DRAFT SCIENTIFIC OPINION

Scientific Opinion on Dietary Reference Values for folate[1]

EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA)[2, 3]

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ABSTRACT

Following a request from the European Commission, the Panel on Dietetic Products, Nutrition and Allergies derived Dietary Reference Values (DRVs) for folate. The Panel concludes that an Average Requirement (AR), as well as a Population Reference Intake (PRI) assuming a coefficient of variation (CV) of 15 % in order to account for the additional variability associated with the higher requirement for folate in individuals with the MTHFR 677TT genotype, can be derived from biomarkers of folate status. Several health outcomes possibly associated with folate intake/status are also considered, but data are found to be insufficient to establish DRVs. For adults, the AR is determined from the folate intake required to maintain functional folate adequacy characterised by serum and red blood cell folate concentrations above 10 and 340 nmol/L, respectively. An AR of 250 µg dietary folate equivalents (DFE)/day and a PRI of 330 µg DFE/day are derived. For infants aged 7-11 months, an Adequate Intake (AI) of 80 µg DFE/day is derived by extrapolating upwards from the estimated folate intake in exclusively breast-fed infants, taking into account differences in reference weights, and considering observed intakes in the only representative survey available. For children, ARs are extrapolated from the AR for adults using isometric scaling and growth factors and considering differences in reference weights. PRIs ranging from 80 µg DFE/day for 1 to 3 year-old children to 330 µg DFE/day for boys and girls aged 15-17 years are derived. For pregnant women, an AI of 600 µg DFE/day is derived based on a study on maintenance of serum and red blood cell folate concentrations in pregnancy. For lactating women, an additional intake of 130 µg DFE/day is considered to cover folate losses with breast milk; this figure is added to the AR for non-lactating women and a PRI of 500 µg DFE/day is derived.

KEY WORDS

folate, folic acid, Average Requirement, Dietary Reference Value, health outcomes

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SUMMARY

Following a request from the European Commission, the EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA) was asked to deliver a scientific opinion on Dietary Reference Values (DRVs) for the European population, including folate.

Folate is a generic term used for a family of compounds which belong to the group of B-vitamins. Naturally occurring food folates are reduced polyglutamates and their chemical structure makes them unstable. In contrast, the synthetic folic acid, which arises in the diet only through ingesting fortified foods or vitamin supplements, is a fully oxidised monoglutamate and the most chemically stable form. Upon ingestion, polyglutamated folate forms are hydrolysed to monoglutamates and actively absorbed by a pH-dependant saturable mechanism in the duodenum and upper jejunum, or by passive diffusion in the ileum if consumed in supraphysiological amounts. Natural food folates have a lower bioavailability than folic acid. In order to take into account these differences, dietary folate equivalents (DFE) have been introduced and defined as 1 µg DFE = 1 µg food folate = 0.6 µg folic acid from fortified food or as a supplement consumed with food = 0.5 µg of a folic acid supplement taken on an empty stomach.

Folates function as cofactors for enzymes involved in one-carbon metabolism. Folate provides one-carbon units for the formation of nucleotides necessary for the synthesis of RNA and DNA. Folate is also fundamental for the normal functioning of the methionine cycle, which is responsible for both the conversion of homocysteine to methionine and the production of the universal methyl donor S-adenosylmethionine (SAM). SAM donates its methyl group to more than 100 methyltransferases for a wide range of substrates such as DNA, hormones, proteins, neurotransmitters and membrane phospholipids, which are regulators of important physiological processes. Folate deficiency impairs DNA replication and cell division, which adversely affects rapidly proliferating tissues such as bone marrow and results in the production of unusually large macrocytic cells with poorly differentiated nuclei. The predominant feature of folate deficiency is megaloblastic anaemia.

Serum and red blood cell folate concentrations are sensitive biomarkers of folate intake and status, and the Panel considers that these are suitable primary criteria for deriving the DRVs for folate. The Panel considers that serum folate concentrations of less than 6.8 nmol/L and red blood cell folate concentrations below 317 nmol/L are suitable cut-off points indicative of folate deficiency. Although plasma total homocysteine on its own is not suitable for use as a biomarker of folate status, the Panel notes that its relationship with folate can be used to define the blood folate concentrations necessary to maintain concentrations of plasma total homocysteine associated with functional folate adequacy. The Panel considers that the previously defined cut-offs for functional folate adequacy (serum folate of 10 nmol/L and red blood cell folate of 340 nmol/L) are suitable criteria for determining folate requirements. Homozygosity for the T allele of the MTHFR 677C→T polymorphism, which has a prevalence of up to 24 % in some European countries, is associated with low folate status and unfavourable health effects. The Panel considers that this polymorphism should be taken into account when determining the requirement for folate. The Panel has also considered several health outcomes possibly associated with folate intake and status, but data are insufficient to establish DRVs.

For healthy adult men and women, an AR of 250 µg DFE/day is proposed based on results of one controlled study showing that an intake of 205-257 µg DFE/day for seven weeks after a depletion phase maintains serum folate concentrations above the cut-off for deficiency in all postmenopausal women studied and above the cut-off for optimal functional folate status in at least about half of the group. These findings are in close agreement with those of two other controlled studies showing that folate intakes of around 200-300 µg/day may be sufficient to maintain serum and red blood cell folate concentrations associated with functional folate adequacy. A Population Reference Intake (PRI) of 330 µg DFE/day is derived assuming a coefficient of variation (CV) of 15 % in order to account for

the additional variability associated with the higher requirement for folate in individuals with the MTHFR 677TT genotype.

For infants aged 7-11 months, an AI of 80 µg DFE/day is derived by extrapolating upwards from the estimated folate intake from breast milk of exclusively breast-fed infants, taking into account differences in reference body weights, and by considering intakes in infants aged 0.5 to < 1 year in the only representative survey available in the EU.

For children and adolescents, the ARs for folate are extrapolated from the AR for adults by isometric scaling and the use of growth factors. The PRIs are derived by assuming a CV of 15 %, and range from 80 µg DFE/day for 1 to 3 year-old children to 330 µg DFE/day for both boys and girls aged 15-17 years.

In pregnancy, intakes of 630-680 µg DFE/day administered in a controlled study to pregnant women during their second and third trimester resulted in concentrations of biomarkers of folate status well above cut-offs for deficiency or functional folate adequacy as established in non-pregnant adults. Acknowledging the weaker data base compared to non-pregnant adults, an AI for folate for pregnancy is proposed at 600 µg DFE/day.

For lactating women, an additional requirement of 130 µg DFE/day is derived in order to compensate for folate losses through breast milk. By adding this additional requirement to account for losses to the AR for non-lactating women, an AR of 380 µg DFE/day is obtained. Assuming a CV of 15 %, a PRI of 500 µg DFE/day is established.

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BACKGROUND AS PROVIDED BY THE EUROPEAN COMMISSION

The scientific advice on nutrient intakes is important as the basis of Community action in the field of nutrition, for example such advice has in the past been used as the basis of nutrition labelling. The Scientific Committee for Food (SCF) report on nutrient and energy intakes for the European Community dates from 1993. There is a need to review and if necessary to update these earlier recommendations to ensure that the Community action in the area of nutrition is underpinned by the latest scientific advice.

In 1993, the SCF adopted an opinion on the nutrient and energy intakes for the European Community[4]. The report provided Reference Intakes for energy, certain macronutrients and micronutrients, but it did not include certain substances of physiological importance, for example dietary fibre.

Since then new scientific data have become available for some of the nutrients, and scientific advisory bodies in many European Union Member States and in the United States have reported on recommended dietary intakes. For a number of nutrients these newly established (national) recommendations differ from the reference intakes in the SCF (1993) report. Although there is considerable consensus between these newly derived (national) recommendations, differing opinions remain on some of the recommendations. Therefore, there is a need to review the existing EU Reference Intakes in the light of new scientific evidence, and taking into account the more recently reported national recommendations. There is also a need to include dietary components that were not covered in the SCF opinion of 1993, such as dietary fibre, and to consider whether it might be appropriate to establish reference intakes for other (essential) substances with a physiological effect.

In this context the EFSA is requested to consider the existing Population Reference Intakes for energy, micro- and macronutrients and certain other dietary components, to review and complete the SCF recommendations, in the light of new evidence, and in addition advise on a Population Reference Intake for dietary fibre.

For communication of nutrition and healthy eating messages to the public it is generally more appropriate to express recommendations for the intake of individual nutrients or substances in food-based terms. In this context the EFSA is asked to provide assistance on the translation of nutrient based recommendations for a healthy diet into food based recommendations intended for the population as a whole.

TERMS OF REFERENCE AS PROVIDED BY THE EUROPEAN COMMISSION

In accordance with Article 29 (1)(a) and Article 31 of Regulation (EC) No. 178/2002,[5] the Commission requests EFSA to review the existing advice of the Scientific Committee for Food on population reference intakes for energy, nutrients and other substances with a nutritional or physiological effect in the context of a balanced diet which, when part of an overall healthy lifestyle, contribute to good health through optimal nutrition.

In the first instance the EFSA is asked to provide advice on energy, macronutrients and dietary fibre. Specifically advice is requested on the following dietary components:

- Carbohydrates, including sugars;

[4] Scientific Committee for Food, Nutrient and energy intakes for the European Community, Reports of the Scientific Committee for Food 31st series, Office for Official Publication of the European Communities, Luxembourg, 1993.

[5] Regulation (EC) No 178/2002 of the European Parliament and of the Council of 28 January 2002 laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety. OJ L 31, 1.2.2002, p. 1-24.

- Fats, including saturated fatty acids, polyunsaturated fatty acids and monounsaturated fatty acids, *trans* fatty acids;
- Protein;
- Dietary fibre.

Following on from the first part of the task, the EFSA is asked to advise on population reference intakes of micronutrients in the diet and, if considered appropriate, other essential substances with a nutritional or physiological effect in the context of a balanced diet which, when part of an overall healthy lifestyle, contribute to good health through optimal nutrition.

Finally, the EFSA is asked to provide guidance on the translation of nutrient based dietary advice into guidance, intended for the European population as a whole, on the contribution of different foods or categories of foods to an overall diet that would help to maintain good health through optimal nutrition (food-based dietary guidelines).

ASSESSMENT

1. Introduction

Folate is a water-soluble organic compound which belongs to the group of B-vitamins. It is an essential micronutrient required for the synthesis of ribo- and deoxyribonucleic acids (RNA and DNA) and consequently for cell division and tissue growth, for methylation reactions and amino acid metabolism.

The Scientific Committee for Food (SCF, 1993) adopted an opinion on the nutrient and energy intakes for the European Community and derived for folate a Lowest Threshold Intake (LTI), an Average Requirement (AR) and a Population Reference Intake (PRI) for adults from data generated by small controlled studies for treatment or prevention of folate deficiency. The SCF also set PRIs for infants aged 6-11 months and for children. The SCF proposed additional intakes for pregnant and lactating women to be added to the PRI for non-pregnant non-lactating women in order to prevent a decrease in red blood cell folate concentration and to compensate for folate secreted in breast milk, respectively.

2. Definition/category

2.1. Chemistry

2.1.1. Folate chemistry

Folate is a generic term used for a group of compounds with a basic structure consisting of a pterine linked through a methylene bridge to p-aminobenzoic acid to which one or more glutamate residues are attached by γ-peptide bonds. The pterine moiety exists in three oxidation states (oxidised, partially reduced as 7,8-dihydrofolate and fully reduced as 5,6,7,8-tetrahydrofolate) and can be substituted at the N-5 or N-10 position by different one-carbon units (Gregory, 1989). Tetrahydrofolate (THF), which is the fully reduced form of the vitamin, carries one-carbon units at one of three different oxidation levels ranging from methanol to formate. In the cell, five different one-carbon substituted forms of THF are present: 10-formyl-THF; 5-formyl-THF; 5,10-methenyl-THF; 5,10-methylene-THF; and 5-methyl-THF; each of these forms is interconverted in the cell through enzyme-mediated catalysis. In the body, addition of glutamate residues to the monoglutamate form increases the affinity of folate cofactors for folate-dependent enzymes and is required to retain folates within the cell and subcellular organelles.

Naturally occurring food folates are reduced vitamers which are usually polyglutamates containing five to seven glutamate residues. Natural folates are unstable and some losses occur in the presence of light, oxygen and at high temperatures. In contrast, the synthetic form of the vitamin, folic acid, is a fully oxidised monoglutamate and is the most chemically stable form. However, folic acid is not a natural component of the diet and is consumed only via fortified foods or food supplements (Brody, 1991). It has vitamin activity after having been fully reduced.

2.1.2. Folate analytical methodology

Folate in plasma/serum, whole blood, tissues and food has been measured by a variety of methods which can be grouped into three main categories: microbiological, protein-binding and chromatographic methods. Microbiological assays are based on folate-sensitive microorganisms (most commonly *Lactobacillus casei* subsp. *rhamnosus*) whose growth is proportional to the amount of folate present in the sample. Although the microbiological assay was first developed more than 50 years ago, it is still considered a very sensitive, robust and accurate method for measurement of total

folate due to the similar growth response of the microorganism to different folate monoglutamates and the considerable technical advancement of the assay with the introduction of the chloramphenicol-resistant strain of *L. casei* subsp. *rhamnosus* (ATCC 7469), use of cryopreserved inoculum and automated microtitre plate technology. However, the most commonly used folate assays nowadays in clinical laboratories are the protein-binding assays (enzyme-linked and chemiluminescent assays) which rely on folate-specific antibodies (folate-binding protein) to capture folate in biological samples. Although protein-binding assays are automated and easy to perform with a high sample throughput and a reasonable level of precision for samples containing a single folate derivative (e.g. as is usually the case in serum/plasma), they are affected by the disadvantage that the binding protein has a different affinity to various folate derivatives (Shane et al., 1980). The chromatographic assays and especially the most technologically advanced isotope dilution-liquid chromatography-tandem mass spectrometry (ID/LC/MS/MS) methods have a high sensitivity and specificity and are able to detect individual folate derivatives at very low concentrations. They are considered as higher–order reference methods for folate analysis and are available mainly in specialised laboratories (Pfeiffer et al., 2010). The Panel notes that this MS method is the method with the highest specificity and sensitivity.

Considerable analytical variability has been shown between different laboratories using similar assays as well as between various methods analysing common sets of serum and red blood cell folate samples (Gunter et al., 1996; Billen et al., 1999; Clifford et al., 2005). A relatively good agreement has been reported between LC/MS/MS methods and the microbiological assay whereas substantial differences have been found between the LC/MS/MS method and some of the protein-binding assays (Fazili et al., 2007; Fazili et al., 2008). Thus, results of folate measurements in biological samples depend on the analytical method used and it is important to consider this fact when comparing results from various studies.

Traditionally, folate in food is measured by microbiological assay with *L. casei* subsp. *rhamnosus* after extraction of folate from the food sample, which involves thermal extraction followed by hydrolysis of polyglutamates with folate conjugase. Improved extraction procedures have been developed and the trienzyme extraction approach (thermal extraction followed by treatment with amylase, protease and folate conjugase) considerably enhances the measurable folate concentration in foods compared with the traditional methodology (Martin et al., 1990; Tamura et al., 1997). This shows that the previously used extraction procedures were insufficient to completely release folate from the food matrix which results in underestimation of food folate content. Although the trienzyme extraction is a recommended procedure for food folate analysis and is included in the internationally approved methodology for determination of total folate in cereal products (AACCI method 86-47), the folate data in the food composition databases have not consistently been updated and detailed information as to the method used for folate analysis is often lacking. Therefore, folate intake of a population calculated using food composition databases may be lower than the actual intake, though it is not possible to quantify the extent of underestimation.

2.2. Functions of folate

Folate functions as a cofactor or cosubstrate in numerous one-carbon transfer reactions important for the synthesis of RNA and DNA, amino acid interconversions and the process of methylation. Different folate forms are involved in specific reactions but all of them are finally metabolised to tetrahydrofolate.

2.2.1. Biochemical functions

Folate is essential for the synthesis of RNA and DNA and consequently for cell division and tissue growth. 10-formyltetrahydofolate provides one-carbon units for the formation of purine nucleotides (adenine and guanine) necessary for both RNA and DNA, whereas 5,10-methylenetetrahydofolate is a

cofactor in the reaction generating thymidine monophosphate, a pyrimidine nucleotide specific for DNA. Folate deficiency impairs DNA replication and cell division, which adversely affects rapidly proliferating tissues such as bone marrow and results in decreased production of blood cells (Selhub et al., 1999). It has also been reported that folate deficiency is associated with structural damage of DNA as a consequence of misincorporation of uracil instead of thymine, which might have implications for cancer development (Blount et al., 1997). Folate is fundamental for the normal functioning of the methionine cycle which is responsible for both the conversion of homocysteine to methionine and the production of the universal methyl donor S-adenosylmethionine (SAM). Folate in the form of 5-methyltetrahydrofolate acts as a co-substrate in the remethylation of homocysteine to methionine in a reaction catalysed by the enzyme methionine synthase, which also requires methylcobalamin as a cofactor. This is an effective way for restoring the essential amino acid methionine, which is used not only for protein synthesis but also for the generation of SAM. In turn, SAM donates its methyl group to more than 100 methyltransferases for a wide range of substrates such as DNA, hormones, proteins, neurotransmitters and membrane phospholipids (Chiang et al., 1996), which are regulators of important physiological processes. As a result of this reaction SAM is converted to S-adenosylhomocysteine and homocysteine. Folate deficiency disturbs the normal function of the methionine cycle, which results in elevation of plasma total homocysteine (Selhub et al., 1993; Ubbink et al., 1993) and insufficient SAM production (Bottiglieri, 1996) with potential impairment of some methylation pathways. For example, reduced global DNA methylation has been reported in folate-depleted individuals (Rampersaud et al., 2000; Pufulete et al., 2005).

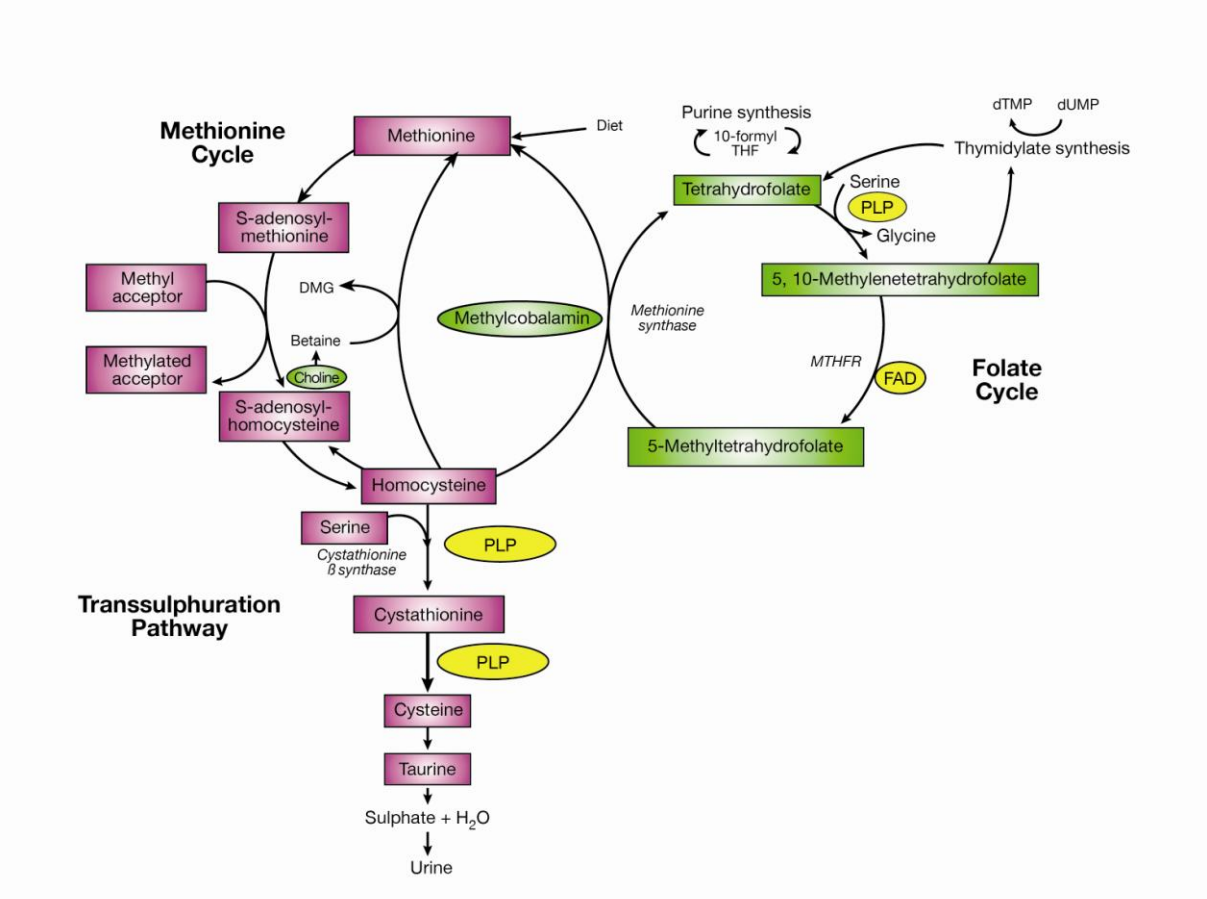

Figure 1: Folate and one-carbon metabolism

Abbreviations: FAD, flavin adenine dinucleotide; PLP, pyridoxal 5´-phosphate; DMG, dimethylglycine; TMP, thymidine monophosphate; UMP, uridine monophosphate (figure kindly provided by JJ Strain).

2.2.2. Health consequences of deficiency and excess

2.2.2.1. Deficiency

Folate deficiency reduces the division rate of all cells in the body, which results in the production of unusually large red blood cells (macrocytic cells) with poorly differentiated nuclei. The predominant feature of folate deficiency is megaloblastic anaemia. An initial fall in serum folate concentration below 6.8 nmol/L (3 ng/mL) followed by a period of progressive depletion of folate stores triggers bone marrow to generate macrocytic cells with abnormal nuclear maturation (Herbert, 1962; Carmel, 2001). As the mean life span of the red blood cells is 120 days, it takes several weeks before the decrease in red blood cell folate concentration, increase in mean cell volume, appearance of irregularly shaped red blood cells in the circulation and decline in both haemoglobin concentration and in red blood cell number can be detected. Granulocyte and platelet counts also fall with the advancement of anaemia. The hypersegmentation (five to six lobes instead of two to four) of neutrophils is considered a very specific sign which appears even before the macrocytosis (Herbert, 1962). Although the megaloblastic anaemia is typical for folate deficiency, the same clinical picture can also occur as a result of cobalamin deficiency alone due to the metabolic interactions of the two vitamins (see Section 2.3.7). The megaloblastosis can also affect the epithelial cells of the entire gastrointestinal tract (Lindenbaum and Allen, 1996) and can impair absorption of folate and exacerbate further the deficiency state (Elsborg, 1976).

Folate deficiency has also been associated with the development of irritability and forgetfulness (Herbert, 1962; Reynolds et al., 1973); however, these complications occur less frequently than megaloblastic anaemia and usually in a mild form.

2.2.2.2. Excess

Natural food folate is considered safe and high intakes have not been associated with any adverse effects (Butterworth and Tamura, 1989; SCF, 2000). A Tolerable Upper Intake Level (UL) has been set by SCF based on safety concerns for high intake of the synthetic form of the vitamin, i.e. folic acid, and these are related mainly to individuals with cobalamin deficiency. Folic acid has the potential to, at least temporarily, reverse the megaloblastic anaemia due to cobalamin deficiency and to delay the timely diagnosis and the appropriate treatment of the disease, thereby allowing the neurological dysfunction as a result of cobalamin deficiency to progress to irreversible subacute combined degeneration of the spinal cord. An evaluation based on the data generated from the case reports on cobalamin-deficient patients treated with folic acid at doses from 1 to 30 mg/day showed a dose-response relationship between the neurological complications and folic acid intake, which was used to set the UL for folic acid (SCF, 2000). It was noted that in nearly all studies showing neurological relapse, doses > 5 mg folic acid/day were administered and data on the effect of doses between 1 mg/day and 5 mg/day are limited to a few cases. Therefore, the Lowest-Observed Adverse Effect Level (LOAEL) was set at 5 mg/day, and using an uncertainty factor of 5, the UL was set at 1 mg/day for adults (SCF, 2000). No data were available to suggest that other life-stage groups have increased susceptibility to adverse effects of high folic acid intake. Thus, the UL also applies to pregnant or lactating women. ULs for children were derived from the adult value on the basis of body weight, ranging from 200 µg/day (1-3 years) to 800 µg/day (15-17 years).

Observational studies have suggested that folic acid supplement use is inversely associated with cancer incidence (Giovannucci et al., 1998; Ericson et al., 2007); however, safety concerns have been voiced with the publication of two studies suggesting that chronic ingestion of folic acid at doses of 1 mg/day or above might increase the risk of colorectal neoplasia in individuals with a recent history of colorectal adenomas (Cole et al., 2007) or increase the risk of development of prostate cancer (Figueiredo et al., 2009). Evidence from animal studies had previously suggested a potential dual role of folic acid, with a protective effect against neoplastic transformations in normal tissue, but

stimulating proliferation of already established neoplastic foci in the colorectal mucosa (Kim, 2004). However, a meta-analysis of 13 randomised controlled trials (RCTs) including almost 50 000 participants showed that folic acid supplementation at a median dose of 2 mg/day and administered with or without other B-vitamins for an average duration of 5.2 years did not significantly increase the overall or site-specific cancer incidence compared with placebo (Vollset et al., 2013). The same conclusions were drawn in a recent meta-analysis which included 26 studies lasting up to 7.3 years and also investigated in sub-analyses the effect of folic acid supplementation alone on overall cancer, selected cancers and all-cause mortality (Mackerras et al., 2014). The Panel notes that the follow-up period of the trials included in these meta-analyses was rather short considering the development of cancer. Thus, the question of the relationship between folic acid and cancer requires to be clarified by studies designed with sufficiently long follow-up addressing the biological hypothesis for the dual effect of folic acid on cancer development (ESCO, 2009). However, the Panel notes that this possible adverse effect of folic acid relates to intakes at or above the currently accepted UL.

Concerns have been raised regarding the potential adverse effects associated with the presence of unmetabolised folic acid in the circulation. Various small and non-representative studies from Europe (Ireland, Germany) (Sweeney et al., 2009; Obeid et al., 2010; Boilson et al., 2012) and a nationally representative study from the US (Bailey et al., 2010) reported that a considerable proportion (40-90 %) of the investigated populations exposed to fortified foods and involving both supplement and non-supplement users had a detectable concentration of unmetabolised folic acid in the blood even in fasting conditions. The metabolic and biological consequences of the presence of unmetabolised folic acid in the circulation are as yet uncertain (Troen et al., 2006; Morris et al., 2010).

2.3. Physiology and metabolism

2.3.1. Intestinal absorption

2.3.1.1. Steps involved during intestinal absorption

Both active and saturable as well as passive and unsaturable mechanisms are involved in folate absorption.

Upon ingestion of polyglutamated forms, hydrolysis to their monoglutamates is required by γ-glutamyl carboxypeptidase (also termed e.g. folate conjugase, γ-glutamyl hydrolase or glutamate carboxypeptidase II) located primarily in the jejunal brush border membrane (Bernstein et al., 1970; Chandler et al., 1986). Subsequently, a folate carrier with a similar affinity for both folic acid and reduced folate forms is involved in transport of monoglutamates across the brush border membrane. After entering the intestinal cells, folates are usually reduced and methylated, followed by a carrier-mediated mechanism exporting the methyl-THF into the blood stream, though there is also evidence that folic acid enters the portal vein unchanged, with reduction and methylation taking place only in the liver (Wright et al., 2005; Patanwala et al., 2014). This active absorption mechanism is pH-dependent and saturable. The body has a limited ability to convert ingested folic acid into reduced folate derivatives and when the capacity for reduction and methylation of folic acid is exceeded unmetabolised folic acid may appear in serum (Kelly et al., 1997; Wright et al., 2003; Sweeney et al., 2007). In contrast, the activity of human jejunal brush border γ-glutamyl carboxypeptidase does not seem to be rate-limiting in the absorption process within the range of usual dietary intakes (Hannon-Fletcher et al., 2004).

For folates not absorbed in the jejunum, unspecific folate absorption takes place predominantly in the ileum involving passive diffusion, in linear proportion to the amount reaching the ileum.

2.3.1.2. Factors influencing intestinal absorption

Incomplete release of folates from plant cellular structures may lower folate bioavailability from plant foods. Whether some types of dietary fibre (e.g. wheat bran) lower folate absorption is unclear, and many types of fibre appear not to reduce folate absorption (IOM, 1998; McNulty and Pentieva, 2010).

It has been suggested that the presence of components with antioxidative properties, such as ascorbic acid, may enhance stability of reduced folates in the digestive tract as shown *in vitro* (Seyoum and Selhub, 1998), and that the addition of milk to the diet may enhance folate bioavailability as shown in *in vivo* and *in vitro* studies (Picciano et al., 2004).

2.3.1.3. Dietary folate equivalents

Because the absorption efficiency of synthetic and natural folates varies, dietary folate equivalents (DFE) have been defined by IOM (1998) to take this into account for the derivation and application of DRVs for folate:

1 µg DFE[6] = 1 µg food folate = 0.6 µg folic acid from fortified food or as a supplement consumed with food = 0.5 µg of a folic acid supplement taken on an empty stomach.

This definition is based on evidence that folic acid has a higher bioavailability than food folate. Food folates are usually reduced, often methylated, typically polyglutamated and eventually protein-bound, and their absorption efficiency has been estimated to be no more than 50 %. This value was suggested in a study aimed at estimating folate requirement in which, after a depletion period of four weeks, increasing amounts of food folate with or without folic acid were given to healthy women (n = 3-4 per group) (Sauberlich et al., 1987). The authors concluded that dietary folates were no more than 50 % available relative to folic acid ingested with a meal. However, it was unclear how this figure was derived.

For the definition of the DFE, the absorption efficiency of folic acid from fortified foods or from a supplement ingested with food was assumed by IOM to be 85 %. This value was based on single-dose absorption studies with stable folic acid isotopes added to white and whole-wheat bread, rice and pasta, with or without co-ingestion of other foods, which showed that bioavailability of folic acid from the fortified cereal grain foods was not different from that of the control (folic acid in water) but showed a non-significantly reduced absorption (difference about 15 %) when consumed in the presence of a light meal (Pfeiffer et al., 1997). Evidence from an intervention for three months with five groups of women receiving either a daily folic acid supplement, foods fortified with folic acid, a diet rich in food folates, dietary advice, or no intervention (Cuskelly et al., 1996) was considered as well by IOM, though this study was not designed as a bioavailability study. Groups consuming supplemental folic acid or folic acid-fortified foods had significant increases in red blood cell folate concentrations, whereas folate status did not improve in the other groups.

In a controlled feeding study for 14 weeks, Yang et al. (2005) aimed to confirm the 1.7 multiplier from the DFE calculation.[6] In this study three groups of 6-8 subjects each received 400 µg DFE/day but with different proportions of folic acid and food folate, and another three groups received 800 µg DFE/day with different proportions of folic acid and food folate. However, the Panel considers that the study was not powered to detect equivalence and that the lack of statistical difference in the outcome parameters serum folate and red blood cell folate for the groups receiving 400 µg DFE/day or 800 µg DFE/day cannot be interpreted as confirming the validity of the 1.7 multiplier.

[6] For combined intakes of food folate and folic acid, DFEs can be computed as follows:
µg DFE = µg food folate + (µg folic acid x 1.7)
This definition was used in the Opinion when there was a need to compute DFEs from separately reported intakes of food folate and folic acid.

2.3.1.4. Studies assessing relative folate bioavailability

Bioavailability of folate is defined as the fraction of ingested folate that is absorbed and can be used for metabolic processes or storage. It has been assessed in short-term and long-term studies, but the results are often difficult to compare because of differences in folate forms (e.g. labelled or not) and doses used, quantification of ingested substances (e.g. via HPLC or microbiological assay), number of study participants, folate status parameters measured or other differences in study protocol. Subsequently, results published after the report by IOM (1998) from long-term studies assessing bioavailability of food folate or L-5-methyl-THF relative to folic acid are presented, as long-term interventions using whole meals are thought to be the most informative and to best reflect the real-life situation. No long-term studies are available assessing bioavailability of folic acid-fortified foods versus that of folic acid alone ingested on an empty stomach.

Relative bioavailability of food folate

Three controlled intervention studies lasting four weeks have assessed bioavailability of food folate from whole meals (Brouwer et al., 1999; Winkels et al., 2007) or from folate-rich food extracts added to a carrier meal (Hannon-Fletcher et al., 2004). Folate content of duplicate diet samples was analysed and relative folate bioavailability assessed based on changes in serum folate (Brouwer et al., 1999; Hannon-Fletcher et al., 2004; Winkels et al., 2007), red blood cell folate (Brouwer et al., 1999) and plasma total homocysteine concentration (Brouwer et al., 1999; Hannon-Fletcher et al., 2004) after four weeks.

Hannon-Fletcher et al. (2004) recruited healthy men (n = 96) with the CC or CT allele of the gene for 5,10-methylene tetrahydrofolate reductase (MTHFR) (see Section 2.5). Subjects received either once daily a folate-depleted meal or a drink to which folates (200 µg/day) extracted from spinach or from yeast were added, or they consumed the meal or the drink together with folic acid (200 µg/day) or placebo. The responses in serum folate (postintervention minus preintervention concentration) did not differ between the yeast folate and the spinach folate groups, but were significantly lower compared to the folic acid group. On the basis of changes in serum folate, the bioavailability of spinach folate (polyglutamate:monoglutamate folate 50:50) relative to folic acid was 36 % (95 % CI 0 %, 90 %), whereas that of yeast folate (polyglutamate:monoglutamate folate 100:0) was 62 % (95 % CI 20 %, 170 %).

Brouwer et al. (1999) found a higher bioavailability of food folate in a study in which three groups of healthy men and women (n = 66) were provided with either a diet high in vegetables and citrus fruits (560 µg folate/day) or a low-folate diet (210 µg/day) plus folic acid (500 µg every other day) or the low-folate diet plus placebo. The bioavailability of food folate relative to folic acid was 78 % based on changes in plasma folate concentration.

In a four-week study with 72 men and women stratified by MTHFR 677C→T genotype, Winkels et al. (2007) found a bioavailability of food folate (measured by HPLC) relative to folic acid (doses of 92, 191, and 289 µg/day taken just before a meal) that amounted to 78 % (95 % CI 48 %, 108 %) when calculating bioavailability based on an isotope method and to 85 % (95 % CI 52 %, 118 %) when calculated based on changes in serum folate. When food folate was analysed with the microbiological assay as in the studies by Brouwer et al. (1999) and Hannon-Fletcher et al. (2004), relative bioavailability of food folate was estimated at 68 % (95 % CI 42 %, 95 %) according to labelled folate data and at 75 % (95 % CI 45 %, 103 %) according to changes in serum folate.

Relative bioavailability of L-5-methyl-THF

The bioavailability of supplemental L-5-methyl-THF (calcium salt of (6*S*)-5-methyltetrahydrofolic acid or calcium-L-methylfolate) has been reported to be similar to folic acid at equimolar doses of supplemental folic acid between 100 µg/day and 400 µg/day used in long-term studies lasting between

16 and 24 weeks (Venn et al., 2002; Venn et al., 2003; Houghton et al., 2006; Lamers et al., 2006; Wright et al., 2010). The bioavailability of folate from (6*S*)-5-methyl-THF, glucosamine salt was considered to be similar to the bioavailability of folate from calcium-L-methylfolate based on a short-term study in humans (EFSA ANS Panel, 2013).

2.3.1.5. Conclusions on folate bioavailability

The Panel notes that the DFE has been designed to take account of the fact that food folate has a lower bioavailability compared to folic acid added to foods or consumed as a supplement, though the evidence base for the figures used by IOM in the DFE definition has been somewhat uncertain. The Panel also notes that the validity of the dietary folate equivalency definition has not been confirmed in studies. The Panel considers that two of three long-term investigations using whole diets indicate that the bioavailability of food folate relative to folic acid may be higher than previously assumed. However, the Panel also considers that results for folate bioavailability in these studies vary and that there is wide variation around estimates. The Panel considers that the difference in bioavailability between food folate and folic acid needs to be accounted for. In the absence of better data, the Panel agrees with the previous definition of the DFE assuming that the bioavailability of food folate is around 50 %, i.e. half that of folic acid taken on an empty stomach, whereas the bioavailability of folic acid from fortified foods or from a supplement ingested with food is about 85 %. The Panel also considers that L-5-methyl-THF has a bioavailability that is similar to that of folic acid.

2.3.2. Transport in blood

The predominant form of folate in the circulation is 5-methyl-THF monoglutamate. It is mainly bound to albumin which is a low affinity folate-binding protein (about 50 % of all bound folate). However, in folate deficiency a higher proportion of folate in plasma is bound to albumin (Ratnam and Freisheim, 1990). Plasma also contains a soluble form of the folate receptor which binds a small proportion of folate; however, in pregnancy its concentration is increased (Ratnam and Freisheim, 1990). One third of folate in plasma is in a free form. The role of both specific and non-specific binding proteins in plasma is unclear but it is believed that they do not have a major influence on tissue folate uptake. After folate ingestion plasma concentration increases and is maintained at an elevated concentration up to approximately four hours followed by a rapid decrease (Shane, 2009).

2.3.3. Distribution to tissues

Folate is delivered to the tissues against a concentration gradient, an energy-dependent process which requires the involvement of folate transporters (reduced folate carrier, proton-coupled folate transporter and folate receptors). The pattern of internalisation of folate is tissue- and cell-specific and depends on the efficiency of the folate transporters and the cellular concentration of folate (Antony, 1996). Once absorbed through the intestine, folate monoglutamates are transferred via portal circulation to the liver where they are retained or released back in the circulation for distribution to other tissues. In order to be retained by the cells, folate monoglutamates are converted to polyglutamates by the enzyme folylpolyglutamate synthase (also termed tetrahydrofolate synthase, EC 6.3.2.17). 5-methyl-THF, the main form of folate entering the cells from the blood, is a very poor substrate for this enzyme (Shane, 1989); thus, it is converted to THF through a reaction involving the cobalamin-dependent enzyme methionine synthase (EC 2.1.1.13, Figure 1). THF has a high affinity for folylpolyglutamate synthase and can be retained by the cells. However, polyglutamated folate is not only a storage form of folate in tissues but also a functional form of the vitamin because only derivatives of folate polyglutamates are able to act as cofactors in folate-dependent enzyme reactions; therefore, polyglutamation is required both for retaining folate within the cells and for the normal function of one-carbon metabolism (Shane, 1989). In addition, some of the polyglutamates in the tissues are bound to folate-binding proteins, but there is a great variability in the expression of these

proteins in different tissues. Although plasma folate increases in parallel with dietary intake, animal studies have shown that tissue folate concentrations saturate at high intakes as a result of decreased ability for polyglutamation (Clifford et al., 1990). Any folate which is not converted to polyglutamate is eliminated from the cells (Shane, 1989). Mature red blood cells do not have mechanisms to transport folate and folate which they contain is accumulated only during erythropoiesis.

Placenta has the ability to concentrate folates due to the abundance of folate receptors (predominantly folate receptor-α), folate-reduced carrier and proton-coupled folate transporter (Prasad et al., 1995; Yasuda et al., 2008; Solanky et al., 2010). This mechanism of folate transport across the placenta is established within the first trimester of pregnancy (Solanky et al., 2010) in order to satisfy the high requirements for folate during fetal development. As a result of the high folate concentration in the intervillous blood, folate in fetal blood is two to four times higher than in maternal blood (Thorand et al., 1996). A high folate concentration in cord blood is reported even in pregnant women with habitually low folate intakes, which is probably maintained at the expense of maternal folate stores (Wallace et al., 2008).

2.3.4. Storage

The ability of tissues to store folates in excess of the amounts required for normal metabolism is limited (Lowe et al., 1993). The exact amount of total body folate content in adults is not precisely known as estimates range from around 22 to 100 mg (Hoppner and Lampi, 1980; Gregory et al., 1998a; Lin et al., 2004). Kinetic studies using deuterium-labelled folic acid have reported the existence of a small folate pool with a fast turnover (half-life of a few hours) associated mainly with the monoglutamyl folates in plasma and large folate pools with a slow turnover (half-life of months) which are composed mainly of the polyglutamates in tissues (Stites et al., 1997; Gregory et al., 1998a). It is estimated that 99 % of total body folate is in the tissues (Lin et al., 2004), with storage taking place predominantly in the liver (Duncan et al., 2013).

There is a strong compartmentalisation of folate within the cell where the following three distinctive folate compartments are identified: cytosolic, mitochondrial and nuclear. Up to 50 % of folate in the cell is in the mitochondria, predominantly in the form of 10-formyltetrahydrofolate, whereas the cytosol contains mainly 5-methyl-THF (Shane, 2009).

2.3.5. Metabolism

The three folate compartments within the cell have specialised metabolic functions but, at the same time, they are interdependent by the exchange of different metabolites (Appling, 1991; Shane, 2009; Stover, 2009). Folate in the mitochondria is involved in the catabolism of serine and glycine generating formate which in turn is utilised in the cytoplasm for the remethylation of homocysteine to methionine and for the synthesis of nucleotides. Folate in the nuclear compartment is responsible for the production of thymidylate for DNA synthesis (see Section 2.2.1).

Folates which are not bound to specific and non-specific binding proteins are subjected to catabolism by oxidative cleavage at the C9-N10 bond, generating *p*-aminobenzoylglutamates which in turn are acetylated in the liver before excretion (Shane, 2009). The whole-body turnover rate of folate is estimated to be 1 % of body folate pools (Stites et al., 1997).

2.3.6. Elimination

2.3.6.1. Urine

Folate is filtered through the kidney glomerulus but most of it is reabsorbed in the proximal tubule with the assistance of folate-binding proteins and specific transporters (Hamid et al., 2009). As a result most of the folate in the urine is in the form of breakdown products with only 1-2 % of the excreted amount being active folate (Scott, 1986; Caudill et al., 1998).

2.3.6.2. Faeces

The majority of faecal folate is synthesised by intestinal microorganisms; however, loss of endogenous folate (biliary folate together with folate from shedded intestinal cells) also occurs through this route. A study in a single human volunteer showed a faecal excretion rate of folate similar to that via urine after administration of labelled folate (Krumdieck et al., 1978). However, it is unknown whether endogenous folate in the faeces is in active forms or breakdown products.

2.3.6.3. Breast milk

During lactation, folate is secreted via breast milk where it is bound to folate-binding proteins. The presence of folate-binding proteins in mammary gland tissue facilitates folate uptake from the circulation, since milk folate concentration is typically 5-10 times higher than that of maternal plasma (Tamura et al., 1980; Smith et al., 1983). Folate-binding proteins are shown to stimulate the absorption of folate by the infant and may preserve folate from degradation and utilisation by the intestinal microflora (Tamura et al., 2009).

Breast milk folate concentrations are maintained at the expense of maternal folate reserves and are not affected by low maternal folate intake (Smith et al., 1983), unless women are severely folate-deficient as suggested by low breast milk folate concentrations reported in two lactating women with megaloblastic anaemia due to folate deficiency (Metz et al., 1968). Folic acid supplementation in well-nourished lactating women does not affect breast milk folate concentration (Smith et al., 1983; Khambalia et al., 2006; Houghton et al., 2009; West et al., 2012), whereas in women with severe folate deficiency supplementation increases folate concentration of breast milk even before any improvement in maternal folate status is seen (Metz et al., 1968).

A wide range of total folate concentration of breast milk (24-141 µg/L) has been reported (SCF, 2003), however, the lower folate values have mainly been reported in the earlier studies and it is considered that they are due to analytical problems associated with inadequate procedures for extraction of folate from milk samples (Tamura et al., 2009). Studies using the most advanced extraction methods (see Section 2.1.2) have shown mean/median folate concentrations of mature breast milk of 45-99 µg/L (Lim et al., 1998; Mackey and Picciano, 1999; Kim et al., 2004; Khambalia et al., 2006; Houghton et al., 2009; West et al., 2012) (Appendix A) or approximately 80 µg/L (about 180 nmol/L) on average.

The Panel notes that the average folate concentration of breast milk is 80 µg/L (about 180 nmol/L) and that this amount is not dependent on dietary folate intake and status of the lactating women.

2.3.7. Interaction with other nutrients

Folate interacts with cobalamin in one of the key reactions in the methionine cycle. Cobalamin functions as a cofactor and 5-methyl-THF acts as a cosubstrate for the enzyme methionine synthase (EC 2.1.1.13) whose main role is to remethylate homocysteine back to methionine for a subsequent production of SAM required for the methylation of various substrates (Chiang et al., 1996). Another

important function of the methionine synthase reaction is to convert 5-methyl-THF to THF which is used either for polyglutamation (THF rather than 5-methyl-THF is a preferable substrate for folylpolyglutamate synthase; see Section 2.3.3) or for nucleotide synthesis. Therefore, cobalamin has a critical role for both the retention of folates in the tissues and for the provision of folate-derived one-carbon units for DNA synthesis or for methylation reactions. In cobalamin deficiency, the methionine synthase reaction is reduced and 5-methyl-THF is trapped in this form, since it cannot be metabolised by any other way and, as a consequence, functional folate deficiency may develop (Savage and Lindenbaum, 1995). This condition is explained by the "methyl-trap hypothesis" and its metabolic and clinical characteristics are well described (Herbert and Zalusky, 1962; Chanarin, 1990; Hoffbrand and Jackson, 1993; Smulders et al., 2006). Clinically, the condition may manifest by haematological and neurological abnormalities but its distinctive metabolic features include high serum folate concentration in combination with low red blood cell folate concentration and high total homocysteine concentration (Chanarin, 1990; Carmel et al., 2003).

Vitamin B6 in the form of pyridoxal 5-phosphate acts as a cofactor for the enzymes hydroxymethyltransferase and glycine decarboxylase, which transfer one-carbon units from serine and glycine, respectively, for the generation of 5,10-methylenetetrahydrofolate in the cytoplasm and mitochondria. These reactions are critical for the normal function of the folate and methionine cycles (see Figure 1). A study using stable isotopes showed that dietary restriction of vitamin B6 (0.5 mg/day for four weeks) in young men and women may cause alterations in the concentrations of some metabolites in the methionine cycle (da Silva et al., 2013), however, it is unknown whether vitamin B6 deficiency might influence the concentration of folate derivatives.

2.4. Biomarkers

2.4.1. Biomarkers of intake and status

2.4.1.1. Serum folate concentration

Folate concentration measured in serum or plasma is considered to be a sensitive marker of recent dietary intake and it is subjected to prandial variation (Green, 2008). However, a single measurement of serum/plasma folate is little informative for assessment of folate status and body stores (Green, 2008). Supplementation studies with folic acid (100-4 000 µg/day) or [6*S*]-5-methyl-THF (113-416 µg/day) found that a steady state in serum/plasma folate concentration (at levels above the cut-off associated with functional folate adequacy) was achieved after as long as 12 to 14 weeks of supplementation with a constant dose (Venn et al., 2002; Lamers et al., 2006; Hao et al., 2008) and, in that case, serum/plasma folate measurement would reflect the status of the vitamin. This comparatively slow response of serum/plasma folate suggests that it is not just a reflection of dietary intake but it is in equilibrium with and controlled by the cellular folate concentration, with a steady state of plasma folate being only reached upon saturation of cellular folate stores (Gregory and Quinlivan, 2002). Based on the microbiological *L. casei* subsp. *rhamnosus* assay a cut-off for folate deficiency has been set at 6.8 nmol/L (3 ng/mL) (Herbert et al., 1962). Serum/plasma folate concentration below this cut-off value confirmed on multiple consecutive occasions during a period of several weeks can be indicative of folate deficiency. A single measurement of serum/plasma folate reflects only the time of blood collection and cannot differentiate between occasionally low dietary intake of the vitamin and folate deficiency (IOM, 1998). Therefore, in order to obtain information on folate status, a single measurement of serum folate should be combined with other biomarkers of folate status. Pregnancy is associated with a decrease in serum folate concentrations (Tamura and Picciano, 2006) but the same criterion for defining folate deficiency as the one adopted for the general population (i.e. serum folate ≤ 6.8 nmol/L) is generally used in pregnancy.

2.4.1.2. Red blood cell folate concentration

Red blood cell folate is considered the most reliable biomarker of folate status as it reflects tissue folate stores (Wu et al., 1975). Folate is incorporated into red blood cells only during their maturation in the bone marrow and folate concentration remains stable throughout the 120 days-life span of the cells (Herbert, 1987a). Red blood cell folate is an indicator of long-term folate status and decreases only months after the initial reduction of folate intake and the fall in serum folate concentration (Eichner and Hillman, 1973). Analytical values of red blood cell folate below 317 nmol/L (140 ng/mL), obtained by microbiological *L. casei* subsp. *rhamnosus* assay, are indicative of folate deficiency. The same criterion for defining folate deficiency is generally used also during pregnancy.

A meta-analysis based on 19 RCTs with a total of 2 341 adult participants showed that folic acid supplementation dose was related to both serum folate and red blood cell folate responses; the regression curves of these relationships were linear within the folic acid intake range of 50 to 400 µg/day (R^2 of 0.31 and 0.54, respectively) (Duffy et al., 2014). This meta-analysis estimated that every doubling of the folic acid dose within the range of 50 to 400 µg/day would increase serum folate by an average of 63 % and red blood cell folate by 31 %.

2.4.1.3. Urinary folate excretion

Metabolic studies showed that 24-hour urinary folate excretion reflects differences in dietary folate intake within the range of 300-1600 µg DFE/day (O'Keefe et al., 1995; Gregory et al., 1998b; West et al., 2012). However, folate continues to be excreted in the urine even in advanced stages of folate depletion (Sauberlich et al., 1987) suggesting that it is not a useful indicator of low dietary intake and status. Moreover, urinary folate excretion is reported to be influenced by the physiologic state, with pregnant women excreting less folate compared with lactating and non-pregnant women after consumption of identical amounts of folate (West et al., 2012). Therefore, urinary folate excretion cannot be considered as a sensitive indicator of folate intake and status.

2.4.2. Biomarkers of function

2.4.2.1. Plasma total homocysteine

In the methionine cycle, folate cofactors are involved in the remethylation of homocysteine to methionine (see Section 2.2.1.). Plasma total homocysteine concentration is used as a biomarker of folate function. Studies have shown that folate is the major nutritional determinant of plasma total homocysteine concentration in healthy people (Selhub et al., 1993; IOM, 1998) and supplementation with folic acid at doses of 200 µg/day provided for 26 weeks can achieve a maximal reduction in total homocysteine (Tighe et al., 2011). However, plasma total homocysteine is not specific for folate function since it is affected also by other B-vitamins participating in one-carbon metabolism (cobalamin, vitamin B6 and riboflavin) as well as renal insufficiency and some lifestyle factors (e.g. alcohol consumption) (Refsum et al., 2004). Low cobalamin status is the dominant nutritional cause for hyperhomocysteinaemia in folate-replete populations (Green and Miller, 2005). Vitamin B6 deficiency has been associated with elevated plasma total homocysteine concentrations (Ubbink et al., 1995; Bates et al., 1999), whereas high total homocysteine concentrations have been reported in individuals homozygous for the MTHFR 677C->T polymorphism with poor riboflavin status (McNulty et al., 2006).

Plasma total homocysteine concentrations increase with age and are higher in men than in women (Selhub et al., 1999). Differences exist between laboratories in relation to the acceptable upper reference limit for plasma total homocysteine (Refsum et al., 2004).

Although plasma total homocysteine lacks specificity for folate and on its own is not suitable to be used for assessing folate status, it can provide information on folate function. The relationship between plasma total homocysteine and serum and red blood cell folate concentrations is reported to be inverse and non-linear; at low folate concentrations total homocysteine increases as folate falls further, but at higher folate concentrations total homocysteine remains unchanged if folate continues to increase (Selhub et al., 2008). This relationship was investigated further based on data from the third National Health and Nutrition Examination Survey (NHANES) of the US population aged 12 years and above, collected before the mandatory folic acid food fortification. Based on a two-phase regression model adjusted for age, sex, serum cobalamin, and creatinine, the minimal total homocysteine concentration was achieved at or above a serum folate concentration of 10 nmol/L (4.4 ng/mL) and a red blood cell folate concentration of 340 nmol/L (150 ng/mL), suggesting that concentrations at or above these cut-off values may be considered indicative of functional folate adequacy. The use of these criteria for assessment of folate status of populations was also recommended by a WHO Technical Consultation on folate and cobalamin deficiencies (de Benoist, 2008).

2.4.2.2. Mean cell volume

Macrocytic cells appear in the bone marrow shortly after initiation of folate depletion and before the fall in red blood cell folate concentration (Eichner et al., 1971). However, given the long life span of the circulating red blood cells (i.e. 120 days) in the peripheral blood, macrocytosis can be detected only at an advanced stage of folate deficiency (Herbert, 1987a).

2.4.3. Conclusion on biomarkers of intake, status and function

The Panel notes that serum/plasma folate concentration is a sensitive marker of recent dietary intake. However, a single measurement of serum/plasma folate cannot be informative of folate status as it reflects the time of blood collection. Thus, for assessment of folate status, multiple measurements of serum folate should be taken over a period of several weeks or a single measurement should be combined with other biomarkers of folate status. Serum folate concentrations of less than 6.8 nmol/L, confirmed on consecutive occasions, indicate folate deficiency.

The Panel considers that red blood cell folate concentration is an indicator of long-term dietary intake and responds slowly to changes in intake. Red blood cell folate is the most reliable biomarker of folate status as it reflects tissue folate stores and concentrations below 317 nmol/L are indicative of folate deficiency.

The Panel notes that plasma total homocysteine is a sensitive but not a specific biomarker of folate status and function since it is influenced by various other factors. Therefore, the Panel considers that plasma total homocysteine is not suitable on its own to be used as a biomarker of folate status and function but its relationship with folate can be used to define the blood folate concentrations necessary to maintain low concentrations of plasma total homocysteine. Controlling for confounders (age, sex, serum/plasma cobalamin and creatinine), the lowest plasma total homocysteine can be achieved in children and adults at or above a serum folate concentration of 10 nmol/L and a red blood cell folate concentration of 340 nmol/L, respectively, and the Panel considers that these concentrations are associated with functional folate adequacy.

The Panel notes that urinary folate concentration cannot be considered a sensitive indicator of folate intake and status as urinary folate excretion continues even in advanced stages of folate depletion and is influenced by the physiologic state. The Panel also notes that the mean cell volume is of limited use as a biomarker since it can be detected only in an advanced stage of folate deficiency and it lacks specificity as it might be also a result of cobalamin deficiency.

2.5. Effects of genotypes

Some polymorphisms of genes encoding enzymes and transport proteins involved in folate metabolism are reported to have an impact on folate status and health consequences (Molloy, 2004; Christensen and Rozen, 2010).

The highest impact on folate metabolism has been reported for the 677C→T polymorphism of the gene encoding the MTHFR enzyme. MTHFR converts 5,10-methylene-THF to 5-methyl-THF providing one-carbon units for the methylation cycle. Homozygosity for the T allele is associated with reduced enzyme activity (up to 70 % lower) and around 20-25 % lower serum folate and higher plasma total homocysteine concentrations compared with the 677CC genotype (Jacques et al., 1996; Davis et al., 2005; Hustad et al., 2007). Biochemical abnormalities in the 677TT genotype are more pronounced in the face of a low folate status and studies have shown that there is no difference in serum folate concentrations between MTHFR 677C→T genotypes when folate intake is above 600 µg DFE/day (Ashfield-Watt et al., 2002; Hung et al., 2006). Reduced global DNA methylation was shown in individuals with the 677TT genotype in one study (Friso et al., 2002), however, the evidence is inconsistent as this was not confirmed in two other studies (Shelnutt et al., 2004; Davis et al., 2005). Meta-analyses have shown that the 677TT genotype is associated with a reduced risk of colorectal cancer in individuals with high folate status (Huang et al., 2007), but with an increased risk of neural tube defect (NTD)-affected pregnancies (Vollset and Botto, 2005), pregnancy complications (Nelen et al., 2000; Kosmas et al., 2004), stroke (Casas et al., 2005; Cronin et al., 2005), schizophrenia (Muntjewerff et al., 2006; Gilbody et al., 2007) and depression (Gilbody et al., 2007). These unfavourable health effects of the MTHFR 677TT variant and its high prevalence among the population in some European countries (12 % in Northern and up to 24 % in Southern Europe (Gueant-Rodriguez et al., 2006)) underline that this polymorphism should be considered in determining the requirements for folate.

The other known genetic polymorphisms related to folate metabolism such as methionine synthase 2756A→G, methionine synthase reductase 66A→G, reduced folate carrier 1 80A→G, dihydrofolate reductase 19-bp deletion, glutamate carboxypeptidase II 1561C→T have been associated with mild disturbances in folate biomarkers, and their impact on health is inconclusive (Molloy, 2004; Christensen and Rozen, 2010).

3. Dietary sources and intake data

3.1. Dietary sources

Naturally occurring folates are found in a wide variety of foods; however, there are few foods which can be considered particularly rich sources. While most fruits and vegetables contain small amounts of folate, the principal sources are dark green leafy vegetables, legumes, orange and grapefruit (juice), peanuts, and almonds (FSA, 2002). Meat generally contains low amounts of folate, with the exception of offal such as liver and kidney, which are particularly high in folate. Another rich source of folate is baker's yeast. Table salt fortified with folic acid to contain 100 µg/g is available in Germany (Gotzfried, 2006).

Contributors to natural folate intakes include foods such as potatoes and dairy products, which are not considered rich sources of naturally occurring folate but are consumed in relatively large quantities (SACN, 2006). For example in Ireland, vegetable and vegetable dishes, potatoes and potato products, and brown bread and rolls were the largest contributors to natural folate intakes (Hopkins, 2013). In European countries with a voluntary folic acid food fortification policy in place, the main contributors to folic acid intake from the diet are fortified foods, such as breakfast cereals and some fat spreads (SACN, 2006; van Rossum et al., 2011; Hopkins, 2013).

Currently, pteroylmonoglutamic acid (folic acid) and calcium-L-methylfolate may be added to foods[7] and food supplements.[8] Recently, the safety of 5-methyl-THF, glucosamine salt was favourably assessed by the EFSA Panel on Food Additives and Nutrient Sources added to Food (ANS) at the proposed use and use levels of up to 1.8 mg/day, which equates to 1 mg 5-methyl-THF and 0.8 mg glucosamine (EFSA ANS Panel, 2013), but has not yet been authorised for addition to food supplements. The folate content of infant and follow-on formulae is regulated.[9]

3.2. Dietary intake

Folate intake data presented in nationally representative surveys lack comparability for various reasons, among which is the lack of differentiation between naturally occurring folates and synthetic folic acid and the diversity of analytical methods for quantifying folate and folic acid in food (Bouckaert et al., 2011).

Concurrently, few representative or country-wide surveys give daily intakes as DFE. Such values are available from surveys in the Netherlands, Ireland, Germany and Austria. However, these surveys differ in the way DFEs were computed, and not all of them take into account folic acid intake from supplements (see Appendix B).

Median DFE intake in German infants aged 0.5 to < 1 year was around 70 µg/day, and median DFE intake ranged between 111 and 128 µg/day in young children (1 to < 4 years, two surveys). In children (4 to < 13 years, three surveys) median/mean DFE intakes ranged from 120 to 272 µg/day and in adolescents (14 to < 18 or 18 years, two surveys), it ranged from 208 to 340 µg/day. In adults (four surveys), median/mean intakes ranged from 170 µg/day to 542 µg/day (Appendix B).

4. Overview of Dietary Reference Values and recommendations

4.1. Adults

The German-speaking countries (D-A-CH, 2013) considered that 200 µg DFE were sufficient to reach target serum folate concentrations ≥ 10 nmol/L and red blood cell concentrations ≥ 340 nmol/L (Milne et al., 1983; Sauberlich et al., 1987). As food folate analysis underestimates the actual folate intake, a value of 10 % was added and an AR of 220 µg DFE/day was derived. By addition of 30 %, the PRI was derived and rounded to 300 µg DFE/day. It was stated that the results by O'Keefe et al. (1995) were no longer taken into account as lower intakes than those observed by these authors seem to be sufficient to ensure an adequate folate supply.

The World Health Organization/Food and Agriculture Organization of the United Nations (WHO/FAO, 2004) adopted the folate values published by the IOM (1998), setting an Estimated Average Requirement (EAR) of 320 µg DFE/day and a recommended nutrient intake of 400 µg DFE/day for adults.

The Nordic countries (NNR, 2004) set a lower level of intake of 100 µg/day based on the criteria of the minimum amount to prevent folate deficiency anaemia (Herbert et al., 1962), daily losses from stores while on a virtually folate-free diet (Zalusky and Herbert, 1961), and the excretion in urine of well-nourished individuals (Herbert, 1987b). Derivation of the AR and Recommended Intake (RI) was based on a combination of indicators reflecting folate status: serum/plasma folate, red blood cell

[7] Regulation (EC) No 1925/2006 of the European Parliament and of the Council of 20 December 2006 on the addition of vitamins and minerals and of certain other substances to foods, OJ L 404, 30.12.2006, p. 26.

[8] Directive 2002/46/EC of the European Parliament and of the Council of 10 June 2002 on the approximation of the laws of the Member States relating to food supplements, OJ L 183, 12.7.2002, p. 51.

[9] Commission Directive 2006/141/EC of 22 December 2006 on infant formulae and follow-on formulae and amending Directive 1999/21/EC, OJ L 401, 30.12.2006, p.1.

folate and serum/plasma total homocysteine. Using dietary studies, it was considered that the average requirement with respect to maintaining normal blood concentrations was 25 to 200 µg/day and that an intake of 300 µg/day seemed to keep folate concentrations in blood above and total homocysteine concentrations below targeted cut-off values (i.e. 6.8 nmol/L and 317 nmol/L for serum and red blood cell folate concentrations, and 12 µmol/L for total homocysteine) (Sauberlich et al., 1987; Jacob et al., 1994; Brussaard et al., 1997; Cuskelly et al., 1999; Rasmussen et al., 2000; Brouwer et al., 2001; Alfthan et al., 2003). Accordingly, the AR for folate for adults was set at 200 µg/day and the RI at 300 µg/day. For women of reproductive age, a folate intake of 400 µg/day was recommended to provide an adequate folate supply to women experiencing unplanned pregnancies. For the 2012 Nordic Nutrition Recommendations (NNR), no scientific evidence was identified to prompt a change in these reference values (Nordic Council of Ministers, 2014).

The Health Council of the Netherlands (2003) based the EAR for adults on the three status parameters, i.e. plasma folate, red blood cell folate, and plasma total homocysteine (Stokes et al., 1975; Milne et al., 1983; McNulty et al., 1987; Sauberlich et al., 1987). A coefficient of variation (CV) of 25 % was used in calculating the Recommended Daily Allowance (RDA) because genetic factors also contribute to the variation in requirement, and individuals with the TT-genotype for 5,10-MTHFR require a higher folate intake. An EAR of 200 µg/day and an RDA of 300 µg/day were set for folate.

To set folate reference values, Afssa (2001) used total homocysteine as a target biomarker and a plasma concentration of 10 µmol/L as a threshold, independent of MTHFR genotype. The folate intakes of a subsample with plasma total homocysteine concentrations below this threshold from the SU.VI.MAX cohort (n = 1 200, aged 35 to 60 years and 50 % of each sex), were used to calculate intakes of 330 µg/day in men and 276 µg/day in women, which were used as the PRIs, except for women, whose PRI was increased to 300 µg/day (+ 10 %) of folate during child-bearing years.

The US Institute of Medicine (IOM, 1998) determined the EAR for adults using a combination of red blood cell folate, plasma total homocysteine, and plasma or serum folate. The focus was on the adequacy of specific quantities of folate, either via food or food plus folic acid, consumed under controlled metabolic conditions to maintain normal blood concentrations of these indicators (Milne et al., 1983; Sauberlich et al., 1987; Jacob et al., 1994; O'Keefe et al., 1995). An EAR of 320 µg DFE/day was derived which was also supported by epidemiological data (Selhub et al., 1993). The RDA was set at 400 µg DFE/day for adults, by assuming a CV of 10 % because information was not available on the standard deviation (SD) of the requirement for folate. Women capable of becoming pregnant were recommended to consume 400 µg/day of folic acid from supplements or fortified food as a preventive measure for NTDs (Mills and Conley, 1996).

Based on depletion-repletion studies with folic acid the SCF (1993) concluded that the mean requirement for an adult was 70 µg/day (Herbert, 1962; Herbert et al., 1962; Banerjee et al., 1975; Sauberlich et al., 1987), and considered that folic acid was twice as bioavailable as food folate (Gregory et al., 1991). Therefore, the AR was set at 140 µg/day. The PRI was calculated assuming a CV of 20 % to give a value of 200 µg/day for adults.

The UK COMA (DH, 1991) considered the folate concentration of autopsied liver samples, the prevalence of 8-10 % of low red blood cell folate concentrations (< 150 µg/mL) and the absence of overt signs of clinical and haematological folate deficiency in Canadian subjects on folate intakes of 150-200 µg/day (Hoppner et al., 1977; Hoppner and Lampi, 1980). Median folate intakes in the UK of 209 µg/day in women and 300 µg/day in men were also considered and the Reference Nutrient Intake (RNI) was set near the median folate intake of British women.

907 **Table 1:** Overview of Dietary Reference Values for folate for adults

	D-A-CH (2013) [a]	NNR (2012)	WHO/FAO (2004) [a]	NL (2003) [b]	Afssa (2001)	IOM (1998) [a]	SCF (1993) [b]	DH (1991)
Age (years)	≥ 19	≥ 18	≥ 19	≥ 19	≥ 20	≥ 19	≥ 18	≥ 19
PRI Men (µg/day)	300	300	400	300	330	400	200	200
PRI Women (µg/day)	300 [c]	300 [f]	400	300	300	400 [d]	200 [e]	200
Age (years)					≥ 75			
PRI Men (µg/day)					330-400			
PRI Women (µg/day)					330-400			

908 NL, Health Council of the Netherlands.
909 (a): Dietary folate equivalents (DFE) defined as follows: 1 µg DFE = 1 µg food folate = 0.6 µg folic acid from fortified food
910 or as a supplement consumed with food = 0.5 µg of a folic acid supplement taken on an empty stomach.
911 (b): Dietary folate.
912 (c): Women capable of or willing to become pregnant should also take a daily supplement containing 400 µg of folic acid
913 during the period four weeks prior to eight weeks after conception, to prevent neural tube defects.
914 (d): Women capable of becoming pregnant are advised to take 400 µg of folic acid daily from fortified foods, supplements,
915 or both to reduce the risk of neural tube defects.
916 (e): Neural tube defects have been shown to be prevented in the offspring by periconceptual ingestion of 400 µg folic
917 acid/day in the form of supplements.
918 (f): Women of reproductive age are recommended to ingest 400 µg/day.

919 ### 4.2. Infants and children

920 The German speaking countries (D-A-CH, 2013) set an AI of 85 µg DFE/day for infants aged four to
921 below 12 months based on the reference energy intake for infants of that age (700 kcal/day) and
922 assuming that breast milk provides 12 µg folate/100 kcal. For children, the AR was extrapolated from
923 that for adults by allometric scaling (i.e. using metabolic weight and growth factors according to age
924 (IOM, 1998)). PRIs were then set by adding 30 % to the age-specific ARs.

925 WHO/FAO (2004) adopted the folate values published by the IOM (1998) for older infants, children
926 and adolescents. A recommended nutrient intake of 80 µg DFE/day was also set for infants aged up to
927 six months, adapting from the EAR set by the IOM (1998).

928 The Nordic countries (NNR, 2004) set the RI at 5 µg/kg body weight per day based on data from
929 Asfour et al. (1977). For the 2012 update, recommended folate intakes remained unchanged, as no
930 new data on folate requirements of children were identified (Nordic Council of Ministers, 2014).

931 The Health Council of the Netherlands (2003) stated that no EAR and consequently no PRI could be
932 determined for children; therefore, Adequate Intakes (AIs) were established. The AI for infants up to
933 six months was based on the average intake of exclusively breast-fed infants (Brown et al., 1986;
934 O'Connor et al., 1991; Fomon and McCormick, 1993; Lim et al., 1997), a mean folate concentration
935 of 60 µg/L and a mean breast milk intake of 0.8 L/day, giving an average folate intake of 48 µg/day.
936 The AIs for children and adolescents were calculated by interpolation of the values for infants.

937 For infants and children, Afssa (2001) extrapolated PRIs from adult values using height squared,
938 which was considered representative of lean mass in children aged four to ten years (Brambilla et al.,
939 1999), and was the variable providing values closest to those derived from breast milk folate for the
940 lowest age ranges.

941 The IOM (1998) considered data of Picciano and colleagues (Brown et al., 1986; O'Connor et al.,
942 1991; Lim et al., 1997) on average folate concentration of human milk of 85 µg/L and observed mean
943 folate intakes of exclusively breast-fed infants. Based on the average milk intake of 0.78 L/day
944 (Hofvander et al., 1982; Butte et al., 1984; Chandra, 1984; Neville et al., 1988; Allen et al., 1991), the
945 AI was rounded to 65 µg DFE/day (approximately 9.4 µg/kg body weight per day). For infants aged

7-12 months, an AI of 80 µg/day (approximately 8.8 µg/kg body weight per day) was set by allometric scaling from the AI for infants from birth to six months. Downward extrapolation from the EAR of adults using allometric scaling and growth factors provided a similar result. These AIs were supported by five studies that assessed folate intake and status (red blood cell folate and/or serum folate) of breast-fed and formula-fed infants (Asfour et al., 1977; Ek and Magnus, 1982; Smith et al., 1983, 1985; Salmenpera et al., 1986). No data were found on which to base an EAR for children aged one to eight years; thus, values were extrapolated from adult values using allometric scaling and growth factors and the resulting EARs were 120 and 160 µg DFE/day for children aged one to three and four to eight years, respectively. The RDAs were set assuming a CV of 10 % because information was not available on the SD for the requirement of folate; the resulting RDAs were 150 µg DFE/day for children aged one to three years and 200 µg DFE/day for children aged four to eight years. EARs and RDAs of 250 and 300 µg DFE/day, respectively, for ages 9 to 13 years, and of 300 and 400 µg DFE/day, respectively, for ages 14 to 18 years were also extrapolated from adult values using allometric scaling and growth factors.

Using data on plasma folate concentrations in infants aged 2 to 11 months receiving folic acid (Asfour et al., 1977), the SCF (1993) set a PRI of 50 µg/day for infants aged 6 to 11 months. In the absence of evidence on folate requirements of children, values were extrapolated from those for adults on the basis of energy expenditure.

The UK COMA (DH, 1991) interpolated RNIs between the value for adults and the one set for formula-fed infants of 50 µg/day. It was stated that the interpolated values were well above the value of 3.6 µg folate/kg body weight per day which had been shown to maintain plasma folate at a concentration considered low but acceptable by the UK COMA and to ensure absence of overt folate deficiency in children under two years of age (Asfour et al., 1977).

Table 2: Overview of Dietary Reference Values for folate for children

	D-A-CH (2013) [a]	NNR (2012)	WHO/FAO (2004) [a]	NL (2003) [b, c]	Afssa (2001)	IOM (1998) [a]	SCF (1993) [c]	DH (1991)
Age (months)	4-<12	6-11	7-12	6-11	Infants	7-12	6-11	7-12
PRI (µg/day)	85 [b]	50	80	60	70	80 [b]	50	50
Age (years)	1-<4	1-<2	1-3	1-3	1-3	1-3	1-3	1-3
PRI (µg/day)	120	60	150	85	100	150	100	70
Age (years)	4-<7	2-5	4-6	4-8	4-6	4-8	4-6	4-6
PRI (µg/day)	140	80	200	150	150	200	130	100
Age (years)	7-<10	6-9	7-9	9-13	7-9	9-13	7-10	7-10
PRI (µg/day)	180	130	300	225	200	300	150	150
Age (years)	10-<13	10-13	10-18	14-18	10-12	14-18	11-14	11-18
PRI (µg/day)	240	200	400	300	250	400 [e]	180	200
Age (years)	13-<15	14-17			13-15		15-17	
PRI (µg/day)	300	300			300		200	
Age (years)	15-<19				16-19			
PRI Boys (µg/day)	300				330			
PRI Girls (µg/day)	300 [d]				300			

NL, Health Council of the Netherlands; PRI, Population Reference Intake
(a): Dietary folate equivalents, for definition see Table 1.
(b): Adequate Intake (AI)
(c): Dietary folate
(d): Women capable of or willing to become pregnant should also take a daily supplement containing 400 µg of folic acid during the period four weeks prior to eight weeks after conception, to prevent neural tube defects

(e): Women capable of becoming pregnant are advised to take 400 µg of folic acid daily from fortified foods, supplements, or both to reduce the risk of neural tube defects

4.3. Pregnancy

The German-speaking countries (D-A-CH, 2013) assumed that the additional folate requirement of the fetus is 200 µg DFE/day (IOM, 1998). Adding this value to the AR for adults (220 µg DFE/day) resulted in the AR for pregnant women, and the PRI was derived by the addition of 30 %.

WHO/FAO (2004) adopted the folate values proposed by the IOM (1998), setting an EAR of 520 µg DFE/day and a recommended nutrient intake of 600 µg DFE/day for pregnant women.

The Nordic countries (NNR, 2004) set the RI for pregnant women at 500 µg/day based on the assumption of women entering pregnancy with moderate folate stores and a dietary study comparing pregnant and non-pregnant women (Caudill et al., 1997). The value remained unchanged in the 2012 update of the Nordic Nutrition recommendations due to absence of new data (Nordic Council of Ministers, 2014).

The Health Council of the Netherlands (2003) estimated an extra requirement of 100 µg/day during pregnancy, setting an AI of 400 µg/day, and advised women wishing to become pregnant to take, besides their intake from food, a supplement containing 400 µg/day of folic acid to prevent NTDs.

Afssa (2001) noted that young (non-pregnant) women did not meet the PRI for folate of 300 µg/day (CREDOC, 1999). Given the health consequences for the fetus of insufficient folate intake particularly at the beginning of pregnancy, Afssa (2001) recommended an increase of 100 µg/day above that of non-pregnant women, setting a PRI of 400 µg/day for pregnant women.

IOM (1998) set an EAR for pregnancy of 520 µg DFE/day, adding to the EAR for non-pregnant women 200 µg DFE/day based on data from supplementation studies (Dawson, 1966; Willoughby and Jewell, 1966; Hansen and Rybo, 1967). Using a CV of 10 %, the RDA was calculated to be 600 µg DFE/day for pregnant women.

As studies have shown that one quarter to one half of women in the later stages of pregnancy show clear signs of deficiency (Chanarin, 1979), and the drop in red blood cell folate could be prevented by a folic acid supplement of 100 µg/day, the SCF (1993) considered 100 µg/day to be a minimum requirement. In order to account for the lower bioavailability of food folate compared to folic acid (Gregory et al., 1991), a dietary increment of 200 µg/day of folate was advised, to be added to the PRI of non-pregnant women. As folic acid has a protective effect on the occurrence of NTDs (Scott et al., 1990), it was considered that, even though some studies used very high doses of folic acid, amounts of 400 µg/day conferred equal protection with a lower risk of side effects (Smithells et al., 1989; MRC Vitamin Study Research Group, 1991).

The UK COMA considered that a mean additional folic acid intake of 100 µg/day maintains plasma and red blood cell folate concentrations at or above those of non-pregnant women (Hansen and Rybo, 1967; Chanarin et al., 1968b). The RNI of non-pregnant women was raised by this amount.

1012 **Table 3:** Overview of Dietary Reference Values for folate for pregnant women

	D-A-CH (2013) [a]	NNR (2012)	WHO/FAO (2004) [a]	NL (2003) [b, c]	Afssa (2001)	IOM (1998) [a]	SCF (1993)	DH (1991)
Age (years)						14-50		
PRI (µg/day)	550 [d]	500	600	400 [d]	400	600 [e]	400 [f]	300

1013 NL, Health Council of the Netherlands; PRI, Population Reference Intake
1014 (a): Dietary folate equivalents, for definition see Table 1.
1015 (b): AI
1016 (c): Dietary folate
1017 (d): Women capable of or willing to become pregnant should also take a daily supplement containing 400 µg of folic acid
1018 during the period four weeks prior to eight weeks after conception, to prevent neural tube defects.
1019 (e): Women capable of becoming pregnant are advised to take 400 µg of folic acid daily from fortified foods, supplements,
1020 or both to reduce the risk of neural tube defects.
1021 (f) Neural tube defects in the offspring have been shown to be prevented by periconceptual ingestion of 400 µg folic
1022 acid/day in the form of supplements.

1023 ### 4.4. Lactation

1024 The German-speaking countries (D-A-CH, 2013) assumed that folate secreted with 0.75 L/day of
1025 human milk amounts to 60 µg/day. Taking into account a folate bioavailability of 50 %, an intake of
1026 120 µg DFE is needed to replace these losses. Adding this value to the AR for non-lactating adults
1027 resulted in an AR for lactating women of 340 µg DFE/day; the PRI was set by adding 30 % to the AR.

1028 WHO/FAO (2004) adopted the folate values published by the IOM (1998), setting an EAR of 450 µg
1029 DFE/day and a recommended nutrient intake of 500 µg DFE/day for lactating women.

1030 The Nordic countries (NNR, 2004) recommended an increase of 100 µg/day of folate based on the
1031 folate concentration of human milk (Ek, 1983; Smith et al., 1985), a secreted volume of 0.75 L/day,
1032 and a bioavailability of 50 %. Therefore, the RI for lactating women was set at 500 µg/day of folate.
1033 This value was kept unchanged for the 2012 update of the Nordic Nutrition recommendations (Nordic
1034 Council of Ministers, 2014).

1035 The Health Council of the Netherlands (2003) based the extra requirement of lactating women on the
1036 average amount secreted via breast milk by mothers who exclusively breast-fed their child and
1037 considering 50 % bioavailability of folate from food, an AI of 400 µg/day of folate occurring
1038 naturally in food was set.

1039 Afssa (2001) recommended an increase in intake of 100 µg/day above that of non-lactating women,
1040 setting a PRI of 400 µg/day for lactating women.

1041 IOM (1998) set an EAR of 450 µg DFE/day for lactating women estimated as the folate intake
1042 necessary to replace the folate secreted daily in human milk[10] plus the amount required by non-
1043 lactating women to maintain folate status. The RDA was stated to have been calculated using a CV of
1044 10 %, and a value of 500 µg DFE/day was given.

1045 The SCF (1993) based their advice for lactating women on the amount of folate in milk (Ek, 1983;
1046 O'Connor et al., 1991) and a daily milk volume of 0.75 L, estimating that between 35 and 75 µg
1047 folate/day is secreted with breast milk. Taking the higher value and allowing for bioavailability, they
1048 advised an increase in intake of 150 µg/day to compensate for losses in breast milk, giving a PRI of
1049 350 µg/day of dietary folate.

[10] 0.78 L (milk volume) x 85 µg/L (folate concentration) x 2 (bioavailability correction factor) = 133 µg

The UK COMA (DH, 1991) estimated that the amount of folate secreted in breast milk amounts to 40 µg/day (Ek, 1983). An additional intake of 60 µg/day was assumed to replace these losses, taking into account incomplete absorption and utilisation of dietary folate.

Table 4: Overview of Dietary Reference Values for folate for lactating women

	D-A-CH (2013) [a]	NNR (2012)	WHO/FAO (2004) [a]	NL (2003) [b,c]	Afssa (2001)	IOM (1998) [a]	SCF (1993) [c]	DH (1991)
Age (years)						14-50		
PRI (µg/day)	450	500	500	400	400	500	350	260

NL, Health Council of the Netherlands; PRI, Population Reference Intake
(a): Dietary folate equivalents, for definition see Table 1.
(b): AI
(c): Dietary folate

5. Criteria (endpoints) on which to base Dietary Reference Values

5.1. Indicators of folate requirement

The Panel considers that serum and red blood cell folate concentrations are sensitive biomarkers of folate intake and status and should be used as primary criteria for deriving the requirement for folate (see Section 2.4.3.). Serum folate concentrations below 6.8 nmol/L and red blood cell folate concentrations below 317 nmol/L should be used as cut-off values indicative of folate deficiency. The cut-offs for deficiency were determined in adults and are also used in pregnancy.

Although plasma total homocysteine on its own is not suitable to be used as a biomarker of folate status, the Panel notes that its relationship with folate can be useful to define the blood folate concentrations necessary to maintain low concentrations of plasma total homocysteine which are associated with functional folate adequacy. The Panel considers that the cut-offs for functional folate adequacy based on plasma total homocysteine, i.e. serum folate at or above 10 nmol/L and red blood cell folate at or above 340 nmol/L derived from data from the third NHANES survey of the US population aged 12 years and above (Selhub et al., 2008), are suitable criteria for deriving the requirement for folate. The application of these criteria in different population groups is discussed below.

5.1.1. Adults

5.1.1.1. Evidence from studies not considering MTHFR genotype

For deriving the folate requirement of adults the SCF (1993) considered the studies by Herbert (1962); Herbert et al. (1962); Banerjee et al. (1975); Sauberlich et al. (1987) (see Section 4.1.). In this section the relevant evidence on the folate requirement of adults will be summarised, with a focus on well-controlled studies in which participants were housed in a metabolic unit (termed metabolic studies) and measurement of the folate content of study diets. No information on MTHFR genotype of the participants is available for all studies described in this section.

A metabolic study conducted in 40 male volunteers showed that a diet containing 200 ± 68 µg/day (range 150 to 250 µg/day) of dietary folate provided for a period of two to eight months was sufficient to maintain folate status within the normal range (12.9 ± 3.1 and 510 ± 98 nmol/L for serum folate and red blood cell folate concentrations, respectively), with only three subjects with serum folate below 9.1 nmol/L and no subject below the functional adequacy cut-off of 340 nmol/L for red blood cell folate at any time during the study (Milne et al., 1983). This is the longest metabolic study and

that with the largest sample size conducted so far, but its results should be interpreted with caution since some of the participants took short breaks (up to ten days) from the study, the diet did not provide a constant amount of folate throughout the whole study and more importantly, the laboratory analysis of the dietary folate content was performed without the procedure required for the complete release of the vitamin from the food matrix (trienzyme extraction, Tamura et al. (1997)) and thus likely underestimated the actual folate intake of study participants.

Underestimation of the folate intake via the assigned diet also occurred in the small depletion-repletion metabolic study of Sauberlich et al. (1987). They found that a diet providing 200 µg/day of food folate for three weeks stabilised plasma folate concentrations just above the deficiency cut-off of 6.8 nmol/L in two women who were kept on a low-folate diet (10 µg/day) for 28 days followed by three sequential periods, each one with a duration of three weeks, in which the women received 50, 100 and 200 µg/day of food folate. In contrast, in another three women who underwent a similar depletion-repletion regimen (repletion with 100 and 150 µg/day of food folate), an intake of 300 µg/day of food folate for the final three weeks of the study resulted in an increase in plasma folate, with concentrations ranging from 11.6-15.0 nmol/L at the end of the last period.

A study by Kauwell et al. (2000) used the recommended methodology for food folate analysis and obtained results similar to those of Milne et al. (1983) and Sauberlich et al. (1987). Kauwell et al. (2000) conducted a metabolic study in elderly women (60-85 years; 7-8 participants in each intervention group) who were subjected to seven weeks of folate depletion (118 ± 25 µg dietary folate/day) followed by a seven-week repletion period with four different diets containing a mixture of dietary folate and folic acid. After depletion, seven of the 32 subjects had serum folate concentrations < 6.8 nmol/L. During repletion, serum folate increased and was maintained at the ranges of 8-15.9, 8.2-38.3, 16.7-45.0 and 14.4-49.4 nmol/L with diets providing 205, 257, 506 and 630 µg DFE/day,[11] respectively. Importantly, at the end of the repletion period, the groups receiving 205 and 257 µg DFE/day had mean serum folate concentrations of 11.7 nmol/L and 16.2 nmol/L, respectively, which were above the functional adequacy cut-off value of 10 nmol/L. The folate repletion period of seven weeks in this metabolic study was not long enough to assess any effects of different folate intakes on red blood cell folate. Total homocysteine concentrations above the cut-off specific for this laboratory (< 16 µmol/L) were found only in one participant from each of the groups receiving 205 and 257 µg DFE/day.

In contrast to these results are the findings of another small metabolic study (O'Keefe et al., 1995) which showed that 320 µg DFE/day (30 µg dietary folate + 170 µg folic acid/day) provided for 70 days to young women maintained serum and red blood cell folate concentrations above the cut-offs for deficiency (6.8 nmol/L and 317 nmol/L for serum and red blood cell folate, respectively) in only two out of the five women.

The effect of diets providing higher amounts of folate on folate status of non-pregnant women (18-35 years, control group) was investigated in another metabolic study (Caudill et al., 1997). This study showed that a diet with a content of 680 µg DFE/day (120 µg food folate + 330 µg folic acid) consumed for 12 weeks by six women resulted in a mean serum folate concentration of 26 ± 11 nmol/L and a mean red blood cell folate concentration of 1 000 ± 387 nmol/L, and blood folate concentrations of all subjects were above the cut-offs for functional folate adequacy.

5.1.1.2. Evidence from studies considering MTHFR genotype

Homozygosity for the T allele of the MTHFR 677C→T polymorphism (677TT genotype) is associated with around 20-25 % lower serum folate and higher plasma total homocysteine concentrations compared with the 677CC genotype (Jacques et al., 1996; Davis et al., 2005; Hustad et

[11] Intakes have been converted into DFE based on the information provided in the study.

al., 2007). In addition, lower serum folate responses to folic acid interventions were shown in individuals with the 677TT genotype compared to those with the CC genotype, suggesting a higher requirement for folate in subjects homozygous for the polymorphism (Guinotte et al., 2003; Shelnutt et al., 2003).

The impact of the MTHFR 677C→T polymorphism on folate requirements was investigated in a metabolic study with 43 Mexican women (14 CC, 12 CT and 17 TT) aged 18-45 years who underwent a depletion period of seven weeks with a folate intake of 135 µg DFE/day followed by seven weeks of repletion with 400 µg DFE/day or 800 µg DFE/day (Guinotte et al., 2003). A higher proportion of women in the 677TT group had serum folate concentrations in the "low-normal" range (6.8–13.6 nmol/L as defined by the authors) compared with the 677CC group (50 % vs 14 %) after the repletion with 400 µg DFE/day and the genotype effect was even evident during the repletion with 800 µg DFE/day (Guinotte et al., 2003). In spite of these differences in serum folate in the 677TT and 677CC genotypes at the end of the repletion period with 400 µg DFE/day, all participants had serum folate concentrations above the deficiency cut-off (i.e. ≥ 6.8 nmol/L), red blood cell folate above the cut-off associated with functional folate adequacy (≥ 340 nmol/L) and plasma total homocysteine concentrations in the "desirable" range (i.e. < 10 µmol/L as defined by the authors) (Guinotte et al., 2003).

Another depletion-repletion metabolic study with a similar design but in 41 non-Hispanic women aged 20-30 years also reported that a repletion with 400 µg DFE/day for seven weeks was able to maintain mean and individual serum folate concentrations above the threshold associated with functional folate adequacy, both in women with the 677CC (n = 22) and the 677TT (n = 19) genotype (Shelnutt et al., 2003).

These results showed that 400 µg DFE/day were sufficient to sustain adequate serum folate concentrations in young women of any MTHFR genotype, but it is unknown whether a lower folate intake may have also been sufficient.

In contrast, a controlled feeding study in Mexican American men (18-55 years), of which 31 had the MTHFR 677CC and 29 had the TT genotype, showed that an intake of 438 µg DFE/day for 12 weeks was insufficient to maintain serum folate concentrations above the deficiency cut-off (≥ 6.8 nmol/L) in 34 % of the men with the 677TT and in 16 % of those with the 677CC genotype (Solis et al., 2008). The Panel notes that the results for serum folate concentrations were in disagreement with the high red blood cell folate concentrations in these subjects at the end of the study (1 233 ± 52 and 1 409 ± 45 nmol/L for subjects with the 677TT and 677CC genotype, respectively) and considers that no conclusions can be drawn from this study.

The influence of the MTHFR 677C→T polymorphism on the responses of biomarkers of folate status to diets with different folate content was also investigated in a non-metabolic cross-over study with 126 men and women (42 CC, 42 CT and 42 TT subjects) aged 20-63 years, who completed in random order three four-month interventions with diets providing 221 ± 93, 660 ± 179 and 814 µg ± 136 DFE/day (Ashfield-Watt et al., 2002). At the end of the intervention with 221 µg DFE/day, the individuals with the 677TT genotype had significantly lower mean plasma folate concentrations compared to those with the 677CC genotype (14.8 ± 7.4 vs. 19.0 ± 7.0 nmol/L) but the mean value of plasma folate of the 677TT group was above the cut-off associated with functional folate adequacy. Although this is not a metabolic study and the folate content of the diet was not determined analytically but was calculated based on a food composition database and semi-quantitative food-frequency questionnaires and thus may have underestimated folate intake, the Panel notes that this study may be considered as supportive, since it is a carefully conducted and sufficiently long intervention involving a relatively large number of participants with the three MTHFR 677C→T genotypes.

5.1.1.3. Conclusions on folate requirement of adults

The Panel considers that a folate intake of 205-257 µg DFE/day, as determined in a metabolic study with women aged 60-85 years with unknown MTHFR genotype, was sufficient for all women in the two groups to achieve a serum folate concentration above the deficiency cut-off, for the groups on average to maintain a serum folate concentration above the cut-off for functional folate adequacy, and for 12 of 14 women to maintain a "normal" plasma total homocysteine concentration (i.e. within the reference range of this laboratory) (Kauwell et al., 2000). The Panel notes the likely underestimation of folate intake in two other metabolic studies in men and women with unknown MTHFR genotype (one small study and one with the largest sample size), but considers that their results also support that a dietary folate/DFE intake around 200-300 µg/day may be sufficient to maintain adequate folate status (Milne et al., 1983; Sauberlich et al., 1987). The Panel decided not to consider the small metabolic study in women with unknown MTHFR genotype (O'Keefe et al., 1995), whose results were in disagreement with other studies presented above.

The Panel also notes that, in individuals with the MTHFR 677TT genotype compared to those with the 677CC genotype, the response of folate biomarkers to folate intervention is lower and that two studies in young women with known MTHFR genotypes have shown that an intake of 400 µg DFE/day maintained serum folate above the cut-offs for deficiency or for functional folate adequacy and red blood cell folate above the cut-off associated with functional folate adequacy (≥ 340 nmol/L) (Guinotte et al., 2003; Shelnutt et al., 2003). Although the effects of lower folate intakes on folate biomarkers in 677TT individuals have not been investigated in controlled metabolic studies, the results of a four-month intervention supports the view that a diet providing less than 400 µg DFE/day (i.e. 221 ± 93 µg DFE/day) can maintain mean plasma folate concentrations of a group of subjects with the MTHFR 677TT genotype at a level above the cut-off for functional folate adequacy (Ashfield-Watt et al., 2002).

The Panel considers that the higher requirements for folate of individuals with the MTHFR 677TT genotype compared to those with the MTHFR 677CC genotype should be taken into account when choosing a CV for deriving the PRI for folate.

5.1.2. Infants aged 7-11 months

Newborn infants have high serum and red blood cell folate concentrations which are maintained up to the age of six months and gradually decline thereafter; at one year of age the serum folate concentrations are 60 % of those at birth (Hay et al., 2008). The high infant folate status is sustained through the consumption of breast milk for which the folate concentration is maintained at the expense of maternal reserves and usually is not affected by low folate intake or status of the mother (Smith et al., 1983). Folate deficiency in exclusively breast-fed infants has not been reported (IOM, 1998). The decline in indicators of folate status after six months of age has been associated with the introduction of weaning foods into the infant's diet (Smith et al., 1985) and consequent changes in the intestinal pH and microflora, which in turn might influence folate bioavailability (Lonnerdal, 2000). In line with IOM (1998), the Panel considers that growth and haematological parameters are not sufficiently specific indicators to be used for deriving the DRVs for folate for infants. Salmenpera et al. (1986) reported that infants fully breast-fed until 12 months of age all maintained adequate plasma folate concentrations with the lowest observed value at 17.9 nmol/L.

In a systematic review, Lohner et al. (2012) identified three intervention studies with folic acid in healthy infants receiving folic acid supplements (5-1 000 µg/day) and measuring either serum folate (Hadler et al., 2008), red blood cell folate (Matoth et al., 1979) or both (Asfour et al., 1977). In a non-randomised controlled trial, Asfour et al. (1977) provided for up to eight months folic acid at 0, 5 or 10 µg/day in addition to a formula diet with a known folate concentration (196 µg/kg formula powder) to 20 Lebanese orphans weighing between about 3.5 and 8 kg and aged 2-11 months at the start of the study. The Panel notes the wide range of ages at baseline, that intakes expressed per kg

body weight did not differentiate between intake of folate and folic acid, that five of 20 infants were below the third percentile of growth standards for North American children of comparable age and sex at the start of the study and that infants were only maintained on a formula diet without access to solid foods throughout the study. In the studies by Hadler et al. (2008) and Matoth et al. (1979) no information is available on intake of dietary folate and thus on total DFE intake. The Panel considers that no conclusions can be drawn from these studies with regard to folate requirements of infants aged 7-11 months.

5.1.3. Children

A systematic review of the available controlled studies on folate intake/folic acid supplementation and status of children concluded that plasma and red blood cell folate concentrations are reliable markers of folate status for this age group (Lohner et al., 2012). However, folate biomarkers in healthy children have been assessed only in supplementation studies (Areekul et al., 1980; Pena et al., 2007; Papandreou et al., 2010) which have used extremely high doses of folic acid (5-15 mg/day) and their relevance for responses to folate intake within the usual dietary range is unknown.

The Panel notes that there is a lack of data on folate requirements of children.

5.1.4. Pregnancy

Pregnant women have higher folate requirements associated with the growth of fetal and maternal tissue and the active transfer of folate to the fetus (see Section 2.3.3.). Several studies investigated the responses of folate biomarkers to supplementation with folic acid in pregnant women but did not assess dietary folate intakes of the women (Dawson, 1966; Hansen and Rybo, 1967; Willoughby and Jewell, 1968). As information on DFE intakes is thus unavailable the Panel considers that no conclusions can be drawn from these studies on the folate requirement in pregnancy.

Caudill et al. (1997) carried out a metabolic study in six women during their second trimester of pregnancy (week 14-25 of gestation) and found that 330 µg/day of folic acid together with 120 µg/day of food folate (i.e. a total intake of 680 µg DFE/day) resulted after 12 weeks in mean serum folate concentrations (27 ± 9 nmol/L) similar to those in six non-pregnant women (26 ± 11 nmol/L) with the same DFE intake. All subjects had serum folate concentrations > 13.6 nmol/L throughout the study period. Mean red blood cell folate concentrations were similar in pregnant and non-pregnant women at baseline (1 383 ± 158 and 1 114 ± 397 nmol/L, respectively) and these values were maintained after 12 weeks with no significant difference between pregnant and non-pregnant women. In a subsample (n = 4) of the participants of this study who were followed up in the third trimester of pregnancy, a daily supplementation of 200 µg of folic acid, in addition to an estimated mean dietary folate intake of 293 µg/day (equivalent to a total intake of about 630 µg DFE/day), also sustained high folate status biomarker values during this period of pregnancy.

An intervention trial in 206 pregnant British women found that folic acid supplementation at 100 µg/day from the 20th week of gestation until the end of pregnancy together with a mean dietary folate intake of 676 µg/day (range 198-1 615 µg/day) (mean total intake equivalent to 850 µg DFE/day) was able to prevent the fall in serum and red blood cell folate concentrations that occurred in the control group during the third trimester of pregnancy (Chanarin et al., 1968b). At 38 weeks of gestation, mean serum and red blood cell folate concentrations in the supplemented group were 14.3 nmol/L and 424 nmol/L, respectively, which were above the cut-offs for folate deficiency and functional folate adequacy in non-pregnant women; however, the variability (SD) was not reported. Folate intake by duplicate diet analysis was measured only once throughout the study period and it was based on a limited number of participants only (16 of 206) (Chanarin et al., 1968a). The Panel notes that intake estimates are only available for about 8 % of the pregnant women in the intervention trial, that this sub-group analysis suggested a rather high mean and a wide range of dietary folate

intakes within and between subjects and considers that no conclusions can be drawn from this study on the folate requirement in pregnancy.

Willoughby and Jewell (1966) investigated the effect of supplementation with folic acid at different doses (0, 100, 300 or 450 µg/day) in addition to intake from the diet on serum folate concentration in 350 pregnant women from about three months of gestation until the end of pregnancy. Random dietary surveys on 150 women allotted to the different supplementation groups suggested that the folate intake was less than 50 µg/day in 60 % of the women. The Panel considers that this is an unrealistically low value for a free-living population and that no conclusions can be drawn from this study on the folate requirement in pregnancy.

The Panel notes that intakes of 630-680 µg DFE/day administered in a small metabolic study resulted in biomarkers of folate status being well above cut-offs for deficiency or functional folate adequacy as established in non-pregnant adults.

An alternative method for deriving folate requirements in pregnancy, which is based on the conversion of the amount of excreted urinary folate catabolites into dietary folate by multiplying for differences in their molecular weight, has been developed initially by McPartlin et al. (1993). Using this approach, Higgins et al. (2000) found a gradual increase of folate catabolites in urine with the progression of pregnancy in 24 women in comparison to 25 non-pregnant women, and estimated that an intake of at least 440 µg DFE/day (the average estimate for the three trimesters, i.e. 340, 430 and 540 µg DFE/day) is needed to compensate for losses in pregnant women. The Panel notes that this approach does not take into account endogenous faecal folate losses and that the approach of deriving requirements in pregnancy solely based on catabolite excretion has not been validated.

5.1.5. Lactation

The folate concentration of breast milk is not influenced by maternal intake and status of the vitamin as it is maintained predominantly at the expense of maternal reserves (Smith et al., 1983). Concentrations (mean ± SEM) of serum folate of 36.8 ± 4.2 nmol/L and red blood cell folate of 667.3 ± 52.3 nmol/L were reported in 21 breastfeeding women with unknown MTHFR genotype. The women were on self-selected diets, did not ingest supplemental folic acid and had a "dietary folate intake" of 401 ± 38 µg/day (measured by a two-day diary) at six months *post partum* (Mackey and Picciano, 1999). Authors mentioned that lactating women obtained 30 % of their total daily dietary folate from fortified, ready-to-eat cereals, but it is unclear whether the differences in bioavailability of folic acid and food folate have been considered in the intake assessment. Thus, the Panel notes that no conclusions can be drawn from this study regarding folate requirements of lactating women.

The Panel considers that lactating women have increased folate requirements compared to non-lactating women, to compensate for folate losses through their milk (see Section 2.3.6.3).

5.2. Folate intake and health consequences

5.2.1. Cardiovascular disease-related outcomes

A meta-analysis of seven observational studies (six prospective and one case-cohort) performed in the US, Finland, Germany, Sweden, the Netherlands, and Japan (n = 2) and including 2 682 cases and 221 009 non-cases showed an inverse relationship between folate intake and cardiovascular disease (CVD). Based on six of the seven studies, it was predicted that an increase in folate intake of 200 µg/day would reduce the risk of coronary heart disease by 12 % (summary RR: 0.88; 95 % CI 0.82, 0.94, p for heterogeneity = 0.219; I^2 = 27.4 %) (Wang et al., 2012). RCTs have usually enrolled patients with pre-existing CVD or other chronic diseases and have investigated the effect of combined B-vitamin supplementation and/or of high folic acid doses (i.e. above the UL) on CVD-

related outcomes (overview in Yang et al. (2012) and Marti-Carvajal et al. (2013)). The Panel considers that no conclusions can be drawn from these studies for deriving the requirement for folate.

An observational study has related the trend for a decrease in stroke mortality in the US and Canada to the introduction of mandatory folic acid fortification in North America (Yang et al., 2006). RCTs investigating the effect of folic acid supplementation alone on stroke prevention in healthy subjects are not available (Huo et al., 2012).

In view of the limited evidence and the absence of a dose-response relationship between folate and CVD-related outcomes, the Panel considers that the data available cannot be used for deriving the requirement for folate.

5.2.2. Cancer and all-cause mortality

Evidence from observational studies suggests that there is an inverse relationship between dietary or total (i.e. from foods and supplements) folate intake and risk of cancer, more specifically breast cancer (Ericson et al., 2007; Larsson et al., 2007) and colon cancer. A meta-analysis of 13 prospective cohort studies, conducted in the US and in Europe and including 725 134 participants, showed that colon cancer risk was reduced by 15 % (multivariate RR 0.85, 95 % CI 0.77, 0.95; $p_{trend} = 0.02$) in the highest quintile of total folate intake, while a dietary folate intake in the highest quintile was not associated with a significantly reduced risk of colon cancer (multivariate RR 0.92, 95 % CI 0.84, 1.00; $p_{trend} = 0.07$) (Kim et al., 2010). RCTs have usually enrolled patients with colon adenoma or other pre-existing diseases and have investigated the effect of combined B-vitamin supplementation and/or of high folic acid doses (i.e. at or above the UL) on recurrence of colorectal adenoma, incidence of selected cancers or all-cause mortality. In a recent systematic review of trials of folic acid supplementation on cancer and all-cause mortality, only 10 of 26 included studies used folic acid alone (at doses of 500-5 000 µg/day) vs. placebo or control or were uncontrolled (Mackerras et al., 2014). In these trials, no effect was observed on total cancer incidence (weighted RR 1.28, 95 % CI 0.95, 1.72; three studies, 500-1 000 µg/day of folic acid alone), colorectal cancer (weighted RR 0.76, 95 % CI 0.32, 1.82; three studies, 500-1 000 µg/day of folic acid alone), and prostate cancer (weighted RR 1.56, 95 % CI 0.45, 4.93; two studies, 1 000 µg/day of folic acid alone). Six studies evaluated the effect of folic acid supplementation alone (with doses of 500-1 000 µg/day) on recurrence of colorectal adenoma and did not observe an effect over one to seven years of follow-up, or when limiting the evaluation to studies following-up for three to seven years or looking at advanced adenoma as an endpoint (Mackerras et al., 2014). Only one study investigated the effect of folic acid alone at a dose of 1 000 µg/day on lung and breast cancer and did not observe an effect (Wu et al., 2009). Five studies on folic acid supplementation alone at doses of 500-1 000 µg/day showed a reduction in all-cause mortality (weighted RR 0.64, 95 % CI 0.43, 0.94), whereas no relationship was observed when three trials were included using doses of 2 500-5 000 µg/day (Mackerras et al., 2014).

The Panel concludes that folate/folic acid has not consistently been associated with the risk of cancer and that the data available on cancer-related outcomes cannot be used for deriving the requirement for folate.

5.2.3. Cognition-related outcomes

A decline in cognitive function in older adults may range in severity from mild memory impairment to Alzheimer's disease. Prospective observational studies have demonstrated that a lower risk of cognitive decline or dementia is associated with higher baseline folate intakes (classified as at/above vs. below the US RDA of 400 µg DFE/day (Corrada et al., 2005)) or serum folate concentrations (Seshadri et al., 2002; Ravaglia et al., 2005). Three RCTs investigating the effect of folic acid supplementation with doses between 750 and 5 000 µg/day on cognitive function in healthy subjects have been conducted (Bryan et al., 2002; Pathansali et al., 2006; Durga et al., 2007). Dietary folate

intake was assessed in only two of these trials. A recent attempt to pool their results for meta-analysis was unsuccessful because the trials assessed different cognitive outcomes (Malouf and Grimley Evans, 2008). In the trial by Durga et al. (2007), 818 healthy men and postmenopausal women (50-70 years) with plasma total homocysteine of 13-25.9 µmol/L were supplemented for three years with folic acid at a dose of 800 µg/day or placebo. Median dietary folate intake at baseline and year 3 ranged between 179 µg/day (interquartile range 152-224 µg/day) and 195 µg/day (interquartile range 158-242 µg/day) in the intervention and placebo groups. After three years, the treatment group compared with placebo showed an improvement of some cognitive domains such as global cognitive function, information-processing speed and memory storage. The two other RCTs (Bryan et al., 2002; Pathansali et al., 2006) in healthy women aged 65-92 years and healthy men and women aged 73 ± 5.6 years did not find any effect of short-term (4-5 weeks) supplementation with folic acid on cognitive processing, memory, executive function, verbal ability, mood measures, reaction time, and attention.

In view of the limited evidence and since a dose-response relationship between folate and cognition-related outcomes cannot be derived, the Panel concludes that the available data cannot be used for deriving the requirement for folate.

5.2.4. Neural tube defects

Neural tube defects (NTD) are a group of congenital malformations which are the result of incomplete closure of the neural tube during early embryonic development (anencephaly and spina bifida). NTD is considered to be of multifactorial aetiology with possible involvement of genetic and environmental factors. Although women with NTD-affected pregnancies are rarely folate-deficient, it was reported that they have lower serum and red blood cell folate (Smithells et al., 1976; Yates et al., 1987) and higher plasma total homocysteine concentrations (Mills et al., 1995) compared to women carrying normal fetuses. Homozygosity for the MTHFR 677C→T polymorphism (TT genotype) was demonstrated to be associated with an increased risk for NTD-affected pregnancies (Vollset and Botto, 2005), which further supports the link between folate status and NTD risk. An inverse dose-response relationship between folate status and risk of NTD has been reported in a case-control study (Daly et al., 1995) with a plateau of the NTD incidence at a serum folate concentration of ≥ 15.9 nmol/L and a red blood cell folate concentration of ≥ 906 nmol/L measured at the 15th gestational week. Although these biomarker values may only be specific for the population investigated, the results showed that achieving much higher folate status than just above the cut-offs for deficiency may be required for NTD prevention.

Periconceptional supplementation with folic acid has a well-established protective role against both first occurrence (Czeizel and Dudas, 1992) and recurrence (MRC Vitamin Study Research Group, 1991) of NTDs, resulting in worldwide consensus on recommendations for the prevention of first occurrence of an NTD, such that women of child-bearing age should consume supplemental folic acid at a dose of 400 µg/day for at least one month before and during the first trimester in addition to consuming food folate from a varied diet (IOM, 1998; NHMRC, 2006; SACN, 2006; D-A-CH, 2013). Observational studies have shown that the risk of NTD also decreases with a dietary folate intake above about 230 µg/day (Shaw et al., 1995), however, the evidence for the protective effect of dietary folate is considered weak due to the observational design of studies and the general inherent inaccuracy of dietary assessment methods. As a result of the mandatory folic acid food fortification policy introduced in 1998 in North America and designed to provide an additional 100 µg/day of folic acid (170 µg DFE/day), the NTD incidence has declined by 27 % and 50 % in the US and in Canada, respectively (Honein et al., 2001; De Wals et al., 2007).

The Panel acknowledges the importance of ingestion of 400 µg/day of supplemental folic acid for at least one month before and during the first trimester of pregnancy for reducing the risk of NTD. The Panel notes that the use of supplemental folic acid is in addition to dietary folate intake and considers

that the available data on folic acid intake and NTD risk cannot be used for deriving the requirement for folate.

6. Data on which to base Dietary Reference Values

6.1. Adults

The Panel considers that new data are available to update the AR and PRI for adults proposed by the SCF (1993). The Panel proposes to base the AR for folate for adults on the results of the small metabolic study by Kauwell et al. (2000) which showed that an intake of 205-257 µg DFE/day for seven weeks after a depletion phase maintains serum folate concentrations above the cut-off for deficiency in all postmenopausal women with unknown MTHFR genotype and above 10 nmol/L (i.e. the cut-off for functional folate adequacy) in at least about half of the group. Moreover, the findings of Kauwell et al. (2000) are in agreement with two earlier metabolic studies in men and women with unknown MTHFR genotype indicating that a dietary folate/DFE intake of around 200-300 µg/day may be sufficient to maintain adequate folate status (Milne et al., 1983; Sauberlich et al., 1987), though intakes in these studies have likely been underestimated.

Therefore, the Panel concludes that an AR for folate can be set at 250 µg DFE/day. As there is no indication that the requirement differs by sex and age, the AR of 250 µg DFE/day is proposed for all adults. In order to account for the additional variability as a result of the higher requirement for folate in individuals with the MTHFR 677TT genotype compared to those with the 677CC genotype, and for the fact that the proportion of subjects with the 677TT genotype in the three key studies was unknown, a CV of 15 % is applied to the AR of 250 µg DFE/day to derive the PRI of 330 µg DFE/day.

6.2. Infants aged 7-11 months

Considering the limitations of available studies on folate intake and status in infants, the Panel concludes that these cannot be used to set an AR and a PRI for folate for infants aged 7-11 months (see Section 5.1.2).

In the absence of data to estimate folate requirements of infants aged 7-11 months, the folate intake of infants may be estimated using upwards extrapolation from the intake of folate in fully breastfed infants aged 0-6 months for which folate deficiency has not been observed. The folate intake of breast-fed infants aged up to six months can be calculated based on the average consumption of breast milk and its folate concentration. Based on seven studies (published between 1998 and 2014) using the most advanced extraction methods for folate (see Section 2.3.6.3 and Appendix A), the mean/median folate concentration of mature breast milk is reported to be in the range of 45-99 µg/L, with an approximate average of 80 µg/L. Mean breast milk intake over the first six months *post partum* is assumed to be 0.8 L/day (Butte et al., 2002; FAO/WHO/UNU, 2004; EFSA NDA Panel, 2009). Thus, the calculated folate intake for infants from birth to six months of age is 64 µg/day.

In order to estimate the folate intake of infants aged 7-11 months from the calculated folate intake for infants from birth to six months, isometric scaling was applied, as the Panel is not aware of evidence relating folate requirement to metabolic rate (EFSA NDA Panel, 2010). Averages of the median weight-for-age of male and female infants aged three months (6.1 kg) and nine months (8.6 kg) according to the WHO Growth Standards (WHO Multicentre Growth Reference Study Group, 2006) were used, and a value of 90 µg/day was calculated.

In the only representative survey available in the EU median DFE intake of infants aged 0.5 to < 1 year was reported to be around 70 µg/day.

The Panel concludes that an AI of folate can be set at 80 µg DFE/day for infants aged 7-11 months.

1456 **Table 5:** Reference body weights and Adequate Intake (AI) of folate for infants aged 7-11 months

Age	Reference body weight (kg)	AI (µg DFE/day)
7-11 months	8.6 [(a)]	80

1457 (a): Average of the median weight-for-age of male or female infants, respectively, aged nine months according to the WHO
1458 Growth Standards (WHO Multicentre Growth Reference Study Group, 2006)

1459 ### 6.3. Children

1460 The Panel considers that there are no reliable data for children and adolescents on which to base an
1461 AR for folate. Therefore, the ARs were calculated by extrapolation from the AR of adults. As there is
1462 no evidence that folate requirement is associated with the metabolic rate (EFSA NDA Panel, 2010),
1463 isometric scaling was applied.

1464 $AR_x = AR_{adults} \times (weight_{child\ x}/weight_{adults}) \times (1 + growth\ factor)$

1465 For the calculations, average of the median weight of boys and girls (van Buuren et al., 2012) and
1466 average of the median body weights of 18 to 79-year-old men and women based on measured body
1467 heights of 16 500 men and 19 969 women in 13 EU Member States and assuming a BMI of 22 kg/m^2
1468 (see Appendix 11 in EFSA NDA Panel (2013)) were used. The following growth factors have been
1469 applied (Table 6).

1470 **Table 6:** Growth factors (EFSA NDA Panel, 2010)

Age	Growth factor
7 months - 3 years	0.30
4 - 8 years	0.15
9 - 13 years	0.15
14 - 18 years, males	0.15
14 - 18 years, females	0.00

1471

1472 For the calculation of the PRI, as for adults, a CV of 15 % was assumed in order to account for the
1473 additional variability as a result of the higher requirements for folate in children with the MTHFR
1474 677TT genotype compared to those with the 677CC genotype. Calculations were done with the
1475 unrounded values, but the values for ARs and PRIs presented in Table 7 were rounded to the nearest
1476 10.

1477 **Table 7:** Reference body weights, Average Requirements (ARs) and Population Reference Intakes
1478 (PRIs) of folate for children and adolescents

Age	Reference body weight (kg)	AR (µg DFE/day)	PRI (µg DFE/day)
1-3 years	11.9 [(a)]	60	80
4-6 years	19.0 [(b)]	90	110
7-10 years	28.7 [(c)]	130	170
11-14 years	44.6 [(d)]	200	260
15-17 years	60.3 [(e)]	250	330

1479 DFE, dietary folate equivalent
1480 (a): Average of the median weight-for-age of male or female children, respectively, aged 24 months according to the WHO
1481 Growth Standards (WHO Multicentre Growth Reference Study Group, 2006)
1482 (b): Average of the median weight of male or female children, respectively, aged 5 years (van Buuren et al., 2012)
1483 (c): Average of the median weight of male or female children, respectively, aged 8.5 years (van Buuren et al., 2012)

(d): Average of the median weight of male or female children, respectively, aged 12.5 years (van Buuren et al., 2012)
(e): Average of the median weight of male or female children, respectively, aged 16 years (van Buuren et al., 2012)
Adult body weight used for calculations: 63.3 kg (average of 68.1 kg for men and 58.5 kg for women).

6.4. Pregnancy

Folate requirement increases during pregnancy because of growth of fetal and maternal tissue and the active transfer of folate to the fetus. The Panel notes the limited evidence base available to assess folate requirements in pregnancy, that intakes of 630-680 µg DFE/day administered in a metabolic study to pregnant women during their second and third trimester resulted in mean concentrations of biomarkers of folate status being well above cut-offs for deficiency or functional folate adequacy as established in non-pregnant adults (Caudill et al., 1997), and that it is unknown whether this may have also been achieved with a lower folate intake.

Acknowledging the weaker data base compared to non-pregnant adults, the Panel considers that it is not possible to set an AR for pregnancy and proposes to set an AI for folate for pregnancy at 600 µg DFE/day.

This DRV does not include the generally accepted public health advice for intake of supplemental folic acid for at least one month before and during the first trimester of pregnancy for NTD prevention (see Section 5.2.5).

6.5. Lactation

Lactating women have increased folate requirements in order to compensate for folate secreted in breast milk and to maintain an adequate folate status. For women exclusively breastfeeding, the mean milk transfer over the first six months *post partum* is assumed to be 0.8 L/day (Butte et al., 2002; FAO/WHO/UNU, 2004; EFSA NDA Panel, 2009). Thus, considering this milk volume and an average breast milk folate concentration of 80 µg/L (see Section 2.3.6.3. and Appendix A), it is estimated that daily folate losses amount to 64 µg in exclusively breastfeeding women. Assuming that the bioavailability of dietary folate is 50 %, a lactating woman would require 128 µg/day of additional folate in order to restore her losses. A value of 130 µg/day is added to the AR for non-lactating women, resulting in an AR of 380 µg DFE/day (i.e. 250 µg DFE/day for non-lactating adults plus 130 µg DFE/day). Assuming a CV of 15 %, and rounding up, a PRI of 500 µg DFE/day is derived.

CONCLUSIONS

The Panel concludes that an AR and a PRI for folate can be derived for adults based on biomarkers of folate status. For adults, there is no indication that the requirement differs by sex and age. In the absence of data on requirements, ARs and PRIs for children were extrapolated from adults using isometric scaling. An AR and a PRI is also derived for lactating women considering their additional needs for compensating for the amount of folate secreted in breast milk. For pregnant women, the Panel proposes to set an AI considering the weaker data base compared to non-pregnant adults. For infants aged 7-11 months, an AI is proposed based on folate intake from breast milk extrapolated from infants aged 0-6 months.

The Panel also considered several health outcomes that may be associated with folate intake; however, the available data were considered insufficient for the setting of DRVs for folate.

1524 **Table 8:** Summary of Dietary Reference Values for folate

Age	AI (µg DFE/day)	
7-11 months	80	
	AR (µg DFE/day)	**PRI (µg DFE/day)**
Age		
1-3 years	60	80
4-6 years	90	110
7-10 years	130	170
11-14 years	200	260
15-17 years	250	330
≥ 18 years	250	330
Pregnancy	-	600 [(a)]
Lactation	380	500

1525 (a): Adequate Intake
1526 For combined intakes of food folate and folic acid, DFEs can be computed as follows: µg DFE = µg food folate + (1.7 x µg
1527 folic acid)
1528

1529 ## RECOMMENDATIONS FOR RESEARCH

1530 The Panel suggests to collate nationally representative folate intake data which differentiate between
1531 natural folate and folic acid, to enable the assessment of folate intakes based on DFE. The Panel also
1532 suggests to review existing food composition databases with regard to inclusion of folate
1533 concentrations based on reliable and appropriate analytical methods.

1534 The Panel suggests to undertake studies to clarify the bioavailability of folic acid and natural food
1535 folates.

1536 The Panel suggests to generate reliable data that can be used for the assessment of folate requirements
1537 of pregnant women as well as adults, infants, children, and individuals homozygous for the
1538 *MTHFR*677C→T polymorphism.

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2210 **APPENDICES**

2211 **Appendix A. Concentrations of total folate in mature breast milk measured by microbiological assay with trienzyme pre-treatment**

Reference	n (number of samples)	Country	Maternal dietary intake (µg/day) Mean	Stage of lactation	Folate concentration (µg/L) Mean ± SD	Median	Range	Analytical method	Comments
Lim et al. (1998)	42(42)	USA	Not reported	3 months	90.6 ± 3.5 [(b)]			Microbiological assay with *L. casei* and trienzyme pre-treatment	Values were also reported for use of conjugase alone and were considerably lower.
				6 months	81.5 ± 3.5 [(b)]				
Mackey and Picciano (1999)	21(21)	USA	Group 1: 337 ± 38 [(b)] + 1 000 µg folic acid/day	3 months	82.2 ± 4.2 [(b)]			Microbiological assay with *L. casei* and trienzyme pre-treatment	In women receiving placebo, milk folate at 6 months was lower than at 3 months ($p<0.02$); in supplemented women, milk folate was inversely correlated with plasma folate ($r=-0.52$, $p<0.01$)
			Group 2: 406 ± 31 [(b)] + placebo		99.0 ± 5.1 [(b)]				
			Group 1: 364 ± 24 [(b)] + 1 000 µg folic acid/day	6 months	80.3 ± 4.7 [(b)]				
			Group 2: 401 ± 38 [(b)] + placebo		82.5 ± 5.3 [(b)]				
Kim et al. (2004)	12(12)	Canada	Not reported; 9 of the 12 women consumed vitamin supplements containing 400-1 000 µg of folic acid	1-6 months	51.5 ± 20.3	53.4	35.4 - 59.9 [(a)]	Microbiological assay with *L. casei* (ATCC 7469) and trienzyme pre-treatment	
Khambalia et al. (2006)	68(68)	Mexico	Dietary intake of all at 22 days: 86 (38, 137) [(a)], Group 1 then received daily folic acid 400 µg + Fe 18 mg + other vitamins, group 2 received daily folic acid 400 µg + other vitamins	22 ± 13 days		45.2	39.5 - 57.0 [(a)]	Microbiological assay with *L. casei* (ATCC 7469) and trienzyme pre-treatment	Otomi women; milk folate concentrations did not differ and thus were combined.
				82 ± 15 days		68.4	56.8 - 78.6 [(a)]		
				138 ± 18 days		63.6	53.9 - 79.1 [(a)]		

Reference	n (number of samples)	Country	Maternal dietary intake (µg/day) Mean	Stage of lactation	Folate concentration (µg/L) Mean ± SD	Median	Range	Analytical method	Comments
Houghton et al. (2009)	55 (55)	Canada	Not reported; Group 1: 416 µg 5-m-THF/day from week 4-16	4 weeks	83.4 ± 22.9			Microbiological assay with *L. casei* (ATCC 7469) and trienzyme pre-treatment	No significant differences between groups over time
			Not reported; Group 2: placebo from week 4-16		85.2 ± 27.4				
			Not reported; Group 3: 400 µg folic acid/day from week 4-16		68.4 ± 24.3				
	53 (53)		Group 1	8 weeks	77.2 ± 19.0				
			Group 2		91.3 ± 33.5				
			Group 3		77.7 ± 35.3				
	57 (57)		Group 1	16 weeks	80.3 ± 45.0				
			Group 2		80.8 ± 25.2				
			Group 3		70.2 ± 34.9				
West et al. (2012)	28 (28)	USA	404 + 750 from supplement (= 1 675 µg DFE/day)	5 weeks	56.2 (48.8 - 64.2) [(c)]			Microbiological assay with *L. casei* (ATCC 7469) and trienzyme pre-treatment	75 % of women used folic acid supplement prior to enrollment; folic acid and 5-m-THF in milk measured by LC-MS/MS
				13-15 weeks	61.8 (54.1 - 70.0) [(c)]				

5-m-THF, 5-methyl-tetrahydrofolate; DFE, dietary folate equivalents.
(a): Median (1^{st}-3^{rd} quartile).
(b): mean ± SE.
(c): 95 % CI.
Note: Trienzyme pre-treatment included α-amylase, protease and folate conjugase treatments. Studies with conjugase pre-treatment only (e.g. Udipi et al. (1987)) were not considered for this table.

Appendix B. Folate intake from foods and supplements in surveys in The Netherlands, Ireland, Germany, and Austria

Study	Country	Age (years)	Number of subjects	Sex	DFE (µg/day) Median	Folic acid intake (µg/day) Median
Westenbrink et al. (2012)	The Netherlands	2-3	327	M	118 [a]	nr
		2-3	313	F	111 [a]	nr
		4-6	327	M	134 [a]	nr
		4-6	312	F	120 [a]	nr
van Rossum et al. (2011)	The Netherlands	7-8	153	M	184 (91-461) [b, c]	20 (0-180) [c]
		7-8	151	F	177 (97-433) [b, c]	14 (0-150) [c]
		9-13	351	M	224 (117-464) [b, c]	18 (0-154) [c]
		9-13	352	F	193 (109-405) [b, c]	13 (0-154) [c]
		14-18	352	M	251 (139-455) [b, c]	9 (0-146) [c]
		14-18	354	F	208 (120-451) [b, c]	14 (0-181) [c]
		19-30	356	M	288 (161-541) [b, c]	14 (0-159) [c]
		19-30	347	F	249 (137-626) [b, c]	31 (0-244) [c]
		31-50	348	M	323 (181-660) [b, c]	22 (0-230) [c]
		31-50	351	F	282 (154-761) [b, c]	45 (0-326) [c]
		51-69	351	M	334 (189-730) [b, c]	50 (0-320) [c]
		51-69	353	F	294 (164-755) [b, c]	55 (0-321) [c]
Hopkins (2013)	Ireland	18-50	350	M	530 ± 303 [b, d]	126 ± 153 [d]
		18-50	335	F	425 ± 305 [b, d]	118 ± 155 [d]
		51-64	98	M	528 ± 303 [b, d]	135 ± 161 [d]
		51-64	106	F	470 ± 327 [b, d]	130 ± 166 [d]
		≥ 65	75	M	528 ± 347 [b, d]	155 ± 180 [d]
		≥ 65	87	F	542 ± 539 [b, d]	183 ± 168 [d]
DGE (2008)	Germany	0.5-<1	52	M	78 [a]	nr
		0.5-<1	43	F	62 [a]	nr
		1-<4	242	M	128 [a]	nr
		1-<4	246	F	116 [a]	nr
		4-<5	74	M	147 [a]	nr
		4-<5	75	F	143 [a]	nr
		6-<7	106	M	190 (118-352) [b, e]	nr
		6-<7	102	F	161 (104-275) [b, e]	nr
		7-<10	321	M	204 (126-374) [b, e]	nr
		7-<10	308	F	188 (111-329) [b, e]	nr
		10-<12	199	M	205 (119-410) [b, e]	nr
		10-<12	198	F	204 (130-324) [b, e]	nr
		12-<13	114	M	272 (145-601) [b, e]	nr
		12-<13	103	F	272 (151-591) [b, e]	nr
		13-<15	214	M	296 (156-619) [b, e]	nr
		13-<15	230	F	273 (170-508) [b, e]	nr
		15-<18	294	M	340 (189-646) [b, e]	nr
		15-<18	317	F	276 (152-558) [b, e]	nr
DGE (2012)	Germany	15-<19	506	M	182 [176; 191] [a, f]	nr
		15-<19	536	F	153 [149; 163] [a, f]	nr
		19-<25	469	M	196 [188; 201] [a, f]	nr
		19-<25	486	F	170 [165; 177] [a, f]	nr
		25-<35	614	M	207 [203; 211] [a, f]	nr
		25-<35	852	F	181 [177; 186] [a, f]	nr
		35-<51	1 946	M	212 [207; 215] [a, f]	nr
		35-<51	2 648	F	185 [182; 188] [a, f]	nr
		51-<65	1 460	M	214 [208; 220] [a, f]	nr
		51-<65	1 740	F	193 [189; 196] [a, f]	nr
		65-80	1 165	M	207 [204; 211] [a, f]	nr
		65-80	1 331	F	189 [185; 192] [a, f]	nr

Elmadfa et al. (2012)	Austria	7-9	67	M	164 [152; 176] (g)	nr
		7-9	57	F	171 [157; 186] (g)	nr
		10-12	83	M	169 [156; 182] (g)	nr
		10-12	81	F	142 [132; 153] (g)	nr
		13-14	19	M	143 [120; 166] (g)	nr
		13-14	25	F	137 [110; 165] (g)	nr
		18-24	17	M	255 [227; 283] (g)	nr
		18-24	37	F	229 [199; 259] (g)	nr
		25-50	87	M	197 [180; 214] (g)	nr
		25-50	143	F	216 [198; 234] (g)	nr
		51-64	44	M	222 [198; 246] (g)	nr
		51-64	52	F	193 [172; 213] (g)	nr
		60-80	76	M	203 [187; 219] (g)	nr
		60-80	100	F	194 [175; 213] (g)	nr

DFE, Dietary folate equivalents calculated as follows in The Netherlands and in Ireland: µg DFE = µg natural folate + (µg folic acid from fortified foods x 1.7) + (µg folic acid from supplements x 2), calculated as follows in Germany: µg DFE = µg natural folate + (µg folic acid from fortified foods x 1.7) + (µg folic acid from supplements x 1.7) and as follows in Austria: µg DFE = µg natural folate + (µg folic acid x 2.0); M, male; F, female; nr, not reported.
(a): Supplements were not taken into account in these calculations.
(b): Intake of folate and folic acid from foods and dietary supplements
(c): Median (P5-P95)
(d): Mean ± SD
(e): Median (P10-P90)
(f): Median [confidence interval of the median]
(g): Mean [confidence interval of the mean]

2232 **ABBREVIATIONS**

Afssa	Agence française de sécurité sanitaire des aliments
AI	Adequate Intake
AR	Average Requirement
BMI	Body mass index
CREDOC	Centre de recherche pour l'étude et l'observation des conditions de vie [*Research Institute for the Study and Monitoring of Living Standards*]
CV	Coefficient of variation
D-A-CH	Deutschland-Austria-Confoederatio Helvetica
DFE	Dietary folate equivalent
DRV	Dietary Reference Value
EAR	Estimated Average Requirement
EC	European Commission
EFSA	European Food Safety Authority
EU	European Union
FAO	Food and Agriculture Organization
IOM	U.S. Institute of Medicine of the National Academy of Sciences
LC/MS/MS	Liquid chromatography-tandem mass spectrometry
MRC	Medical Research Council
MTHFR	5,10-methylene tetrahydrofolate reductase
NHANES	National Health and Nutrition Examination Survey
NL	Health Council of the Netherlands
NNR	Nordic Nutrition Recommendations
NTD	Neural tube defect
PRI	Population Reference Intake
RDA	Recommended Dietary Allowance
RI	Recommended Intake
RNI	Reference Nutrient Intake

SAM	S-adenosylmethionine
SCF	Scientific Committee on Food
SD	Standard deviation
SU.VI.MAX	SUpplementation en VItamines et Minéraux AntoXidants [*French prospective study on supplementation with vitamins and minerals*]
THF	Tetrahydrofolate
WHO	World Health Organization

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