Addressing optimal folate and related B vitamin status through the lifecycle: health impacts and challenges.

Helene McNulty, Mary Ward, Leane Hoey, Catherine F Hughes and Kristina Pentieva

Author Affiliation: Nutrition Innovation Centre for Food and Health (NICHE), School of Biomedical Sciences, Ulster University, Coleraine, Northern Ireland, BT52 1SA

Authors' last names: McNulty, Ward, Hoey, Hughes and Pentieva

Corresponding Author: Professor Helene McNulty, Nutrition Innovation Centre for Food and Health (NICHE), School of Biomedical Sciences, Ulster University, Coleraine, Northern Ireland, BT52 1SA. Tel: +44 (0)28 7012 4583, E-mail: h.mcnulty@ulster.ac.uk.

Shortened title (45 characters): Folate and related B vitamins throughout life

Abbreviations: CVD, cardiovascular disease; DFEs, Dietary Folate Equivalents; DHFR, dihydrofolate reductase; EGRac, erythrocyte glutathione activation coefficient; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; GWAS, genome-wide association studies; HoloTC, holotranscobalamin; MCI, mild cognitive impairment; MMA, methylmalonic acid; MTHFR, methylenetetrahydrofolate reductase; NTDs, neural tube defects; PLP, pyridoxal 5'phosphate; PPO, pyridoxine-phosphate oxidase; SAM, S-adenosylmethionine; THF, tetrahydrofolate.

Abstract

The functional effects of folate within one-carbon metabolism involve interrelationships with vitamin B12, vitamin B6 and riboflavin and related gene-nutrient interactions. These B vitamins have important roles throughout life, from pregnancy, through childhood, to middle and older age. Achieving optimal nutritional status for preventing folate-related disease is challenging, however, primarily as a result of the poor stability and incomplete bioavailability of folate from natural food sources when compared with the synthetic vitamin form, folic acid. Thus, in European countries, measures to prevent neural tube defects (NTD) have been largely ineffective because of the generally poor compliance of women with folic acid supplementation as recommended before and in early pregnancy. In contrast, countries worldwide with mandatory folic acid fortification policies have experienced marked reductions in NTD. Low vitamin B12 status is associated with increased risk of cognitive dysfunction, cardiovascular disease and osteoporosis. Achieving optimal B12 status can be problematic for older people, however, primarily owing to food-bound B12 malabsorption which leads to sub-clinical deficiency even with high dietary B12 intakes. Optimising B vitamin intake may be particularly important for sub-populations with impaired folate metabolism owing to genetic characteristics, most notably the 677C→T variant in the gene encoding the enzyme MTHFR. This common folate polymorphism is linked with several adverse health outcomes, including stroke, however, recent evidence has identified its novel interaction with riboflavin (the MTHFR co-factor) in relation to blood pressure and risk of developing hypertension. This review addresses why and how optimal status of folate-related B vitamins should be achieved through the lifecycle.

Introduction

Folate is required for one-carbon metabolism and is thus essential for important biological pathways including DNA and RNA biosynthesis and methylation reactions. The functional effects of folate within the one-carbon network involve close interaction with vitamin B12, vitamin B6 and riboflavin. This review will address why these B vitamins are key in human health and how optimal status can be achieved. To illustrate the role of these metabolically interrelated vitamins through the lifecycle, case studies highlighting their health impacts at early, middle and late life will be reviewed. The significant public health challenges in

achieving optimal nutritional status, and thus potentially preventing folate-related disease, will also be considered.

Functional role of folate and metabolic interaction with other B vitamins

Folates function as cofactors within one-carbon metabolism (**Figure 1**). This involves the transfer and utilisation of one-carbon units in a network of pathways required for DNA and RNA biosynthesis, amino acid metabolism and methylation processes. For folate to function effectively within this network, it interacts closely with vitamin B12, vitamin B6 and riboflavin[1]. Reduced folates enter the one-carbon cycle as tetrahydrofolate (THF) which acquires a carbon unit from serine in a vitamin B6-dependent reaction to form 5,10 methyleneTHF. This co-factor form, in turn, is either converted to 5 methylTHF or serves as the one-carbon donor in the synthesis of nucleic acids, where it is required by thymidylate synthetase in the conversion of deoyxuridine (dUMP) to deoxythymidine (dTMP) for pyrimidine biosynthesis, or is converted to other folate co-factor forms required for purine biosynthesis. Methylenetetrahydrofolate reductase (MTHFR) is a riboflavin-dependent enzyme that catalyses the reduction of 5,10 methyleneTHF to 5 methylTHF. Once formed, 5 methylTHF is used by methionine synthase for the vitamin B12-dependent conversion of homocysteine to methionine and the formation of THF. Methionine is activated by ATP to form S-adenosylmethionine (SAM), which then donates its methyl group to more than 100 methyltransferases for a wide range of substrates such as DNA, hormones, proteins, neurotransmitters and membrane phospholipids, all of which are regulators of important physiological processes[1]. SAM is typically referred to as 'the universal methyl donor' as it is used for a great number of methylation reactions; its generation in tissues is dependent on an adequate supply of folate and vitamin B12. In summary, effective folate functioning requires essential metabolic interaction with vitamins B12, B6 and riboflavin, and therefore, sub-optimal status of one or more of these B vitamins, or polymorphisms in folate genes, can impair one-carbon metabolism, even if folate intakes are adequate.

Health impacts of folate and related B vitamins through the lifecycle

Folate and the related B vitamins have essential roles in human health. Although the preventative role of folate in neural tube defects (NTD) has been the major focus of public health efforts worldwide, international research is addressing other roles of folate in early,

middle and late life and indeed the intergenerational effects of maternal folate status during pregnancy in relation to health outcomes in the offspring during childhood and beyond.

Early life: Effects of folate in preconception, pregnancy and offspring health

Given that folate is essential for cell division and tissue growth, this vitamin plays a particularly important role in pregnancy and fetal development. Over 25 years ago it was proven beyond doubt that maternal folic acid supplementation in early pregnancy could protect against NTDs[2,3] including spina bifida, anencephaly and related defects. NTDs are the most common major malformations of the central nervous system and occur as a result of failure of the neural tube to close properly in the first few weeks of pregnancy, leading to death of the fetus or newborn, or to various disabilities involving the spinal cord. The conclusive evidence of the role of folate in preventing NTD has led to folic acid recommendations for women of reproductive age which are in place worldwide[4,5]. As will be discussed later in this review, however, achieving optimal folate status for preventing NTD can be challenging for individuals and populations. Of note, rates of NTD in Ireland are among the highest in the world. A recent report from the Food Safety Authority of Ireland shows that there was a large decrease in the incidence rate of NTDs in Ireland from the early 1980s (about 3.5 per 1,000 births) to the mid-1990s; however, the rate has remained relatively stable since then at about 1 per 1,000 births (about 80 cases per annum), similar to the UK.

Apart from its well established role in preventing NTD in very early pregnancy, folate has other essential roles throughout pregnancy, with impacts in early life and beyond. The importance of folate for pregnancy was in fact recognised in historical reports of the discovery of human folate deficiency dating back to the early 1930s, when a fatal anaemia of pregnancy first described in India was proven to be responsive to treatment with food sources of the vitamin[6]. Clinical folate deficiency is now known to cause megaloblastic anaemia, a condition characterised by immature, enlarged blood cells (reflecting impaired DNA synthesis), which is reversible with folic acid treatment[7]. In the absence of maternal folic acid supplementation, folate-related anaemia of pregnancy has been reported to occur in up to 24% of unsupplemented pregnancies in parts of Asia, Africa, and South America and in up to 5% of those in well-nourished populations[7]. Red blood cell and serum folate concentrations typically decrease throughout pregnancy[8], while folic acid supplementation prevents this decline[9] and can thus prevent the occurrence of megaloblastic anaemia of pregnancy[10,11]. As folate is

required for the remethylation of homocysteine to methionine, plasma homocysteine is invariably elevated with low folate status[12]. Elevated homocysteine, in turn, is associated with increased risk of a number of pregnancy complications including NTD,[13,14] preeclampsia,[15,16] placental abruption,[17] recurrent early pregnancy loss[18] low birth weight and intrauterine growth retardation[19].

As reviewed elsewhere in this journal[20], in addition to protecting against the development of megaloblastic anaemia in the mother, there is emerging evidence linking maternal folate during pregnancy with offspring neurodevelopment and cognitive function in childhood. Evidence in this area is predominantly based on observational studies reporting significant associations between maternal folate concentrations, or use of folic acid supplements by mothers in pregnancy, and the cognitive performance of children at critical stages of childhood[21–23]. However, an intervention study from this centre, the Folic Acid Supplementation in the Second and Third Trimesters (FASSTT) trial[9], provided a unique opportunity to follow up the children of trial participants and found direct evidence of beneficial effects of folic acid provided to mothers during pregnancy on the cognitive performance of their children at age 3 and 6 years (i.e. as measured by validated tools, the Bayley Scales of Infant and Toddler Development and Weschler Preschool and Primary Scale of Intelligence, respectively) [24,25]. Thus maintaining optimal maternal folate throughout pregnancy, well beyond the early period known to be protective against NTD, may be beneficial for brain development and functioning in the child.

The biological mechanism linking maternal folate with the offspring brain is likely to involve folate-mediated epigenetic changes related to brain development and function. DNA methylation, the most widely studied epigenetic mechanism for gene regulation, is dependent upon the supply of methyl donors provided by folate and related B vitamins via SAM[1]. Folate deficiency could thus lead to aberrant gene expression with consequential health outcomes[26]. As reviewed elsewhere in this journal, most epigenetic studies in humans have used a candidate gene approach to link maternal status of folate, or reported usage of folic acid supplements, with DNA methylation in offspring genes involved in folate biology and neurodevelopmental processes[20]. The only randomised trial to date to investigate the effect of maternal folic acid was carried out at this centre and showed that folic acid supplementation through trimesters 2 and 3 of pregnancy resulted in significant changes in DNA methylation in cord blood of *LINE-1* and candidate genes related to brain development, *IGF2* and *BDNF*[27]. Likewise, animal studies found that folic acid supplementation throughout pregnancy significantly increased

brain folate concentrations in the newborn pups, while brain global DNA methylation decreased[28]. The findings offer a potential biological basis to link maternal folate status with neurodevelopment of the offspring, but this requires further investigation using a genome-wide approach to more thoroughly explore the role of DNA methylation in mediating these effects.

Apart from maternal folate status during pregnancy, the child's folate status also will have important health impacts. Folate biomarkers in children are reported to decline progressively with age in British and American children, possibly indicating that folate requirements of older children are higher to sustain the increased metabolic demands for growth from childhood to adolescence[29,30]. Such findings have not been taken into consideration in setting current folate recommendations for adolescents, but should perhaps be considered by future panels tasked with evaluating the evidence to support dietary reference values for folate in this population sub-group.

Middle age: Effects of MTHFR and riboflavin on blood pressure and hypertension

Optimal B vitamin intake may be particularly important for sub-populations with impaired folate metabolism owing to genetic characteristics, most notably the 677C→T variant in the gene encoding the enzyme MTHFR. Since this common folate polymorphism was first described over 20 years ago, it has been linked with several adverse health outcomes, including stroke[31]. Although the health concerns in relation to this polymorphism have predominantly focussed on the well described homocysteine phenotype, arguably of greater relevance to public health is the more recent emergence of a blood pressure phenotype, and a modulating role of riboflavin (MTHFR co-factor), in determining the risk of hypertension[31].

Hypertension is a significant health concern because it is the leading risk factor contributing to mortality worldwide, accounting for 17% of all deaths (9.4 million each year), most notably from cardiovascular disease (CVD), with 45% of deaths from heart disease and 51% of deaths from stroke estimated to result from hypertension[32,33]. Reducing blood pressure is however proven to decrease CVD[34] and it is estimated that lowering of systolic BP by as little as 2 mmHg can decrease cardiovascular risk by as much as 10%[35]. The risk of hypertension increases with age and occurs as a result of a complex interaction of lifestyle, dietary and environmental factors. Genetic factors are also implicated in the development and progression of hypertension. Of note, a region near the gene encoding the folate-metabolising enzyme MTHFR has been identified by genome-wide association studies (GWAS) among a

small number of loci associated with blood pressure in European, American and Asian populations[36,37]. Epidemiological studies have also linked this folate gene with blood pressure, with the 677C→T polymorphism in *MTHFR* estimated to increase the risk of hypertension by up to 87% [38], and CVD by up to 40%[39]. However, there is large geographical variability in estimates of the excess risk of CVD owing to this polymorphism[31], suggesting the involvement of a gene-environment interaction. Folate only was previously considered as the relevant environmental factor, but evidence from our centre suggests that riboflavin may be a more important modulating factor via a novel effect on blood pressure[31].

Riboflavin (vitamin B2) plays an important role in folate recycling within one-carbon metabolism where it acts in the form of flavin adenine dinucleotide (FAD) as a cofactor for MTHFR, the enzyme which catalyses the conversion of 5,10 methyleneTHF to 5 methylTHF; the latter co-factor then serves as the methyl donor in the B12-dependent re-methylation of homocysteine to methionine (by methionine synthase). The role of riboflavin within the one-carbon metabolic network is often overlooked in human health, but it is most evident in individuals with the homozygous mutant 677TT genotype in *MTHFR*, resulting in a thermolabile enzyme with reduced activity[40]. The decreased activity of the variant MTHFR enzyme was demonstrated in molecular studies to be the result of a reduced affinity for its FAD cofactor[41,42]. In human studies, individuals with the *MTHFR* 677TT genotype typically present with elevated plasma homocysteine[40], along with low folate concentrations [43]. The homocysteine phenotype is known to be most pronounced if the TT genotype occurs in combination with poor nutritional status of either folate [44] or riboflavin[45,46]. Furthermore, riboflavin supplementation results in marked lowering of homocysteine concentrations in individuals with the TT genotype, but not in those with CC or CT genotypes[47], suggesting that enhancing riboflavin status may stabilise the variant enzyme and thus restore MTHFR activity *in vivo*.

Recent studies indicate that riboflavin interacts with MTHFR to influence blood pressure and hypertension risk[31]. We have been studying the modulating effect of riboflavin on blood pressure in hypertensive patients (with and without overt CVD) pre-screened for *MTHFR* genotype and published three randomised trials to date showing that the blood pressure phenotype is highly responsive to lowering by intervention with riboflavin[48–50]. In the first of these trials, we investigated a cohort of over 400 premature CVD patients with a mean age of 53 years (and 47 years at time of event)[48]. At baseline, patients with the variant TT genotype had significantly higher systolic and diastolic blood pressure compared to their age-matched counterparts with CC or CT genotypes, with mean systolic/diastolic blood pressure (mmHg)

of 131/80, 133/83 and 143/86 for CC, CT and TT genotypes, respectively. Of greater note, riboflavin supplementation (1.6 mg/d for 16 weeks) resulted in significant blood pressure-lowering in patients with the TT genotype, but no effect in those with CC or CT genotypes[48]. When these high-risk patients were followed up 4 years later, those with TT genotype were again found to be hypertensive, despite marked changes in the number and type of antihypertensive drugs being prescribed (following changes in clinical guidelines for hypertension since the initial investigation), and target blood pressure was again achieved only in response to riboflavin[49]. In a third trial, we demonstrated that the responsiveness of blood pressure to riboflavin intervention in the genetically at-risk group was not confined to high-risk CVD patients but was also achievable in hypertensive adults without overt CVD[50]. At baseline, despite being prescribed multiple classes of antihypertensive drugs, over 60% of participants with this genotype had failed to reach target blood pressure ($\leq$140/90mmHg), but riboflavin supplementation for 16 weeks (when antihypertensive drugs remained unchanged) resulted in a significant decrease in blood pressure[50].

These randomised trials show that riboflavin has a novel and genotype-specific role in lowering blood pressure (by an average of 6 to 14 mmHg in systolic blood pressure), which occurs independently of antihypertensive medication[48-50]. Furthermore, antihypertensive therapy as currently prescribed (typically, polytherapy with 2 or more drugs used in combination) appears to be associated with poorer blood pressure control in patients with the *MTHFR* 677TT genotype, while the achievement of target blood pressure can be greatly enhanced with supplemental riboflavin at the dietary range of 1.6 mg/d. Given recent calls for more personalised approaches to lower blood pressure and thus improve cardiovascular health[34], there are clinical and public health implications arising from the finding that the under-recognised blood pressure phenotype associated with this common folate polymorphism is modifiable by riboflavin. For hypertensive patients with the *MTHFR* 677TT genotype, supplementation with riboflavin could offer a personalised, non-drug treatment to lower blood pressure. For sub-populations worldwide, enhancing riboflavin status could prevent or delay the development of high blood pressure in those with this genetic risk factor. Mechanistic studies are however required to elucidate the precise link between one-carbon metabolism and blood pressure and to understand the biological perturbation leading to higher blood pressure in the TT genotype and how riboflavin corrects it. Randomised trials investigating the role of this novel gene-nutrient interaction in hypertension through the lifecycle are also required. In any case, at this time while other genetic factors appear to play a role in the development of

hypertension[36,37], the common 677C→T polymorphism in *MTHFR* is the only genetic factor linked with hypertension that offers a personalised management option, via optimising riboflavin, the MTHFR co-factor.

Older age: Effects of folate and related B vitamins on the ageing brain

Dementia and depression are recognised as major disorders of ageing with profound human[51] and economic[52–54] costs. Dementia affects an estimated 46.8 million people worldwide, with projections that this will increase to over 131 million people by 2050[52]. Cognitive function typically declines with age, but in some cases the decline occurs at a greater rate than expected, ranging in severity from relatively minor slips in performing activities, through to subjective cognitive decline, mild cognitive impairment (MCI) and dementia[55]. Activities of daily living are profoundly affected in dementia, whereas in MCI, although cognitive decline is greater than expected for an individual's age and education level, there is no notable interference in activities of daily life, albeit 50% of those with MCI can be predicted to develop dementia within 5 years.[56] Depression is the most frequent psychiatric disease, and 'late–life depression' is reported more commonly in females, affecting an estimated 28% of females compared with 22% of males, over the age of 65 years[57]. In addition, depression has been shown to increase cognitive dysfunction, while poorer cognitive health can also predispose older adults to depression[58,59], suggesting a bi-directional relationship between the two conditions. A recent comprehensive report identified a model of modifiable risk factors for dementia across the lifespan, highlighting the potential for effective prevention through early interventions that target these risk factors[28]. In this context, as recently reviewed extensively in this journal[60], nutrition can play an important preventative role, with emerging evidence linking certain dietary patterns (particularly the 'Mediterranean' diet) or specific dietary components, including omega-3 polyunsaturated fatty acids, polyphenols, vitamin D and particularly B-vitamins, with a reduced risk of dementia and depression.

Folate, vitamin B12 and vitamin B6 play important roles in the nervous system at all ages, from neural development in early life through to the maintenance of mental health and cognitive function in older age. Thus B vitamin deficiencies can manifest with significant neurological and neuropsychiatric disturbances. Historically, deficiencies of folate[61,62] and vitamin B12[63,64] have been linked with psychiatric illness and poorer mental wellbeing. The reported neuropsychiatric effects of deficiency of either nutrient include cognitive decline, depression and peripheral neuropathy, although the latter is more commonly found in vitamin

B12 deficiency[65]. Folate and vitamin B12 are required for the activity of methionine synthase within one-carbon metabolism and therefore the synthesis of SAM, which in turn provides methyl groups for numerous central nervous system methylation reactions involving neurotransmitter and membrane phospholipid synthesis and myelin methylation[66,67]. Thus, with folate or vitamin B12 deficiency, the reduction in tissue levels of SAM may contribute to cognitive dysfunction by impairing these methylation processes. Reduced tissue concentration of SAM may be linked to depression through perturbing monoamine (serotonin, dopamine and noradrenaline) synthesis and methylation[68]. Additionally, vitamin B6 plays an essential role in transamination and decarboxylation reactions, which in turn are involved in neurotransmitter synthesis, metabolism and release; deficiency of vitamin B6 is associated with deficits in nerve conduction[69]. The metabolism of homocysteine requires folate along with vitamin B12, and to a lesser extent vitamin B6; when the status of these vitamins is low or deficient, plasma homocysteine concentration will invariably be elevated. Homocysteine concentrations are typically higher in patients with Alzheimer's disease (the most common form of dementia), and are strongly related to the rate of cognitive decline in patients with mild cognitive impairment and Alzheimer's disease[68,70] Thus, there has been considerable interest in plasma homocysteine as a potential risk factor for cognitive dysfunction, but it remains unconfirmed whether homocysteine is a true disease risk factor or merely a marker. In any case however, homocysteine measurement provides a sensitive functional biomarker of folate and related B-vitamin status, and plasma homocysteine shows significant lowering in response to B vitamin intervention[1].

Lower status of folate, vitamin B12 and /or vitamin B6 (or a higher concentration of the related metabolite homocysteine) is associated in observational studies with cognitive dysfunction[68]. Observational data supporting a role for these nutrients, or any nutritional factor, can however be complicated by the fact that poor diet may be both a cause and a consequence of impaired cognitive function. Only randomised trials can confirm whether a causative relationship exists. Although several randomised controlled trials have investigated the potential benefits of B-vitamin supplementation on cognitive function, many of these were of insufficient duration to provide clear evidence, while others intervened in patients with confirmed Alzheimer's disease where a beneficial effect is highly unlikely. One well designed randomised trial of healthy older adults in New Zealand reported no benefit of high dose combined folic acid/vitamin B12/vitamin B6 for two years on any cognitive function parameter examined[71], whereas another similar study from the Netherlands showed that supplementation

with folic acid alone for three years significantly improved a number of cognitive parameters including memory, information-processing speed and sensorimotor function[72]. An important difference between these two studies was baseline folate status which was far lower in the Dutch trial[72], suggesting that any benefit of folic acid on cognitive function arises through correction of sub-optimal folate status, whereas providing additional folic acid to those with already optimal status may have no effect on cognition. More recent randomised trials, showing that intervention with B vitamins prevented cognitive decline in free-living older adults with depressive symptoms, or improved cognitive performance in participants with mild cognitive impairment, provide convincing evidence that low B vitamin status may be causatively linked with cognitive dysfunction in ageing. Research in this area has been very substantially underpinned by the work of Smith and colleagues on the VITACOG trial which showed that B vitamin intervention not only improved cognitive performance[73], but also slowed the rate of global and regional brain atrophy as determined using magnetic resonance imaging (MRI) in participants with mild cognitive impairment [74,75]. Not all studies support the role of B vitamins, however, and one notable meta-analysis concluded that there was no beneficial effect of either folic acid or vitamin B12 on cognition in older adults[76]. The latter report has however been widely criticised by experts in this area, primarily owing to the inclusion criteria used to select the trials for investigation in the meta-analysis[77,78].

Apart from memory deficits and cognitive dysfunction, depressive symptoms are well described in both folate and vitamin B12 deficiency[65]. Furthermore, folate deficiency can affect the duration and clinical severity of depression, and is associated with poorer response to antidepressant medication[65]. Low folate status was associated with a significantly greater risk of depression in one meta-analysis of observational studies[79]. Likewise, low concentrations of vitamin B12[80–82] and vitamin B6 (required as a cofactor in the metabolism of tryptophan and serotonin)[83,84] have also been linked with depression. Randomised trials, investigating the role of B-vitamin supplements alone or as an adjunct to anti-depressant medications, have however produced conflicting results. The balance of available evidence in this area would appear to suggest that folate and vitamin B12 may have roles in the longer-term management of depression[85,86].

Overall, there is good evidence to suggest that optimal B vitamin status has protective effects on cognitive function, and potentially against depressive symptoms, in ageing[60]. To address the gaps in the evidence-base in this area, however, appropriately designed randomised trials are required, targeting participants with low B-vitamin status and thus most likely to

benefit from optimising B-vitamin status to achieve better cognitive and mental health in ageing.

Considerations and challenges in achieving optimal status of B vitamins

Folate

Folic acid refers to the synthetic form of the B vitamin known as folate, whereas the natural folate forms are found in plant, animal and human tissues. Food folates occur naturally in richest supply in green leafy vegetables, asparagus, beans, legumes, liver and yeast. Folic acid is found in the human diet only in fortified foods and supplements but is readily converted to the natural cofactor forms of folate after its ingestion[87]. There are however chemical differences between folic acid and the natural folate forms which have important nutrition consequences. Folic acid is a fully oxidised molecule and is a monoglutamate, meaning that it contains just one glutamate moiety in its structure. Naturally occurring food folates on the other hand are a mixture of reduced folate forms (predominantly 5 methyTHF) and typically found as polyglutamates, containing a variable number of glutamate residues[88]. As reduced molecules, natural food folates are inherently unstable outside living cells and tend to have poor bioavailability[87]. In addition to their limited bioavailability, food folates (particularly green vegetables) can be unstable during cooking, and this will substantially reduce the folate content of the food before it is even ingested[89]. Folic acid is more stable and more bioavailable compared to an equivalent amount of the vitamin eaten as naturally occurring food folates[87]. As a result of the poor stability and limited bioavailability of folate from natural food sources, achieving optimal folate status is challenging for individuals and populations.

It is important to appreciate that the absence of folate deficiency (i.e. megaloblastic anaemia) does not necessarily mean that folate status is optimal in terms of maintaining health and preventing folate-related disease such as NTD. Thus in many developed countries folate deficiency may be relatively rare, but sub-optimal folate status is commonly encountered[1]. Folate status and response to intervention are routinely assessed by measuring folate concentrations in serum/plasma or in red blood cells[90,91]. Serum folate reflects recent dietary intake and is the earliest indicator of altered folate exposure[92]. Red blood cell folate parallels liver concentrations (accounting for about 50% of total body folate) and is thus considered to represent tissue folate stores[93]. Compared with serum folate, red blood cell folate responds slowly to changes in dietary folate intake and is a better indicator of folate intake over the

previous 3-4 months, when circulating folate is incorporated into the maturing red cells[92,94]. On the basis that homocysteine metabolism requires an adequate supply of folate, the measurement of plasma homocysteine provides a functional biomarker of folate status and is invariably found to be elevated with low or deficient folate status.

Folate intakes and recommendations in the United States and certain other countries are now expressed as Dietary Folate Equivalents (DFEs), a calculation which was devised to take into account the greater bioavailability of folic acid from fortified foods compared to naturally occurring dietary folates[95,96]. Specifically for the prevention of NTD, women are recommended to take 400 μg/d folic acid as a supplement from preconception until the end of the first trimester of pregnancy[4,5]. Folic acid supplementation is a highly effective means to optimise folate status in women if the recommendations are followed[97,98]. However, it is not an effective public health strategy for populations because in practice very few women take folic acid as recommended before and in early pregnancy and this means that maternal biomarker status of folate is often found to be suboptimal in achieving concentrations known to be protective against NTD[98,99]. Fortification of food with folic acid, like supplementation, is very effective in optimising dietary intake and biomarker status of folate in those who choose to eat fortified food products[100,101]. However, fortification has the advantage over supplementation that it can also be highly effective for populations. When folic acid fortification is undertaken on a population-wide basis (via a policy of mandatory fortification), it results in significant increases in folate biomarker concentrations and marked reductions in NTD[1]. Thus reported rates of NTD have declined by between 27% and 50% in the USA, Canada and Chile in response to mandatory folic acid fortification of food[102–104]. In contrast, in the UK, Ireland and other European countries, policy to prevent NTD (i.e. based on folic acid supplementation) has had little or no impact in preventing NTD, despite active health promotion campaigns over many years[105,106]. This is primarily because the neural tube closes by day 28 post-conception and therefore the timing of folic acid usage by women is critical. For many women, the early period of pregnancy when folic acid is protective against NTD may have passed before folic acid supplements are started. This has resulted in an unacceptably high rate of NTD in European countries, recently estimated to be 1.6 times higher than in regions of the world with mandatory folic acid-fortification policies in place[107]. Of particular concern are reports that the incidence of NTD in Ireland is increasing in recent years[108].

Over 80 countries worldwide to date (including the United States, Canada and Australia) have passed regulations for the mandatory fortification of staple foods with folic

acid in order to prevent NTD. Other countries including the UK and Ireland have delayed decisions to introduce mandatory fortification on the basis of concerns relating to possible health risks. Once ingested, folic acid is reduced by dihydrofolate reductase (DHFR) and methylated, then released into the systemic circulation as 5 methylTHF. However, the capacity of DHFR in humans to efficiently metabolise folic acid is limited and thus exposure to high oral doses can result in the appearance of unmetabolised folic acid in plasma[1]. On the basis that the latter is not a normal constituent of plasma or other tissues, concerns have arisen regarding potential adverse health effects of unmetabolised folic acid in the circulation arising through high folic acid exposures from supplements and fortified foods. Traditionally this related to the potential risk that long-term exposure to high dose folic acid might mask the anaemia of vitamin B12 deficiency in older people and allow the associated irreversible neurological symptoms to progress, but this is no longer considered to be a public health issue[1]. A more recent concern has arisen from reports that the presence of unmetabolised folic acid in plasma in elderly people with low vitamin B12 status is associated with worse cognitive performance compared to those with low B12 status and no detectable folic acid in the circulation[109]; some subsequent studies have not been able to confirm such findings and therefore this issue remains somewhat controversial[1]. Other evidence suggested that folic acid doses in excess of 1mg/d could potentially promote the growth of undiagnosed colorectal adenomas in those with pre-existing lesions[110]. However one recent meta-analysis (involving 50,000 individuals) concluded that folic acid supplementation neither increased nor decreased site-specific cancer within the first 5 years of treatment[111]. One recent study from this centre investigated the effects on circulating unmetabolised folic acid concentrations, in pregnant women and their newborns, of folic acid supplements at a dose of 400 µg/d continued beyond the period of pregnancy currently recommended (i.e. to the end of trimester 1). On the basis that folic acid intervention improved maternal and neonatal folate status[9], but did not cause higher concentrations of unmetabolised folic acid[112], it was concluded that there was no adverse impact from the exposure of pregnant women to 400 µg/d supplemental folic acid, over and above typical intakes through fortified foods. It remains to be confirmed however whether plasma unmetabolised folic acid arising from higher folic acid intakes is a cause for concern. In the meantime, given the uncertainty regarding the long term effects of exposure to high dose folic acid, it is important to avoid population-wide chronic exposures to folic acid at levels higher than are necessary, with evidence that beneficial effects are likely to be achievable at low intakes[90].

In summary, it is unlikely that there are adverse effects associated with the presence of unmetabolised folic acid in the circulation at the generally low concentrations arising through food fortification. Indeed an expert international panel tasked with reviewing all aspects of folate biology and biomarkers recently concluded that it was 'not aware of any toxic or abnormal effects of circulating folic acid' even from much higher exposures than those obtained by food fortification[1]. The risk-benefit debate surrounding food fortification with folic acid is continuing among scientists and policymakers, but the balance of evidence at this time appears to indicate that the proven benefits of folic acid fortification would more than outweigh any potential risks.

Vitamin B12

Low status of B12 is associated with a higher risk of cognitive dysfunction, cardiovascular disease and osteoporosis[70,113]. There are however important public health considerations in relation to vitamin B12. First, the ability to achieve optimal B12 status in practice can be problematic for older people even with high dietary B12 intakes. Second, accurately assessing vitamin B12 status, and thus detecting deficient and low status, is difficult.

A severe form of vitamin B12 deficiency arises in pernicious anaemia. This is an autoimmune gastritis characterised by profound B12 malabsorption owing to loss of intrinsic factor, leading to haematological signs (megaloblastic anaemia) and irreversible neurological disease which is fatal if untreated[114]. Once diagnosed, clinical deficiency is readily treatable with regular B12 injections delivered intramuscularly. A much more subtle depletion of vitamin B12 arises as a result of food-bound B12 malabsorption from mild atrophic gastritis which diminishes gastric acid production (i.e. hypochlorhydria). This in turn reduces B12 absorption from food because of the essential role of gastric acid in the release of B12 from proteins in the food matrix[115]. Food-bound B12 malabsorption commonly occurs in older adults, reported to affect up to 20%[114], and leads to sub-clinical deficiency, where there is metabolic evidence of deficiency but without the classical haematological or neurological signs[116]. Thus the low B12 status found in older adults is primarily the result of food-bound B12 malabsorption related to atrophic gastritis rather than inadequate dietary intake[113]. Similarly, the use of proton pump inhibitors or other gastric acid suppressant drugs can lower B12 status through malabsorption owing to hypochlorhydria[113]

Accurate assessment of vitamin B12 status in order to identify and correct low or deficient status is problematic[117]. Up to 4 biomarkers are used to assess B12 status, both direct (total B12 and holotranscobalamin; holoTC) and functional (homocysteine and methylmalonic acid; MMA), but each of these has limitations. Serum total vitamin B12 is the standard test used in clinical settings, with deficiency typically identified as B12 concentrations <148 pmol/L. On the basis that 80% of total vitamin B12 concentrations is metabolically inert, the measurement of HoloTC has attracted much interest in recent years because it represents the metabolically active fraction of B12 available for cellular processes. HoloTC shows promise as a reliable biomarker of vitamin B12 status, but the influence of confounding factors needs to be more fully explored[117]. Measurement of metabolites of vitamin B12-dependent reactions can also provide useful functional indicators of B12 status. With B12 depletion, the activity of B12-dependent enzyme methionine synthase will be impaired leading to an elevation of total homocysteine that can be readily measured in plasma. Plasma homocysteine is however not specific to vitamin B12 as it is influenced by folate, other B vitamins and non-nutrient factors including renal function, limiting its use as a biomarker of B12 status[117]. Vitamin B12 depletion also leads to reduced activity of methylmalonyl CoA mutase and an accumulation of the by-product MMA which can be measured in plasma. Measurement of MMA, unlike homocysteine, provides a specific biomarker for vitamin B12. Serum MMA is invariably elevated in patients with B12 deficiency and it also provides a useful biomarker of B12 status in population-based studies[118].

In summary, accurate assessment of vitamin B12 is problematic and there is no consensus as to the best biomarker to use in clinical or research settings. It is now recommended that more than one biomarker be used to accurately diagnose B12 deficiency, and recently approaches that identify deficient status using combinations of two or more biomarkers have emerged[117]. Ensuring that vitamin B12 deficiency is diagnosed and treated in patients, and B12 status optimised in older populations generally, should be prioritised in order to ensure that any adverse health consequences of deficient and low B12 status are prevented.

Riboflavin and its metabolic interaction with vitamin B6

Riboflavin, in its cofactor forms flavin mononucleotide (FMN) and flavin dinucleotide (FAD), is essential for numerous oxidation-reduction reactions and plays a fundamental role in the metabolism of energy, certain drugs and toxins and in supporting cellular antioxidant

potential[119,120]. Riboflavin-dependent metabolism involves interaction with a number of other nutrients. Riboflavin deficiency in animals is associated with impaired iron absorption, whilst riboflavin supplementation in humans was shown to enhance circulating haemoglobin concentrations and improve the response of iron deficiency anaemia to iron therapy[121]. In addition, riboflavin involves close metabolic interaction with vitamin B6, in that it is required (as FMN) for the generation of pyridoxal 5' phosphate (PLP; the active vitamin B6 coenzyme form) in tissues from pyridoxine phosphate by pyridoxine-phosphate oxidase (PPO). Animal studies show that PPO activity is sensitive to changes in dietary riboflavin intake, with evidence that riboflavin deficiency can alter PLP levels[122]. In humans, research from our centre demonstrated the metabolic dependency of vitamin B6 on riboflavin by showing that supplementing older adults with riboflavin not only improved biomarker status of riboflavin, but also led to a significant increase in blood PLP concentrations[123].

As described earlier in this review, emerging evidence points to a novel role of riboflavin as an important modulator of blood pressure specifically in genetically at-risk individuals owing to the 677C→T polymorphism in *MTHFR*[31]. The precise biological mechanism explaining this novel gene-nutrient interaction in blood pressure is unclear at this time, however, MTHFR activity in people with the TT genotype appears to be particularly sensitive to riboflavin status[45]. One could speculate that people with the variant TT genotype who have optimal riboflavin status may have a higher capacity to replace inactivated enzyme than TT genotype individuals with low riboflavin status. Alternatively, a higher riboflavin status may prevent the FAD cofactor from leaving the active site or may allow its quick replacement, thus stabilising the variant form of the enzyme. Overall the evidence indicates that these genetically at-risk adults have higher riboflavin requirements in order to sustain normal MTHFR activity although this remains to be specifically demonstrated[45,47].

On the limited evidence available, riboflavin deficiency is a significant problem in developing countries[124]. Across the developed world also, sub-optimal riboflavin status may be widespread, but this is largely undocumented as biomarker status is rarely measured in population-based studies[125]. The UK is in fact one of the very few countries worldwide to have included a riboflavin biomarker as part of its rolling NDNS survey[126]. Much more recently Ireland, in its National Adult Nutrition Survey (NANS), has measured biomarker status of riboflavin for the first time on a population-wide basis and the results are in close agreement with those of NDNS[126,127].

In the British and Irish population-wide nutrition surveys, biomarker status of riboflavin was measured using erythrocyte glutathione activation coefficient (EGRac), widely accepted

as the gold-standard measure of status[128]. This coefficient is expressed as the ratio of the activity of the enzyme glutathione reductase in lysed red cells with and without addition of the cofactor FAD. EGRac, therefore, is a measure of glutathione reductase enzyme saturation with its riboflavin-derived cofactor; a low coefficient is generally considered to be normal, while higher values are indicative of suboptimal riboflavin status, although there is no universal agreement as to the cut-off points indicative of deficient and low status. Advantages of EGRac include stability and high sensitivity to small degrees of cofactor desaturation, while the lack of accessibility of this assay worldwide and very specific pre-analysis processing (including the need for washed red blood cells) makes this assay unfeasible in many settings[128]. Concern has been expressed regarding the large proportion of adults, as assessed in both the British and Irish population-based surveys, showing low biomarker status of riboflavin using EGRac. The functional significance of such findings is unclear however since in general, with the exception of younger women, mean dietary riboflavin intakes of British and Irish adults compared favourably with dietary reference ranges[126,127]. Elsewhere in the world (including Canada and US), the situation is much less clear as riboflavin biomarkers are not measured (by any method) in nutrition surveys.

In summary, on the limited available evidence, sub-optimal riboflavin status appears to be a more widespread problem than is generally recognised across the world because of the current reliance on dietary data only in nutrition surveys, without corresponding information on riboflavin biomarker status. There is a need to measure riboflavin biomarkers in population surveys, and to demonstrate the functional, gene-nutrient and health effects of riboflavin across the range of values, from deficient to optional biomarker status.

Summary and Conclusions

The health impacts, challenges and research priorities in relation to folate and related B vitamin status at key stages of lifecycle were reviewed using case studies to illustrate the roles of these vitamins in early, middle and late life (**Table 1**). To summarise:

Optimal maternal folate status prevents megaloblastic anaemia in mothers during pregnancy and protects against the occurrence of NTD in the child. Folic acid, the vitamin form found in fortified foods and supplements, provides a highly bioavailable source of folate. Folic acid supplementation as a policy to prevent NTD has however proven to be largely ineffective

in the UK, Ireland and elsewhere in Europe primarily because women typically start taking folic acid after the period of neural tube closure (3rd to 4th week of pregnancy) when folic acid is protective. Apart from preventing NTD, the human *in utero* environment may influence offspring brain health in the longer term via DNA methylation, which is dependent on an adequate supply of folate, but this aspect requires further investigation.

Optimal riboflavin status is required for the generation of the active form of B6 (PLP) in tissues, with some evidence that riboflavin may be the more limiting nutrient in humans with low PLP concentrations. Suboptimal riboflavin may be a widespread problem in all populations worldwide, but this is not well recognised because riboflavin status biomarkers are rarely measured. A novel gene-nutrient interaction, involving riboflavin and *MTHFR* has emerged in recent years, with important effects on blood pressure. Emerging evidence shows that targeted riboflavin supplementation could offer a personalised, non-drug treatment to lower blood pressure and improve blood pressure control in hypertensive patients with the variant *MTHFR* 677TT genotype. In a wider public health context, sub-populations worldwide with this genotype may benefit from optimising riboflavin status to prevent or delay the development of high blood pressure in middle age. This in turn could potentially reduce the risk of stroke; however, large clinical trials are required to investigate effects on stroke and other disease end-points.

Folate and vitamin B12 are particularly important in later life because they are essential for the brain to support numerous central nervous system methylation reactions involving neurotransmitter and membrane phospholipid synthesis and myelin methylation. Subclinical deficiencies of these and related B vitamins are implicated in cognitive decline and depression in older adults, while optimal B vitamin status may help in maintaining better brain health in ageing. Well-designed randomised trials are required to confirm whether improving B-vitamin status can achieve better mental and cognitive health in older adults. Any intervention with B vitamins is likely to be most effective before overt signs of neuropsychiatric disease has occurred and in people with low B vitamin status. Given some concerns regarding potentially harmful effects of folic acid at high exposure levels in older people with low vitamin B12 status, future trials should investigate the effects of intervention at low-dose intakes.

In conclusion, there are important health impacts of folate and metabolically related B vitamins through the lifecycle. There are also significant public health challenges to be overcome in order to achieve optimal status, and thus potentially prevent folate-related disease

at a population level. For governments worldwide considering the relevant policy issues, there is a need for a balanced approach, and an emphasis on maintaining an optimal status of all relevant B vitamins throughout life.

.

Figure Legends

Figure 1. (Colour online) Overview of B vitamins in One-carbon Metabolism. Abbreviations. DHF, dihydrofolate; DHFR, dihydrofolate reductase; DMNT, DNA methyltransferases; dTMP, deoxythymidine monophosphate; dUMP, deoxyuridine monophosphate; MTHFR; methylenetetrahydrofolate reductase; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine; THF, tetrahydrofolate. (Adapted from Linus Pauling Institute at https://lpi.oregonstate.edu/mic/vitamins/folate; Accessed November 2018).

References

1. Bailey LB, Stover PJ, McNulty H *et al*. (2015) Biomarkers of nutrition for development— folate review. *J Nutr* **147**, 1636S–1680S.

2. MRC Vitamin Study Research Group (1991) Prevention of neural tube defects: results of the Medical Research Council Vitamin Study. *Lancet* **338**, 131–137.

3. Czeizel AE & Dudas I (1992) Prevention of the first occurrence of neural-tube defects by periconceptional vitamin supplementation. *N Engl J Med* **327**, 1832–1835.

4. Department of Health (1992) *Folic acid and the prevention of neural tube defects. Report of an Expert Advisory Group for the Department of Health*. London.

5. CDC (1992) Recommendations for the use of folic acid to reduce the number of cases of spina bifida and other neural tube defects. *MMWR Recomm Rep* **41**, 1–7.

6. Wills L & Evans B (1938) Tropical macrocytic anaemia: its relation to pernicious anaemia. *Lancet* **ii**, 416–421.

7. Chanarin I (1985) Folate and cobalamin. *Clin Haematol* **14**, 629–641.

8. Hall MH, Pirani BBK & Campbell D (1976) The cause of the fall in serum folate in normal pregnancy. *Br J Obs Gynaecol* **83**, 132–136.

9. McNulty B, McNulty H, Marshall B *et al*. (2013) Impact of continuing folic acid after the first trimester of pregnancy: findings of a randomized trial of folic acid supplementation in the second and third trimesters. *Am J Clin Nutr* **98**, 92–98.

10. Fletcher J, Gurr A, Fellingham F *et al*. (1971) The value of folic acid supplements in pregnancy. *J Obs Gynaecol Br Commonw* **78**, 781–785.

11. Blot I, Papiernik E, Kaltwasser JP *et al*. (1981) Influence of routine administration of folic acid and iron during pregnancy. *Gynecol Obstet Invest* **12**, 294–304.

12. Homocysteine Lowering Trialists' Collaboration (2005) Dose-dependent effects of folic acid on blood concentrations of homocysteine: a meta-analysis of the randomized trials. *Am J Clin Nutr* **82**, 806–812.

13. Mills JL, Lee YJ, Conley MR *et al*. (1995) Homocysteine metabolism in pregnancies complicated by neural-tube defects. *Lancet* **345**, 149–151.

14. Felkner M, Suarez L, Canfield MA *et al*. (2009) Maternal serum homocysteine and risk for neural tube defects in a Texas-Mexico border population. *Birth Defects Res Part A - Clin Mol Teratol* **85**, 574–581.

15. Cotter AM, Molloy AM, Scott JM *et al*. (2001) Elevated plasma homocysteine in early pregnancy: A risk factor for the development of severe preeclampsia. *Am J Obstet Gynecol* **185**, 781–785.

16. Cotter AM, Molloy AM, Scott JM *et al*. (2003) Elevated plasma homocysteine in early pregnancy: a risk factor for the development of nonsevere preeclampsia. *American Journal of Obstetrics and Gynecology*, pp 391–394.

17. Goddijn-Wessel TA, Wouters MG, van de Molen EF *et al*. (1996) Hyperhomocysteinemia: a risk factor for placental abruption or infarction. *Eur J Obstet Gynecol Reprod Biol* **66**, 23–29.

18. Nelen WLDM, Blom HJ, Steegers EAP *et al*. (2000) Hyperhomocysteinemia and recurrent early pregnancy loss: a meta-analysis. *Fertil Steril* **74**, 1196–1199.

19. Vollset SE, Refsum H, Irgens LM *et al*. (2000) Plasma total homocysteine, pregnancy complications, and adverse pregnancy outcomes: the Hordaland Homocysteine Study. *Am J Clin Nutr* **71**, 962–968.

20. Caffrey A, McNulty H, Irwin RE *et al*. (2018) Maternal folate nutrition and offspring health: evidence and current controversies . *Proc Nutr Soc* [In Press].

21. Julvez J, Fortuny J, Mendez M *et al*. (2009) Maternal use of folic acid supplements during pregnancy and four-year-old neurodevelopment in a population-based birth cohort. *Paediatr Perinat Epidemiol* **23**, 199–206.

22. Veena SR, Krishnaveni G V, Srinivasan K *et al*. (2010) Higher maternal plasma folate but not vitamin B-12 concentrations during pregnancy are associated with better cognitive function scores in 9- to 10- year-old children in South India. *J Nutr* **140**, 1014–1022.

23. Polańska K, Muszyński P, Sobala W *et al*. (2015) Maternal lifestyle during pregnancy and child psychomotor development - Polish Mother and Child Cohort study. *Early Hum Dev* **91**, 317–325.

24. Pentieva K, McGarel C, McNulty BA *et al*. (2012) Effect of folic acid supplementation during pregnancy on growth and cognitive development of the offspring: a pilot follow-up investigation of children of FASSTT study participants. *Proc Nutr Soc* **71 (OCE2)**, E139 (Abstr).

25. McGarel C, Pentieva K, Strain JJ *et al*. (2015) Emerging roles for folate and related B-vitamins in brain health across the lifecycle. *Proc Nutr Soc* **74**, 46–55.

26. James P, Sajjadi S, Tomar AS *et al*. (2018) Candidate genes linking maternal nutrient exposure to offspring health via DNA methylation: a review of existing evidence in humans with specific focus on one-carbon metabolism. *Int J Epidemiol* [Epublication ahead of print version].

27. Caffrey A, Irwin RE, McNulty H *et al*. (2018) Gene-specific DNA methylation in newborns in response to folic acid supplementation during the second and third trimesters of pregnancy: epigenetic analysis from a randomized controlled trial. *Am J Clin Nutr* **107**, 566–575.

28. Ly A, Ishiguro L, Kim D *et al*. (2016) Maternal folic acid supplementation modulates DNA methylation and gene expression in the rat offspring in a gestation period-dependent and organ-specific manner. *J Nutr Biochem* **33**, 103–110.

29. Kerr MA, Livingstone B, Bates CJ *et al*. (2009) Folate, related B vitamins, and homocysteine in childhood and adolescence: potential implications for disease risk in later life. *Pediatrics* **123**, 627–635.

30. Pfeiffer CM, Hughes JP, Lacher DA *et al*. (2012) Estimation of trends in serum and RBC folate in the U.S. population from pre- to postfortification using assay-adjusted data from the NHANES 1988-2010. *J Nutr* **142**, 886–893.

31. McNulty H, Strain JJ, Hughes CF *et al*. (2017) Riboflavin, MTHFR genotype and blood pressure: a personalized approach to prevention and treatment of hypertension. *Mol Aspects Med* **53**, 2–9.

32. Lim SS, Vos T, Flaxman AD *et al*. (2012) A comparative risk assessment of burden of disease and injury attributable to 67 risk factors and risk factor clusters in 21 regions, 1990-2010: a systematic analysis for the Global Burden of Disease Study 2010. *Lancet* **380**, 2224–2260.

33. World Health Organisation (2013) *A global brief on hypertension*. WHO/DCO/WHD/2013.2.April 3.

34. Mozaffarian D, Benjamin EJ, Go AS *et al*. (2016) Heart disease and stroke statistics - 2016 update a report from the American Heart Association. *Circulation* **133**, e38–e48.

35. Lewington S, Clarke R, Qizilbash N *et al*. (2002) Age-specific relevance of usual blood pressure to vascular mortality: a meta-analysis of individual data for one million adults in 61 prospective studies. *Lancet* **360**, 1903–1913.

36. Newton-Cheh C, Johnson T, Gateva V *et al*. (2009) Genome-wide association study identifies eight loci associated with blood pressure. *Nat Genet* **41**, 666–676.

37. Ehret GB, Munroe PB, Rice KM *et al*. (2011) Genetic variants in novel pathways influence blood pressure and cardiovascular disease risk. *Nature* **478**, 103–109.

38. Yang B, Fan S, Zhi X *et al*. (2014) Associations of MTHFR gene polymorphisms with hypertension and hypertension in pregnancy: a meta-analysis from 114 studies with 15411 cases and 21970 controls. *PLoS One* **9**, e87497.

39. Holmes M V, Newcombe P, Hubacek JA *et al*. (2011) Effect modification by population dietary folate on the association between MTHFR genotype, homocysteine, and stroke risk: a meta-analysis of genetic studies and randomised trials. *Lancet* **378**, 584–594.

40. Frosst P, Blom HJ, Milos R *et al*. (1995) A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. *Nat Genet* **10**, 111–113.

41. Yamada K, Chen Z, Rozen R *et al*. (2001) Effects of common polymorphisms on the properties of recombinant human methylenetetrahydrofolate reductase. *Proc Natl Acad Sci* **98**, 14853–14858.

42. Pejchal R, Campbell E, Guenther BD *et al*. (2006) Structural perturbations in the Ala → Val polymorphism of methylenetetrahydrofolate reductase: how binding of folates may protect against inactivation. *Biochemistry* **45**, 4808–4818.

43. Molloy AM, Daly S, Mills JL *et al*. (1997) Thermolabile variant of 5,10-methylenetetrahydrofolate reductase associated with low red-cell folates: implications for folate intake recommendations. *Lancet* **349**, 1591–1593.

44. Jacques PF, Bostom AG, Williams RR *et al*. (1996) Relation between folate status, a common mutation in methylenetetrahydrofolate reductase, and plasma homocysteine concentrations. *Circulation* **93**, 7–9.

45. McNulty H, McKinley MC, Wilson B *et al*. (2002) Impaired functioning of thermolabile methylenetetrahydrofolate reductase is dependent on riboflavin status: implications for riboflavin requirements. *Am J Clin Nutr* **76**, 436–441.

46. Hustad S, Midttun Ø, Schneede J *et al*. (2007) The methylenetetrahydrofolate reductase 677C→T polymorphism as a modulator of a B-vitamin network with major effects on homocysteine metabolism. *Am J Hum Genet* **80**, 846–855.

47. McNulty H, Dowey LRC, Strain JJ *et al*. (2006) Riboflavin lowers homocysteine in individuals homozygous for the MTHFR 677C→T polymorphism. *Circulation* **113**, 74–80.

48. Horigan G, McNulty H, Ward M *et al*. (2010) Riboflavin lowers blood pressure in cardiovascular disease patients homozygous for the 677C→T polymorphism in MTHFR. *J Hypertens* **28**, 478–486.

49. Wilson CP, Ward M, McNulty H *et al*. (2012) Riboflavin offers a targeted strategy for managing hypertension in patients with the MTHFR 677TT genotype: a 4-y follow-up. *Am J Clin Nutr* **95**, 766–772.

50. Wilson CP, McNulty H, Ward M *et al*. (2013) Blood pressure in treated hypertensive individuals with the mthfr 677TT genotype is responsive to intervention with riboflavin: findings of a targeted randomized trial. *Hypertension* **61**, 1302–1308.

51. World Health Organisation (2016) *Mental health and older adults*. WHO Available at: http://www.who.int/mediacentre/factsheets/fs381/en/.

52. Prince MJ, Comas-Herrera A, Knapp M *et al*. (2016) *World Alzheimer Report 2016 Improving Healthcare for People Living with Dementia Coverage, Quality and Costs Now and in the Future*. London: Alzheimer's Disease International.

53. National Collaborating Centre for Mental Health (2010) *The Treatment and Management of Depression in Adults (Updated Editition) National Clinical Practice Guideline 90*. Leicester: The British Psychological Society and The Royal College of Psychiatrists.

54. O'Shea E & Kennelly E (2008) *The economics of mental healthcare Ireland*. NUI Galway: Mental Health Commission.

55. Calder PC, Carding SR, Christopher G *et al*. (2018) A holistic approach to healthy ageing: how can people live longer, healthier lives? *J Hum Nutr Diet* **31**, 439–450.

56. Gauthier S, Reisberg B, Zaudig M *et al*. (2006) Mild cognitive impairment. *Lancet* **367**, 1262–1270.

57. Craig R & Mindell J (2007) *Health survey for England 2005: The health of Older People*. London: The Information Centre.

58. Panza F, Frisardi V, Capurso C *et al*. (2010) Late-life depression, mild cognitive impairment, and dementia: possible continuum? *Am J Geriatr Psychiatry* **18**, 98–116.

59. Brailean A, Aartsen MJ, Muniz-Terrera G *et al*. (2017) Longitudinal associations between late-life depression dimensions and cognitive functioning: a cross-domain latent growth curve analysis. *Psychol Med* **47**, 690–702.

60. Moore K, Hughes CF, Ward M *et al*. (2018) Diet, nutrition and the ageing brain: Current evidence and new directions. *Proceedings of the Nutrition Society*, pp 152–163.

61. Carney MWP (1967) Serum folate values in 423 psychiatric patients. *Br Med J* **4**, 512–516.

62. Reynolds EH, Preece JM, Bailey J *et al*. (1970) Folate deficiency in depressive illness. *Br J Psychiatry* **117**, 287–292.

63. Strachan RW & Henderson JG (1965) Psychiatric syndromes due to avitaminosis B 12

with normal blood and marrow. *Q J Med* **34**, 303–317.

64. Shorvon SD, Carney MWP, Chanarin I *et al*. (1980) The neuropsychiatry of megaloblastic anaemia. *Br Med J* **281**, 1036–1038.

65. Reynolds E (2006) Vitamin B12, folic acid, and the nervous system. *Lancet Neurol* **5**, 949–960.

66. Selhub J, Bagley LC, Miller J *et al*. (2000) B vitamins, homocysteine, and neurocognitive function in the elderly. *Am J Clin Nutr* **71**, 614S–620S.

67. Bottiglieri T, Laundy M, Crellin R *et al*. (2000) Homocysteine, folate, methylation, and monoamine metabolism in depression. *J Neurol Neurosurg Psychiatry* **69**, 228–232.

68. Smith AD *&* Refsum H (2016) Homocysteine, B-vitamins, and cognitive impairment. *Annu Rev Nutr* **36**, 211–239.

69. Carney MW, Ravindran A, Rinsler MG *et al*. (1982) Thiamine, riboflavin and pyridoxine deficiency in psychiatric in-patients. *Br J Psychiatry* **141**, 271–272.

70. Porter K, Hoey L, Hughes CF *et al*. (2016) Causes, consequences and public health implications of low B-vitamin status in ageing. *Nutrients* **8**, 1–29.

71. McMahon JA, Green TJ, Skeaff CM *et al*. (2006) A controlled trial of homocysteine lowering and cognitive performance. *N Engl J Med* **354**, 2764–2772.

72. Durga J, van Boxtel MP, Schouten EG *et al*. (2007) Effect of 3-year folic acid supplementation on cognitive function in older adults in the FACIT trial: a randomised, double blind, controlled trial. *Lancet* **369**, 208–216.

73. De Jager CA, Oulhaj A, Jacoby R *et al*. (2012) Cognitive and clinical outcomes of homocysteine-lowering B-vitamin treatment in mild cognitive impairment: a randomized controlled trial. *Int J Geriatr Psychiatry* **27**, 592–600.

74. Smith AD, Smith SM, de Jager CA *et al*. (2010) Homocysteine-lowering by B-vitamins slows the rate of accelerated brain atrophy in mild cognitive impairment: a randomized controlled trial. *PLoS One* **5**, e12244.

75. Douaud G, Refsum H, de Jager CA *et al*. (2013) Preventing Alzheimer's disease-related gray matter atrophy by B-vitamin treatment. *Proc Natl Acad Sci* **110**, 9523–9528.

76. Clarke R, Bennett D, Parish S *et al*. (2014) Effects of homocysteine lowering with B vitamins on cognitive aging: meta-analysis of 11 trials with cognitive data on 22,000 individuals. *Am J Clin Nutr* **100**, 657–666.

77. Garrard P *&* Jacoby R (2015) B-vitamin trials meta-analysis: less than meets the eye. *Am J Clin Nutr* **101**, 414–415.

78. Smith AD, De Jager CA, Refsum H *et al*. (2015) Homocysteine lowering, B vitamins, and cognitive aging. *Am J Clin Nutr* **101**, 415–416.

79. Gilbody S, Lightfoot T *&* Sheldon T (2007) Is low folate a risk factor for depression? A meta-analysis and exploration of heterogeneity. *J Epidemiol Community Health* **61**, 631–637.

80. Kim J-M, Stewart R, Kim S-W *et al*. (2008) Predictive value of folate, vitamin B12 and homocysteine levels in late-life depression. *Br J Psychiatry* **192**, 268–274.

81. Ng T-P, Feng L, Niti M *et al*. (2009) Folate, vitamin B12, homocysteine, and depressive symptoms in a population sample of older Chinese adults. *J Am Geriatr Soc* **57**, 871–876.

82. Robinson DJ, O'Luanaigh C, Tehee E *et al*. (2011) Associations between holotranscobalamin, vitamin B12, homocysteine and depressive symptoms in community-dwelling elders. *Int J Geriatr Psychiatry* **26**, 307–313.

83. Merete C, Tucker KL & Falcon LM (2008) Vitamin B6 is associated with depressive symptomatology in Massachusetts elders. *J Am Coll Nutr* **27**, 421–427.

84. Skarupski KA, Tangney C, Li H *et al*. (2010) Longitudinal association of vitamin B-6, folate, and vitamin B-12 with depressive symptoms among older adults over time. *Am J Clin Nutr* **92**, 330–335.

85. Taylor MJ, Carney SM, Goodwin GM *et al*. (2004) Folate for depressive disorders: systematic review and meta-analysis of randomized controlled trials. *J Psychopharmacol* **18**, 251–256.

86. Almeida OP, Ford AH & Flicker L (2015) Systematic review and meta-analysis of randomized placebo-controlled trials of folate and vitamin B12 for depression. *Int Psychogeriatrics* **27**, 727–737.

87. McNulty H and Pentieva K (2010) Folate bioavailability. In: Bailey L.B, editor. *Folate in Health and Disease*, 2nd ed. Boca Raton (FL): CRC Press, Taylor and Francis Group; pp.25-47.

88. McKillop DJ, McNulty H, Scott JM *et al*. (2006) The rate of intestinal absorption of natural food folates is not related to the extent of folate conjugation. *Am J Clin Nutr* **84**, 167–173.

89. McKillop DJ, Pentieva K, Daly D *et al*. (2002) The effect of different cooking methods on folate retention in various foods that are amongst the major contributors to folate intake in the UK diet. *Br J Nutr* **88**, 681.

90. Tighe P, Ward M, McNulty H *et al*. (2011) A dose-finding trial of the effect of long-term folic acid intervention: implications for food fortification policy. *Am J Clin Nutr* **93**, 11–18.

91. Duffy ME, Hoey L, Hughes CF *et al*. (2014) Biomarker responses to folic acid intervention in healthy adults: a meta-analysis of randomized controlled trials. *Am J Clin Nutr* **99**, 96–106.

92. Gibson RS (2005) Assessment of folate and vitamin B12 status. *Principles of Nutritional Assessment. 2nd Ed.* New York: Oxford University Press, pp 595–640.

93. Wu A, Chanarin I, Slavin G *et al*. (1975) Folate deficiency in the alcoholic—its relationship to clinical and haematological abnormalities, liver disease and folate stores. *Br J Haematol* **29**, 469–478.

94. Shane B (2011) Folate status assessment history: implications for measurement of biomarkers in NHANES. *American Journal of Clinical Nutrition*, p 337S–342S.

95. IOM (1998) Folate. In*: Dietary Reference Intakes for Thiamin, Riboflavin, Niacin, Vitamin B6, Folate, Vitamin B12, Pantothenic Acid, Biotin, and Choline* . Washington: National Academy Press, pp 196–305.

96. EFSA NDA Panel (2014) Scientific Opinion on Dietary Reference Values for folate. *EFSA J* **12**, 3893.

97. Cuskelly GJ, McNulty H & Scott JM (1996) Effect of increasing dietary folate on red-cell folate: implications for prevention of neural tube defects. *Lancet* **347**, 657–659.

98. McNulty B, Pentieva K, Marshall B *et al*. (2011) Womens compliance with current folic acid recommendations and achievement of optimal vitamin status for preventing neural tube defects. *Hum Reprod* **26**, 1530–1536.

99. Daly L., Kirke PM, Molloy A *et al*. (1995) Folate levels and nerual tube defects. *JAMA* **274**, 1698–1702.

100. Hoey L, McNulty H, Askin N *et al*. (2007) Effect of a voluntary food fortification policy on folate, related B vitamin status, and homocysteine in healthy adults. *Am J Clin Nutr* **86**, 1405–1413.

101. Hopkins SM, Gibney MJ, Nugent AP *et al*. (2015) Impact of voluntary fortification and supplement use on dietary intakes and biomarker status of folate and vitamin B-12 in Irish adults. *Am J Clin Nutr* **101**, 1163–1172.

102. Williams J, Mai CT, Mulinare J *et al*. (2015) Updated estimates of neural tube defects prevented by mandatory folic acid fortification-United States, 1995-2011. *MMWR Morb Mortal Wkly Rep* **64**, 1–5.

103. De Wals P, Tairou F, Van Allen MI *et al*. (2007) Reduction in neural-tube defects after folic acid fortification in Canada. *N Engl J Med* **357**, 135–142.

104. Cortés F, Mellado C, Pardo RA *et al*. (2012) Wheat flour fortification with folic acid: changes in neural tube defects rates in Chile. *Am J Med Genet Part A* **158A**, 1885–1890.

105. FSAI (2016) *Report of the Scientific Committee of the Food Safety Authority of Ireland: Update report on Folic Acid and the Prevention of Birth Defects in Ireland*. Food Safety Authority of Ireland (FSAI): Dublin. Available at: www.fsai.ie/news_centre/press_releases/folic_acid_report_04052016.html.

106. SACN (2017) *Folic acid: updated recommendations issued by the Scientific Advisory Committee on Nutrition (SACN)*. Public Health England: London.

107. Khoshnood B, Loane M, Walle H De *et al*. (2015) Long term trends in prevalence of neural tube defects in Europe: population based study. *Br Med J* **351**, h5949.

108. McDonnell R, Delany V, O'Mahony MT *et al*. (2015) Neural tube defects in the Republic of Ireland in 2009-11. *J Public Health (Bangkok)* **37**, 57–63.

109. Morris MS, Jacques PF, Rosenberg IH *et al*. (2010) Circulating unmetabolized folic acid and 5-methyltetrahydrofolate in relation to anemia, macrocytosis, and cognitive test performance in American seniors. *Am J Clin Nutr* **91**, 1733–1744.

110. Cole BF, Baron JA, Sandler RS *et al*. (2007) Folic Acid for the Prevention of Colorectal Adenomas. *JAMA* **297**, 2351–2359.

111. Vollset SE, Clarke R, Lewington S *et al*. (2013) Effects of folic acid on overall and site-specific cancer incidence during the randomised trials: meta-analyses of data on 50 000 individuals. *Lancet* **381**, 1029–1036.

112. Pentieva K, Selhub J, Paul L *et al*. (2016) Evidence from a randomized trial that

exposure to supplemental folic acid at recommended levels during pregnancy does not lead to increased unmetabolized folic acid concentrations in maternal or cord blood. *J Nutr* **146**, 494–500.

113. Hughes CF, Ward M, Hoey L *et al*. (2013) Vitamin B12 and ageing: current issues and interaction with folate. *Ann Clin Biochem* **50**, 315–329.

114. Stabler SP (2013) Vitamin B12 deficiency. *New England Journal of Medicine*, pp 2040–2042.

115. Carmel R (2011) Biomarkers of cobalamin (vitamin B-12) status in the epidemiologic setting: a critical overview of context, applications, and performance characteristics of cobalamin, methylmalonic acid, and holotranscobalamin II. *Am J Clin Nutr* **94**, 348S–358S.

116. Carmel R (2013) Diagnosis and management of clinical and subclinical cobalamin deficiencies: why controversies persist in the age of sensitive metabolic testing. *Biochimie* **95**, 1047–1055.

117. Hughes CF & McNulty H (2018) Assessing biomarker status of vitamin B12 in the laboratory: no simple solution. *Ann Clin Biochem* **55**, 188–189.

118. Valente E, Scott JM, Ueland PM *et al*. (2011) Diagnostic accuracy of holotranscobalamin, methylmalonic acid, serum cobalamin, and other indicators of tissue vitamin B12 status in the elderly. *Clin Chem* **57**, 856–863.

119. McCormick DB (2006) Riboflavin. *In Modern Nutrition in Health and Disease*, ed ME Shills . New York: Williams & Wilkins, pp 391–399.

120. Powers HJ (1999) Current knowledge concerning optimum nutritional status of riboflavin, niacin and pyridoxine. *Proceedings of the Nutrition Society*, pp 435–440.

121. Powers HJ, Hill MH, Mushtaq S *et al*. (2011) Correcting a marginal riboflavin deficiency improves hematologic status in young women in the United Kingdom (RIBOFEM). *Am J Clin Nutr* **93**, 1274–1284.

122. Rasmussen KM, Barsa PM & McCormick DB (1979) Pyridoxamine (pyridoxine) 5'-phosphate oxidase activity in rat tissues during development of riboflavin or pyridoxine deficiency. *Proc Soc Exp Biol Med* **161**, 527–530.

123. Madigan SM, Tracey F, McNulty H *et al*. (1998) Riboflavin and vitamin B-6 intakes and status and biochemical response to riboflavin supplementation in free-living elderly people. *Am J Clin Nutr* **68**, 389–395.

124. Whitfield KC, Karakochuk CD, Liu Y *et al*. (2015) Poor thiamin and riboflavin status is common among women of childbearing age in rural and urban Cambodia. *J Nutr* **145**, 628–633.

125. McAuley E, McNulty H, Hughes C *et al*. (2016) Riboflavin status, MTHFR genotype and blood pressure: current evidence and implications for personalised nutrition. *Proc Nutr Soc* **75**, 405–414.

126. National Diet and Nutrition Survey UK (2016) *Results from years 5-6 (combined) of the rolling programme (2012/13-2013/14)*. Available at www.gov.uk/government/statistics/ndns-results-from-years-5-and-6-combined. Accessed 20th November 2018.

127. Kehoe L, Walton J, Hopkins SM *et al*. (2018) Intake, status and dietary sources of riboflavin in a representative sample of Irish adults aged 18–90 years. *Proc Nutr Soc* **77(OCE3)**, E66.

128. Hoey L, McNulty H & Strain JJ (2009) Studies of biomarker responses to intervention with riboflavin: a systematic review. *American Journal of Clinical Nutrition*, p 1960S–1980S.

Table 1. Health impacts and challenges in relation to folate and related B vitamins at key stages of the lifecycle

	Health Impact	Strength of Evidence	Challenge or Research Gap
Folate in pregnancy and early life	• Peri-conceptional folic acid use by mothers protects against the occurrence of NTD in the child.	Conclusive	Women typically start taking supplements after the period of neural tube closure when folic acid is protective. Mandatory folic acid food fortification is highly effective but controversial.
	• Maternal folate may influence brain development and subsequent cognitive function in the offspring.	New	More epigenetic studies needed to investigate effect of folic acid in humans.
Riboflavin in middle life	• Optimal riboflavin is required to generate the active form of B6 (PLP) in tissues	Established functional role	Sub-optimal riboflavin status occurs more commonly than is generally recognised.
	• Riboflavin interacts with MTHFR within one-carbon metabolism to influence blood pressure	New	Mechanistic studies needed to explain role of MTHFR in blood pressure and modulating effect of riboflavin.
	• Supplemental riboflavin can lower blood pressure if targeted at adults genetically at risk of developing hypertension owing to the *MTHFR* C677T polymorphism	Convincing	More RCTs needed to investigate effect of riboflavin in genetically at risk adults with and without existing hypertension and in pregnancy.
Folate, vitamin B12 and related B vitamins in later life	• Folate and B12 essential for functioning of central nervous system	Established functional role	More RCTs needed to confirm whether improving B-vitamin status can achieve better mental and cognitive health in older adults. Such trials should target those with suboptimal B vitamin status and test effects of low-dose B vitamin intervention.
	• Optimal B vitamin status may help in maintaining better brain health in ageing	Convincing	