Changes in basal and postmethionine load concentrations of total homocysteine and cystathionine after B vitamin intervention[1–3]

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ABSTRACT

Background: Vitamin B-6 is necessary for the metabolism of homocysteine and is often used in combination with folic acid and vitamin B-12 in clinical trials that investigate whether the lowering of plasma total homocysteine (tHcy) can prevent vascular disease.

Objective: We compared the effects of vitamin B-6 with the effects of folic acid and vitamin B-12, as used in the Western Norway B-vitamin Intervention Trial (WENBIT), on basal and postmethionine load (PML) tHcy and cystathionine concentrations.

Design: Ninety patients with suspected coronary artery disease were randomly assigned to 1 of 4 groups to receive daily oral treatment with *1*) 0.8 mg folic acid, 0.4 mg vitamin B-12, and 40 mg vitamin B-6 (group A); *2*) 0.8 mg folic acid and 0.4 mg vitamin B-12 (group B); *3*) 40 mg vitamin B-6 (group C); or *4*) placebo (group D). For the first 2 wk, groups A and B received additional folic acid (5 mg/d). A methionine-loading test was performed at baseline and after 3 mo.

Results: Treatment with folic acid and vitamin B-12 caused a rapid and significant lowering of basal (31%) and PML tHcy concentrations (22%), with no effect on cystathionine. Vitamin B-6 did not change basal tHcy and had a significant but limited effect on PML tHcy concentrations. However, vitamin B-6 treatment markedly lowered basal and PML cystathionine by 31% and 42%, respectively.

Conclusion: The folic acid and vitamin B-12 combination applied in WENBIT provides rapid, substantial, and long-term tHcy-lowering effects, whereas the effect of vitamin B-6 on tHcy was relatively small and confined to PML tHcy. However, vitamin B-6 treatment caused a marked reduction in plasma cystathionine. Cystathionine could be a useful marker for assessment of the vitamin B-6 effect and should, together with tHcy, be related to clinical outcome in ongoing trials. *Am J Clin Nutr* 2004;80:641–8.

KEY WORDS Homocysteine, cystathionine, folate, vitamin B-6, vitamin B-12, vascular disease, methionine loading

INTRODUCTION

Epidemiologic and experimental evidence suggest that the concentration of total homocysteine (tHcy) is an independent and important risk factor for cardiovascular disease (CVD) (1–4). The precise underlying mechanism is, however, unknown (5, 6). Some data suggest that important determinants of the tHcy concentration, such as folate and vitamin B-6, could be associated with CVD and vascular function independently of the tHcy concentration (7–10).

Most studies on tHcy and CVD involve measurement of tHcy in the fasting or basal state. Measuring the concentration of tHcy after methionine loading could identify additional people with hyperhomocysteinemia (11). Data from cross-sectional studies indicate that postmethionine load (PML) hyperhomocysteinemia predicts risk of CVD independently from fasting or basal tHcy concentrations (2, 12). Prospective cohort studies that evaluate the predictive role of PML tHcy, however, have not been performed.

The tHcy concentration can be effectively lowered by B vitamins. A 25% reduction is achieved by a low daily dose of 0.4–0.5 mg folic acid (13). Oral treatment with vitamin B-12 or vitamin B-6 could have some effect in selected subjects (14, 15), and vitamin B-6 reduces the PML increase in tHcy (16).

Several large-scale clinical trials were initiated to test whether lowering tHcy prevents (recurrent) occlusive vascular disease (17). Results from one trial were published and suggest that tHcy lowering decreases the incidence of cardiovascular events after coronary angioplasty (18). A combination of folic acid, vitamin B-6, and vitamin B-12 was used in this study, and such triple combinations are used in most of the other ongoing tHcy-lowering trials (17). Notably, these B vitamins influence other metabolic pathways in addition to homocysteine. Vitamin B-6 alone acts as a cofactor of more than 100 different reactions (19). If trials with the triple combinations turn out positive, it could, therefore, be controversial to what extent a reduction in homocysteine itself could account for vascular and clinical effects, at least from a mechanistic point of view.

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TABLE 1
Characteristics of the study population at baseline by total group and treatment category

		Treatment groups				P^1		
	Total group (n = 90)	A: folic acid + B-12 + B-6 (n = 22)	B: folic acid + B-12 (n = 23)	C: B-6 (n = 21)	D: placebo (n = 24)	All groups	Groups A + B vs groups C + D	Groups A + C vs groups B + D
Age (y)	61.5 ± 9.8[2]	62.0 ± 10.3	58.8 ± 9.1	63.0 ± 8.8	62.5 ± 11.0	0.48	0.23	0.39
Women (%)	23	27	13	33	21	0.43	0.46	0.14
Ever smokers (%)	83	77	96	71	86	0.14	0.40	0.03
Diabetes (%)	7	9	5	10	4	0.83	0.82	0.44
Prior myocardial infarction (%)	60	68	57	62	54	0.79	0.67	0.35
Prior revascularization (%)	34	36	35	14	50	0.10	0.83	0.09
BMI (kg/m^2)	26.5 ± 3.7	26.3 ± 2.7	27.2 ± 3.7	25.9 ± 3.6	26.5 ± 4.6	0.73	0.52	0.35
Systolic blood pressure (mm Hg)	145 ± 20.0	141 ± 16.2	147 ± 19.5	139 ± 18.4	150 ± 23.8	0.20	0.75	0.04
Diastolic blood pressure (mm Hg)	82 ± 9.6	81 ± 8.7	85 ± 8.9	79 ± 8.0	84 ± 11.6	0.18	0.47	0.04
Total cholesterol (mmol/L)	5.6 ± 1.5	5.4 ± 1.3	5.4 ± 0.7	5.7 ± 1.5	5.8 ± 2.2	0.77	0.30	0.87
LDL cholesterol (mmol/L)	3.5 ± 1.4	3.4 ± 1.1	3.4 ± 0.7	3.6 ± 1.3	3.8 ± 2.1	0.78	0.33	0.80
HDL cholesterol (mmol/L)	1.18 ± 0.33	1.19 ± 0.34	1.13 ± 0.30	1.29 ± 0.41	1.13 ± 0.26	0.35	0.54	0.12
Triacylglycerol (mmol/L)	1.90 ± 0.90	1.82 ± 1.06	1.99 ± 0.78	1.85 ± 0.95	2.02 ± 0.84	0.85	0.86	0.37
Creatinine (μmol/L)	96 ± 19.1	91 ± 11.4	96 ± 9.5	90 ± 12.6	104 ± 31.0	0.04	0.29	0.02

[1] One-factor ANOVA across and between groups.
[2] $\bar{x}$ ± SD (all such values).

The current investigation is part of the Western Norway B-vitamin Intervention Trial (WENBIT) and was designed to evaluate acute and long-term effects of the intervention on tHcy and related metabolites in the basal state and after repeated methionine loading. The 2 × 2 factorial design of folic acid and vitamin B-12 combination and vitamin B-6 is similar to that applied in the other Norwegian tHcy-lowering trial, NORVIT. This design allows us to investigate the separate biochemical effects of both folic acid and vitamin B-12 combination and vitamin B-6.

SUBJECTS AND METHODS

Patients and recruitment

WENBIT is a prospective, randomized, double-blind study on the effects of tHcy-lowering therapy on mortality and cardiovascular events. Adult patients (>18 y) undergoing coronary angiography for suspected coronary artery disease or aortic valvular stenosis are eligible, independent of subsequent therapy. Exclusion criteria are malignant disease, alcohol abuse, mental illness, reluctance to long-term follow-up, and participation in other clinical trials. Recruitment to the main study started in 2000 and finished March 2004 with the aim of 3000 included patients.

The present study examined the biochemical response to B vitamin intervention in a total of 90 consecutive patients (**Table 1**) recruited at Haukeland University Hospital in the period of April 1999 to September 1999. Written informed consent was obtained from all patients. The study protocol was approved by the regional ethics committee and by the Norwegian Medicines Agency.

Follow-up was not complete. One patient died after the visit at 1 mo, and 2 patients withdrew their consent after 3 d and 1 mo, respectively. A total of 81 patients (90%) attended the second methionine-loading session at 3 mo, and 74 patients (82%) attended all 6 sessions.

Study design

With use of a 2 × 2 factorial block design, the recruited patients were randomly assigned into 4 groups for daily oral treatment: group A received folic acid (0.8 mg), vitamin B-12 (cyanocobalamin; 0.4 mg), and vitamin B-6 (40 mg); group B received folic acid and vitamin B-12; group C received vitamin B-6; and group D received placebo. For the first 2 wk, the folic acid groups (A and B) received an additional loading dose of folic acid (5 mg/d); the other 2 groups (C and D) received additional placebo capsules. All capsules, both for long-term treatment (red capsules) and for the loading period (white capsules), contained 0.4 mg silica, 0.7 mg magnesium stearate, and 56.8–138.9 mg lactose monohydrate capsulated in gelatin. Capsules were colored red with 0.2 mg azorubin and 0.6 mg titanium dioxide or white with 1.0 mg titanium dioxide. Packages of trial capsules were prepared and given serial numbers in random order in blocks of 20 by Alpharma A/S (Copenhagen).

Patient data were collected from patient-administered questionnaires, and a full routine medical examination was done at baseline before vitamin therapy started. Coronary angiography was performed 3 d later.

Blood collection and biochemical analyses

Nonfasting (basal) blood samples were collected at baseline and after 3 d, 2 wk, 1 mo, 3 mo, and 6 mo of B vitamin intervention. Times since last meal and last big meal were noted. An oral methionine-loading test (0.1 g/kg body weight) was done at baseline and after 3 mo. PML blood samples were drawn 4 h after methionine intake (20).

Routine blood analyses, including hematologic indicators, renal function markers, and lipid-related factors, were analyzed at

TABLE 2
Baseline values of B vitamins, total homocysteine (tHcy), and related metabolites before treatment[1]

		Treatment groups[2]				P[3]		
	Total group[2] (n = 90)	A: folic acid + B-12 + B-6 (n = 22)	B: folic acid + B-12 (n = 23)	C: B-6 (n = 21)	D: placebo (n = 24)	All groups	Groups A + B vs groups C + D	Groups A + C vs groups B + D
Plasma folate (nmol/L)	8.3 (3.1–22.3)	8.8 (3.3–23.8)	7.5 (3.0–18.7)	8.6 (3.3–22.8)	8.4 (2.8–25.2)	0.73	0.68	0.39
RBC folate (nmol/L)	263 (126–547)	257 (111–591)	282 (125–637)	249 (130–477)	263 (139–501)	0.73	0.55	0.36
Cobalamine (pmol/L)	373 (177–788)	359 (234–552)	388 (222–678)	403 (170–954)	349 (129–945)	0.56	0.99	0.67
Vitamin B-6, PLP (nmol/L)	24.4 (7.6–78.7)	22.1 (8.3–59.3)	26.1 (6.8–101.2)	24.1 (6.2–93.9)	25.4 (9.4–68.5)	0.80	0.83	0.38
tHcy (μmol/L)	11.1 (6.4–19.4)	10.1 (6.8–15.2)	11.7 (7.7–17.8)	10.3 (5.9–17.7)	12.3 (6.0–25.3)	0.05	0.52	0.01
PML tHcy (μmol/L)	31.3 (19.0–51.8)	29.4 (17.4–49.7)	31.8 (22.5–45.0)	29.3 (18.2–47.4)	34.8 (19.5–62.1)	0.08	0.38	0.02
MMA (μmol/L)	0.16 (0.07–0.36)	0.16 (0.08–0.31)	0.14 (0.07–0.29)	0.14 (0.09–0.21)	0.19 (0.06–0.60)	0.03	0.28	0.19
Cystathionine (μmol/L)	0.31 (0.09–0.99)	0.26 (0.07–0.93)	0.28 (0.11–0.70)	0.33 (0.08–1.45)	0.36 (0.14–0.93)	0.24	0.05	0.57
PML cystathionine (μmol/L)	2.90 (0.73–11.5)	2.57 (0.65–10.1)	2.45 (0.78–7.67)	3.49 (1.04–11.8)	3.25 (0.62–16.9)	0.26	0.05	0.72
Cysteine (μmol/L)	292 (227–376)	282 (218–366)	287 (239–345)	298 (240–369)	301 (219–414)	0.29	0.06	0.57
PML cysteine (μmol/L)	279 (216–361)	264 (216–323)	277 (224–344)	281 (217–363)	294 (217–398)	0.05	0.03	0.08

[1] RBC, red blood cell; PLP, pyridoxal 5-phosphate; PML, postmethionine load; MMA, methylmalonic acid.
[2] Geometric $\bar{x}$; geometric reference range of population in parentheses (antilog: mean log-tHcy ± 1.96 SD).
[3] One-factor ANOVA across and between groups.

the central laboratory of the Haukeland University Hospital, with use of Technicon Chem 1 (Bayer, Leverkusen, Germany) and CELL-DYN 4000 (Abbot, Abbott Park, IL) platforms.

Blood samples containing EDTA for analysis of vitamins, tHcy, and metabolites were immediately placed on ice, centrifuged within <30 min, and stored at −80 °C until further analyzed.

Plasma tHcy, total cysteine, and methylmalonic acid (MMA) were determined with use of a modification of a gas chromatography–mass spectroscopy (GC-MS) method that involves ethylchloroformate derivatization as described by Husek (21). Cystathionine (and tHcy) was determined with use of a tandem mass spectrometry method (AB Guttormsen, H Refsum, E Solheim, unpublished observations, 1998). Briefly, after addition of reductant and deuterated standards (cystathionine and homocystine), the sample was acid precipitated and the supernatant fluid was injected on a reversed-phase column. The sulfur amino acids were eluted with use of an ethanol gradient in acetic acid, then detected, and quantified with use of the transition from the precursor to the product ion for each of the amino acids and their deuterated standards. The between-day CV for cystathionine is between 5% and 10%, depending on the concentration. Plasma tHcy was also determined with use of a fluorescence polarization immunoassay adapted to the Abbot IMx analyzer (Abbot Laboratories, Abbott Park, IL) (22). Both tandem mass spectroscopy (r = 0.94) and IMx (r = 0.95) methods correlated well with the GC-MS method. On the basis of an evaluation of the data with use of Bland-Altmann plots (23), the tHcy concentrations measured by the GC-MS method are reported.

Folate and cobalamin were determined by microbiologic assays with use of a chloramphenicol-resistant strain of *Lactobacillus casei* and colistin sulfate–resistant strain of *Lactobacillus leichmannii*, respectively (24, 25). Both the folate and cobalamin assays were adapted to a microtiter plate format (26) and carried out by a robotic workstation (Microlab AT plus 2; Hamilton, Bonadus AG, Switzerland). Plasma concentrations of pyridoxal 5-phosphate (PLP), pyridoxal, pyridoxine, and 4-pyridoxic acid were analyzed by an ion-pair reversed-phase chromatography (27). Data on these B-6 vitamers are reported in a separate paper (28); plasma concentrations of PLP are used in this report.

Statistical analysis

Summary measures for continuous variables are reported as means and categorical variables as proportions (%). Vitamin and metabolite data were logarithmically transformed before further analysis and are presented as geometric mean. Associations were assessed by Pearson correlation coefficients. Analysis of variance (ANOVA) was used for comparison of continuous variables between treatment groups at baseline. Possible interaction of folic acid and vitamin B-12 combination and vitamin B-6 treatments was tested by repeated measures ANOVA. When the treatment effect of either folic acid or vitamin B-6 was studied, the 2 patient groups with and without active treatment were combined if no significant interaction was observed. A 2-tailed $P < 0.05$ was considered statistically significant. Data were analyzed with use of SPSS 11.0 (SPSS Inc, Chicago).

RESULTS

Among the 90 patients aged 38–80 y (21 women and 69 men), 22 patients were randomly assigned to folic acid, vitamin B-12, and vitamin B-6 (group A); 23 patients to folic acid and vitamin B-12 (group B); 21 patients to B-6 alone (group C); and 24 patients to placebo (group D) (Table 1). The groups were well matched, but creatinine, tHcy, and MMA concentrations were somewhat higher in the placebo group (Table 1 and **Table 2**).

We observed no significant interaction of the folic acid and vitamin B-12 combination and vitamin B-6 treatments for any of the vitamins or metabolites studied (plasma folate, P = 0.9; blood folate, P = 0.07; plasma cobalamin, P = 0.3; plasma MMA, P = 0.4; plasma tHcy, P = 0.1; plasma cystathionine, P = 0.4). Consequently, the 2 patient groups with and without the

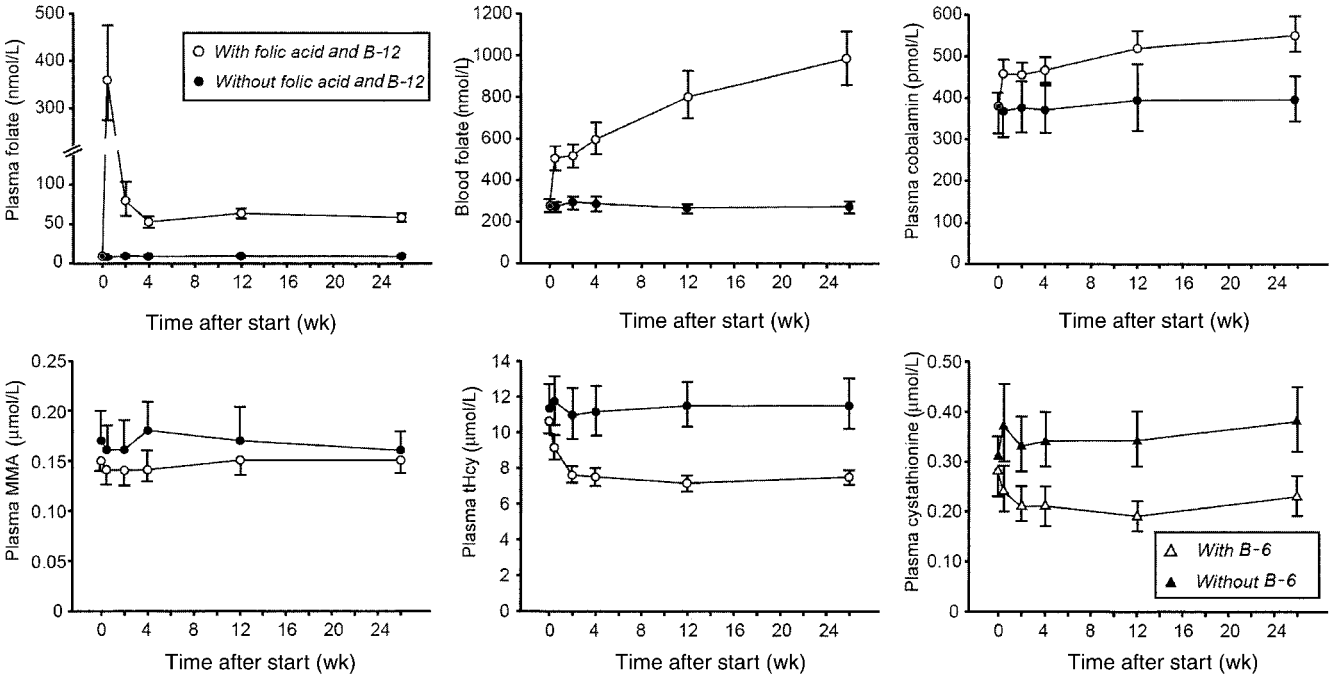

FIGURE 1. Geometric mean changes in vitamin and metabolite concentrations during 6 mo of B vitamin supplementation. Error bars represent 95% CIs. Only patients attending all 6 sessions are included in these analyzes ($n = 74$). The 2 patient groups with and without the actual vitamins were combined in the analysis when the treatment effect of either combination of folic acid and vitamin B-12 [groups A + B ($n = 40$) compared with groups C + D ($n = 34$)] or vitamin B-6 [groups A + C ($n = 36$) compared with groups B + D ($n = 38$)] was studied because there were no significant interactions. Regimens containing folic acid and vitamin B-12 had significant effects on plasma folate ($P < 0.001$), blood folate ($P < 0.001$), plasma cobalamin ($P < 0.001$), and plasma total homocysteine (tHcy) ($P < 0.001$) but not on plasma methylmalonic acid (MMA) ($P = 0.18$), whereas vitamin B-6 had significant effects on plasma cystathionine ($P = 0.001$) by repeated-measures ANOVA.

actual vitamins were pooled in the analysis of changes over time (**Figure 1**).

Vitamin status

Mean plasma folate concentrations increased from 8.1 nmol/L to a peak concentration of 334 nmol/L at day 3 and remained elevated for the next 6 mo (57.0 nmol/L) in the combined group of patients treated with folic acid (groups A and B). Blood folate concentration was significantly increased after 3 d (from 269 nmol/L to 486 nmol/L, $P < 0.001$) and continued to increase over the next 6 mo (to 996 nmol/L, $P < 0.001$). No change in folate status was observed in the groups not treated with folic acid (Figure 1).

Mean plasma cobalamin was significantly increased already at day 3 in the groups (A and B) treated with vitamin B-12 (from 374 pmol/L to 448 pmol/L, $P < 0.001$), and a moderate additional increase was observed during the next 6 mo (to 546 pmol/L, $P < 0.001$; Figure 1). Further details on vitamin B-12 and fluctuations in its binding proteins were reported in a separate paper (29).

After vitamin B-6 treatment, concentrations of PLP were increased 10-fold within 3 d, and PLP remained at this concentration during the observation period. The responses of the different B-6 vitamers were described in a separate paper (28).

Methylmalonic acid, homocysteine, and cystathionine

Overall, the vitamin B-12 treatment caused no significant change in MMA during the study (Figure 1). Eight patients had elevated MMA ≥ 0.28 μmol/L at baseline. Only one patient [in group D (placebo)] had plasma cobalamin <200 pmol/L and was classified as vitamin B-12 deficient (MMA, 0.77 μmol/L; plasma cobalamin, 117 pmol/L). Vitamin B-12 injections were started after 3 mo, and a decline in MMA to 0.15 μmol/L was observed 3 mo later. Four subjects receiving vitamin B-12 (group A or B) had marginally elevated MMA (<0.32 μmol/L). Three of them responded with ≈50% reduction in MMA within the next few months. However, 1 of the 3 subjects not receiving vitamin B-12 experienced a marked decline in MMA.

Treatment with folate and vitamin B-12 (in groups A and B) was associated with a significant 14% reduction (−1.5 μmol, $P < 0.001$) in plasma tHcy by day 3. The full effect was obtained after 2 wk with 31% reduction (−3.3 μmol, $P < 0.001$). Thereafter, no further reduction was observed, and tHcy remained at this low concentration (Figure 1). Repeated measures ANOVA confirmed a significant effect of treatment across the entire period ($P < 0.001$). The degree of reduction in tHcy observed after 3 mo was strongly related to initial concentrations of plasma tHcy ($r = 0.775$, $P < 0.001$) and plasma folate ($r = -0.478$, $P = 0.001$). Cumulative frequency plots show reduction in tHcy by folate and vitamin B-12 treatment compared with the groups given vitamin B-6 or placebo over the whole tHcy distribution, but more pronounced effect at high concentrations (**Figure 2**, upper left panel).

Vitamin B-6 treatment had no effect on basal tHcy concentration but was associated with a significant 14% decrease (−0.042 μmol/L, $P = 0.03$) in basal plasma cystathionine already at day 3. Maximum reduction of 31% (−0.090 μmol/L, $P = 0.001$) was reached at 3 mo and correlated strongly with cystathionine concentrations before treatment ($r = 0.95$, $P < 0.001$). Repeated measures ANOVA verified a significant reduction of plasma cystathionine by intervention throughout the entire treatment

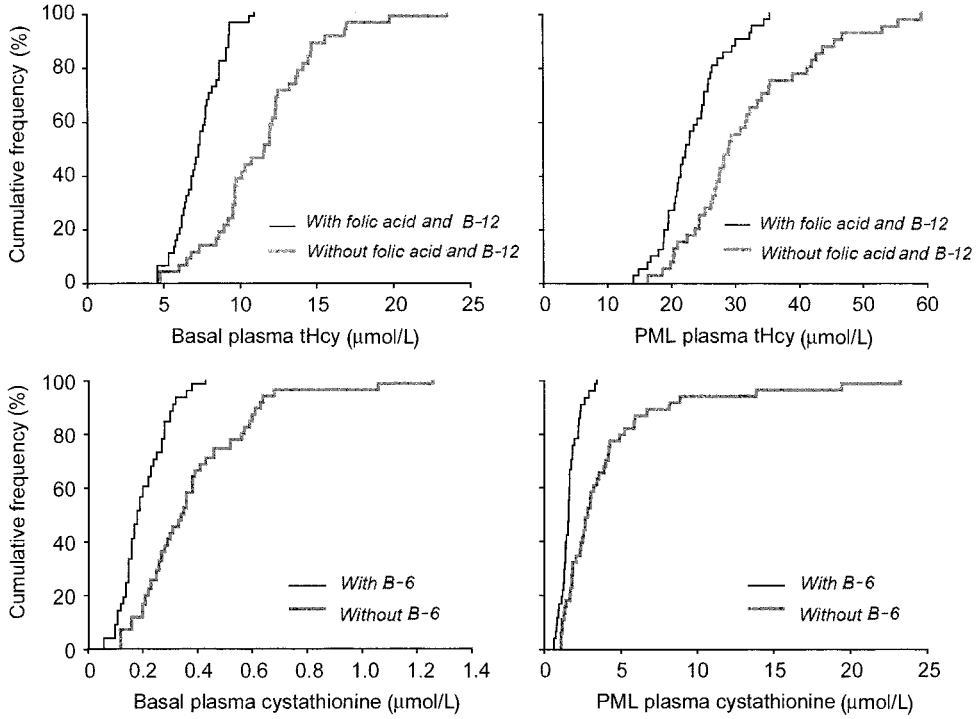

FIGURE 2. Cumulative frequency plots of plasma total homocysteine (tHcy) and plasma cystathionine after 3 mo of B vitamin intervention ($n = 81$). The effectiveness of folic acid and vitamin B-12 ($n = 41$) in reducing both high and low basal plasma and postmethionine load (PML) tHcy compared with vitamin B-6 alone or placebo ($n = 40$) is shown in the upper panels. The effect was particularly pronounced in subjects with high baseline values. Likewise, the plots for cystathionine and PML cystathionine illustrate the effectiveness of vitamin B-6 ($n = 38$) in reducing especially high concentrations of cystathionine compared with the concentrations observed in subjects given folic acid and vitamin B-12 in combination or placebo ($n = 43$).

period ($P = 0.005$). We observed no significant change in basal cystathionine concentrations by folic acid and vitamin B-12 or placebo (Figure 1). Cumulative frequency plots of cystathionine with and without vitamin B-6 treatment demonstrated that vitamin B-6 affected the whole range of cystathionine concentrations and displaced the distribution curve to the left (Figure 2, bottom left panel).

Methionine, homocysteine, and cystathionine after methionine loading

Vitamin treatment had no influence on basal plasma methionine or the increase in methionine observed after methionine loading (from 18 ± 3.4 μmol/L to 659 ± 104 μmol/L at baseline; from 22 ± 4.8 μmol/L to 661 ± 105 μmol/L after 3 mo).

At baseline, both PML tHcy and the increase in tHcy after methionine loading (ΔPML tHcy) were strongly related to basal tHcy concentrations ($r = 0.739, P < 0.001$; $r = 0.439, P < 0.001$, respectively). After 3 mo of treatment, PML tHcy was significantly reduced compared with baseline, both in group B treated with folic acid and vitamin B-12 (22%; $P = 0.001$) and in group C treated with vitamin B-6 (10%; $P = 0.02$). The greatest reduction was observed in group A treated with all vitamins (27%; $P < 0.001$; data not shown). Comparable response to vitamin treatment was seen for ΔPML tHcy (**Table 3**). The cumulative frequency plots of PML tHcy after 3 mo (Figure 2, upper right panel) illustrate that folic acid and vitamin B-12 treatment was associated with a shift of the PML tHcy distribution to the left compared with vitamin B-6 or placebo.

Similar to tHcy, baseline PML cystathionine and Δ PML cystathionine were strongly related to the basal concentration ($r = 0.578$, $P < 0.001$; $r = 0.567$, $P < 0.001$, respectively). The associations were attenuated by vitamin B-6 treatment and no longer significant after 3 mo ($r = 0.143$, $P = 0.39$; $r = 0.005$, $P = 0.98$, respectively). Overall, vitamin B-6 treatment for 3 mo reduced PML cystathionine by 42% and Δ PML cystathionine by 44% compared with baseline, whereas folate and vitamin B-12 had no such reducing effect (Table 3). The cumulative frequency plot of PML cystathionine (Figure 2, bottom right) after 3 mo of intervention illustrates the ability of vitamin B-6 treatment to suppress high PML cystathionine concentrations.

DISCUSSION

We studied the plasma concentrations of tHcy and a panel of related biochemical markers before and during treatment with a B vitamin intervention regimen that is currently used in 2 Norwegian clinical trials. Frequent initial blood sampling enabled us to study the early effects, and the influence on metabolite concentrations after methionine loading was re-investigated after 3 mo. Unlike most other ongoing clinical trials (17), the present study used a 2×2 factorial design, which also allowed us to discriminate between the effects of folic acid and vitamin B-12 combination and vitamin B-6 on the concentration of metabolites.

Previous studies have shown that daily supplementation with 0.5–5 mg folic acid leads to a reduction in tHcy of about 25% (14). The dose required for effective and safe tHcy reduction during long-term supplementation for years is, however, debated, and there are safety concerns about daily doses >1 mg (30). A high folic acid dose of 2–5 mg is used in many ongoing trials (17), probably because such a dose can have additional

TABLE 3
Increases in total homocysteine (tHcy) and cystathionine after methionine loading and before and after 3 mo of B vitamin intervention[1]

	Treatment groups			
	A: folic acid + B-12 + B-6 (n = 21)	B: folic acid + B-12 (n = 20)	C: B-6 (n = 18)	D: placebo (n = 22)
ΔPML tHcy[2]		*μmol/L*		
Before	19.0 (16.4–22.0)	19.7 (17.6–22.0)	18.4 (16.1–21.1)	21.6 (18.9–24.5)
After 3 mo[3]	14.4 (12.8–16.1)	16.6 (14.9–18.5)	15.8 (13.9–17.8)	21.9 (18.7–25.6)
ΔPML cystathionine[2]				
Before	2.13 (1.55–2.92)	2.02 (1.55–2.62)	2.80 (2.13–3.67)	2.58 (1.81–3.69)
After 3 mo[4]	1.39 (1.15–1.68)	2.61 (2.04–3.36)	1.38 (1.14–1.66)	2.88 (1.94–4.29)

[1] Geometric $\bar{x}$ in natural units; geometric reference range in parentheses (antilog: mean log-tHcy ± 1.96 SEM). ΔPML, increase postmethionine loading.
[2] There was no significant interaction between folic acid B-12 and B-6 treatment on ΔPML tHcy or on ΔPML cystathionine by repeated-measures ANOVA.
[3] Main effect of folic acid + B-12 (groups A + B versus C + D), P = 0.006, and of vitamin B-6 (groups A + C versus B + D), $P = 0.015$, on ΔPML tHcy by repeated-measures ANOVA.
[4] Main effect of folic acid + B-12 (groups A + B versus C + D), $P = 0.7$, and of vitamin B-6 (groups A + C versus B + D), $P < 0.001$, on ΔPML cystathionine by repeated-measures ANOVA.

tHcy-reducing effects in some patients, such as patients with renal impairment (31).

In WENBIT and NORVIT, a relatively low daily dose of folic acid of 0.8 mg was chosen for long-term treatment. A recent meta-analysis confirms that 0.8 mg is sufficient to obtain maximal tHcy-reducing effect in most subjects (32). To ensure rapid effect, our regimen also includes a high additional folic acid dose of 5 mg/d given for the first 2 wk. This loading dose caused an immediate tHcy response with a significant reduction at 3 d, and a maximum reduction of 31% was reached within 2 wk. Also, the effect was maintained for at least 6 mo despite the low long-term dose.

Because folic acid therapy can mask symptoms of vitamin B-12 deficiency by correcting the megaloblastic anemia but allowing the neuropathy to develop (33), vitamin B-12 was added mainly for safety reasons (34). The daily requirement of cobalamin is only 2–6 μg, but in patients with pernicious anemia intrinsic factor is missing and cobalamin is not absorbed (35). About 1% of a high oral dose is absorbed by passive diffusion independent of intrinsic factor; hence, 0.4 mg was considered adequate for the prevention of vitamin B-12 deficiency (36). We observed increasing plasma cobalamin concentrations already at day 3. No associated decline in mean MMA was observed, but no patient except one (in the placebo group) was considered vitamin B-12 deficient. Three subjects with marginally elevated MMA responded with a reduction of MMA concentrations on vitamin B-12 treatment, suggesting that this oral B-12 treatment is sufficient to normalize MMA. However, larger studies are necessary to document the effect of such a low-dose treatment of oral vitamin B-12 on MMA status. Previous studies have shown that vitamin B-12 can add to the tHcy-lowering effect of folic acid (14). Because of the design, a potential tHcy-lowering effect of vitamin B-12 cannot be assessed in the present study.

Vitamin B-6 is independently associated with the risk of CVD (9). It is, however, not known how and whether vitamin B-6 is causally related to CVD, and the dose required is uncertain. In our study, vitamin B-6 was given in a relative high dose of 40 mg/d. This dose is available over the counter in Norway, and it is considered nontoxic (37). The theoretical basis for including vitamin B-6 is that PLP is required by the enzymes cystathionine β-synthase and cystathionine-γ-lyase that catalyze the transsulfuration of homocysteine by way of cystathionine to cysteine (38). In a rat model, however, vitamin B-6 deficiency is associated with both decreased remethylation and transsulfuration (39). Population-based observational studies revealed associations between vitamin B-6 status and plasma tHcy (40, 41). In contrast, treatment studies with vitamin B-6 suggest that the effect on basal tHcy concentrations is modest or absent (14, 42–45).

Our study confirms previous reports that treatment with vitamin B-6 had no significant effect on basal tHcy (42–46) but caused a significant reduction in PML tHcy (47, 48). In our patients, the vitamin B-6 effect on PML tHcy was independent of and added to the effect of the folic acid and vitamin B-12 combination. The most striking effect of the vitamin B-6 supplementation was a rapid and pronounced reduction in the plasma cystathionine concentrations. Vitamin B-6 treatment eliminated high concentrations of cystathionine after methionine loading with no change in PML methionine. Our findings are in agreement with published data showing that cystathionine concentrations are elevated in patients with B vitamin deficiency (49, 50); that a triple combination with folic acid, vitamin B-12, and vitamin B-6 lowers basal cystathionine concentrations in elderly people (43); and that vitamin B-6 alone lowers basal as well as PML cystathionine concentrations in both healthy and vitamin B-6–deficient subjects (51). The vitamin B-6 effect on cystathionine concentrations probably reflect an enhanced activity of the cystathionine-γ-lyase, which is known to be very sensitive to PLP depletion (39, 51, 52). In our study population, we found that only vitamin B-6 treatment influences cystathionine concentrations, suggesting that cystathionine concentrations could be a unique marker for identifying subjects that will respond to vitamin B-6 intervention.

Methionine intake causes an increase in *S*-adenosylmethionine, which is known to stimulate the transsulfuration pathway by activating cystathionine β-synthase (38) and thereby the synthesis and excretion (53) of cystathionine. In line with this process, we found that cystathionine was increased 10-fold after methionine loading. The relative increase in cystathionine was

much higher than the 3-fold increase observed for tHcy. Recently, we found that an ordinary meal resulted in a 3-fold increase of cystathionine, whereas tHcy did not increase significantly (54). Hence, the cystathionine response to methionine loading, the changes observed after an ordinary meal, and the effect of vitamin B-6 treatment suggest that plasma cystathionine is more sensitive than plasma tHcy to a changed flux from methionine by way of homocysteine through the transsulfuration pathway. This finding is supported by previous findings in subjects fed a protein-rich diet depleted of vitamin B-6 in which homocysteine was not detected in the urine until a marked increase in cystathionine excretion had occurred (52). Thus, increased cystathionine concentrations could reflect elevated intracellular concentrations of homocysteine or increased conversion from homocysteine to cystathionine that is not associated with changes in plasma tHcy concentrations. Because low vitamin B-6 intake and PLP concentrations were associated with CVD risk (9, 55), future studies should further evaluate elevated concentrations of cystathionine as a possible marker of intracellular depletion of PLP or intracellular increased tHcy.

In conclusion, we have shown that the folic acid and vitamin B-12 combination used in 2 Norwegian clinical intervention trials provides a rapid, substantial, and long-term tHcy-lowering effect. Vitamin B-6 does not influence basal tHcy but reduces tHcy after methionine loading. The most pronounced effect of vitamin B-6, however, is a marked reduction in basal and PML cystathionine concentrations. Cystathionine could serve as a marker of intracellular vitamin B-6 depletion and relate vitamin B-6 to clinical effects of homocysteine. The clinical implications of these different metabolic effects should be evaluated in ongoing trials.

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