normal in a study of HIV infected children in New York (74).

Inflammation	It is difficult to conclude from published data whether folate biomarkers are related to inflammation, because the data are inconsistent, and the underlying condition leading to inflammation may itself affect folate status. It has been stated that serum folate and to a lesser extent RBC folate are low during acute inflammation (75), but the supportive data are not convincing. Plasma/serum folate and Hcy are not related to C-reactive protein (CRP) and other inflammatory markers in healthy middle aged subjects in the Atherosclerosis Risk in Communities (ARIC) study (76) or to inflammation (as assessed by CRP) in participants from the population-based Framingham Heart Study cohort (77). Supplementation with folic acid does not affect the level of inflammatory biomarkers in most studies (78, 79) but did so in one study (80). It is possible that folate status is related to expression of proteins involved in activation and regulation of immune function that are not captured by CRP (81).
Disease	*Cancer:* Patients with established cancer, particularly at advanced stages may have low folate status, as measured by low circulating folate and high Hcy (82, 83). The mechanisms involved may include inadequate folate intake, increased folate requirements due to accelerated DNA synthesis, increased folate catabolism by cancer cells (45, 83) and antifolate chemotherapy (82, 84). *Psoriasis:* Patients with psoriasis have lower folate levels and higher Hcy than healthy controls. Notably, blood levels of other B-vitamins are often normal (85). Concentrations of Hcy are related to disease severity but also to low folate levels in psoriatics (86). The most likely explanation is increased folate requirement due to increased keratinocyte turnover, but lifestyle factors, obesity (85) and methotrexate therapy (87) may contribute as well. *Inflammatory bowel disease:* Blood levels of several micronutrients, including folate, are often low in patients with inflammatory bowel disease (IBD), in particular Crohn's disease (CD). Most studies on folate status involve measurement of serum folate, which demonstrate a prevalence of deficiency of about 25%. A few studies based on RBC folate demonstrate a lower prevalence, and RBC folate may be a more accurate test since it reflects long term-status (88). Notably, it has been recommended that low circulating folate in IBD should be confirmed by Hcy measurement, regarded as a more sensitive test in these

	patients (88). The most important mechanisms behind impaired folate status are enteric loss, malaborption, inadequate intake of folate, and treatment with folate antagonists like sulfasalazine (88). *Sickle cell disease (SCD):* Low serum and RBC folate (89, 90) have been reported in SCD in most but not all studies (91-93). Low RBC folate has been detected even in subjects prescribed folic acid supplements (90). RBC folate, however, does not seem to be an adequate measure of folate status in SCD patients because RBC folate increases with decreasing RBC age; therefore serum folate and Hcy have been recommended for assessment of folate status in these patients (94). However, the accuracy of folate biomarkers to assess folate status in SCD may vary according to intake of folic acid supplements, age and renal function (93). *Thyroid Disease*: Folate and Hcy status change according to thyroid state. Hypothyroid patients have low RBC and serum folate and elevated Hcy, whereas hyperthyroid patients have an opposite profile with elevated RBC and serum folate and low Hcy (95-97). These changes have been documented in longitudinal studies of patients during treatment, which normalizes thyroid state (95, 98-101). Altered folate status has been attributed to effects of thyroid hormones on folate metabolism including altered riboflavin status (100, 102) that in turn affects the FAD-dependent MTHFR. *Diabetes:* Studies have demonstrated moderately elevated serum/plasma folate in type 1 and type 2 diabetic patients (103-105), adequate serum and RBC folate in type 1 and type 2 diabetic patients (106, 107), and lower serum folate in type 2 diabetics than in healthy controls (108). Thus, no equivocal conclusion can be made on the effect of the diabetic state itself on circulating folate. *Renal failure:* Patients with renal failure have deficiencies of several water soluble micronutrients and B-vitamins, including folate (109). Serum and RBC folate are often below normal values in chronic renal impairment (110), and folate deficiency is more frequent in hemodialysis than peritoneal dialysis patients (111, 112). Treatment of renal patients with folic acid normalizes circulating folate. These observations suggest impaired folate function in renal patients, who might require larger folate intake than healthy subjects.
Contextual Factors; impact of the following on performance of each biomarker:	

Sample source	While most laboratories prefer serum over plasma, both matrices generally produce comparable results for serum total folate (113-115), as long as the sample processing is not delayed (116).
Bioavailability	Serum folate is often used in acute studies in research settings to reflect differences in folate bioavailability when provided as a bolus dose/meal.
Fasting; time of day; time of exposure/meal/intervention	Data from several thousand U.S. adults participating in NHANES 2003–2006 have shown 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 compared to samples from non-fasted (<3 h) participants, but the difference was relatively small, indicating that fasting may not be essential when assessing the folate status of populations (44). 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 h after the dose, with peak concentrations dependent on the size of the dose, the baseline folate status, and the vehicle in which folate was administered. The variability of biomarkers over time is critical for their use in epidemiological studies and for being able to judge if or to what extent a single measurement reflects long-term exposure. Reliability of plasma folate was determined in 40 Nurses' Health Study (NHS) participants over 1 -2 years and in 551 patients with stable angina pectoris from the WENBIT study over 3.5 years, and the results were good (ICCs of 0.61 and 0.50, respectively). Notably, in the WENBIT population reproducibility for plasma folate showed a stronger relation to time between measurements than for other nutritional biomarkers, and ICC decreased from 0.71 over 1 month to 0.61 over 1 year and 0.50 over 3.5 years (117). To our knowledge, no data on reliability of RBC folate measurement have been published. The reproducibility of serum/plasma folate over time allows one-time assessment of biomarker status.
Drug use (in context of acute or chronic treatment for disease; recreational)	Some drugs have a negative effect on folate status and thereby increase plasma Hcy, but many drugs affect plasma Hcy by mechanisms independent of folate. *Folate antagonists:* Methotrexate (MTX), the most widely known antifolate, has been used to combat cancer since the 1950s and has been especially effective in alleviating

	inflammation in patients with rheumatoid arthritis. Structurally, MTX is an analog of folic acid with modifications that result in a higher affinity for the drug's enzyme target, dihydrofolate reductase (DHFR) (118). Accordingly, methotrexate decreases circulating folate and increases Hcy. This response is observed at low doses used in patients with psoriasis (87) or rheumatoid arthritis (119, 120) to high doses given to cancer patients (82), and is explained by inhibition DHFR. The antibiotic, trimethoprim, is also a DHFR inhibitor, and has a similar effect on folate (121). *Anticonvulsants:* Conventional antiepileptic drugs (AEDs) like carbamazepine, phenobarbital, primidone and phenytoin are associated with reduced plasma folate and markedly elevated Hcy (122, 123). This effect has been explained by increased degradation and elimination of folate, secondary to the marked induction of liver enzymes caused by these drugs (45). New AEDs such as levetiracetam and lamotrigine have no or less inductive potential and no effect on folate and Hcy status (122, 123). *Antihypertensive drugs:* Therapy with diuretics including hydrochlorothiazid is associated with decreased circulating folate and elevated Hcy. The underlying mechanisms may involve folate depletion and impaired renal function (124). *Warfarin:* RBC but not serum folate is decreased in patients after 6 months on warfarin therapy. Whether altered folate status is caused by warfarin itself, or is secondary to dietary advice in these patients, is uncertain. There is no increase in Hcy, which may reflect increased baseline Hcy after the acute event preceding therapy (125).
Coffee Consumption	Coffee consumption is associated with a decrease in plasma folate (126, 127), vitamin B6 and riboflavin (but not vitamin B12) and parallel increase in Hcy (127). Thus, coffee drinkers have lower mean folate and higher mean Hcy than non-drinkers, but the differences are only observed at the higher end of the folate distribution (127, 128) and at the lower end of the Hcy distribution (129). Possible mechanisms involved are increased renal excretion of folate (at high plasma folate) mediated by caffeine (127).
Smoking tobacco	Smoking is associated with deficiencies of several micronutrients and B-vitamins, including folate. Smokers have lower RBC and plasma/serum folate and higher Hcy than non-smokers (29, 130, 131). Folate and Hcy status improve somewhat within days of smoking cessation, but

	there is a long-term effect in ex-smokers lasting for years with ex-smokers having lower folate and higher Hcy than never smokers (132). It has been suggested that the acute effect of smoking is related to increased folate breakdown or utilization caused by toxic (prooxidant) chemicals, which is in agreement with persistence of low folate after adjustment for dietary intake. The chronic effect may be explained by imprudent dietary habits of ex-smokers in combination with the time required to replenish folate stores (132).
Alcohol consumption	The associations between folate and Hcy status and alcohol intake are inconsistent and complex and related to type and amount of alcohol. Intake of beer and to a lesser extent wine may be positively associated with circulating folate and inversely related to Hcy, which may partly be related to vitamin content in beer (128). However, these associations could be confounded by nutrition and lifestyle factors. In a controlled intervention study, 2 weeks with red wine or vodka (24 g ethanol daily) decreased serum folate and increased Hcy (133). Excessive alcohol intake or alcoholism is associated with B-vitamin deficiencies including impaired folate status (45, 134). The ethanol related folate-deficiency has been explained by low intake, malabsorption, altered liver metabolism, increased catabolism and renal excretion of folate (45, 135).
Exercise	Data on associations between exercise in leisure time and folate are limited (136), but there are reports on higher levels of serum folate in physically active persons (137, 138). Physical activity is associated with several potential confounders, including nutritional and life-style factors and physiological and metabolic changes. Thus, from published results one cannot conclude that physical exercise has a direct effect on biomarkers of folate status. In a recent analysis of NHANES 2003-2006 data, US adults who expended 750 vs. 150 total metabolic equivalent tasks minutes/week from leisure-time physical activity had slightly higher serum (1.4%) and RBC folate (0.6%) concentrations after adjustment for sociodemographic and lifestyle variables (29).
Socioeconomic (e.g. education; income)	A recent report from NHANES 2003-2006 (31) indicated that the socioeconomic variables of education and family poverty-income ratio were significantly associated with serum and RBC folate, however they did not account for much of the variability in biomarker concentration.

Seasonal variations	A cross-sectional study indicated seasonal variation in serum and RBC folate (139) whereas a longitudinal study on an Irish population demonstrated a moderate reduction in RBC folate in spring compared with autumn; no seasonal changes were observed for serum folate and Hcy (140). In a large epidemiological study from China, serum and RBC folate were lower in the spring than in the fall in the North and lower in the fall than in the spring in the South (141). Plasma Hcy was inversely associated with circulating folate across regions and seasons (142). Thus, the seasonal variations in folate biomarker seem to be moderate, might be concealed in a fortified population, are different between geographical regions, and probably reflect seasonal differences in the availability of fresh fruit and vegetables.

Red Blood Cell Folate: General Characteristics

Humans versus animal models versus cell/molecular studies?	See serum folate (humans versus animal models versus cell/molecular studies) for more information.
Exposure (short-/long-term?)	RBC folate is a sensitive indicator of longterm folate status. RBC folate compared with serum folate will respond more slowly to changes in dietary folate intake and is a better indicator of folate intake over the previous 3-4 months when circulating folate is incorporated into the maturating red cells (143).
Status: are there validated norms to define deficiency/adequacy?	During the late 1960's, biological cut-off points for sequential stages of folate deficiency were established through depletion/repletion experiments. A RBC folate concentration <363 nmol/L (160 ng/mL) indicated the onset of folate depletion, concentrations <272 nmol/L (120 ng/mL) marked the beginning of folate-deficient erythropoiesis, and concentrations <227 nmol/L (100 ng/mL) marked folate-deficient anemia (5). It was more common though for investigators to use a single cutoff point for RBC folate to designate deficiency: <317 nmol/L (140 ng/mL) (144). More recently, cut-off points for folate deficiency (RBC folate <340 nmol/L) were defined based on a metabolic indicator (increased plasma total Hcy) (6). These cut-off points have been recommended by the 2005 WHO Technical Consultation on Folate and Vitamin B12 Deficiencies for the assessment of folate status of populations (7).

Function: does biomarker reflect direct function, e.g, enzyme stimulation assays or indirectly reflect function of biological systems, e.g. growth	RBC folate parallels liver concentrations (accounting for about 50% of total body folate) and is thus considered to reflect tissue folate stores (145). RBC folate represents the amount of folate that accumulates in blood cells during erythropoiesis and reflects folate status during the preceding 120 days, i.e. the half-life of red cells (146, 147). RBC folate and serum folate reflect different folate pools; RBC shows a moderate but variable correlation with serum folate (r in the range 0.41 to 0.63) in different populations (9).
Effect: does the biomarker directly reflect a response to an intervention either positive or negative?	RBC folate is highly responsive to intervention with folic acid, with natural food folates typically resulting in a poorer RBC folate response compared to folic acid at similar intervention levels. Likewise, population data show that RBC folate concentrations are highly reflective of exposure to folic acid, with the highest concentrations observed in people who consume folic acid in both supplements and fortified foods, both in regions with mandatory fortification and voluntary-only fortification (11, 12). As circulating folate is incorporated into red cells during erythropoiesis and the average life-span of red cells is 120 days (143), intervention trial periods of 3-4 months are considered necessary in order to allow an optimal RBC folate response to an increase in folate intake to be observed. The RBC folate response to intervention with folic acid (irrespective of dose) is strongly affected by the common 677 C>T polymorphism in *MTHFR*.
User groups considerations for biomarker with regard to RBC folate:	
Population (e.g. policy makers assessing status of population; agencies conducting national surveys; agencies responsible for development, implementation, and evaluation of food/nutrient based programs) Different environment and resources: • developed countries • developing-low resource countries	Red cell folate is a marker for longer term (months) folate exposure. There are, however, obstacles to using it in population studies because the specimen preparation (generation of a whole blood hemolysate using accurate pipetting) is more difficult than for serum folate, and red cell folate assays are prone to more variability and less agreement across assay platforms. 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 and compare folate status between population groups in different countries (14). Detailed specimen processing protocols and thorough training of field staffs are essential if this marker is to be used successfully in large scale population studies, particularly those in low

	resource areas. Alternatively, the MBA for dried blood spots (DBS) developed by O'Broin *et al.* (148, 149) and implemented at the CDC (150) is a suitable tool to assess folate status in a population when no venous sample can be collected. The great sensitivity of the MBA is of particular benefit when only a small sample volume is available, such as for samples collected from a finger-stick or as a DBS. No other type of method has so far been applied successfully to DBS. Concentrations thought to be protective of neural tube birth defects (8, 151, 152) have also been published.
Research settings (e.g. researchers and educators involved in studies of nutrition/health including development and utilization of biomarkers and training students and research staff)	In research, RBC folate is measured in human participants and animal models to investigate the effects of genetics on folate status and requirements, the effects of other nutrients and diets on folate status, and to study folate bioavailability. RBC folate reflects long-term status (*exposure*) and has wide utility in the research setting because there are established reference values and cut-offs (*status*) based on haematological indices (*function*) published in the folate DRI (16). RBC folate is responsive to folate intake (*effect*); as a long-term marker of folate status, it is often considered in concert with serum folate, a biomarker that reflects short-term folate status. It is important that interpretation of RBC folate values in research settings fully consider the variety of biological and contextual factors (covered in detail below) that can impact circulating concentrations. RBC total folate is the most widely utilized RBC folate biomarker; however, RBC folate vitamers, including 5-methyltetrahydrofolate and formyl folate (51, 153) are also important folate biomarkers in the research setting.
Clinical settings	Folate enters red blood cells as they form in the bone marrow. The concentration is determined by exposure before they are released into the circulation. When RBC folate is measured clinically, it reflects the availability of folate when the circulating red blood cells were developing. Because the life of these cells is approximately 90 to 120 days, measuring red cell folate provides a long term marker of folate status. Thus, it is useful in conjunction with serum folate, a short term marker of folate status. Because vitamin B12 is required for folate retention in developing red blood cells, RBC folate concentrations are dependent on vitamin B12 as well as folate availability and low levels may reflect vitamin B12 deficiency as well as folate deficiency. The same factors that influence serum folate (pregnancy, alcohol, anti-folate drugs, etc.) can influence RBC folate concentrations.

Biological Factors; impact of the following on interpretation of each biomarker:	
Race/ethnicity	See above (serum folate)
Gender	See above (serum folate)
Age-life stage/endocrinology	See above (serum folate)
Genetics	See above (serum folate)
Body mass index	See above (serum folate)
Endemic disease (e.g. malaria; HIV)	See above (serum folate)
Inflammation	See above (serum folate)
Non-communicable disease (e.g. cancer)	See above (serum folate)
Pharmacology (treatment interactions including traditional therapies)	See above (serum folate)
Nutrient interactions	See Table 12
Contextual Factors; impact of the following on performance of each biomarker:	
Sample source	See above (serum folate)
Bioavailability	Red blood cell folate reflects bioavailability if measured longitudinally where long term changes are reflected over several months.
Fasting; time of day; time of exposure/meal/intervention	See above (serum folate)
Drug use (in context of acute or chronic treatment for disease; recreational)	See above (serum folate)
Coffee consumption	See above (serum folate)
Smoking tobacco	See above (serum folate)
Alcohol consumption	See above (serum folate)
Exercise	See above (serum folate)
Socioeconomic (e.g. education; income)	See above (serum folate)

Plasma Homocysteine: General Characteristics

It has been recommended that Hcy should 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 (154).

Humans versus animal models versus cell/molecular studies?	Functional biomarkers of folate metabolism, including Hcy concentrations, were shown to vary among 13 different mouse strains (155). See serum folate (humans versus animal models versus cell/molecular studies) for more information.
Exposure (short-/long-term?)	Plasma Hcy decreases at a rapid rate of 0.08 h^{-1} and reaches a plateau 24 hours after iv administration of high dose 5-formylTHF (156). This is explained by the role of 5-methyl-THF as a methyl donor in the remethylation of Hcy catalyzed by the enzyme methionine synthase (154). Plasma Hcy responds within 3-4 weeks of folate depletion (increases) and subsequent repletion (declines) in healthy subjects (157). The fast response probably reflects that methyl groups for Hcy remethylation are dependent on "shallow" folate pool(s) with a fast turnover rate (158).
Status: are there validated norms to define deficiency/adequacy?	A multitude of factors affecting Hcy concentrations complicate the establishment of reference ranges and cut-off levels. Traditionally, Hcy below 15 µmol/L was considered as normal, 15–30 µmol/L as moderate hyperhomocysteinemia and >30 µmol/L as severe hyperhomocysteinemia (154, 159). Optimized Hcy in the range 5-10 µmol/L were reported in several studies (160-163). Apart from providing an indication of functional folate deficiency, elevated plasma Hcy concentrations are associated with an increased risk of cardiovascular diseases. The 2009 US National Academy of Clinical Biochemistry (NACB) Laboratory Medicine Practice Guidelines on "Emerging Biomarkers of Cardiovascular Disease and Stroke" categorized Hcy 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 (164).
Function: does biomarker reflect direct function (e.g, enzyme stimulation assays)	The measurement of plasma Hcy provides a sensitive functional biomarker of folate status. When the status of folate is low or deficient, plasma Hcy is invariably found to be

or indirectly reflect function of biological systems, (e.g. growth)	elevated. Plasma Hcy will thus be inversely related to folate status in population data (whether measured as serum or RBC folate), and highly responsive to intervention with folate. Plasma Hcy is not however a specific marker of folate status, as it will also be elevated with other B-vitamin deficiencies, lifestyle factors, renal insufficiency and drug treatments (154, 157). Most importantly, Hcy is also an indicator of vitamin B12 status, which is explained by methylcobalamin serving as a co-factor in the methionine synthase reaction which remethylates Hcy to methionine. In population groups that consume folic acid fortified foods or folic acid supplements, Hcy is considered a more reliable biomarker of vitamin B12 status than of folate status (154). Plasma Hcy is an indicator of overall methyl status *in vivo* and is influenced by several vitamins and methyl donors involved in one-carbon metabolism, including vitamins B2, B6, betaine (and choline), in addition to B12 (165). This is demonstrated by the inverse association between plasma Hcy and serum folate, which is strongest when the other nutrients are low (166, 167). The most important genetic determinant of elevated plasma Hcy in the general population is the 677C>T polymorphism in *MTHFR*; individuals with the homozygous mutant *MTHFR* 677TT genotype will typically have significantly higher plasma Hcy compared to those with the CC or CT genotypes.
Effect: does the biomarker directly reflect a response to an intervention either positive or negative?	Plasma Hcy decreases in response to intervention with folate, alone or in combination with the other methyl donors involved in one-carbon metabolism: vitamin B12, vitamin B6, vitamin B2 and betaine (or choline). Plasma Hcy was previously reported to decrease in a dose-responsive manner with folic acid supplementation, reaching a maximum reduction of 23% at ≥800 μg/d, an effect that was most pronounced in subjects with high Hcy and/or low blood folate at baseline (168). More recent evidence however showed that a dose of folic acid as low as 200 μg/d can, if administered for a prolonged period of 6 months, effectively lower Hcy concentrations regardless of initial plasma Hcy or folate concentrations, suggesting that higher folic acid doses were not necessary (13). Several previous trials probably overestimated the folic acid dose required for maximal lowering of plasma Hcy because of treatment durations that were too short to allow the maximal plasma Hcy response to be observed (168).

	Mandatory folic acid fortification implemented in North America in 1998 was associated with a reduction in Hcy of about 7-10% (169, 170). Additional supplementation with folic acid appears to result in a further lowering of Hcy by about 15%, as indicated by intervention trials conducted after the introduction of mandatory folic acid-fortification in North America (171). The plasma Hcy response to folic acid (irrespective of dose or duration of intervention) is however strongly affected by the common 677C>T polymorphism in *MTHFR*.
User groups considerations for homocyteine with regard to:	
Population (e.g. policy makers assessing status of population; agencies conducting national surveys; agencies responsible for development, implementation, and evaluation of food/nutrient based programs) Different environment and resources: • developed countries • developing-low resource countries	Theoretically, Hcy is an attractive candidate for population use because it reflects suboptimal levels of one or more B vitamins (folate, B12, B6, or B2) and is considered a functional marker of folate status. If the primary goal is to investigate folate status, however, the effect of other vitamins on Hcy status would be confounding. Moreover, other factors such as renal status, age and sex affect Hcy status as well.
Research settings (e.g. researchers and educators involved in studies of nutrition/health including development and utilization of biomarkers and training students and research staff)	In research, total plasma Hcy is measured in human participants and animal models as a functional marker of folate status, to investigate the effects of genetics on folate function and requirements, and the effects of other nutrients and diets on folate function. Plasma Hcy is a functional biomarker as it is related to methionine cycle activity and methyl group availability (*function*). There is an inverse relationship between folate intake and plasma Hcy (*effect*); however, there is a point at which increased folate intake will not continue to lower plasma Hcy (16). Cut-offs indicating elevated plasma Hcy for various populations have been suggested (*status*) (16, 154). Plasma Hcy is an ancillary indicator of folate status and should be considered in concert with serum and/or RBC folate

	concentrations. Biological and contextual factors (covered in detail below) should always be considered in the interpretation of plasma Hcy concentrations.
Clinical settings	Plasma Hcy is a valuable marker of folate status because 5-methylTHF is required to convert Hcy to methionine. It is important to recognize that vitamin B12 is required for this reaction as well; therefore, elevated Hcy concentrations may result from folate deficiency, B12 deficiency, or a combination of the two. It is also important to note that other factors including age, sex, renal function, genetic variants such as *MTHFR* 677C>T and other vitamin concentrations (B6 and B2) have to be taken into account in interpreting Hcy results. In clinical settings Hcy can be used to confirm a suspected diagnosis of folate deficiency based on low serum folate or red cell folate. Because Hcy may be elevated by either folate or B12 deficiency it is often advisable to include a measure of B12 status when measuring Hcy. MMA is useful because unlike Hcy, it reflects B12, but not folate, status. In the clinical setting, patients with extremely high Hcy (50 - 200 µmol/L) but with normal B-vitamin status and renal function are occasionally encountered. High Hcy could be due to inborn error homocystinuria, which is more common than usually reported (172) and has variable phenotypic expression. Since mutation(s) in cystathionine ß-synthase, the most common cause of homocystinuria, also causes a substantial increase in plasma methionine (often > 100 µmol/L (173), the combined measurement of plasma Hcy and methionine could be diagnostic (174).
Biological Factors; impact of the following on interpretation of each biomarker:	
Race/ethnicity	Folic acid fortification in the US, enacted in 1998, has markedly improved all markers of folate status across all ethnicities. Comparing data from NHANES III 1988—1994 with NHANES 1999-2000, NHANES 2001-2002 and NHANES 2003-2006, shows a moderate decline in Hcy of 20% post-fortification (26, 27). Notably, both before and after fortification, Non-Hispanic Whites (NHW) had slightly higher Hcy than Mexican Americans (MA). In a recent analysis of NHANES 2003-2006 data, race-ethnic differentials in Hcy remained significant after adjustment for socioeconomic and lifestyle variables (29).
Age-life stage/endocrinology	<u>Age and gender</u>: Folate biomarkers, including Hcy, and their interrelations change markedly from birth to senescence.

(infants/children; adolescents; women of reproductive age; pregnancy; elderly)	Infants (< 1 year of age) have a relatively high Hcy (~ 7 µmol/L) (30) which declines markedly after one year of age. No differences in folate biomarkers were observed between girls and boys aged < 15 years (31-34), but in older age groups (32), males attained a higher Hcy than females (26, 27, 130). The lowest values for Hcy (~5.5 µmol/L) were observed in the age range of 1 - 10 years (26, 30). Thereafter, the biomarker increased in both genders (26). Notably, in infants cobalamin rather than folate status predicts Hcy (34, 42), and high serum folate is attributed to methyl folate trapping as demonstrated by reduction of both Hcy and serum folate following cobalamin supplementation (34). In children and adolescents, folate becomes a stronger predictor of Hcy than cobalamin (33), whereas in the elderly (> 60 years) Hcy correlates more strongly with cobalamin than with folate (6, 175), in particular when including subjects taking supplements (176). Pregnancy: Plasma Hcy is relatively low in fertile women with mean preconceptional plasma levels of about 8.5 µmol/L. It declines within 8 weeks of pregnancy, reaches a nadir corresponding to about 30% decline during the second trimester and thereafter approaches preconceptional levels at labor. The decline is not offset by folic acid intake (43) but is less pronounced in supplement users than non-users (177). A between-subject variability, however, is maintained throughout pregnancy with strong to moderate correlations of Hcy during pregnancy with preconceptional levels (r of 0.71(gw 8) to 0.54 (gw 32)) (177). Oral contraceptives: Relatively low Hcy has been consistently demonstrated in women on estrogen replacement therapy (178). It has been concluded that higher estrogen status is associated with decreased Hcy, independent of nutritional status and muscle mass, and probably reflects hormonal effects on Hcy metabolizing enzymes (179). Male sex hormones: Plasma Hcy is higher in men than in women, and higher in women with polycystic ovary syndrome (characterized by androgen excess) than controls; however, Hcy shows no relation with circulating levels of testosterone or dehydroepiandrosterone in middle aged men in a study adjusting for potential confounders (180).
Genetics	There are consistent results demonstrating that Hcy increases and serum folate decreases in a dose-response manner according to the number of *MTHFR* 677-T alleles. These effects are most pronounced in subjects/populations with low

	folate status, and the inverse association between Hcy and serum folate is strongest in subjects with the TT genotype (50). In the prefortification US population (NHANES III), plasma Hcy was about 25% higher (54) in subjects with the TT genotype compared with the CC genotype; the Hcy difference was only moderately reduced by supplementation with > 400 µg/d of folic acid or moderate intake of folic acid (55). Plasma Hcy is higher in Asia and Europe as compared to the US and ANZ (54). In the prefortification US population, differences in Hcy by *MTHFR* 677C->T genotype were noted for all race-ethnicity groups, with Hcy values lower among NHB (40%), NHW (26%) and MA (21%) of the TT as compared with the CT genotype (55). See Table 12 for a description of the impact of poor riboflavin status on plasma Hcy in individuals with the *MTHFR* 677 TT genotype.
Body mass index	Hcy shows no relation with BMI in most (60, 62, 181, 182) but not all studies (59).
Endemic disease (e.g. malaria; HIV)	*Malaria:* Plasma Hcy has been reported to be normal in some studies on malarial infections (68, 183), but elevated in one study on acute malaria (184). In the latter study, hyperhomocysteinemia was associated with disease severity and attributed to oxidative stress (184). *HIV/AIDS:* There are several studies on Hcy in HIV-infected patients, but the results are inconsistent (185, 186). In a recent study (187) the authors conclude that HIV and combination antiretroviral therapy (cART) do not influence the levels of Hcy; main determinants of hyperhomocysteinemia are deficiencies of folate and/or cobalamin (186, 187), and elevated Hcy may be effectively treated by B-vitamins (70).
Inflammation	Plasma Hcy is not related to CRP and other inflammatory markers in healthy middle aged subjects in the ARIC study (76) or to inflammation (as assessed by CRP) in participants from the population-based Framingham Heart Study cohort (77). Cardiovascular patients with high Hcy have higher levels of some inflammatory markers than patients with low Hcy, but CRP did not differ between the groups (188). Plasma Hcy shows a moderate to strong positive association with markers of cellular Th1 immune activation, like neopterin and the kynurenine/tryptophan ratio. Such associations have been observed in the elderly (189), in patients with rheumatoid

	arthritis (190), cardiovascular disease (191) and cancer (192). But the associations may reflect development of hyperhomocysteinemia as a consequence of immune activation rather than impaired folate status (191).
Disease	Cancer: Patients with established cancer, particularly at advanced stages may have high Hcy (82, 83). The mechanisms involved may include increased folate requirements due to accelerated DNA synthesis, increased folate catabolism by cancer cells (45, 83) and antifolate chemotherapy (82), (84). *Psoriasis*: Patients with psoriasis have higher Hcy than healthy controls. Concentrations of Hcy are related to disease severity but also to low folate levels in psoriatics (86). *Inflammatory bowel disease:* A recent metaanalysis demonstrated a substantially higher Hcy in patients with inflammatory bowel disease than controls, but with no differences between patients with ulcerative colitis versus Crohn's disease (193). Hyperhomocysteinemia is associated with impaired folate and cobalamin status, erythrocyte sedimentation rate and disease severity (193). Notably, it has been recommended that low circulating folate in IBD should be confirmed by Hcy measurement, regarded as a more sensitive test in these patients (88). Sickle cell disease (SCD): Elevated Hcy (91, 92, 194, 195), (93) has been reported in SCD in most but not all studies (92). As RBC folate does not seem to be an adequate measure of folate status in SCD patients, serum folate and Hcy have been recommended for assessment of folate status in these patients (94). However, there have been uncertainties whether elevated Hcy reflects impaired folate status in SCD patients (93, 195). In a large study including 90 adult patients and 76 controls, creatinine rather than folate was a significant predictor of hyperhomocysteinemia (93). *Thyroid Disease:* Hcy status changes according to thyroid state. Hypothyroid patients have elevated Hcy, whereas hyperthyroid patients have low Hcy (95-97). These changes have been documented in longitudinal studies of patients during treatment, which normalizes thyroid state (95, 98-100). Renal function and folate status are both determinants of plasma Hcy in studies of thyroid patients (99-101, 196), suggesting that changes in folate status cannot be followed by measuring Hcy. *Diabetes*: In diabetics, serum or RBC folate and kidney function are determinants of Hcy (106, 197), but in some studies renal function is the strongest or sole determinant (198),

	in particular when renal dysfunction evolves (103). In diabetics (type I and II) with no complications, Hcy is lower than in healthy controls, which has been explained by hyperfiltration and hormonal effects on Hcy metabolizing enzymes (197, 199, 200). Likewise, insulin and hyperinsulinism decrease whereas insulin resistance (in type II diabetes) increases plasma Hcy (201). *Kidney Disease/ Renal failure:* Hyperhomocysteinemia is observed in patients with nephropathy (201), which can be explained by impaired Hcy metabolism in kidney and liver (200). Plasma Hcy is markedly elevated even in mild renal impairment (111, 112), and there is an inverse association between Hcy and glomerular filtration rate (GFR) across the whole range of GFR values from above normal (as in early diabetes with hyperfiltration) to low GFR (renal dysfunction) (197), (203). Treatment of renal patients with folic acid normalizes circulating folate and also lowers (by about 30%), but does not normalize, Hcy (204). Higher ($\geq$ 2.5 mg/d) than the standard folic acid dose (0.4 mg/d) effective in healthy subjects are required (205, 206) to normalize Hcy in these patients. In renal patients Hcy shows a strong, inverse association with serum and RBC folate even in folate replete patients treated with folic acid (207, 208). These observations suggest impaired folate function in renal patients, who might require larger folate intake than healthy subjects. In addition to folate inadequacy, hyperhomocysteinemia may reflect reduced renal clearance of Hcy and impaired Hcy remethylation (209).
Pharmacology (treatment interactions including traditional therapies)	Some drugs have a negative effect on folate status and thereby increase plasma Hcy, but other drugs affect plasma Hcy by mechanisms independent of folate. See: Drug Use – below.
Contextual Factors; impact of the following on performance of each biomarker:	
Sample source	EDTA plasma is preferred over serum for Hcy analysis because the vacutainer can be immediately centrifuged.
Fasting; time of day; time of exposure/meal/intervention	Fasting is generally not required (154); however, variations in Hcy concentrations have been observed in response to a high protein meal (210). The variability of plasma Hcy in healthy subjects (n=96) aged 65-75 years over a 1 year period was investigated 15 years ago (210). The reliability was found to be excellent with an (adjusted) ICC of 0.88. Recent assessments of within-subject stability of plasma Hcy in 40 Nurses' Health Study (NHS) participants over 1 -2 years and in 551 patients with stable

	angina pectoris from the WENBIT study over 3.5 years demonstrated a somewhat lower but still good reproducibility (ICCs of 0.71 and 0.73, respectively) (117). The reproducibility of plasma Hcy over time allows one-exposure assessment of biomarker status.
Drug use (in context of acute or chronic treatment for disease; recreational)	*Lipid-lowering drugs:* Marked elevations of Hcy by 20-50% are observed in patients treated with fibric acid derivatives, like fenofibrate and bezafibrate. Folate and vitamin B12 are not affected. Suggested mechanisms involve enhanced creatine synthesis, renal cyclooxygenase (COX-2) down-regulation and PPARα activation (124). There is some evidence that nicotinic acid (niacin) moderately increases Hcy, probably by enhanced S-adenosylhomocysteine production during methylation of nicotinamide. HMG-CoA reductase inhibitors have essentially no effect on plasma Hcy (124, 212). *Antihypertensive drugs:* Therapy with diuretics including hydrochlorothiazid is associated with decreased circulating folate and elevated Hcy. The underlying mechanisms may involve folate depletion and impaired renal function (124). In contrast, beta blockers seem to reduce Hcy in hypertensive patients by unknown mechanisms (124). *Vitamin B6 and cobalamin antagonists:* Elevated levels of Hcy have been reported in subjects treated with azauridine, isoniazid and theophylline (213). These drugs are inhibitors of the enzyme pyridoxal kinase and thereby may interfere with vitamin B6 function. Plasma Hcy increases within hours in patients exposed to the anaesthetic gas, nitrous oxide, which is explained by oxidation of cobalamin bound to methionine synthase. Inhibition of the enzyme leads to a 5-methyltetrahydrofolate trap and thereby a transient increase in serum folate (214). A slow increase in Hcy over months reflecting cobalamin deficiency is observed in patients treated with drugs interfering with cobalamin absorption, which include H2-receptor antagonists (215), proton-pump inhibitors (216) and metformin (201, 217, 218). *Others:* Parkinson patients treated with levodopa are at increased risk of elevated Hcy as a consequence of levodopa methylation by catechol-O-methyltransferase (COMT). Accordingly, the ensuing hyperhomocysteinemia is prevented by peripherally acting COMT inhibitors (212, 219). The immunosuppressive drug cyclosporine A (CyA) seems to increase plasma Hcy (213, 220), probably by its adverse effect on renal function, which may be difficult to distinguish from renal impairment from other causes in (cardiac and renal)

	transplant recipients (221).
Coffee consumption	Coffee consumption is a strong determinant of plasma Hcy, which shows a dose-response relationship with coffee intake. This relationship is observed for filtered coffee, boiled coffee but not for decaffeinated coffee, in smokers and non-smokers, and is only slightly attenuated after adjustment for vitamin intake (129, 222). The increase in Hcy is paralleled by a decrease in plasma folate (126, 127), but also a decrease in vitamin B6 and riboflavin (but not vitamin B12) (127). Thus, coffee drinkers have higher mean Hcy and lower mean folate than non-drinkers, but the differences are only observed at the lower end of the Hcy distribution (129) and at the higher end of the folate distribution (127, 128). It seems that plasma folate (127) and caffeine (223) are main determinants of Hcy. Possible mechanisms involved are increased renal excretion of folate (at high plasma folate) mediated by caffeine (127) and metabolism of chlorogenic acid in coffee by methylation, leading to increased Hcy production (224).
Smoking tobacco	Smoking is associated with deficiencies of several micronutrients and B-vitamins, including folate. Smokers have lower RBC and plasma/serum folate and higher Hcy than non-smoker (130, 131), and Hcy shows a positive association with cotinine, a marker of tobacco exposure, in passive smokers (225). Folate and Hcy status improve somewhat within days of smoking cessation, but there is a long-term effect in ex-smokers lasting for years with ex-smokers having lower folate and higher Hcy than never smokers (132). It has been suggested that the acute effect of smoking is related to increased folate breakdown or utilization caused by toxic (prooxidant) chemicals, which is in agreement with persistence of low folate after adjustment for dietary intake. The chronic effect may be explained by imprudent dietary habits of ex-smokers in combination with the time required to replenish folate stores (132).
Alcohol consumption	The association between Hcy status and alcohol intake is inconsistent, complex and related to type and amount of alcohol. Intake of beer and to a lesser extent wine may be inversely related to Hcy, which may partly be related to vitamin content in beer (128). Intake of liquor shows a positive association with Hcy (222). However, these associations could be confounded by nutrition and lifestyle factors. In a controlled intervention study, 2 weeks with red wine or vodka (24 g ethanol daily) decreased serum folate and increased Hcy (133). Excessive alcohol intake or alcoholism

	is associated with Hcy concentrations twice normal, which are normalized in abstinent alcoholics (135). Thus, a J-shaped association between alcohol intake and Hcy seems to exist. The ethanol related folate-deficiency has been explained by low intake, malabsorption, altered liver metabolism, increased catabolism and renal excretion of folate (45, 135).
Exercise	More than 15 years ago, Nygard et al. (226) reported an overall inverse association between exercise in leisure time and plasma Hcy in a large epidemiological study of about 16000 men and women. Physical activity is associated with several potential confounders, including nutritional and life-style factors and physiological and metabolic changes, and cross sectional and intervention studies (137) on exercise and Hcy have provided inconsistent results (227). Thus, from published results one cannot conclude that physical exercise has a direct effect on biomarkers of folate status, including plasma Hcy.
Socioeconomic (e.g. education; income)	In a recent analysis of data from NHANES 2003-2006, the socioeconomic variables of education and family poverty-income ratio were significantly associated with Hcy, however they did not account for much of the variability in biomarker concentration (29).

Biomarker utility of serum folic acid concentration: Depending on the research question, unmetabolized folic acid in serum or plasma may be considered an exposure, status, functional, and/or effect folate biomarker.	
Exposure:	The appearance and quantity of unmetabolized folic acid in circulation has been associated with folic acid exposure via fortified foods, dietary supplements, and a combination of both (22, 23, 228).
Status:	There are no concentration cut-offs for unmetabolized folic acid in blood; however, in the research setting, cases are often grouped and analyzed as those without detectable unmetabolized folic acid versus those with detectable folic acid in circulation. In addition, greater concentrations of unmetabolized folic acid are associated with higher serum folate concentrations (20, 23), suggesting that the amount of folic acid in blood is related to whole body folate status.
Function:	Dihydrofolate reductase enzyme activity is variable (25), thus unmetabolized folic acid in blood may also be considered a

	functional indicator of the body's ability to metabolize folic acid to a coenzymatic form.
Effect:	With bolus doses above 200 µg, which appear to exceed the capacity of the dihydrofolate reductase enzyme to reduce FA during intestinal absorption to the bioactive folate vitamer THF, unmetabolized folic acid appears in post-prandial circulation (229). Ingestion of folic acid, either by consumption of fortified foods or dietary supplements, increases the prevalence of detectable unmetabolized folic acid in blood (22, 228). However, there is large variation in folic acid concentrations and the dose response relationship 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.
Biological and contextual factors:	
Genetics	Genetic variation in dihydrofolate reductase is a biological factor that may contribute to the variation in unmetabolized folic acid concentrations (24, 25).
Fasted vs. non-fasted blood collection	As with serum folate concentrations, fasted versus non-fasted blood collection is an important contextual factor in the interpretation of unmetabolized folic acid concentrations.

Biomarker utility of urinary folate/folic acid concentration:	
Intact 24-hour urinary folate excretion is not a commonly utilized folate biomarker; however, in research settings it can provide unique information about folate status and metabolism when used in concert with serum and/or RBC folate levels.	
Exposure:	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-hour period (18). This is in contrast to serum folate concentration which reflects a single time point on the 24-h kinetic curve.
Status:	There are not validated norms to define deficiency/adequacy; however, historical/pre-fortification levels published in the folate DRI (16) can be used for comparison in assessing exposure and bodily folate stores.
Function:	Folate is reabsorbed from the kidney filtrate; however, the process is saturable, thus, intact urinary folate excretion is a

	functional indicator that the folate concentration of the filtrate exceeded this capacity (230). Similarly, excretion of unmetabolized folic acid may be a functional indicator that the capacity of DHFR to reduce folic acid to a physiologic form was exceeded.
Effect:	Urinary folate excretion is responsive to folate intake (231, 232); however, intact urinary folate excretion exhibits a large degree of inter- and intra-individual variability (233).
Biological factors	Although less explored, biological factors that impact serum folate likely affect urinary folate excretion as urinary folate is folate which is filtered from blood. Specifically, pregnancy (18, 234) and race/ethnicity (28) have been shown to affect urinary folate excretion.
Contextual factors	Study design and methods as well as companion folate biomarkers (i.e. serum and RBC folate), are important in the interpretation of urinary folate. Urinary folate excretion as a folate biomarker is most useful when folate intake is known or controlled. Incomplete or improper 24-hour urine collection by study participants is of concern and can be corrected with measurements of urinary creatinine. In the quantification of urinary folate, the large variation in collection volumes and concentrations may present challenges for method development.

Biomarker utility of urinary and serum pABG and apABG:	
The oxidative folate catabolites *p*-aminobenzoylglutamate (pABG) and *p*-acetamidobenzoylglutamte (apABG) are biomarkers of folate status and turnover. While pABG and apABG are found in blood, urinary pABG and apABG are studied most often.	
Status:	Total urinary catabolite excretion (i.e. pABG plus apABG) is positively correlated with serum total folate and RBC folate and negatively correlated with plasma Hcy (235). Similarly, pABG and apABG in serum are positively correlated with serum total folate concentrations (21). But these catabolites have high renal clearance, and their serum levels increase up to 30-fold in patients with impaired renal function (21). In contrast, urinary excretion of catabolites reflect net production rate, and are most likely not influenced by renal function, unless severely impaired.

Exposure:	Urinary pABG and apABG reflect turnover in endogenous folate pools rather than excretion of ingested pABG (236).
Function:	Folate catabolism and excretion represent an obligatory route of folate loss (237). Importantly, the quantity of urinary pABG and apABG are related to the size and turn-over rates of body folate pools which is unique among folate biomarkers (235, 238).
Effect:	Urinary excretion of pABG and apABG is positively associated with folate intake (235); however, it is not as sensitive to folate intake as urinary folate excretion (239), serum folate (238), and plasma Hcy (238).
Biological and contextual factors	
Pregnancy	Pregnancy is an important biological factor that may alter the rate of folate catabolism, and therefore concentrations of urinary pABG and apABG (240-242).
Ferritin	The iron storage protein ferritin catalyzes the oxidation of 5-formyltetrahydrofolate, thus biological states that impact ferritin concentrations may also effect folate catabolism, production of pABG and apABG, and folate status (243).
Other	Additional biological and contextual factors that may also increase folate catabolism include cancer and anticonvulsant and contraceptive drug use (45).

Supplemental Table 2

Nutrient Review Outline

I. Background about the Nutrient
- Historical overview
- Exposure: food sources
- Public Health significance: including major causes of deficiency/excess
- Current guidelines for use

II. Biology of the Nutrient
- Current understanding of the biology: dependent systems
- Homeostatic controls/metabolism including nutrient/nutrient interactions
- Role in health and disease

III. Currently Available Biomarkers: Overview
- Exposure
- Status: current cut-offs, how to derive at them and relevance to global/population/individuals
- Function:
    - direct: biomarkers of function of the micronutrients within relevant biological systems;
    - indirect: surrogate markers of function
- Effect: markers that respond to intervention (supplement and/or food based)

IV. Biomarker Specific issues: for each biomarker listed in III address:
- Humans versus animal models versus cell/molecular studies?
    - Exposure (short-/long-term?)
    - Status: are there validated norms to define deficiency/adequacy?
    - Function: do biomarkers reflect direct function, e.g, enzyme stimulation assays or indirectly reflect function of biological systems, e.g., vision, cognition/behavior, growth, immune-competence?
    - Effect: are there markers that directly reflect a response to an intervention either positive or negative?
    - Need for use of surrogate markers of all of the above?

- Population: considerations for each biomarker with regard to:

- Environment: low/middle income, food insecure etc.
- Life stage/gender considerations
- Health considerations: prevalence of infection, NCDs

- Confounders; impact of the following on performance of each biomarker
  - Bioavailability (in case of use in context of exposure)
  - Time of day/time of exposure/meal/intervention
  - Inflammation
  - Sample source (urine, plasma/serum, RBC etc.)
  - Loss (excretion, secretion etc.),
  - Endocrinology (life stage, stress etc.),
  - Pharmacology (treatment interactions including traditional therapies)
  - Nutrient interactions

V. Assay specific queries

Once a candidate is identified based on the above questions, specific details regarding the assay, methods and technology requirements would be provided to the user.

- Specificity/Sensitivity
- Multiple use? (e.g, can it reflect exposure, status, function, effect?)
- Sample collection considerations
- Optimal cut-off points
- Life stage sensitivity?
  - Infants/Children
  - Adolescents
  - Women of reproductive age
  - Pregnancy
  - Elderly
- Laboratory methodology
  - Reagents
  - Laboratory conditions (temp/humidity etc)
  - Equipment
- Field applicability (technical requirements, capacity/resource needs etc.)
- Interpretation in

    - Isolated nutritional deficiency
    - The setting of endemic disease, e.g., malaria
    - Interpretation in the presence of other infections/conditions
- Utility for targeting interventions

VI. New Direction/technologies: “omics” etc.
VII. Research Gaps/Needs
VIII. Conclusions

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