Lowering homocysteine with B vitamins has no effect on biomarkers of bone turnover in older persons: a 2-y randomized controlled trial[1–3]

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ABSTRACT

Background: In recent prospective studies, higher homocysteine concentrations were shown to be a risk factor for osteoporotic fractures in older persons. Supplements containing folate and vitamins B-12 and B-6 lower homocysteine concentrations.

Objective: The objective of the study was to determine in healthy older persons whether lowering homocysteine with B vitamins affects plasma biomarkers of bone turnover.

Design: Healthy older persons ($n = 276$; aged ≥65 y) were randomly assigned to receive either a daily supplement containing folate (1 mg), vitamin B-12 (500 μg), and vitamin B-6 (10 mg) or a placebo for 2 y. Of these participants, we selected 135 with baseline homocysteine concentrations >15.0 μmol/L, and we measured serum bone-specific alkaline phosphatase, a marker of bone formation, and bone-derived collagen fragments, a marker of bone resorption, at baseline and 2 y later.

Results: At 2 y, plasma homocysteine concentrations were 5.2 μmol/L (95% CI: 3.9, 6.6 μmol/L; $P < 0.001$) lower in the vitamin than in the placebo group. No significant differences were found in either serum bone-specific alkaline phosphatase (−0.3 μg/L; 95% CI: −2.8, 2.1 μg/L; $P = 0.79$) or bone-derived collagen fragments (−0.0 μg/L; 95% CI: −0.1, 0.1 μg/L; $P = 0.76$) between the vitamin and placebo groups, respectively, with 2 y of supplementation.

Conclusion: Supplementation with folate and vitamins B-6 and B-12 lowered plasma homocysteine but had no beneficial effect on bone turnover at the end of 2 y, as assessed by biomarkers of bone formation and resorption. *Am J Clin Nutr* 2007;85:460–4.

KEY WORDS Homocysteine, bone biomarkers, folate, vitamin B-12, vitamin B-6, clinical trial, older persons

INTRODUCTION

A high circulating homocysteine concentration is an independent risk factor for several chronic conditions, including cardiovascular (1) and Alzheimer (2) disease. A high homocysteine concentration has also been identified as a risk factor for osteoporosis. Classic homocystinuria, an inborn error of metabolism, is characterized by a very high blood homocysteine concentration. In addition to vascular complications, persons with this condition are at greater risk of premature osteoporosis and fracture than are persons without the condition (3). A mildly elevated homocysteine concentration is common in older persons (4, 5). In 2 prospective studies, high homocysteine concentrations were a risk factor for osteoporotic fractures in older persons (6, 7). In one of these studies, men ($n = 825$) and women ($n = 1174$) with a mean age of 70 y in the highest quartile of homocysteine concentrations were at a risk of hip fracture 3.8 and 1.9 times, respectively, that for men and women in the lowest quartile (6). In the Hordaland Homocysteine Study, a large cross-sectional study of 5338 participants aged 47–50 y and 71–75 y, plasma homocysteine concentrations were inversely associated with bone mineral density (BMD) in both younger and older women but not in men of any age (8). The mechanism by which high homocysteine could be detrimental to bone is not clear. It has been proposed that high homocysteine concentrations may interfere with collagen cross-linking. Impaired collagen cross-linking would decrease the stability and strength of the collagen network, which would decrease bone strength and increase fracture risk (9). In addition, at physiologic concentrations, homocysteine may increase osteoclast formation and activity (10, 11).

Circulating concentrations of folate, vitamin B-12, and vitamin B-6 are inversely associated with plasma homocysteine (4); furthermore, results of randomized controlled trials show that supplementation with folic acid and vitamins B-12 and B-6 is an effective way of lowering homocysteine (12). However, little is known about the effect of homocysteine-lowering therapy on fracture risk, BMD, or markers of bone turnover. Sato et al (13) reported that stroke patients in Japan who received folate and vitamin B-12 supplements over 2 y had a significantly lower rate of hip fracture than did a placebo group. However, the generalizibility of these findings to healthy persons is clearly limited, especially given the high rate of hip fracture (4.5%/y) in the placebo group in the study of Sato et al.

We recently conducted a 2-y placebo-controlled randomized trial to assess the effect of homocysteine-lowering vitamins (folate and vitamins B-12 and B-6) on cognitive performance in older persons with homocysteine concentrations of ≥13 μmol/L

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(14). This trial provided an opportunity to examine whether a reduction in homocysteine concentrations affects plasma biomarkers of bone turnover—in particular, serum bone-specific alkaline phosphatase (BSAP), a marker of bone formation, and bone-derived collagen fragments (β-CTX), a marker of bone resorption.

SUBJECTS AND METHODS

Participants

Volunteers aged ≥65 y were recruited into our cognition study from local service clubs (eg, Rotary International) and by direct mail and advertising in newspapers. Participants were not eligible to participate if they were taking medications known to interfere with folate metabolism; had suspected dementia; were taking vitamin supplements containing folic acid, vitamin B-12, or vitamin B-6; were being treated for depression; had a history of stroke or transient ischemic attacks; or had diabetes. Volunteers were asked to attend an early-morning clinic at which a fasting blood sample was collected for measurements of plasma homocysteine and creatinine concentrations. Those with a fasting plasma homocysteine concentration <13 μmol/L or creatinine concentration >133 μmol/L (men) and >115 μmol/L (women) were excluded.

Written informed consent was obtained from all subjects. The Human Ethics Committee of the University of Otago approved the study.

Study design

Eligible participants were stratified by using the median values of age and homocysteine concentration from the screening population. A random decimal between 0 and 1 was generated for each person in each of the 4 strata. Those with a random decimal below the median of the random decimal in each stratum were assigned to one group, and the remainder were assigned to the other group. Participants were asked to consume one capsule daily for 2 y. Compliance was assessed by counting returned capsules.

Supplements

The treatment capsules contained microcrystalline cellulose as a filler (Merck Eprova, Schaffhausen, Switzerland) plus 1000 μg folate (L-5-methyltetrahydrofolate, calcium salt), 500 μg vitamin B-12 (cyanocobalamin), and 10 mg vitamin B-6. The placebo capsules contained a blend of magnesium stearate and the filler. Neither the investigators nor the participants were aware of the contents of the supplements.

Blood collection and laboratory methods

Plasma and serum were obtained by centrifuging the whole blood at 1650 × *g* for 15 min at 4 °C within 2 h of collection. Blood samples were stored at −80 °C until they were analyzed. Total homocysteine was measured by using the IMx fluorescence polarization immunoassay (FPIA; Abbott Laboratories, Abbott Park, IL) to measure total L-homocysteine in plasma. Plasma folate concentrations were measured by using a microbiological method on 96-well microplates, as described by O'Broin and Kelleher (15), with chloramphenicol-resistant *Lactobacillus* casei used as the test microorganism. Plasma vitamin B-12 was measured by using the ADVIA Centaur vitamin B-12 assay (Bayer Diagnostics, Tarrytown, NY), a competitive immunoassay using direct chemiluminescent technology. Serum holo-transcobalamin II (holo-TCII) was measured by using a competitive binding radioimmunoassay kit (Axis Shield, Oslo, Norway). Plasma creatinine was measured colorimetrically by using diagnostic kits on a Cobas Mira Analyzer (both: Roche Diagnostics, Basel, Switzerland). CVs for these assays were 6.7% for plasma homocysteine, 7.7% for plasma folate, 5.6% for plasma vitamin B-12, 8.3% for serum serum holo-TCII, and 7.2% for plasma creatinine.

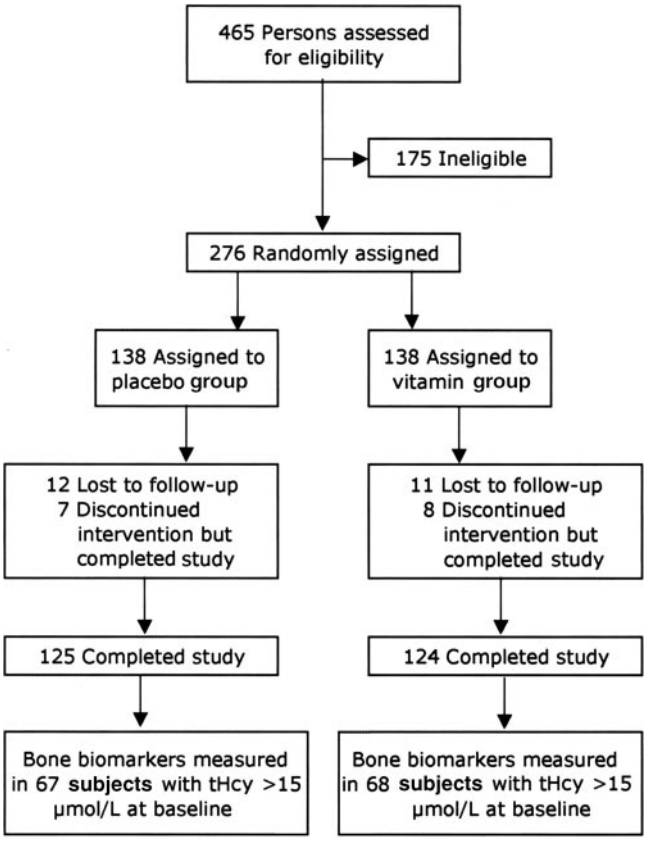

FIGURE 1. Participant flow and follow-up. tHcy, total homocysteine.

Bone biomarkers

Serum BSAP and β-CTX were measured at baseline and at 2 y in a subset of 135 participants with baseline homocysteine concentrations > 15.0 μmol/L (**Figure 1**). We estimated that detection of a minimum treatment effect size of 0.5 to 0.6 for serum β-CTX (CV: 13%)—the more variable of the 2 bone biomarkers, with a power of 90% and 2-sided alpha at 0.05 (16, 17)—would require a total of 65 participants in each of the 2 subject groups. Serum BSAP and β-CTX were measured by the Endocrinology Laboratory at Canterbury Health Laboratories (Christchurch, New Zealand). Serum BSAP was measured on an Access analyzer (Beckman Coulter Inc, Fullerton CA) with the use of the Ostase assay (Beckman Coulter), an immunoassay with chemiluminescent detection. BSAP is a glycoprotein localized in the plasma membrane of osteoblasts, which reflects bone formation. Serum β-CTX was measured by using an electrochemiluminescence method on an Elecys 2010 analyzer (Roche Diagnostics). This assay is specific for an octapeptide in the C-terminus of the

TABLE 1
Baseline characteristics of bone biomarker study participants in each treatment group[1]

Baseline characteristic	Placebo group ($n = 67$)	Vitamin group ($n = 68$)
Age at screening (y)	74.6 ± 5.6[2]	74.1 ± 5.9
Women [*n* (%)]	40 (60)	29 (43)[3]
Current smoker [*n* (%)]	1 (1.5)	2 (2.9)
BMI (kg/m^2)	26.3 ± 3.8	26.7 ± 4.3
Current hormone replacement therapy [*n* (%)]	4 (3)	3 (2)
Plasma cholesterol (mmol/L)	6.0 ± 1.3	6.4 ± 1.4
Serum holo-transcobalamin II (pmol/L)	76 ± 37	79 ± 41
MTHFR C677T homozygous *TT* genotype (*n*)	5	15[3]

[1] MTHFR, methylenetetrahydrofolate reductase.
[2] $\bar{x}$ ± SD (all such values).
[3] Significantly different from placebo group, $P < 0.05$ (Fisher's exact test).

α1 chain of type 1 collagen, and it reflects osteoclast-mediated bone resorption. CVs for serum BSAP and β-CTX were 5.9% and 9.4%, respectively.

Statistical analysis

Differences in baseline characteristics of participants in the subject groups were determined by using Fisher's exact test for categorical variables and Student's *t* test for continuous variables. Multiple regression with adjustment for the baseline values was used to estimate the differences at 2 y between the placebo and treatment groups. The difference was adjusted for baseline blood concentrations of bone biomarkers, homocysteine, or vitamins; sex; and *TT* genotype for the MTHFR polymorphism in a second model. We also tested for an interaction effect between treatment and each of baseline plasma homocysteine, folate, and vitamin B-12 for BSAP and β-CTX. Results were considered significant at $P < 0.05$. All analyses were performed with STATA for MACINTOSH software (version 9.0; Stata Corp, College Station, TX).

RESULTS

The flow of participants in the original randomized control trial is shown in Figure 1. Of the 465 screened participants, we excluded 172 because they had fasting plasma homocysteine concentrations <13 μmol/L and 3 because of an elevated plasma creatinine. In the cognition study, 276 participants were randomly assigned to the placebo or vitamin group. Three participants withdrew before baseline measures were collected. Twenty participants were lost to follow-up—11 in the placebo group and 9 in the vitamin group. Fifteen participants discontinued taking the supplements but completed the study. The subgroup in which bone biomarker concentrations were measured consisted of 135 participants—67 in the placebo group and 68 in the vitamin group—who had baseline homocysteine concentrations >15 μmol/L. Participants who discontinued taking the supplements were not included in the subgroup.

Selected baseline characteristics of the particpants in the bone biomarker substudy are presented in **Table 1**. The placebo group had a significantly greater proportion of women than did the vitamin group (60% and 43%, respectively; $P < 0.05$). Moreover, the *TT* genotype of the MTHFR polymorphism occurred significantly more frequently in the vitamin group than in the placebo group (15% and 5%, respectively; $P < 0.05$). Overall, 85% of participants took ≥95% of their study capsules.

The main biochemical results of the bone biomarker substudy are presented in **Table 2**. Plasma homocysteine concentration at 2 y, after adjustment for baseline values, was 5.4 (95% CI: 4.2,

TABLE 2
Plasma total homocysteine (tHcy), plasma folate, plasma vitamin B-12, serum bone-specific alkaline phosphatase (BSAP), and serum bone-derived collagen fragments (β-CTX) in each treatment group[1]

	Baseline ($n = 67$)	Values at 2 y ($n = 68$)	Difference between groups[2]	
			Adjusted for baseline values	Fully adjusted[3]
Plasma tHcy (μmol/L)				
Placebo group	19.3 ± 3.6[4]	16.4 ± 4.7		
Vitamin group	19.7 ± 5.1	11.1 ± 2.8	−5.4 (−6.6, −4.2)[5]	−5.2 (−6.4, −3.9)[5]
Plasma folate (nmol/L)				
Placebo group	20.6 ± 12.0	21.0 ± 14.1		
Vitamin group	20.3 ± 10.2	73.0 ± 21.4	52.1 (45.9, 58.3)[5]	52.7 (46.4, 59.1)[5]
Plasma vitamin B-12 (nmol/L)				
Placebo group	282 ± 99	254 ± 90		
Vitamin group	264 ± 85	564 ± 227	327 (275, 378)[5]	317 (264, 371)[5]
Serum BSAP (μg/L)				
Placebo group	13.6 ± 6.8	14.9 ± 15.2		
Vitamin group	13.5 ± 7.0	14.0 ± 7.2	−0.8 (−3.2, 1.6)	−0.3 (−2.8, 2.1)
Serum β-CTX (μg/L)				
Placebo group	0.42 ± 0.20	0.47 ± 0.27		
Vitamin group	0.43 ± 0.23	0.47 ± 0.28	−0.01 (−0.08, 0.06)	−0.01 (−0.09, 0.06)

[1] No significant differences were seen between the subject groups at baseline (Student's *t* test).
[2] All values are $\bar{x}$; 95% CI in parentheses. Differences at 2 y (multiple regression analysis).
[3] Adjusted for baseline values, age, sex, and *MTHFR* genotype.
[4] $\bar{x}$ ± SD (all such values).
[5] Significantly different from placebo, $P < 0.01$.

6.6) μmol/L lower in the vitamin group than in the placebo group ($P < 0.001$). Plasma folate and vitamin B-12 were 52.1 (95% CI: 45.9, 58.3; $P < 0.001$) nmol/L and 327 (95% CI: 275, 378; $P < 0.001$) pmol/L higher, respectively, in the vitamin group than in the placebo group. At 2 y, no significant difference in serum BSAP was found between the vitamin and placebo groups (−0.8 μg/L; 95% CI: −3.2, 1.6 μg/L; $P = 0.50$). Likewise, no significant difference in serum β-CTX was found between the vitamins and placebo groups (−0.01 μg/L; 95% CI: −0.08, 0.06 μg/L; $P = 0.83$). The inclusion of age, sex, and MTHFR genotype in the regression model had a negligible effect on the adjusted differences: −0.3 μg/L (95% CI: −2.8, 2.1 μg/L; $P = 0.79$) and −0.0 μg/L (95% CI: −0.1, 0.1 μg/L; $P = 0.76$) for serum BSAP and β-CTX, respectively. No significant interaction was found between treatment and the baseline plasma homocysteine, folate, and vitamin B-12 concentrations for either bone biomarker ($P > 0.05$).

DISCUSSION

We tested the hypothesis that reducing homocysteine with B vitamins would favorably affect biomarkers of bone turnover. Despite a significant lowering of homocysteine concentration with B vitamin supplements, no effect on bone biomarkers of resorption or formation over 2 y was found. This trial is the first long-term randomized trial to examine the effect of folate, vitamin B-12, and vitamin B-6 on bone biomarkers in healthy older persons with elevated baseline homocysteine concentrations. Our findings are consistent with the results of a recent, small, short-term study of folic acid alone (18). In that study, 61 participants ($\bar{x}$ age: 58 y) were randomly assigned to receive placebo or 400, 1000 or 5000 μg folic acid daily for 2 mo. All doses of folic acid lowered homocysteine and increased plasma folate, but no effect on bone markers of formation (serum osteocalcin) or resorption (serum procollagen type I N-terminal propeptide and β-CTX) was found. Our findings are in contrast with those of a randomized controlled trial in Japan, in which folate and vitamin B-12 supplementation for 2 y led to a significantly lower fracture rate than that in the placebo group (13). However, that study's subjects were patients who were recovering from stroke and who were osteoporotic and at greater risk of hip fracture due to falls. The fracture rate in the placebo group was 4.5% per year, whereas the subjects in the current study had no fractures.

Our results are also at odds with most of the observational evidence. Most notably, results from 2 large prospective studies indicate that elevated homocysteine concentrations are an independent risk factor for osteoporotic fractures in older persons (6, 7). Moreover, a common polymorphism for the methylenetetrahydrofolate reductase enzyme has been associated in some studies with higher homocysteine, lower BMD, and greater fracture risk (19, 20). The suggestion that homocysteine is causally related to bone density and fracture risk would be enhanced if homocysteine were also associated with relevant changes in markers of bone turnover. The validity of bone biomarkers as indicators of bone health has been well established in randomized controlled drug trials for the treatment of osteoporosis (21). However, few studies have examined the relation between bone biomarkers and homocysteine concentrations (22–26), and those studies have produced conflicting results. For example, Dhonuskhe-Rutten et al (23) reported in women but not men that high homocysteine combined with low serum B-12 was associated with high concentrations of serum osteocalcin and urinary deoxypyridinoline. Our findings do not support the notion that the relation between bone health and homocysteine is causal, because homocysteine lowering did not alter bone turnover in a way that is consistent with increased bone strength; however, we cannot exclude the possibility that elevated homocysteine concentrations may adversely effect bone strength through means that are not related to bone turnover and mass.

The strengths of our study include its 2-y duration, which exceeds the time required to observe changes in studies of bone biomarkers with pharmacologic antiresorptive therapy, a low dropout rate, and high compliance (27). Furthermore, the study included only persons with elevated homocysteine concentrations; thus, the homocysteine lowering achieved with the B-vitamins was large at 5.2 μmol/L. However, the current study also has several limitations. We did not include measurements of BMD, and the study was not powered to assess the effect of homocysteine-lowering vitamins on fracture rates. Furthermore, we cannot generalize our results to patients with very high homocysteine concentrations or with established osteoporosis.

Low vitamin B-12 status has been associated with low BMD (23, 24). For example, Tucker el al (24) reported that significantly lower BMD at the hip (men) and spine (women) was seen in subjects in the Framingham Study cohort who had plasma vitamin B-12 concentration <148 pmol/L than in subjects who had higher vitamin B-12 concentrations. Moreover, patients with pernicious anemia (severe vitamin B-12 deficiency) are at greater risk of bone fracture than are patients without pernicious anemia (28). These observations suggest that increasing vitamin B-12 status may improve bone health, independent of any effect on homocysteine concentrations. However, we found no evidence that biomarkers of bone turnover in participants with low baseline vitamin B-12 status responded any differently to vitamin supplementation (including vitamin B-12) than did those biomarkers in participants with high baseline vitamin B-12 status (ie, no interaction was seen between treatment and baseline plasma B-12 concentrations). However, very few of the participants ($n = 7$; 5%) had low vitamin B-12 status (<150 pmol/L) at the beginning of the study, and the power of our study to examine this effect was low.

In conclusion, supplementation with folate and vitamins B-6 and B-12 lowered plasma homocysteine but had no effect on biomarkers of bone resorption and formation. These results suggest that, if the inverse association reported in observational studies between homocysteine and bone health is causal—and our results do not support this interpretation—it is not mediated by effects of homocysteine on bone turnover.

TJG, CMS, and SJW conceived of the idea of the bone-biomarker substudy and obtained funding; TJG, CMS, and JAM conceived of the design of the larger randomized controlled trial; JAM recruited the participants and carried out the intervention study; SMW performed the statistical analyses; and all authors contributed to writing the manuscript. None of the authors had a personal or financial conflict of interest.

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