Proceedings of the Nutrition Society (2008), **67**, 232–237 doi:10.1017/S0029665108007076

The Summer Meeting of the Nutrition Society, hosted by the Irish Section, was held at the University of Ulster, Coleraine on 16–19 July 2007

Symposium on 'Diet and CVD'

Homocysteine, B-vitamins and CVD

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There is considerable interest in plasma homocysteine (tHcy) as a CVD risk factor. Although the secondary prevention trials published to date have been inconclusive in confirming a benefit of tHcy-lowering treatment with B-vitamins on CVD events generally, such studies are widely recognised to have been insufficiently powered to detect a significant effect for the predicted magnitude of association between tHcy and heart disease risk, and therefore cannot be interpreted as evidence that no relationship exists. In fact, a recent meta-analysis of clinical trials has confirmed that folic acid supplementation reduces the risk of stroke, particularly in individuals without a history of stroke. Evidence supporting a causal relationship between elevated tHcy and heart disease also comes from genetic studies. The most important genetic determinant of tHcy in the general population is the common C677T variant in methylenetetrahydrofolate reductase (MTHFR) that results in higher tHcy. Individuals with the homozygous mutant (TT) genotype have a significantly higher (14–21 %) risk of heart disease. Plasma tHcy is very responsive to intervention with the B-vitamins required for its metabolism, in particular folic acid, and to a lesser extent vitamins B_{12} and B_6. Thus, although primarily aimed at reducing neural-tube defects, folic acid fortification may have an important role in the primary prevention of CVD via tHcy lowering. Besides folate, riboflavin is required as a cofactor for MTHFR and enhanced riboflavin status results in a marked lowering in tHcy specifically in individuals with the TT genotype, presumably by neutralising the variant form of the enzyme. About 10 % of the UK and Irish populations have the TT genotype. In the present paper the potential role of folate and related B-vitamins in the primary prevention of CVD and the implications for nutrition policy are explored.

B-vitamins: Folate: Homocysteine: CVD

Elevated homocysteine as a risk factor for CVD

Evidence from numerous prospective and retrospective case–control studies has emerged in recent years to link elevated plasma homocysteine (tHcy) levels with an increased risk of CVD. Meta-analyses of prospective studies have predicted that lowering tHcy by 3 µmol/l (or a reduction of 25 % based on an average tHcy of 12 µmol/l) would reduce the risk of heart disease by 11–16 % and stroke by 19–24 %[1,2]. Although none of the secondary prevention trials published in more recent years have been able to confirm the benefit of tHcy-lowering therapy on CVD events generally[3–5], it should not be assumed that no relationship exists. It is now generally recognised that these trials lacked sufficient statistical power to detect an effect for the predicted magnitude of association between tHcy and heart disease[6]. In support of this viewpoint, a clear benefit in reducing stroke was shown in one of the previously mentioned 'negative' trials, although this result was not explicit in the conclusions[4]. Moreover, evidence just published from a meta-analysis of clinical trials in relation to stroke outcome has confirmed that folic acid supplementation reduces the risk of stroke by 18 % overall, by 29 % in trials with a treatment duration of >36 months and by 25 % in those trials involving individuals without a history of stroke[7]. Such evidence is consistent with the

Abbreviations: MTHFR, methylenetetrahydrofolate reductase; tHcy, plasma homocysteine.

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findings of a population-based study that has shown that the temporal decline in stroke-related mortality in the USA and Canada coincided with the introduction of folic acid fortification[8]. Thus, the case for tHcy as a risk factor in CVD is stronger for stroke than for heart disease, and possibly strongest for the primary prevention of stroke.

Another important line of evidence supporting a causal relationship between elevated tHcy and heart disease comes from genetic studies. The common C677T variant in the gene coding for the folate-metabolising enzyme methylenetetrahydrofolate reductase (MTHFR) is the most important genetic determinant of tHcy in the general population. The homozygous mutant TT genotype for the MTHFR C677T polymorphism typically affects about 10% of individuals worldwide but can be as high as 26% (southern Italy) and 32% (Mexico) in some areas[9]. MTHFR is required for the formation of 5-methyltetrahydrofolate, which in turn is required to convert homocysteine to methionine. Individuals with the TT genotype have reduced MTHFR activity, which results in impaired folate metabolism and elevated tHcy levels[10]. Three recent meta-analyses involving >25 000 cases have examined the impact on heart disease risk of this genetic variant and have shown an overall significantly higher (14–21%) risk of heart disease in individuals with this polymorphism as compared with those without this polymorphism[2,11,12]. The trend toward increasing risk of disease among individuals with no (CC genotype), one (CT genotype) or two copies (TT genotype) of the defective gene is consistent with the increasing gradient in tHcy typically found among the three genotypes (as would be expected if there is a causal relationship between elevated tHcy and risk of disease). However, analysis of the OR among countries shows a large geographical variation in the association between this polymorphism and heart disease risk[11,12].

Homocysteine-lowering with B-vitamins

The B-vitamins folate, vitamin B_{12} and vitamin B_6 are required for homocysteine metabolism and several studies have examined their potential to lower tHcy.

Folate

Folate, the focus of much current debate on food fortification worldwide, is the major determinant of tHcy. The first meta-analysis to assess the tHcy-lowering effect of folic acid has concluded that doses in the range of 0·5 to 5 mg/d could lower tHcy by about 20–25%[13]. The effect of doses <0·5 mg/d is much less clear but is arguably the more relevant question for emerging food fortification policy. Some earlier evidence has suggested that doses in the range of 0·2–0·4 mg/d can achieve a maximum reduction in tHcy in young healthy populations[14,15], while another study has shown that doses as high as 0·8 mg folic acid/d are required for maximal lowering of tHcy in those individuals with established heart disease[16]. Similarly, a meta-analysis of twenty-five randomised trials involving 2596 subjects (both healthy and patient groups) has concluded that a daily dose of 0·8 mg/d is required to achieve a maximal reduction in tHcy, while doses of 0·2 and 0·4 mg/d are associated with only 60 and 90% of this maximal effect respectively[17]. However, one limitation of the latter meta-analysis is treatment duration; of the twenty-five studies examined the treatment in eighteen was of ≤8 weeks duration, six were of 12 weeks duration and only one was of long-term duration (i.e. 6 months). Some preliminary evidence suggests that longer treatment duration may be necessary to allow a complete tHcy-lowering effect to be observed in response to the lower doses of folic acid[18]. In addition, details on subject compliance were rarely reported in the original studies that examined the effect of different doses[17], but this information is critical to the interpretation of the results because incomplete compliance by participants may result in an underestimation of the full extent of the tHcy response to a given dose. Food fortification, unlike supplementation, automatically provides maximal participant compliance; therefore, ensuring high subject compliance to the treatments should be a key aspect of any dose-finding study aimed at developing food fortification policies for the prevention of CVD.

Thus, some published reports may have overestimated the folic acid dose required to achieve maximal tHcy lowering, because of either incomplete or unmonitored subject compliance or, more importantly, an intervention period that was too short to observe the full extent of the tHcy response to lower folic acid doses in the range 0·2–0·4 mg/d. Clearly, the issue as to the lowest dose of folic acid associated with a maximum reduction in tHcy remains controversial and requires further investigation, especially in light of recent reports that highlight potential adverse effects of overexposure to higher intakes of folic acid[19,20].

Vitamin B_{12}

In addition to folate, the enzyme methionine synthase involved in the remethylation of homocysteine to methionine requires vitamin B_{12} as cofactor. Although a number of studies in healthy populations and in patients have investigated the effect of various doses of folic acid supplementation in lowering tHcy, few have examined the independent effects of vitamin B_{12}. In those studies that have administered low-dose vitamin B_{12} as a separate treatment, the response of vitamin B_{12} biomarkers was the primary outcome and the tHcy response was measured as a secondary objective[21,22]. Furthermore, the study populations were selected for mild vitamin B_{12} deficiency and were not pretreated with folic acid before vitamin B_{12} supplementation. Of even greater importance, vitamin B_{12} was not administered with food, the presence of which is required to stimulate the normal vitamin B_{12} absorptive mechanisms. The tHcy-lowering response observed may therefore have been confounded by differences in folate status or an incomplete absorption of the administered dose.

The reason for the limited number of intervention studies with vitamin B_{12} is that this vitamin is generally considered to be a far-less-effective determinant of tHcy concentrations compared with folate. However, evidence from a study of healthy subjects supplemented with

https://doi.org/10.1017/S0029665108007076

low-dose folic acid[23] and from studies in the era of mandatory folic acid fortification of cereal grains in the USA[24–26] shows that vitamin B_{12} becomes the main nutritional determinant of tHcy once folate status is optimised. Furthermore, meta-analyses of intervention studies that have examined the effect of B-vitamins on tHcy lowering have shown that including vitamin B_{12} along with folate produces an additional one-third (7 %) lowering of tHcy above that achieved with folate alone (typically 25 % lowering[13,17]). As the relationship between tHcy and CVD is graded, any small but significant further decrease in tHcy concentrations could be predicted to confer an additional benefit in terms of CVD risk. Although the results of the randomised clinical trials published so far are disappointing in that they have failed to show a benefit of tHcy-lowering therapy on CVD events generally, re-analysis of data from the negative Vitamin Intervention for Stroke Prevention trial[3] has shown the importance of vitamin B_{12} in relation to tHcy and CVD risk. When subjects who were considered to receive no benefit from vitamin therapy were excluded (i.e. patients believed to be on vitamin B_{12} supplements and patients with renal failure), the group receiving the high-dose therapy were found to have a 21 % reduced risk of CVD events compared with the low-dose group[27]. Those patients with higher baseline vitamin B_{12} levels who received the high-dose B-vitamin therapy during intervention were shown to have the best outcome for survival free of a CVD event[27]. Thus, the inclusion of vitamin B_{12} along with folic acid may be a more effective therapy for reducing the risk of CVD via tHcy lowering than folic acid alone. Such a therapy could be of particular benefit to older adults, as suboptimal vitamin B_{12} status (mostly as a result of food-bound vitamin B_{12} malabsorption) is highly prevalent[28] and CVD is a common cause of morbidity and mortality in this subgroup of the population. However, further supplementation studies with vitamin B_{12} are required to determine the optimal dose for lowering tHcy.

Vitamin B_6

The transulfuration of homocysteine to cysteine by cystathione β-synthase requires vitamin B_6 (pyridoxal phosphate) as a cofactor, and studies have considered the potential of vitamin B_6 to lower tHcy. Although the literature generally suggests either that vitamin B_6 does not have a tHcy-lowering effect[13,17] or that it lowers tHcy only in exceptional circumstances (e.g. in pyridoxine-responsive homocystinuria or in patients with severe vitamin B_6 deficiency), there is some evidence of a small but significant tHcy response (lowered by 7·5 %) to low-dose vitamin B_6[29]. However, the latter intervention was conducted in healthy older subjects whose status of both folate and riboflavin was optimised before intervention with vitamin B_6; this study design may explain why a significant tHcy response was observed in this study but not in other studies[13,17].

Apart from its role in homocysteine metabolism, vitamin B_6 is also required for the metabolism of *n*-3 PUFA[30]. Thus, independently of any tHcy-lowering effect, some studies have investigated the potential ability of vitamin B_6 to suppress some of the underlying mechanisms involved in the atherosclerotic process such as platelet aggregation[31] and the proliferation of endothelial cells[32]. The findings so far from various studies are inconsistent. The results from the Nurses' Health Study of 80 000 women followed for 14 years have demonstrated that a higher dietary intake of vitamin B_6 (i.e. >3 mg/d) is associated with a decreased risk of heart disease[33]. Furthermore, retrospective and prospective case–control studies have related low plasma concentrations of vitamin B_6-status indices to increased risk of stroke, peripheral vascular disease and coronary artery disease[34–37]. However, after adjusting for established CVD risk factors the relationship between vitamin B_6 indices and CVD in some of these studies was found to be no longer significant[34,38]. More-recent investigations have reported that low vitamin B_6 status is related to chronic inflammation[39], which in turn is known to promote atherosclerosis and the development of CVD. This finding might suggest that low vitamin B_6 status is not an independent risk factor for CVD and stroke but merely a marker of chronic inflammation. Evidence as to whether low vitamin B_6 status is a causal factor for CVD can only be provided by controlled intervention studies. So far, the results of four randomised controlled trials that included vitamin B_6 supplementation (as an independent treatment or in combination with folic acid and vitamin B_{12}) for ≤5 years in patients with pre-existing CVD have been published, but they have failed to detect any impact of vitamin B_6 on the risk of a recurrent cardiovascular event[40]. However, it is widely acknowledged that these trials were underpowered, and even after combining them it appears that there is insufficient power to detect a significant effect on the risk of CVD[40]. Moreover, the doses of vitamin B_6 used in these trials were pharmacological levels (40 mg/d), the long-term effects of which remain unknown. Finally, these trials were conducted in individuals with pre-existing disease and it is not known whether vitamin B_6 would have a role in the primary prevention of CVD. Carefully-designed and sufficiently-powered long-term intervention studies examining the effect on CVD of low doses of vitamin B_6 (within the dietary range) are clearly warranted.

Gene–nutrient interactions

The typical phenotype associated with homozygosity for the MTHFR C677T polymorphism is elevated tHcy levels[10]. Individuals with the TT genotype are considered to have increased dietary folate requirements on the basis that they have lower erythrocyte folate levels compared with those without this genetic variant[41], and the increase in tHcy is found to be most marked among those with lower folate status[42,43].

A fourth, much overlooked, B-vitamin is also involved in homocysteine metabolism. In addition to folate, riboflavin (in its co-enzymic form FAD) is required as a cofactor for the MTHFR enzyme. The reduced activity of the MTHFR 677TT variant of MTHFR has been shown to result from the inappropriate loss of its FAD cofactor[44]. Observational studies in human subjects that have

investigated the relationship between riboflavin status and tHcy in individuals with different MTHFR genotypes have reported somewhat inconsistent results[45–47]. In the USA an association between riboflavin status and tHcy has been reported in individuals with the TT genotype in the Framingham Cohort[45], but this association is confined only to those with the TT genotype and low folate status; thus, riboflavin does not seem to be the limiting nutrient. The two studies conducted in healthy European populations (Norway and Northern Ireland), however, have both identified riboflavin as an important determinant of tHcy among individuals with the TT genotype and have indicated that this effect is not explained by folate[46,47]. The inconsistency between studies is probably explained by the mandatory fortification of flour with riboflavin in the USA, which would have the effect of optimising riboflavin status in the general US population, thereby reducing the extent to which riboflavin is found to be a limiting nutrient in determining tHcy levels in individuals with the TT genotype. Recent results that show a genotype-specific response of tHcy to riboflavin supplementation now confirm that riboflavin is an independent modifier of tHcy in individuals with the TT genotype. Significant lowering of tHcy in response to riboflavin supplementation was observed in healthy individuals with the TT genotype, with levels decreasing by as much as 22% overall, and markedly so (by 40%) in those with lower riboflavin status at baseline[48]. No tHcy response to intervention was observed in those with CC or CT genotypes, despite a significant improvement in riboflavin status in both cases and the pre-selection of subjects with suboptimal riboflavin status at baseline. The lack of a tHcy-lowering effect of riboflavin in the absence of this polymorphism was observed previously, in an earlier intervention study of healthy elderly individuals who were also pre-screened for suboptimal riboflavin status but not for MTHFR genotype[49]. The responsiveness of tHcy to riboflavin is therefore specific to individuals with the MTHFR 677TT genotype and represents a new gene–nutrient interaction.

The inconsistencies in the literature as to whether the MTHFR C677T polymorphism is associated with a higher risk of heart disease may also relate to the role of riboflavin. Although meta-analyses have reported an overall 14–21% higher risk of heart disease in individuals with the TT genotype[2,11,12], analysis of the OR between continents shows that the excess heart disease risk associated with this polymorphism is significant in Europe but not in North America. This geographical variation is generally assumed to be the result of differences in folate status[11,12], while riboflavin, the cofactor for MTHFR, is generally overlooked as a potential modulator of the disease risk associated with this polymorphism. However, the policy of mandatory riboflavin fortification has existed for >50 years in the USA, and given the marked genotype-specific decrease in tHcy with riboflavin intervention[48], it is not unreasonable to suggest that it might also modulate heart disease risk in this subpopulation with genetic predisposition to elevated tHcy. Thus, the reported differences among countries as to whether this polymorphism represents an increased risk of heart disease may relate not only to differences in folate as commonly suggested[11,12], but also to differences in the prevailing riboflavin status. It could be predicted that individuals with the TT genotype who also have low riboflavin status would have an excess risk of heart disease, whereas with optimal riboflavin status they would not carry the expected risk. Notably, support for this viewpoint is offered by very recent evidence of higher blood pressure at baseline and a marked lowering of both systolic and diastolic blood pressure in response to riboflavin intervention specifically in patients with the TT genotype[50].

Implications for nutrition policy

Folate is the major determinant of tHcy. As has been experienced in the USA since its introduction[51], there is no doubt that folic acid fortification, if introduced in a population, would have a major tHcy-lowering effect irrespective of genotype. Vitamins B_{12} and B_6 also play key roles in homocysteine metabolism, with evidence showing that both vitamins may be important determinants of tHcy, particularly when folate status has been optimised[23,24,29]. Thus, all three vitamins could arguably be included in any fortification policy aimed at lowering tHcy in the general population, with potential benefits in preventing CVD and possibly stroke in particular[7]. Emerging food policy in different countries should also consider the riboflavin requirements of individuals homozygous for the common MTHFR C677T polymorphism. Riboflavin status appears to be a potent modulator of the expected (high tHcy) phenotype among individuals with the TT genotype. Of greater importance, new evidence shows marked lowering of blood pressure in response to riboflavin intervention specifically in patients with the TT genotype, which may or may not be independent of the tHcy-lowering effect of riboflavin also seen only in the TT genotype[48,50]. Importantly, the fact that these effects of riboflavin are achievable with a very modest increase in riboflavin intake (1·6 mg/d) suggests that there are important implications for dietary riboflavin requirements in individuals with this common genetic variant and for food-fortification policy aimed at the primary prevention of CVD. In order to cover the needs of appreciable subgroups (3–32%) of populations worldwide with this genotype[7] riboflavin may need to be considered for inclusion together with folic acid in fortification programmes under discussion.

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https://doi.org/10.1017/S0029665108007076