# Folate

## Abstract

The function of folate, in its various cofactor forms, is in mediating one-carbon metabolism, a network of pathways involving the transfer and utilization of single-carbon units, including methylene, forminino, methyl, methenyl, and formyl groups. Folate is thus essential for key biological functions, including the biosynthesis of DNA, serine and glycine metabolism, and methionine synthesis. Folate, in the form of 5-methyltetrahydrofolate, along with vitamin B12 (as methylcobalamin), is required for the synthesis of methionine from homocysteine, and in turn the generation of S-adenosylmethionine, a methyl group donor used in numerous reactions, including the methylation of DNA, RNA, proteins, and phospholipids. Clinical deficiency of folate is manifested as megaloblastic anemia, characterized by abnormal cell replication in the hematopoietic system, megaloblasts in the bone marrow and macrocytes in the peripheral blood. The megaloblastic anemia of folate deficiency is identical to that of vitamin B12 deficiency, and specific biomarker testing is essential to provide a differential diagnosis. Folate-related anemia occurs commonly in pregnant and lactating women in low- and middle-income countries. Clinical folate deficiency is less common in high-income countries, but subclinical deficiency is widespread, especially in women of reproductive age and in the presence of certain diseases and drugs. Notably, maternal folate nutrition before and in early pregnancy plays a critical role in fetal development, with conclusive scientific evidence that folic acid supplementation in early pregnancy protects against the occurrence of neural tube defects. Serum and red blood cell (RBC) folate are the biomarkers used to assess folate status, whilst plasma homocysteine provides a functional indictor of status. 255 words


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## Contents

## 22a.1 Folate

Folate is a generic term referring to both natural folates and folic acid (pteroylmonoglutamic acid, PGA), the synthetic form used in supplements and fortified food. All folate forms comprise three moieties: a pteridine; a p-aminobenzoic acid (PABA) and a glutamate residue (Figure 1). The parent compound, PGA, is completely oxidized and not found in nature. The natural folate forms are reduced molecules, with the addition of 2 or 4 hydrogen atoms to the pteridine, giving rise to dihydrofolate or the various tetrahydrofolate (THF) forms. THF can carry one-carbon groups attached at the N-5 (methyl, formyl or formimino), the N-10 (formyl) or bridging N-5 and N-10 (methylene or methenyl) positions of the pteridine ring, giving rise to a number of different cofactor forms of folate. Also, whereas folic acid is a monoglutamate, containing only one glutamic acid, most natural food folates exist as polyglutamate derivatives containing additional glutamate residues bound in peptide linkage to the gamma-carboxyl group.

### 22a.1.1 Functions of folate

Folate coenzymes are required for one-carbon metabolism involving the transfer and ulilization of single-carbon atom units, including methylene ($CH_2$), forminino (CH=NH), methyl ($CH_3$), methenyl (CH), and formyl (CHO) groups (Figure 22a.2). Folate is thus essential for key biological functions, including, serine and glycine metabolism, histidine catabolism, methionine synthesis and in thymidylate and purine biosynthesis, precursors of DNA. Folate, in the form of 5 methylTHF, along with vitamin B12, is required for the synthesis of methionine from homocysteine (catalyzed by the enzyme methionine synthase), and in turn, the synthesis of S-adenosylmethionine (SAM), a methyl group donor used in numerous biological methylation reactions, including the methylation of a number of sites within DNA, RNA, proteins, and phospholipids. The reader is referred to Bailey et al (2015) for a detailed review of the functions of folate.

***Folate interaction with other B vitamins***. For folate to function within one-carbon metabolism, it interacts closely with vitamin B12, vitamin B6 and riboflavin (McNulty 2019). Reduced folates enter the one-carbon cycle as THF which acquires a carbon unit from serine in a vitamin B6-dependent reaction to form 5,10 methyleneTHF. This cofactor form, once formed either converted to 5 methylTHF or serves as the one-carbon donor in the synthesis of nucleic acids, where it is required by thymidylate synthetase in the conversion of deoxuridine (dUMP) to deoxythymidine monophosphate (dTMP) for pyrimidine biosynthesis, or is converted to other folate forms required for purine biosynthesis. Methylenetetrahydrofolate reductase (MTHFR) is a riboflavin-dependent enzyme that catalyzes the reduction of 5,10 methyleneTHF to 5 methylTHF, the folate form used by methionine synthase for the vitamin B12-dependent conversion of homocysteine to methionine and the formation of THF. Methionine is activated by ATP to form S-adenosylmethionine (SAM), which then donates its methyl group to more than 100 methyltransferases for a wide range of substrates such as DNA, hormones, proteins, neurotransmitters and membrane phospholipids, all of which are regulators of important physiological processes (Bailey et al 2015). In summary, effective folate functioning requires essential metabolic interaction with vitamins B12, B6 and riboflavin. Thus sub-optimal status

of one or more of these B vitamins, or polymorphisms in folate genes, can impair one-carbon metabolism, even if folate status is sufficient.

***Absorption and transport of folate.*** Folate absorption takes place by an active process, primarily from the proximal part of the jejunum. Before absorption, the polyglutamate forms of folate are deconjugated to the monoglutamate form in the gut by the intestinal brush-border enzyme glutamate carboxypeptidase II (GCPII), more commonly termed 'folate conjugase'. The monoglutamates are then taken up by the mucosal cells. Folates require transporters to cross cell membranes; these include the reduced folate carrier (RFC), the proton-coupled folate transporter (PCFT), and the folate receptor proteins, FRα and FRβ. Folic acid is a monoglutamate and thus does not require deconjugation before uptake by intestinal cells.

Most of the folate monoglutamates absorbed from the gut are transported to the liver (the major storage organ for folate) and are re-converted intracellularly within hepatocytes to polyglutamate derivatives by the enzyme folylpolyglutamate synthetase. These folate polyglutamates are stored in the liver or converted to 5 methylTHF for secretion into the bile, and then reabsorbed by way of the enterohepatic circulation. This recirculation process may account for as much as 50% of the total folate that reaches the peripheral tissues. Circulating folate in blood is found in the monoglutamate form, predominantly as 5 methylTHF

**22a.1.2 Deficiency of folate in humans**

Severe deficiency of folate leads to megaloblastic anemia, presenting as fatigue, weakness, and shortness of breath owing to a low red blood cell count. This condition is hematologically characterized by the presence of immature, enlarged nucleated cells, reflecting impaired DNA synthesis as a result of folate depletion. Rapidly proliferating cells are especially sensitive to abnormalities in DNA synthesis. Hence, the manifestations of folate deficiency appear in the hematopoietic system, the epithelial cell surfaces and the gonads. Abnormal cell replication in the hematopoietic system, manifested by hypersegmented neutrophils, is one of the earliest morphological changes. Later, megaloblasts appear in the bone marrow and macrocytes in the peripheral blood. Some additional signs and symptoms that have been reported with clinical folate deficiency include fatigue, angular cheilosis, anorexia, insomnia, glossitis, recurrent aphthous ulcers, and pallor of the skin and mucous membranes. Of note, the megaloblastic anemia resulting from folate deficiency is identical to that resulting from vitamin B12 deficiency, and therefore specific biomarker testing is essential to provide a differential diagnosis (See section **22a.2**).

***Causes of low and deficient folate status.*** Folate deficiency arises from various causes, relating either to increased requirements, reduced availability or both. Pregnancy is a time when folate requirement is greatly increased in order to sustain the demand for rapid cell replication and growth of fetal, placental and maternal tissue (McNulty et al 2019). Gastrointestinal conditions, such as celiac disease, can also lead to deficient folate status through chronic malabsorption. Certain drugs, including phenytoin and primidone (anticonvulsants) and sulfasalazine (used in inflammatory bowel disease), are also associated with folate deficiency through adversely affecting folate metabolism. Likewise, heavy alcohol consumption and smoking are also linked with lower folate status (Bailey et al 2015).

***Prevalence of deficiency***. Folate deficiency is common in pregnant and lactating women in low- and middle-income countries, where it is reported to occur in >20% of women of reproductive age (Rogers et al 2018). Here, dietary intakes of folate are often inadequate to meet the high requirements of pregnancy.

In high-income countries, clinical folate deficiency is less common (due largely to the beneficial effects on folate status of folic acid supplementation and/or food fortification), but subclinical deficiency (indicated by low serum and RBC folate concentrations) is widespread in women of reproductive age, particularly in European countries where food fortification can be rather limited. Low folate status has also been reported in low birth weight and premature infants (Scholl and Johnson 2000) and adolescents of low socioeconomic status (Bailey et al 2015). Serum and RBC folate concentrations typically decrease throughout pregnancy; however, supplementation with folic acid prevents this decline and can thus prevent the occurrence of megaloblastic anemia of pregnancy (Blot et al 1981; McNulty et al 2013). Estimates of folate deficiency can however vary considerably across different populations and population sub-groups, depending on the method used and the cut-points applied to biomarkers measures of folate status Bailey et al., 2015.

***Neural tube defects***. Maternal folate nutrition before and in early pregnancy is known to play a critical role in fetal development. Notably, conclusive scientific evidence published 30 years ago shows that supplementation with folic acid (the synthetic form of the vitamin) in early pregnancy protects against both first occurrence (Czeizel and Dudas I992) and recurrence (MRC 1991) of neural tube defects (NTDs). These major birth defects occur as a result of a failure of the neural tube to close properly in the first few weeks of pregnancy, leading to death of the fetus or newborn, or to various disabilities involving the spinal cord, the most common form of which is spina bifida. The conclusive evidence that folic acid can prevent NTD has led to clear folic acid recommendations for women of reproductive age which are in place worldwide. It is important to appreciate that the risk of NTD will be increased when maternal folate status is low, though not necessarily within the range typically classed as deficiency. In a large prospective study in Ireland (where rates of NTD are among the highest in the world), a woman's risk of having a child with an NTD was found to be strongly associated with pregnancy concentrations of RBC folate in a continuous dose-response inverse relationship (Daly et al 1995).

In practice, implementing folic acid recommendations into practice, so that women and their babies can benefit, is challenging. Although mandatory folic acid fortification of foods has proved to be highly effective in reducing NTD wherever it has been introduced (to date in over 80 countries worldwide), elsewhere preventable NTDs are not being prevented including in European countries. Notably, one recent study estimated that from 1998 to 2017, a total of 95,213 NTD pregnancies have occurred amongst 104 million births in 28 countries European countries; a prevalence of 0.92 per 1,000 births (Morris et al 2021). This study concluded that failure to implement mandatory folic acid fortification in the 28 European countries continues to cause NTD to occur in almost 1,000 pregnancies every year.

The precise mechanism explaining the beneficial effects of periconceptional folic acid against NTD remains uncertain, though proposed mechanisms have focused on factors that impair normal folate metabolism, including polymorphisms in folate genes. Among the latter, an increased risk of NTD is most strongly associated with the 677C→T variant in the gene encoding the folate-metabolizing enzyme MTHFR, as reported in most studies including meta-analyses (Botto and Yang 2000; Vollset et al 2004). Autoantibodies against folate receptors are also implicated in pregnancies affected by NTD (Rothenberg et al 2004). Also, although low maternal folate is considered the major contributing factor in NTD, convincing evidence shows that low vitamin B12 is an independent risk factor in NTD (Molloy et al 2009). Apart from preventing NTD, there is good evidence that periconceptional folic acid use may prevent congenital heart defects in infants (Van Beynum et al 2010), and possibly, orofacial clefts (Bailey et al 2015).

### 22a.1.3 Food folate sources, bioavailability and dietary intakes

***Food folates.*** Folates are widely distributed in animal and plant foods, but only certain foods provide a rich source. These include liver, yeast, green leafy vegetables, asparagus, beans, legumes, where folates exist primarily as polyglutamates, containing several glutamate residues attached to the *p*-aminobenzoic group of the molecule (Figure 1). In contrast, folic acid, the synthetic vitamin, is a monoglutamate, containing only one glutamate moiety. Also, unlike folic acid, which is a fully oxidized molecule, natural folates are reduced at the 5, 6, 7 and 8 positions of the pteridine ring and so are prone to oxidative cleavage at the C9–N10 bond producing two degradation products, a pteridine and p-aminobenzoylglutamate, both inactive and cannot be biologically converted to any active folate form.

***Folate bioavailability***. Bioavailability refers to the proportion of ingested nutrient that is absorbed and available for metabolic processes. Naturally-occurring food folates are widely recognized to have limited bioavailability (McNulty & Pentieva 2004). These folates are reduced molecules, and therefore are inherently unstable outside living cells. In addition, the ease with which folates are released from different food matrices and their conversion to the monoglutamate form before uptake by intestinal cells can vary greatly. Folate bioavailability from different food sources is also dependent on the presence of certain dietary constituents, that may affect folate stability during digestion, or inhibit bioavailability owing to specific inhibitors of deconjugation. Thus, the bioavailability of food folates from a mixed diet is limited and highly variable. In addition, natural food folates (particularly green vegetables) are unstable during cooking, and this can substantially reduce the folate content of a food before it is even ingested (McKillop et al 2002). This is an additional factor, often overlooked, that can further limit the ability of naturally-occurring food folates to positively influence folate status. Notably, a controlled 12-week feeding study in young women showed that, whereas RBC folate was found to be highly responsive to intervention with folic acid supplements or fortified foods, increased intakes of natural food folates at equivalent levels showed no significant RBC folate responses (Cuskelly et al., 1996). (Cuskelly et al 1996).

***Dietary folates.*** The bioavailability of folic acid is assumed to be 100% when ingested as a supplement, while folic acid in fortified food is estimated to have about 85% the bioavailability of supplemental folic acid (Pfeiffer et al., 1997). These differences have led to the development

of 'dietary folate equivalents' or DFE values; more details are given in Section 8a. Briefly, expressing dietary folate intakes and recommendations in DFE terms, enables an adjustment for the differences in bioavailability between natural food folates and the synthetic vitamin. The DFE is defined as the quantity of natural food folate plus 1.7 times the quantity of folic acid in the diet; this definition is based on the assumption that the bioavailability of folic acid added to food is greater than that of natural food folate by a factor of 1.7 (Institute of Medicine, 1998. This estimation is largely dependent on a metabolic study in non-pregnant women that estimated the bioavailability of food folates to be 50% relative to that of folic acid (Sauberlich et al. 1987), and other evidence, mentioned above, showing that folic acid added to food has 85% of the bioavailability of free folic acid (Pfeiffer et al. 1997).

***Fortified foods as a source of folate.*** Food fortification, the process of adding essential micronutrients to food, plays an important role in facilitating more optimal nutritional status in individuals and populations. In the case of folate, it plays a crucial role. Folic acid, the folate form used for food fortification, is cheap to produce, very stable once added to foods and highly bioavailable when ingested. Thus, depending on national fortification policy and/or access to folic acid-fortified food, the folate status of populations can vary greatly from one country to the next. This in turn will be reflected in differences in health outcomes, most notably in relation to NTD risk. Food fortification may be undertaken on a voluntary or mandatory basis. Voluntary fortification, whereby folic acid is added to foods such as breakfast cereals at the discretion of the manufacturer, is permitted in most European countries. This results in higher folate intake and status (Hopkins et al 2015), but the benefit is limited only to consumers who choose to eat the fortified food products. However, when folic acid fortification is undertaken on a mandatory (population-wide) basis, including in the USA, Canada, Australia and Chile, it has proved to be highly effective, not only in increasing folate status and reducing folate deficiency (Yang et al 2010), but also in reducing NTD in that country (Hoein et al 2001, Lopez-Camelo et al 2005, De Wals et al 2007, Sayed et al 2008). Mandatory fortification is now in place in 85 countries worldwide, both high- and low-middle income countries.

***Effects of high intakes of folate.*** High dietary folate intakes are generally not associated with any adverse effects. However, there are concerns of potential adverse effects of excess intakes of folic acid, the synthetic vitamin form. Excessive folic acid intake constitutes exposure doses that exceed the Tolerable Upper Intake Level (UL) of 1000µg/d for adults, as set by the U.S. Institute of Medicine (1998). Historically the concern regarding excess folic acid focused on the potential to mask pernicious anemia and exacerbation of the clinical effects of vitamin B-12 deficiency. More recently other concerns have been raised, including potential adverse effects on cancer risk, birth outcomes, and other diseases. However, a recent report (in 2019) from an expert working group tasked with reviewing this research area, as convened by the U.S. National Institutes of Health, recently concluded that there is an insufficient body of evidence to support adverse human health outcomes as a result of high intakes of folic acid. Nonetheless, these experts called for further high quality research to determine the safety of excess folic acid intake (Maruvada et al 2020).

**22a.2 Biomarkers of folate status**

**Overview.** In 2015, an expert international panel produced a comprehensive review of folate biology and biomarkers as part of the Biomarkers of Nutrition for Development (BOND) project, as convened by the U.S. National Institutes of Health (Bailey et al 2015). This identified serum folate, red blood cell (RBC) folate and plasma homocysteine as the 'Priority Folate Biomarkers' for assessing folate status. Serum folate reflects recent dietary intake. RBC folate, compared with serum folate, is a better indicator of folate intake and status over the previous 3-4 months. Plasma homocysteine provides a functional indicator of folate status and will be elevated with deficient folate status on the basis that normal homocysteine metabolism requires an adequate supply of folate. Concentrations of folate in erythrocytes, but not serum, also fall in vitamin B12 deficiency; ideally, therefore, both serum and RBC folate concentrations should be measured.

**Cut-off values for assessment of folate status**. The sequential stages in the development of folate deficiency were originally established through detailed depletion/repletion experiments conducted during the late 1960's, proving a basis for setting cut-off values for serum and RBC folate for the assessment of status (Herbert 1987; these sequential stages of folate deficiency and related cut-off values are summarized in Box 22a.1. *Box to be inserted here*

The initial stage of folate deficiency, termed negative folate balance, is consistent with serum folate values of <7 nmol/L (Herbert 1987). If the negative balance persists, tissue folate becomes depleted, as indicated by RBC folate falling below the normal range to <363 nmol/L. At this second stage, there is little evidence that biochemical function is impaired, although plasma homocysteine concentrations may be slightly elevated. By the third stage, termed folate-deficient erythropoiesis, functional impairment is usually evident, with erythrocyte folate values <272 nmol/L. Tissue folate stores are severely depleted in the fourth and final stage where the classical hematological changes occur, manifested as folate-deficiency anemia. These include macro-ovalocytic erythrocytes in the circulating blood and megaloblasts in the bone marrow. At this stage, hypersegmented neutrophils in the peripheral blood smear and abnormal RBC indices are also apparent; mean red cell volume and mean cell hemoglobin are elevated and hemoglobin concentration is low

As described below, more recently, revised cut-off values for folate deficiency (serum folate <10 nmol/L and RBC folate <340 nmol/L) have been defined based on the metabolic indicator, plasma homocysteine (Selhub et al 2008), as summarized in Table 22a.1.

### 22a.2.1 Serum and RBC folate concentrations

Serum folate represents the sum of several folate forms circulating in blood. The main circulating folate is 5-methyl-THF; other reduced forms such as THF and formyl-THF may also be present, but in very small concentrations (Bailey et al 2015). As will be discussed later (section 22a.2.4), unmetabolized folic acid can also be present in varying concentrations in plasma (Bailey RL et al 2010).

Red cells contain much higher folate concentrations than serum. 5-methylTHF polyglutamates are the main RBC folate forms. The measurement of RBC folate is more complex than that of

serum folate, because of the need to convert polyglutamates to monoglutamates prior to analysis. In individuals homozygous for the *MTHFR* C677T polymorphism, a portion of the 5-methylTHF polyglutamates is replaced by formyl-folates (Bagley and Selhub 1998).

Serum folate is the earliest indicator of altered folate exposure and reflects recent dietary intake (Pfeiffer et al 2010). In the individual, serum folate concentrations can increase markedly in response to dietary folate, reaching a peak concentration 90 min after ingestion. RBC folate is a sensitive indicator of long-term folate status. RBC folate parallels liver concentrations, accounting for about 50% of total body folate, and thus reflects tissue folate stores (Wu 1975). RBC folate, compared with serum folate, responds 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 during erythropoiesis, thereby reflecting folate status during the preceding 120 days, the half-life of the RBC (Mason 2003).

**Factors affecting serum and RBC folate concentrations**

***Dietary folate intakes.*** Blood folate concentrations are primarily affected by dietary folate intakes. Serum and RBC folate are highly responsive to intervention with folic acid (Duffy et al 2014), with natural food folates typically resulting in poorer folate responses compared to folic acid at similar intervention levels (Cuskelly et al 1996). Likewise, population data show that both serum and 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 (Yang et al 2010; Hopkins et al 2015).

***Vitamin B12 status***. Because vitamin B12 is required for normal folate re-cycling and folate retention within cells, vitamin B12 deficiency leads to a failure to retain folate within cells (Hoffbrand and Weir 2001). Consequently, RBC folate concentrations fall, despite the presence of normal (or sometimes even elevated) serum folate. Thus, low RBC folate may reflect vitamin B12 deficiency as well as folate deficiency.

***Fasting versus non fasting samples***. Samples from fasted individuals, on average, have lower concentrations of serum (by 10%) and RBC folate (by 5%) compared with samples from nonfasted (<3 h) participants, but the small differences generally indicate that fasting is not essential when assessing the folate status of populations.

***Important preanalytical factors.*** Detailed information on preanalytical factors affecting serum and RBC folate is described in a comprehensive review article on analytical approaches by Pfeiffer et al. (2010). Folate is the least stable of the B vitamins and is susceptible to oxidative degradation during preanalytical sample handling and storage. Careful sample handling and use of antioxidants are therefore required to maintain sample integrity. Blood for serum folate analysis should be processed and frozen promptly. Although serum is generally preferred over plasma in most laboratories, both matrices generally produce comparable results for serum folate, as long as the sample processing is not delayed. If delays are unavoidable, the sample should be protected from light, kept cool and processed within a few days of collection. Folate in serum and hemolysates (but not in whole blood) can withstand a few short freeze/thawing cycles, particularly if the vials are kept closed as much as possible to minimize the exposure of

the sample to air. Folate in serum/plasma degrades rapidly at room temperature, particularly in the presence of EDTA.

**Analytical methodologies for measurement of serum and RBC folate**

Over the past 50y, analytical 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. The within-person variability for serum folate is about twice that for RBC folate (CV of 21.5% and 9.1%, respectively (Bailey et al 2015).

***Critical considerations in folate assessment.*** Assessing folate status is complicated by the large number of folate forms that may be readily interconverted. To overcome this problem, 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, i.e. L. rhamnosus responds to all active monoglutamate forms (see below). Subsequently, assays using competitive protein binding became common because of their simplicity. In more recent years, the use of LC-MS/MS has emerged, enabling investigation of relevant research findings related to folate metabolism. These include alterations in the relative proportion of different folate forms in red cells owing to the common polymorphism in *MTHFR* C677T along with an overall lower RBC folate (Molloy et al 1997; Bagley and Selhub 1998; Shane et al 2011), and the presence of free ('unmetabolized') folic acid in the blood as a result of high intakes of folic acid (Pfeiffer 2010).

There are three main method types for measurement of serum and RBC folate, each with advantages and disadvantages (Bailey et al 2015).

***Microbiological assay.*** This is widely considered to be the gold standard assay for serum and RBC total folate because it measures all biologically active forms equally and does not measure folate species that lack vitamin activity. The underlying principle of the microbiological assay 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 the folate concentration can be quantified by measuring the turbidity of the inoculated medium after a 2-d incubation. A chloramphenicol-resistant strain of L. rhamnosus is used and the assay is performed using automated microtiter plate technology.

The key advantages of the microbiological assay include its excellent sensitivity, low cost and simple instrumentation (thus suited for low-resource settings), and the fact that it can be used with dried blood spot samples. The high sensitivity is a particular advantage when limited volume is available, such as for samples collected from a fingerstick or dried blood spot. The disadvantages are that the microbiological assay is relatively laborious unless automated liquid handling is introduced, lengthy assay time with limited throughput and limited linear range (thus requiring dilution of samples). It is also prone to contamination issues and potential interference by the presence of antibiotics or antifolates. The latter limitation is well recognized, but in practice may not be such an issue, in that analysts in the US reported that <1% of samples from the population-based NHANES cohort exhibited a pattern of interference due to the presence of antibiotics or antifolates (Pfeiffer et al 2010).

***Protein-binding assays.*** These assays were developed primarily for clinical settings, to enable the diagnosis of folate deficiency. Protein-binding assays use the highly specific folate-binding protein to extract folate from the sample. The strengths of this approach include the high sample throughput, quick turnaround time, availability in commercial kit form and minimum operator involvement. The disadvantages are that the various folate forms have different affinities to folate-binding protein, the questionable accuracy when mixtures of folate are present, the limited linear range (thus requiring dilution of samples), matrix effects when sample is diluted, and lot-to-lot variability of commercial kits. Also, although not sensitive to antibiotics, protein-binding assays are influenced by certain antifolates such as methotrexate.

***Chromatography-based assays (HPLC-FD, LC-MS/MS).*** Chromatography-based methods typically provide information on individual folate forms based on measurement of intact folates via HPLC. More recently, LC-tandem mass spectrometry (LC-MS/MS) is now the preferred detection for HPLC-based methods in specialized laboratories. Advantages of chromatography-based approaches are that they measure all folate forms, are highly selective and specific, have good sensitivity and precision, enable in-house control of performance and use of stable-isotope-labeled internal standards to compensate for procedural losses. Ensuring accurate calibration is a big task for chromatography-based methods, due to the high number of folate forms and also because of their instability. Disadvantages include the high costs and requirement for expensive instrumentation, experienced operator and frequent technical service, along with being a relatively laborious approach unless automated liquid handling is introduced and the requirement for complex sample extraction/cleanup. Also interconversions of folate forms during the assay procedure need to be considered in interpretation of data.

**Cut-off values and Interpretation of Serum and RBC folate**

The measurement of total folate provides information on the folate status of the individual, either in the short-term through serum folate, or in the long-term through RBC folate. An historical perspective on the use of folate -cut-offs is provided in Table 22a.1. However, the inconsistent use of cut-off values over time to assess the proportion of populations with deficient or low folate status led to a certain degree of confusion. More recently, revised cut-off points for folate deficiency (serum folate <10 nmol/L and RBC folate <340 nmol/L) have been defined based on the metabolic indicator, plasma homocysteine (Selhub et al 2008), as summarized in Table 22a.1. These cut-off values have been recommended by the WHO for assessing folate status of populations (de Benoist 2008). Of note, the values were derived from data generated using the microbiological assay.

As discussed earlier, the risk of NTD is associated with a maternal folate status that would not conventionally be classed as deficient. Although there is no cut-off value established by international organizations for folate concentrations to define NTD risk in populations, it is accepted that the number of NTDs that can be prevented in a population is dependent on maternal folate status. Specifically, RBC folate concentration in the mother has been shown to be a sensitive biomarker of NTD risk. In the one and only prospective study that has been conducted to date, Daly et al. (1995) found that the prevalence of NTD in an Irish population was lowest when maternal RBC folate concentrations were ≥906 nmol/L (400 ng/mL). Data

modeled from folic acid intervention studies in China by Crider et al. (2014) were consistent with the dose response between RBC folate concentrations and NTD risk as reported in the Irish study (Daly et al 1995)

### 22a.2.2 Plasma homocysteine concentrations

**Background**. Plasma homocysteine provides a sensitive functional indicator of folate status. On the basis that normal homocysteine metabolism requires an adequate supply of folate, plasma homocysteine becomes elevated when folate status is low.

Homocysteine is a four-carbon, thiol-containing amino acid found in human plasma, mostly present in the form of various disulfides, such as homocysteine-cysteine disulfide. About 75% of total homocysteine is bound to protein (mainly albumin), whereas the remainder occurs in nonprotein-bound 'free' forms. Total homocysteine is defined as the sum of all homocysteine species in serum or plasma, including free and protein-bound forms. Only a very small portion (1–2%) of plasma homocysteine is present as the thiol, however the relative contribution of the thiol to total homocysteine increases to 10–25% in patients with abnormally elevated plasma homocysteine (Bailey et al 2015).

Homocysteine is derived from the essential amino acid, methionine. It is metabolized by one of the two pathways: remethylation to methionine (by methionine synthase) or transsulfuration to cystathionine (by cystathionine beta-synthase; CBS) then to cysteine (Figure 22a.2). Both pathways require adequate status of folate and the metabolically related B vitamins. The remethylation pathway, whereby methionine is synthesized from homocysteine by methionine synthase, is dependent on both folate and vitamin B12 as cofactors, whilst the transsulfuration pathway that converts homocysteine to cysteine is catalyzed by two vitamin B-6 dependent enzymes. A fourth B vitamin, riboflavin, is required in its cofactor form flavin adenine dinucleotide (FAD) for the activity of MTHFR, the enzyme that catalyzes the reduction of 5,10 methyleneTHF to 5 methylTHF. Once formed, 5 methylTHF is used by methionine synthase for the vitamin B12-dependent conversion of homocysteine to methionine and the formation of THF. Thus, the concentration of homocysteine in plasma/serum is determined by four B-vitamins: folate, vitamin B12, vitamin B6 and riboflavin.

Apart from providing a functional biomarker of folate, higher plasma homocysteine is associated with a number of chronic diseases of ageing, including an increased risk of cardiovascular disease (CVD; Graham et al 1997), cognitive impairment and dementia (Smith and Refsum 2016). It remains to be established, however, whether plasma homocysteine is a risk factor for these or other diseases. In the case of CVD, this issue is particularly controversial. Despite strong and consistent evidence from observational studies over many years, several secondary prevention trialpublished between 2004 and 2014 failed to demonstrate a benefit of homocysteine-lowering therapy against the recurrence of CVD events in patients with existing disease. The evidence is however stronger for the relationship of homocysteine with stroke than heart disease, with good evidence from both population data and randomized trials that folic acid intervention and/or homocysteine-lowering can significantly reduce the risk of stroke, and particularly so in people with no previous history of stroke

(McNulty et al 2017). Of note, although the literature in this area focuses on homocysteine as the CVD risk factor, it is possible that folate and related B-vitamins have roles in CVD that are independent of their homocysteine-lowering effects. Thus, plasma homocysteine may be associated with CVD as a functional marker of low B-vitamin status which reliably reflects perturbed one-carbon metabolism, rather than being causatively related to CVD *per se* (McNulty et al 2017).

**Factors affecting homocysteine concentrations**

***Folate status***. On the basis that normal homocysteine metabolism requires an adequate supply of folate, plasma homocysteine is first and foremost affected by folate status. Thus, plasma homocysteine was shown to respond within 3-4 weeks of folate depletion (increase) and subsequent repletion (decrease) in a controlled metabolic study in healthy women (Jacob 1998). Likewise, in observational studies, plasma homocysteine is invariably found to be inversely associated with folate status, whether measured as serum or RBC folate.

***Intervention with folic acid.*** Plasma homocysteine is highly responsive to intervention with folic acid (the synthetic vitamin form), alone or in combination with vitamin B12, vitamin B6, riboflavin and betaine (or choline). Thus, food fortification with folic acid has marked effects on homocysteine concentrations. Using population-based data from the US, Pfeiffer et al (2008) reported a 10% decrease in plasma homocysteine when comparing values pre-fortification (1991–1994) to post-fortification (1999–2004) with folic acid, as implemented on a mandatory basis in 1996-1998. Folic acid-fortification on a voluntary basis (i.e. added to food at the manufacturer's discretion) also affects plasma homocysteine. In a convenience sample of nearly 500 healthy adults in Northern Ireland aged 18–92 years, who were not taking folic acid supplements, homocysteine concentrations were lower by 2 μmol/L in high consumers compared to non-consumers of fortified foods (providing >100 μg/d and 0 mg/d folic acid, respectively) (Hoey et al 2007).

***Folate-related B vitamins***. Because normal homocysteine metabolism is dependent on four B vitamins, homocysteine concentrations will be elevated with other B vitamin deficiencies apart from folate, notably vitamin B12 (Allen et al 2018). Thus whilst plasma homocysteine is primarily a folate biomarker, once folate status is optimized, a much greater dependency on vitamin B12 emerges (Quinlivan et al 2002). Likewise, in population groups who consume folic acid-fortified foods or folic acid supplements, homocysteine is considered to be a more reliable biomarker of vitamin B12 than folate (Refsum et al 2004).

***Disease and lifestyle***. Apart from inversely reflecting folate and related B vitamin status, plasma homocysteine is found to be elevated in patients with impaired renal function (Yetley et al 2011; Allen et al 2018) and with certain drug treatments (Refsum et al 2004). Alcohol intake, coffee-drinking and smoking are also associated with higher plasma homocysteine.

***Age, sex and lifecycle stage.*** Homocysteine concentrations increase throughout life and are higher in males compared to females at all ages. Although plasma homocysteine decreases in pregnancy (by about 50%), concentrations are reported to normalize within a few days postpartum (Murphy et al 2004).

***Ethnic and genetic effects.*** Plasma homocysteine may also differ among ethnic groups, but these differences appear to be less important than the influence of B vitamin status. Notably, the most common genetic cause of elevated homocysteine in the general population is homozygosity for the *MTHFR* C677T polymorphism (affecting an estimated 10% of people worldwide but much higher in some populations, including Mexico and Northern China). This folate gene variant also contributes to a higher risk of blood pressure throughout the lifecycle, particularly when combined with deficient status of riboflavin, which is required as a cofactor for MTHFR, a key folate metabolizing enzyme (Psara et al 2020; Ward et al 2020). Notably, both phenotypes associated with this common polymorphism (high homocysteine and high blood pressure) appear to be modifiable with better riboflavin status (McNulty et al 2020). Most other genetic polymorphisms in enzymes related to one-carbon metabolism have little effect on homocysteine concentrations (Refsum et al 2004).

***Inborn errors of metabolism***. Homocystinuria is a rare inborn error of metabolism which leads to severely increased homocysteine concentrations in plasma, usually >100 mmol/L, along with large amounts excreted in urine. The most common cause of homocystinuria is deficiency of the enzyme cystathionine beta synthase (CBS). CBS deficiency, an autosomal recessive condition, has a reported worldwide birth prevalence of 1 in 344,000, while that in Ireland the frequency is much higher, at 1 in 65,000, based on newborn screening and cases detected clinically. These patients have a high risk of premature, frequently fatal, thromboembolic events. Early diagnosis and treatment with pyridoxine and/or folic acid and betaine, preferably from infancy, can however prevent CVD events and most of the clinical symptoms (Refsum et al 2004).

**Analytical methodologies for measurement of plasma homocysteine**

Plasma total homocysteine is a very stable analyte as long as the plasma is separated from the red blood cells within 1 hour of blood collection (or within 8 hours if the whole blood is kept on ice). As described in detail by Refsum et al (2004), various method types are available for homocysteine determination. These range from fully automated commercial kits (immunoassay or enzymatic methods) to chromatographic assays with mass spectrometry detection, overall providing comparable results and good assay performance. All methods require the reduction of the disulfide bonds to allow measurement of total homocysteine. The reported within-person variability CV for plasma homocysteine is 12.2% (Refsum et al 2004).

***Choice of method.*** Measurement of plasma homocysteine produces comparable results across different method types, the choice of method is mainly dependent on available instrumentation and technical expertise. Bailey et al (2015) the following recommendations were put forward to help to inform this decision:

- Using 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. The main disadvantage is the relatively high reagent costs, which 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 that the number of samples that can be run is quite limited (typically to about 50-70 unknown samples per analytical run).

- If a laboratory has access to an existing LC-MS/MS system and the required technical knowhow, plasma homocysteine can be measured in a high throughput semi-automated manner, with quite low reagent costs. Due to the high initial cost, however, an LC-MS/MS system is economical only if a large number of samples are measured regularly.
- GC-MS or GC-MS/MS have also been used in high throughput research settings, particularly when the co-determination of methylmalonic acid and methionine is of interest.
- GC-based methods provide better precision, higher resolution, and longer column life compared to LC-based methods and are not subject to ion suppression issues, which can be a problem in LC-MS/MS methods.

**Cut-off values and Interpretation**

In one of the most comprehensive reviews of all relevant aspects of homocysteine measurement, it is proposed that reference values for homocysteine are established for different populations to account for the influence of both nonmodifiable and modifiable factors (Refsum et al 2004; Table 22a.2). The following recommendations were put forward for setting reference ranges:

- Each laboratory should establish reference limits for its region.
- The reference population should exclude individuals with folate or cobalamin deficiency or increased creatinine, and patients with diseases or who are taking drugs that increase homocysteine concentrations.
- Separate reference limits for children, adults, the elderly, and pregnant women should be used (Table 22a.2).
- In populations consuming folic acid supplements or folic acid-fortified foods, the upper reference limit is usually 20-25% lower than in nonfortified populations (Table 22a.2).
- The upper homocysteine reference limit should be interpreted with age, sex, and other determinants in mind, particularly prevailing food fortification practices.

### 22a.2.3 Other biomarkers of folate status

**Neutrophil lobe count**

Usually, neutrophils have three or four segments, but in megaloblastic anemia (owing to folate or vitamin B12 deficiency), this number increases. Neutrophil hypersegmentation can be evaluated in smears of peripheral blood or in white blood cells obtained from the buffy coat (i.e. interface between the serum / plasma and the sedimented red cells), and may be the earliest morphological change to appear in the blood in folate and B12 deficiency. Neutrophil hypersegmentation however is not specific to folate or B12 deficiency as it also occurs in other conditions including uremia, myeloproliferative disorders, myelofibrosis and as a congenital lesion in approximately 1% of the population, even when the status of folate and vitamin B12 is adequate. 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 (Westerman 1999; Metz 2008).

**Serum folic acid**

Unmetabolized (or 'free') folic acid can be present in varying concentrations in plasma and is typically associated with higher folic acid intake via fortified foods, supplements, or a combination of both (Bailey et al 2010). Whilst greater concentrations of unmetabolized folic acid are generally associated with higher serum folate concentrations, there is large variation in reported values and no clear dose response relationship exists between folic acid intake and unmetabolized folic acid in plasma (Pfeiffer et al 2004; Bailey RL et al 2010; Obeid et al 2011). Thus, cut-off values or desirable ranges have yet to be identified. Of note, the biological impact and the significance for health of unmetabolized folic acid in blood remain to be elucidated.

**Genomic biomarkers of folate status**

***DNA cytosine methylation***. Apart from its role in DNA synthesis, folate plays an important role in DNA metabolism as it is required for the synthesis of methionine and thus SAM, which in turn is required as a methyl donor for the maintenance of cytosine methylation, essential for silencing of genes or structural integrity of specific regions of chromosomes (Fenech, 2012). When SAM is depleted, the maintenance of DNA methylation may become compromised, leading to hypomethylation of cytosine and structural changes in chromatin. Some studies suggest that global DNA methylation status is reduced when folate is deficient (Kim, 2005; Crider et al 2012), but findings in this regard are somewhat inconsistent.

***Uracil misincorporation into DNA***. Measurement of uracil content in DNA may also provide a biomarker of folate status, on the basis that adequate amounts of folate as 5,10-methyleneTHF are required by thymidylate synthase to convert dUMP to dTMP in pyrimidine synthesis and thus DNA biosynthesis. If 5,10-methyleneTHF is limiting as a substrate of thymidylate synthase, dUMP accumulates and it becomes more probable that uracil is incorporated into DNA instead of thymidine during DNA synthesis (Stover 2009; Fenech, 2012).

***Micronuclei***. Micronuclei have the same morphological features as normal nuclei but are much smaller. Excessive uracil misincorporation into DNA and hypomethylation of DNA can lead to the formation of micronuclei from chromosome fragments which can be measured in lymphocytes or in erythrocytes (Blount 1997; Fenech, 2012). Observational studies show that micronuclei in lymphocytes or erythrocytes are inversely associated with dietary folate intake and RBC folate (Blount et al 1997), whilst intervention studies have reported a significant reduction in micronuclei frequency in lymphocytes with folic acid supplementation (Fenech et al, 1997). It is now possible to score micronuclei automatically and reliably using a wide range of image cytometry platforms, making this technique amenable to mass screening. Given its sensitivity to folate deficiency, micronuclei measurement in combination with uracil and DNA methylation measurements, can potentially provide a reliable assessment of genome pathology resulting from deficient folate status (Bailey et al 2015).

### 22a.2.4 Assessment of folate status in children

Most studies assessing folate status have focused on adults. Far fewer studies provide folate biomarker data for children, with notable exceptions being published reports using data from population-based surveys conducted in the United States (Pfeiffer et al 2012) and the United Kingdom (Kerr et al 2009). The latter report proposes normal ranges for folate biomarkers for use in clinical pediatric settings (Figure 3).

The population-based data in British and American children, both show progressive declines in folate (and correspondingly, increases in homocysteine) concentrations with age from childhood to adolescence. Consistent with these reports are the findings from convenience cohorts of Belgian, Dutch, and Greek children, also showing age-related decreases in folate concentrations in childhood (De Laet 1999; van Beynum et al 2005; Papandreou et al 2006). Of note, where dietary intakes were also measured, the data showed that folate intakes generally compared favorably with dietary reference values across all age groups and were not lower in the older children (Papandreou et al 2006; Kerr et al 2009; Pfeiffer et al 2012). The explanation for the decline in folate status biomarkers with age in children, despite no corresponding decline in dietary folate intakes, is not entirely clear but likely reflects the higher folate requirements of older children related to increased metabolic demands for growth from childhood to adolescence (Bailey et al 2015).

## 22a.3 Conclusions

Folate is required for one-carbon metabolism and thus plays a critical role in essential biological processes, including amino acid metabolism, DNA synthesis and repair and methylation reactions. Low folate status leads to adverse health effects throughout the lifecycle, even if not severe enough to cause megaloblastic anemia, the clinical manifestation of folate deficiency. Thus, accurate assessment of folate status is essential. Serum and RBC folate concentrations are sensitive biomarkers used widely to assess folate status. Serum folate is the earliest indicator of altered folate exposure and reflects recent dietary intake, whilst RBC folate reflects tissue folate stores and is a better indicator of folate intake over the previous 3-4 months when circulating folate is incorporated into the maturating red cells. On the basis that normal homocysteine metabolism requires an adequate supply of folate, the measurement of plasma homocysteine provides a sensitive functional indicator that will be elevated with deficient or low folate status, but it is not a specific folate biomarker as it is influenced by other nutrient (most notably vitamin B12 deficiency) and non-nutrient factors including renal function. Emerging genomic biomarkers may provide a reliable assessment of genome pathology resulting from deficient status to folate, to add to the core biomarkers of status, serum folate, RBC folate, and plasma homocysteine.

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