Cardiovascular Calcification and Bone: A Comparison of the Effects of Homocysteine and Dietary and Serum B Vitamins

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Abstract

Several studies show the co-existence of cardiovascular calcification and osteoporosis and we have sought to determine whether dietary factors may be associated. In this review of B vitamins (principally B6, B12 and folate) and homocysteine, elevated homocysteine, a known risk factor for Alzheimer’s disease and stroke, proved also to be a significant risk factor for the presence and extent of arterial, but not valvular, calcification, with a plasma concentration threshold of 12 μmol/L. The MTHFR reductase C677T genotype, although associated with homocysteine concentrations, was not an independent predictor of arterial calcification. Homocysteine is also associated with osteoporosis and bone fracture but the lack of association with BMD may be because homocysteine interferes with collagen cross-linking, causing fragility, which increases fracture risk but does not affect BMD. Although trials of B vitamins consistently demonstrate an ability to lower homocysteine, there is only one arterial calcification observational study, which showed a lower carotid calcification score with plasma folate concentrations >39.4nmol/l. There are more studies of supplementation of B vitamins and bone, where they have proved beneficial, particularly with elevated homocysteine in osteoporosis. Homocysteine appears to act principally through its pro-oxidant properties and many of its effects may be inhibited by supplementing antioxidants rather than B vitamins. This suggests that although B vitamins can prevent homocysteine concentrations from rising or rising further, once its pro-oxidant effects have been initiated, then dietary antioxidants should be increased as well. We recommend that trials of B vitamins and antioxidants should be carried out in patients with arterial calcification and osteoporosis.

INTRODUCTION

The role of the water-soluble B vitamins is principally energy production, cell metabolism and nerve function. It is thought likely that a further function of certain B vitamins is to lower homocysteine, a sulphur-containing amino acid and excitatory neurotransmitter produced naturally in the body, whose metabolism is implicated in two biochemical pathways: remethylation to methionine, which requires folic acid and vitamin B12 coenzymes, and trans-sulphuration to cystathionine, which requires pyridoxal-5’-phosphate, the vitamin B6 coenzyme. The two pathways are coordinated by S-adenosylmethionine which acts as an allosteric inhibitor of the methylenetetrahydrofolate reductase (MTHFR) enzyme and as an activator of cystathionine beta-synthase (CBS) [1-4]. Osteoporosis and atherosclerosis are leading causes of morbidity and mortality in the Western world. Although these conditions commonly co-occur in older adults, growing evidence suggests an association between vascular calcification and skeletal fragility that is independent of age and other shared risk factors. Postmenopausal women with the greatest bone loss have the greatest progression of vascular calcification [5,6], and the incidence of cardiovascular events is greater in women with lower bone mass [7] and in men with higher levels of bone resorption [8-10]. Vitamin B6 (pyridoxine), is found in whole grains, fortified cereals, liver and beans, vitamin B12 (cobalamin) is found in meat, fish, shell fish, cheese and eggs, and folic acid is found in green leafy vegetables, wholegrain, nuts and fortified cereals [11].

Association of homocysteine with CV calcification

A 2007 systematic review of investigations of asymptomatic subjects revealed a significant positive association between elevated levels of homocysteine and presence of coronary artery calcification (CAC) and other measures of subclinical atherosclerosis in most studies [12]. These findings have generally been confirmed in later studies, which show a positive association between plasma homocysteine levels and the presence and extent of CAC and severity of coronary disease [13,14], the abdominal aortic calcification (AAC) score [15] and the carotid calcification score, which increased across quartiles of plasma homocysteine (>9.4 μmol/l vs >13.4 μmol/l) [16], while CAC progression was twice as rapid in individuals with a plasma homocysteine concentrations of ≥ 12 μmol/L [17]. Homocysteine was also found in endarterectomy atheroma biopsies, with higher concentrations in those which were calcified and a correlation between tissue homocysteine and calcium [15]. Nevertheless, no association was seen with the incidence of aortic valve calcification (AVC) in older symptomatic patients [18]. In CKD patients, who are particularly prone to calcified
arteries and valves, there appears to be much less association of homocysteine with calcification, with studies showing little association with CAC presence, extent or progression [19-22], AVG presence [23] or peripheral arterial calcification scores [24], although an association with CAC extent was seen in young adults [25]. Similarly there are mixed results in systemic lupus erythematosus (SLE) patients [26-28].

Association of homocysteine with bone

A 2013 systematic review and meta-analysis of observational studies found an increased fracture risk with increased homocysteine concentrations but with respect to BMD, a meta-analysis was only possible in female populations and showed no association [29]. Nevertheless, a 2014 meta-analysis found that homocysteine levels were significantly higher in postmenopausal osteoporosis [30] and other studies have found an association among both elderly females and males [31]. Later studies have shown mixed results with respect to the association of elevated homocysteine and fractures [32,33] and no association with BMD [33], although using log-transformed homocysteine levels there is generally an inverse association with BMD [34,35]. A Korean study found that homocysteine concentrations were dose-dependently associated with bone loss in premenopausal women and men but not postmenopausal women [36] but this is not borne out in all studies of males [37]. Where an association with homocysteine levels has been found significant, the threshold level seems to be around 15 μmol/l [37-39]. Interestingly, however, where subjects have a pre-existing condition, the association between homocysteine and BMD appears more consistent [38,40,41], with the threshold concentration for osteoporosis in Chinese postmenopausal women with T2D being only 10.18 μmol/l [41].

Relationship between homocysteine concentrations and B-vitamins

Most studies show that plasma homocysteine levels are inversely associated with dietary folate [40,42,43], vitamin B6 and vitamin B12 intake [43], with similar associations for serum [40,42,44-46] and erythrocyte [47] folate, serum vitamin B6 [45,47] and serum vitamin B12 [44-47] concentrations. Deficiencies in these vitamins inhibit the breakdown of homocysteine, thus increasing the intracellular homocysteine concentration [3]. An inverse association was also found between homocysteine and vitamins B1 and B2 intake [43], as well as intake of non-haem iron and magnesium [48] and betaine (trimethylglycine) [4]. Furthermore, a calcium intake of >536mg/d was associated with lowered plasma homocysteine in postmenopausal Japanese women, regardless of folate concentrations [49]. Another factor is the MTHFR C677T genotype, which may in some studies be an independent determinant of homocysteine concentrations [28,42], although in SLE patients the C677T genotype was not a predictor of CAC status [28].

Meta-analyses consistently show that folic acid and vitamin B12 supplementation can lower homocysteine [50,51]. Up to 5mg/d folic acid supplemented alone dose-dependently reduced homocysteine by up to 25% [52,53], but even a dose of 0.5mg/d proved effective [54]. The greatest reduction was seen among females and in those with the highest baseline homocysteine and lowest folate concentrations; the addition of 0.4mg/d vitamin B12 produced a further 7% reduction [55,56]. Vitamin B12 [50] and vitamin B6 alone proved ineffective and there was no benefit to adding vitamin B6 to other B vitamins [55,56]. A combination of folic acid plus vitamin B6 lowered serum vitamin B12 as well as homocysteine levels in haemodialysis patients [57], suggesting that the three should be supplemented together to avoid vitamin B12 depletion. Furthermore there is concern that a vitamin B12 deficiency could be masked by folic acid supplementation [58].

Association of B vitamins and CV calcification

Despite the association of high homocysteine with CV calcification and the evidence that B vitamins are associated with lower homocysteine, there have been no observational studies investigating intake of B vitamins and incidence of CV calcification and only one study investigating serum levels. Here, the carotid calcification score decreased across quartiles of plasma folate concentrations (>39.4nmol/l vs <23nmol/l) in patients with vascular disease or diabetes but there was no association with vitamin B6 or B12 concentrations [16]. This study suggests that in vascular disease and diabetic patients, the normal reference range for plasma folate (7-36nmol/l) may be too low.

Association of B vitamins with bone

Most studies have shown little association between folate and vitamin B12 intake and fracture risk or BMD in either the elderly [59,60] or the young [61], although in perimenopausal women followed up after 10 years, there was a positive correlation between BMD, but not fracture incidence, and intake of folate, but not intake of vitamin B12 [62]. Among the other B vitamins, older female fracture patients had lower intake of vitamins B1 and B6 [63], while a large study of older Chinese adults found a significant inverse relationship between vitamin B6 intake and hip fracture risk after 14 years among postmenopausal women, but not men, with no association for intake of vitamins B1, B2, B3, B12 and folate [64]. Although some studies have shown a positive correlation between intake of vitamins B1, B2 or B3 and BMD [65-67], others have shown no association [62,66,68]. A large Scottish study, however, showed that while B vitamins were not associated with BMD after seven years, when stratified by MTHFR genotype, those homozygous for the TT allele showed a positive correlation between vitamin B2 intake and BMD [69]. Animal studies found that a vitamin B2 deficiency decreases bone calcium and magnesium [70,71], while vitamin B6 deficiency was associated with degraded bone morphology [72].

With respect to serum concentrations, a 2013 systematic review and meta-analysis found a modest decrease in fracture risk with increase in vitamin B12 levels but no association with BMD in female populations [29]. Since then a Spanish study showed that fracture risk was not related to erythrocyte folate or serum vitamin B12 [60]. In general serum vitamin B12 was dose-dependently associated with BMD only up to about 200 pmol/L in older women [39,73,74], although another large study suggested that the threshold may be 148pmol/l [75]. A recent vitamin B12 study confirmed that concentrations predicted fracture incidence in elderly men [32], although confusingly, a 2014 meta-analysis found that vitamin B12 levels were significantly higher in

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postmenopausal osteoporosis than in controls [30]. Most serum, red blood cell or erythrocyte folate studies show no association with fracture in postmenopausal women [60,76,77], with BMD or with osteoporosis [30,73,78]. Where an association is found, those with serum folate concentrations of ≤ 9.3 nmol/L were at increased risk of fracture [79]. Nevertheless when stratified by the MTHFR genotype, subjects homozygous for the TT allele had significantly higher BMD [80], particularly where plasma folate concentrations were ≥0.06 nmol/L [81]. In the only serum vitamin B6 study, low concentrations were associated with fracture in postmenopausal women [82], although any association with BMD was inconclusive [45,83].

B vitamin intervention studies and CV calcification

In the only clinical trial of B vitamins alone, subjects with baseline plasma homocysteine levels of >8.5 µmol/L were given 5 mg/d folic acid, 0.4 mg/d vitamin B12 and 50 mg/d vitamin B6 for three years but there was no significant effect on progression of coronary or aortic calcification or carotid intima/media thickness (cIMT), another measure of subclinical atherosclerosis. Although homocysteine levels were significantly lowered from mean 9.5 µmol/L to 8.8 µmol/L, a mean homocysteine level of 9.5 µmol/L is possibly too low for it to be responsible for inducing subclinical atherosclerosis, although among subjects with baseline homocysteine ≥9.1 µmol/L, B vitamins generated a significantly lower rate of cIMT progression [84]. Nevertheless, two trials showed that a combination of 250 mg/d aged garlic extract, 0.1 mg/d vitamin B12, 0.3 mg/d folic acid, 12.5 mg/d vitamin B6 and 100 mg/d L-arginine for 12 months produced significantly lower homocysteine and cAC progression, while also significantly lowering total and LDL cholesterol and significantly raising HDL [85,86].

B vitamin intervention studies and bone

Randomized controlled trials of B vitamins have generally shown no effect on BMD overall, although in osteoporosis patients with plasma homocysteine >15 µmol/L, BMD was improved with supplementation of 2.5 mg/d folic acid, 0.5 mg/d vitamin B12 and 25 mg/d vitamin B6 for one year [87]. Lower levels and lesser combinations of B vitamins in healthy subjects have similarly had no effect on markers of bone formation or turnover [88-92], even when vitamin D and calcium were also supplemented in both the treatment and placebo groups [92]. Only in subjects with pre-existing disease did B vitamins have a beneficial effect on hip fracture risk and BMD [87,93,94].

Mechanisms

Animal and in vitro studies suggest there may be several means by which high homocysteine is associated with CV calcification, although none has yet been shown to be definitive. Firstly, homocysteine, or its metabolite homocysteic acid (HCA), is a pro-oxidant, promoting lipid peroxidation and generating production of hydrogen peroxide, superoxide anions and other reactive oxygen species (ROS) in myocardial mitochondria, endothelial cells and mesenchymal stem cells, which were inhibited by antioxidants, so preventing apoptosis [95-100]. Associated with the oxidative process, elevated homocysteine induces differentiation of inflammatory monocytes and their accumulation in atherosclerotic lesions [101], while dose- and time-dependently increasing the expression of cyclooxygenase (COX) 2, thromboxane A2 and B2, nuclear factor kappa-B (NF-kB) and pro-inflammatory cytokines and increasing arachidonic acid (AA) release in macrophages, platelets and lymphocytes [102-104]; COX 2 activation and NF-kB and ROS production could be attenuated by calcium chelators and the antioxidant N-acetylcysteine (NAC), suggesting that homocysteine-induced COX 2 expression may be mediated through ROS generated by calcium-dependent signalling pathways [102,105]. B vitamins also appear to have an antioxidant effect, with cells cultivated in a folate-deficient medium showing a significant increase in lipid per oxidation and hydrogen peroxide with activation of NF-kappaB [106].

Additional mechanisms include a dose-dependent induction of elevated intracellular calcium concentrations in VSMCs, with enhanced allaline phosphatase activity [15,107], which was unaffected by lowering extracellular calcium, although lowering extracellular sodium significantly increased intracellular calcium, suggesting a possible role of sodium-calcium exchange in the process. Pre-treatment with a calcium channel blocker completely inhibited the increased intracellular calcium in VSMCs [108,109]. Cytoplasmic calcium influx leads to depletion of cellular adenosine triphosphate (ATP) by reaction with cytoplasmic phosphate, leading to calcium apatite deposition [2]. Intracellular calcium was also raised in macrophages, lymphocytes, cardiac myocytes, platelets and mitochondria [3,102-104,110-113]; in platelets this induces aggregation, which could be attenuated by the antioxidant N-acetylcysteine [112].

It is thought that increased intracellular calcium, ROS and COX-2 production is generated by homocysteine binding and activating the N-methyl-D-aspartate (NMDA) receptor. In cardiac tissue, this contributed to a decline in mechanoelectrical function and arrhythmogenesis [2,3,110,111,114]. In macrophages, this effect could be attenuated by an NMDA receptor inhibitor [102,109], suggesting that homocysteine may act through NMDA receptor-mediated calcium signalling pathways [102,103]. Homocysteine has the same effect through binding to glutamate receptors in neurons, which may contribute to the neuronal pathology and immunosenescence that occur in Alzheimer’s disease (AD), as well as the increased ROS and intracellular calcium [115,116]. Homocysteine could also induce membrane hyperpolarization and actin polymerization, which could be prevented by glutathione and other antioxidants [117].

In addition, homocysteine can affect collagen production, causing VSMCs to become extremely reactive to angiotensin II, at concentrations well below the physiologic range [108]. Many patients with hyperhomocystaemia have connective tissue abnormalities, similar to those with Marfan’s syndrome, suggesting that the structure and function of fibrillin-1 is compromised in these patients [118]. Homocysteine can also induce matrix metalloproteinases (MMPs), particularly MMP9, through disturbed mitochondrial membrane permeability, which further induces ROS, degrades cell membrane and proteins and leads to cardiac tissue remodelling, mechano-electrical dysfunction, arrhythmogenesis and sudden cardiac death [94,8,105,108,110,111,119].
Although it has been suggested that homocysteine affects nitric oxide (NO) production, the study results are inconclusive [97], although it is generally believed that increased oxidative stress reduces NO bioavailability and decreases blood flow [105]. Two studies show that homocysteine can inhibit endothelial nitric oxide synthase (eNOS) activity and hence NO production, which could be reversed by folates and peroxynitrite scavengers [97,120], but others, found that homocysteine could increase eNOS activity in coronary and aortic endothelial cells, which could ameliorate endothelial cell injury and thereby decrease the atherothrombotic risk of hyperhomocysteinaemia [108,121,122]. There is also an interaction between homocysteine and vitamin D, which up regulates the CBS enzyme to speed homocysteine clearance [105].

Similarly in bone, elevated homocysteine disturbs mitochondrial membrane permeability via the NMDA receptor, leading to release of mitochondrial MMPs which cause further oxidative stress [105]. Homocysteine also interferes with collagen cross-linking, altering the matrix and causing fragility. Since cross-linking does not affect BMD, this may explain why elevated homocysteine is more associated with fracture risk [29,105]. Furthermore homocysteine alters blood flow, depriving bone of vital nutrients, upregulating osteoblast activation, modulating osteocalcin and osteoprotegerin, increasing urinary markers of resorption and decreasing osteoblast activity [105]. Vitamin B12 deficiency is associated with impaired maturation of osteoblasts, while folate can aid bone metabolism by protein and nucleic acid methylation, although decreased vitamin B12 had limited effect on osteoclastogenesis [29]. Although no clear mechanism has yet been defined to explain any link between B vitamins and bone health [123], it nevertheless seems likely that they act on bone via both homocysteine-dependent and homocysteine-independent pathways [105,124] and may affect the bioavailability of each other [125]. Nevertheless, in several bone studies, the association of plasma homocysteine with bone parameters was no longer significant when adjusted for folate and vitamin B12 [35], while an association between B vitamins and bone parameters lost significance with adjustment for homocysteine [76,81], suggesting that there are other associations which have not yet been identified.

**DISCUSSION**

Homocysteine concentrations generally appear to be positively correlated with the presence and extent of arterial calcification, although the association is less clear in CKD or SLE patients and there seems to be no association with aortic valve calcification. Interestingly, the MTHFR reductase C677T genotype, although associated with homocysteine, was not a predictor of calcification or CVD. Although there is no determined threshold for homocysteine concentration, risk of arterial calcification, ischemic stroke and mortality is minimised when homocysteine is <12 µmol/l. Although meta-analyses consistently show that homocysteine concentrations can be lowered by folic acid and vitamin B12, there have been no observational studies of intake and the only serum study found that the carotid calcification score was significantly reduced with plasma folate concentrations of >39.4nmol/l vs <23nmol/l, the upper limit of the reference range being 36.0nmol/l. There is only one clinical trial of B vitamins and arterial calcification, which showed no effect, although since mean baseline homocysteine concentrations were 9.5 µmol/l, this was possibly too low for homocysteine to have been the cause of the calcification.

The association of homocysteine concentrations and B vitamins with bone is clearer, with a generally significant positive association of homocysteine with fracture risk and osteoporosis incidence in the elderly but no association with BMD. The threshold homocysteine concentration appears to be around 15 µmol/l in some studies but could be as low as 10.2 µmol/l. Consistent with the CV studies, most show little association of B vitamin intake and bone. In contrast to the CV studies, however, the MTHFR genotype may play a role in bone, with those with the TT allele having significantly higher BMD, particularly where plasma folate concentrations were ≥ 9.06nmol/l. Clinical trials of B vitamins have similarly shown little effect on BMD, except in osteoporosis patients where plasma homocysteine was >15 µmol/l, although both fracture risk and BMD were improved in subjects with pre-existing disease.

There have been many meta-analyses of observational studies investigating the association between cardio- and cerebrovascular disease and homocysteine levels. These show a strong correlation between homocysteine concentrations and stroke [126-128], with a 25% (3 µmol/l) lower homocysteine concentration being associated with a 19% lower risk of stroke after adjustment for CV risk factors [129]. The association with CVD is weaker but still significant, including in CKD. Meta-analyses have shown a 20-50% increased risk of CHD for each increase of 5 µmol/l in homocysteine level [130] and an independent association of elevated homocysteine with CV events, ischaemic heart disease, venous thrombosis, pulmonary embolism and >50% occlusion of the coronary or carotid artery [127,131-135].

Supplementation of B vitamins for stroke prevention also appear effective. Meta-analyses showed that stroke risk was significantly reduced in those supplementing B vitamins for ≥ 3 years but there was no association for vitamin B12 supplementation alone, although folic acid was beneficial for primary, but not secondary stroke prevention, and was most effective among men, who tended to have higher baseline homocysteine concentrations [50,136,137]. In other CVD, however, B vitamins show less effect. A 2013 Cochrane Review showed that after excluding studies of CKD patients, B vitamins failed to lower risk of non-fatal or fatal myocardial infarction, stroke or all-cause mortality by 20% or more, although they were unable to conclude on a lesser risk reduction [138]. It has been suggested that most trials are powered to detect a 20% reduction in CV events but if the reduction is 15% then the power is likely to be inadequate, despite the fact that a 15% reduction in CV events might be extremely clinically relevant [139]. It has been found, however, that patients with a vitamin B12 deficiency had a higher prevalence of CV risk factors and a higher SYNTAX score, used to assess the complexity of coronary artery disease [140]. The associations of homocysteine concentrations and B vitamins with CVD are echoed in studies of Alzheimer’s disease, for which the ApoE4 genotype is a shared risk factor. Here, elevated plasma homocysteine is a known predictor of AD, vascular dementia and degree and rate of cognitive decline in both cross-sectional...
[116,141-149] and prospective studies [150-155]. AD is also associated with decreased serum folate concentrations (≤ 5.7 ng/ml), with mixed results for vitamin B12 [143,149-155].

Although no definitive mechanism for the detrimental effects of homocysteine has yet been proposed, there are many potential candidates. Firstly homocysteine is a pro-oxidant and hence pro-inflammatory, with antioxidants, including B vitamins, reducing the effect. Secondly, homocysteine induces mitochondrial membrane hyperpolarisation and increased permeability, with induction of MMP-9, which can be inhibited by antioxidants and it also raises intracellular calcium, inducing platelet aggregation, which was inhibited by calcium channel blockers and antioxidants. Both effects are likely mediated via NMDA receptor binding in the CV system. Homocysteine may affect collagen production, making VSMCs reactive to angiotensin II, and interfering with cross-linking in bone, possibly explaining why elevated homocysteine is more associated with fracture than BMD. A parallel mechanism may be occurring in AD, with homocysteine binding to the glutamate receptors in neurons, increasing oxidative stress, inflammation and intracellular calcium. Possibly the reason that B vitamins, although beneficial for lowering homocysteine, have little effect on CVD is because once homocysteine concentrations are elevated, the chain of events leading to increased intracellular calcium, oxidative stress and inflammation has already been set in train and can only be reversed by antioxidants. This would suggest that B vitamins should be supplemented both to prevent elevated homocysteine and to prevent already elevated homocysteine from causing more damage, but once injury to the CV system, brain and bone have been initiated then dietary antioxidants should be increased as well.

CONCLUSION

Elevated homocysteine is a risk factor for arterial, but not valvular, calcification, as well as for osteoporosis and bone fracture but not BMD loss. The MTHFR reductase C677T genotype, although associated with homocysteine concentrations, was not an independent predictor of calcification. Although trials of B vitamins, particularly folate, consistently fail, despite the evidence for lowering homocysteine, their association with arterial calcification has generally not been investigated. Nevertheless, in bone, B vitamin supplementation has proved beneficial, particularly with elevated homocysteine in osteoporosis. Homocysteine appears to increase arterial calcification and raise fracture risk through generating oxidative stress and increasing inflammation and intracellular calcium, which may be mediated through binding to NMDA receptors and inducing MMP-9. Many of these effects may be inhibited by supplementing antioxidants rather than B vitamins.

REFERENCES


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