Studies on Homocysteine-Lowering B-Vitamin Therapy in Patients with Coronary Artery Disease

Sub-studies from the Western Norway B-vitamin Intervention Trial (WENBIT)

Øyvind Bleie

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Institute of Medicine,
University of Bergen
and
Department of Heart Disease,
Haukeland University Hospital,
Bergen, Norway

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The present thesis is based on sub-studies of the Western Norway B-Vitamin Intervention Trial, WENBIT, a randomised clinical trial started at Department of Heart disease, Haukeland University Hospital, Bergen and Stavanger University Hospital, Stavanger, Norway, in close cooperation with Section of Pharmacology, Institute of Medicine and Locus for Homocysteine and related Vitamins, University of Bergen, Norway. The Norwegian Research Council granted a three-year's research fellowship, with additional support from Helse Vest RHF.

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LIST OF PAPERS

1. Bleie Ø, Refsum H, Ueland PM, Vollset SE, Guttormsen AB, Nexo E, Schneede J, Nordrehaug JE, Nygard O. Changes in basal and postmethionine load concentrations of total homocysteine and cystathionine after B vitamin intervention. *Am J Clin Nutr* 2004;80(3):641-8.

2. Holm PI, Bleie Ø, Ueland PM, Lien EA, Refsum H, Nordrehaug JE, Nygard O. Betaine as a Determinant of Postmethionine Load Total Plasma Homocysteine Before and After B-Vitamin Supplementation. *Arterioscler Thromb Vasc Biol* 2004;24(2):301-7.

3. Bleie Ø, Semb AG, Grundt H, Nordrehaug JE, Vollset SE, Ueland PM, Nilsen DW, Bakken AM, Refsum H, Nygard OK. Homocysteine-lowering therapy does not affect inflammatory markers of atherosclerosis in patients with stable coronary artery disease. *J Intern Med* 2007; 262:244-253.

4. Bleie Ø, Strand E, Ueland PM, Vollset SE, Refsum H, Igland J, Nordrehaug JE, Nygård OK. Folic acid intervention increases coronary blood flow in patients with stable coronary artery disease. *Submitted.*

ABBREVIATIONS

ANOVA	Analysis of variance
ApoA1	Apolipoprotein A-1
APV	Average peak velocity
BHMT	Betaine-homocysteine methyltransferase
CBF	Coronary blood flow
CBS	Cystathionine ß-synthase
CL	Cystathionine γ-lyase
CAD	Coronary artery disease
CD40L	CD40 ligand
CHD	Coronary heart disease
CRP	C-reactive proteine
CVD	Cardiovascular disease
FMD	Flow mediated dilatation
ELISA	Enzyme-linked immunosorbent assay
GFR	Glomerular filtration rate
HDL	High density lipoproteine
IFN-γ	Interferon gamma
IL-6	Interleukin 6
LDL	Low density lipoproteine
MS	Methionine synthase
MTHFR	Methyltetrahydrofolate reductase
PLP	Pyridoxal 5-phosphate
PML	Post methionine load
QCA	Quantitive coronary angiography
ROS	Reactive oxygen species
sCD40L	soluble CD40L
SD	Standard deviation
tHcy	total homocysteine concentration
WENBIT	Western Norway B-Vitamin Intervention Trial

ABSTRACT

Background. A high plasma level of total homocysteine (tHcy) is a risk factor for cardiovascular disease, and is related to important components of atherosclerosis such as inflammation and endothelial dysfunction.

Objectives. To test the effect of homocysteine-lowering B-vitamin therapy on 1) tHcy, and metabolites and determinants of tHcy, 2) inflammatory markers associated with atherosclerosis and 3) coronary endothelial and vascular function.

Design. Single centre, double-blind clinical interventional study, randomised in a 2x2 factorial design into daily oral treatment with A) folic acid (0.8 mg)/vitamin B12 (0.4 mg)/vitamin B6 (40 mg), B) folic acid/vitamin B12, C) vitamin B6 alone or D) placebo. For the first two weeks, groups A and B received additional folic acid 5 mg/day.

Subjects and methods. Two sub-groups of patients participating in the Western Norway B-vitamin Intervention Trial (WENBIT); *Patient group 1*: Ninety patients (21 female, aged 38-80 years) with suspected coronary artery disease (CAD). Blood samples were collected at baseline, after 3 days, 2 weeks, 1, 3, 6 months and one year of B-vitamin intervention. An oral methionine loading test (0.1 g/kg body weight) was done at baseline and after 3 months. *Patient group 2:* Forty patients (8 female, aged 39-74 years) with CAD. They were examined at baseline, and after 9 and 24 months, coronary blood flow (CBF) was assessed by coronary angiography and Doppler flow-wire measurements during intra-coronary infusion of saline (basal), incremental (0.72 μg/min, 7.2 μg/min and 36.0 μg/min) doses of acetylcholine, 2.4 mg/min adenosine and nitroglycerin.

Results. In patient group 1, we documented a reduction of 31% on plasma tHcy and a pronounced reduction of post methionine load (PML) tHcy by folic acid/vitamin B12. Vitamin B6 reduced cystathionine and particularly PML cystathionine. There was a strong inverse relation between PML betaine and PML increase in tHcy, a relation that was abolished by folic acid/vitamin B12 treatment for 3 months. Treatment with folic acid/vitamin B12 or vitamin B6 for 6 months had no effects on levels of neopterin, sCD40L, IL 6 or CRP. During the two years of follow-up of patient group 2, basal CBF and adenosine-stimulated CBF increased among patients treated with folic acid /B12 as opposed to those not receiving folic acid /B12.

Conclusions. The doses of folic acid/B12 applied in WENBIT give adequate tHcy lowering effect. Cystathionine may be a useful marker for assessment of the vitamin B6 effect. Plasma betaine is a strong determinant of the PML increase in tHcy in patients not supplemented with

B-vitamins which emphasizes the complementary relationship between betaine and folate metabolism. Although we observed improved coronary vascular function after treatment with folic acid/vitamin B12, failure to reverse inflammatory processes associated with atherosclerosis may partly explain the negative results of previous B-vitamin intervention trials among patients with established CVD.

INTRODUCTION

Cardiovascular disease

Cardiovascular disease (CVD) is a major cause of morbidity and mortality in Norway (**Figure1**) and most other countries, although age-adjusted mortality rates have been declining in the last 30 years. Epidemiologic studies have demonstrated important modifiable risk factors for ischemic heart disease, such as smoking, diabetes, hypertension, dyslipidemia and obesity. It has been suggested that these conventional risk factors only explain about half of the variance in coronary heart disease, but the role of these risk factors is probably underestimated [1]. A meta-analyses of studies on coronary heart disease (CHD) shows that more than 80 % of patients have at least one of these conventional risk factors [2]. It is, however, difficult to predict the risk for CHD on individual basis, even with sophisticated algorithms [3]. In order to improve risk assessment and more accurately distinguish between moderate and high risk of CHD, research has focused on new risk markers associated with CVD.

The subject of this thesis is homocysteine metabolism and its relation to CHD.

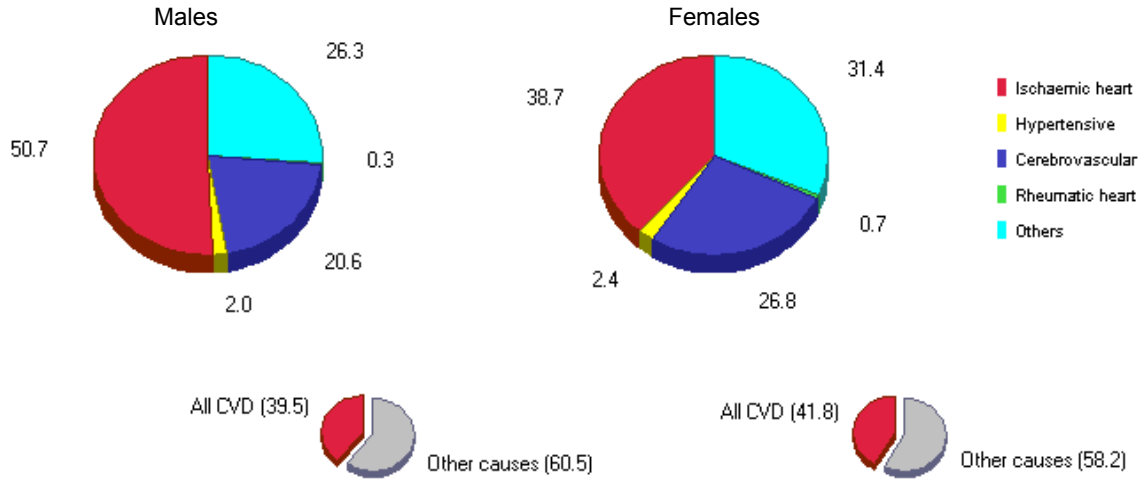

Figure 1. Proportional mortality (%) from CVD in Norway 2001. From Global Cardiovascular InfoBase (www.cvdinfobase.ca)

Homocysteine

History

A new sulphur amino acid was discovered in 1932 and called homocysteine [4] (**Figure 2**). One year later, a case report of a retarded boy with dislocated ocular lenses and skeletal abnormalities, who died of a massive cerebral infarction with arteriosclerosis and thrombosis of the carotid artery, was published [5]. Later, excretion of homocystine in urine, homocystinuria, was described in children with mental retardation, growth disturbances and thrombosis of arteries and veins [6,7]. A metabolic defect in the homocysteine metabolism affecting the vitamin B6-dependent enzyme, cystathionine ß-synthase (CBS), was discovered in these children [8]. New cases lead to the discovery of other enzymatic defects of the homocysteine metabolism; deficiency of the vitamin B12-dependent enzyme methionine synthase (MS) [9] and deficiency of methylenetetrahydrofolate reductase (MTHFR) [10].

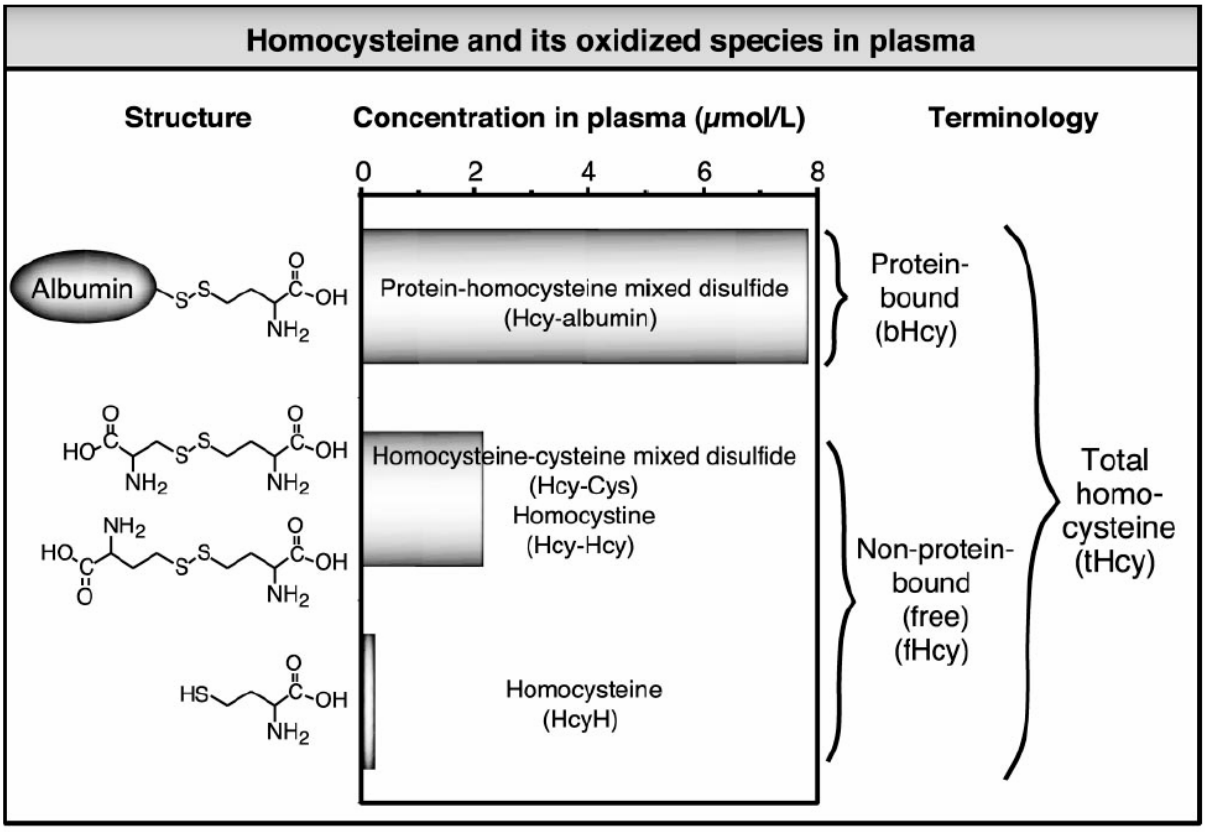

Figure 2. Structure and distribution of different homocysteine forms in plasma. From [11], adapted from Ueland [12].

Homocysteine and atherosclerosis, background

In 1949 atherosclerotic lesions was described in vitamin B6 deficient monkeys [13] and later in choline deficient rats [14]. With the presentation of patients suffering from inborn enzyme defects leading to homocystinuria and premature atherothrombotic disease, the link between homocysteine and B-vitamin status and CVD was established [15]. Without treatment, about 50 % of these patients with homocystinuria suffered from a thromboembolic event and 20 % died before the age of 30 years [16]. In 1976, a study of Wilcken and Wilcken showed significantly higher homocysteine levels among patients with coronary artery disease before the age of 50 years compared to a control group [17].

Early meta-analysis of retrospective observational studies on the effect of moderate elevated total homocysteine concentration (tHcy), found tHcy as a graded independent risk factor of CHD and estimated a relative risk of 1.7 associated with a 5 μmol/L increment in tHcy level [18]. Later, meta-analysis of prospective observational studies reported that after adjustments for conventional risk factors, a 25 % lower plasma tHcy concentration was associated with only 11 % lower risk of CHD [19].

The association between tHcy and CHD in prospective studies is supported by observations from investigations using Mendelian randomisation. These studies are based on the occurrence of 25 % higher tHcy level among subjects with a homozygous (TT) mutation in the MTHFR gene compared with the common (CC) variant. Analysing 80 studies with 26000 cases, those who were homozygous TT had an overall 14 % higher risk of CHD and a 2.2 μmol/L higher level of tHcy compared to those who had the CC genotype, although with some heterogeneity between geographically regions [20]. All together, cohort and MTHFR 677C → T polymorphism studies show a significant association between tHcy level and risk of CHD [21], and warrant mechanistic and clinical intervention studies to explore the effects of homocysteine-lowering therapy.

Biochemistry and measurements

Homocysteine is a sulphur amino acid, generated through demethylation of the essential amino acid methionine. The concentration in plasma of its free reduced form is, under normal conditions, very low, only 1-2 % of tHcy. Most of homocysteine in plasma is oxidized and bound to albumin or exists as a disulfide (homocystine or homocysteine-cysteine) (Figure 2). The sum of all these homocysteine forms is denoted tHcy [11].

Methods for measuring tHcy were introduced more than 20 years ago. All assays are based on treating plasma or serum with a reducing agent to convert all homocysteine forms to its reduced thiol, which is then measured directly or after derivatization [11].

Methionine loading test

To increase the sensitivity of diagnosing hyperhomocysteinemia, a methionine loading test has been used. After overnight fasting, oral methionine (0.1 g/kg body weight) is administered and post methionine load (PML) blood samples are drawn 4 (6) hours after methionine intake [22,23]. Plasma PML samples in patients with CHD show a 30-times increase of methionine, thus stressing the metabolic pathways resulting in a 3-times increase of tHcy and 9-times increase of cystathionine [24]. Originally, this was done to detect heterozygosity for CBS deficiency, but also subjects without CBS mutations and with normal fasting tHcy demonstrate elevated PML tHcy [25], suggesting that additional information is obtained with the test. In contrast, a light meal will not significantly influence tHcy level, whereas a heavy protein rich meal may increase tHcy by 10 – 15 % [26].

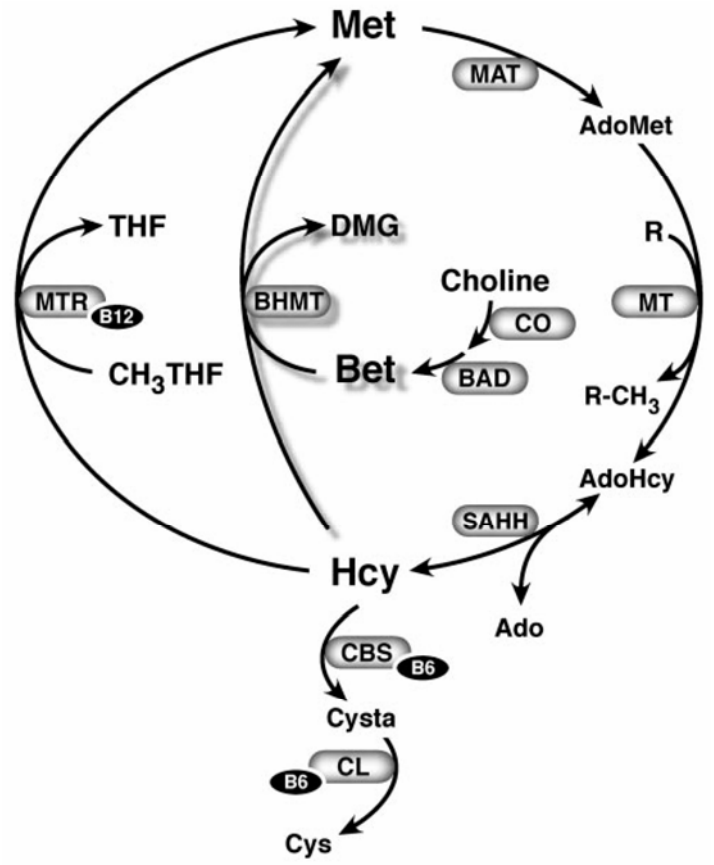

Figure 3. Betaine metabolism, homocysteine remethylation, and transsulfuration.
Ado indicates adenosine; AdoHcy, S-adenosylhomocysteine; AdoMet, S-adenosylmethionine; BAD, betaine aldehyde dehydrogenase; Bet, betaine; BHMT, betaine-homocysteine methyltransferase; CBS, cystathionine ß-synthase; CL, cystathionine γ-lyase; CH3THF, methyltetrahydrofolate; CO, choline oxidase; DMG, dimethylglycine; Cys, cysteine; Cysta, cystathionine; Hcy, homocysteine; MAT, methionine adenosyltransferase; Met, methionine; MT, methyltransferase; MTR, methionine synthase; SAHH, S-adenosylhomocysteine hydrolase; THF, tetrahydrofolate.
Adapted from paper 2

Metabolism

Homocysteine content in foods are very small [27].Almost all Hcy is formed as a metabolic product of the essential amino acid methionine, which donate a methyl group, and through several enzymatic steps is converted to homocysteine. The metabolism of homocysteine and its relation to B-vitamins are summarised in **Figure 3**.

The concentration of tHcy is dependent on the balance between formation, as described above, and salvage through *remethylation* to methionine or degraded through *transsulfuration*.

Remethylation to methionine is in most tissues catalysed by the folate-dependent enzyme methionine synthase (MS) with vitamin B12 as cofactor and 5-methyltetrahydrofolate (5-MTHF) as methyl donor. Alternatively, in liver and kidney homocysteine is remethylated to methionine by betaine-homocysteine methyltransferase (BHMT) with betaine as a methyl donor.

Degradation through transsulfuration is catalyzed first by cystathionine ß-synthase (CBS) to cystathionine, and then by cystathionine γ-lyase (CL) to cysteine; both enzymes dependent on vitamin B6 as cofactor.

Determinants of homocysteine level

Genetic disorders inactivating key enzymes (CBS [8], MS [28] or MTHFR [29]) of homocysteine metabolism may cause serve hyperhomocysteinemia. Other genetic variants, such as MTHFR 677C → T polymorphism, may cause a mild to moderate elevation of tHcy [30], especially in subjects with impaired folate status [31]. Other factors associated with tHcy level include age, gender, kidney function, folate deficiency, vitamin B12 deficiency, smoking, coffee intake, physical activity, blood pressure, and malignant diseases [32].

Mechanisms relating homocysteine to cardiovascular disease

Numerous studies demonstrate that hyperhomocysteinemia is related to an increased risk of the existence, extent, progression and prognosis of CVD [33,34]. The mechanisms of this association are not fully understood [33,35]. Recent data indicate that homocysteine is associated with several important components of atherogenesis, including endothelial function, platelet- and immune activation and inflammation [36-38].

Intact endothelium is important for the maintenance of vascular integrity and regulates vasomotor tone through release of nitric oxide to meet increased blood flow demands during

physical strain. Endothelial dysfunction is an early marker of atherosclerotic disease, it is involved in the pathogenesis, and predicts future cardiac events [39,40]. Various treatment strategies have been shown to improve endothelial function and cardiovascular prognosis, including statins, angiotensin converting enzyme inhibitors and exercise [39]. High doses of folic acid given to patients with CVD improve endothelial function measured as flow-mediated dilatation (FMD) in forearm arteries in some, but not in all studies [41]. However, low dose (0.4 mg/d) folate therapy seems to be ineffective [42,43]. Typically, the duration of the studies was for a few weeks to months [41].

Inflammation and persistent immune activation are hallmarks of the atherosclerotic process [44]. The markers, C-reactive protein (CRP), CD40 ligand (CD40L) and neopterin, represent different inflammatory pathways related to atherogenesis. CRP is an acute phase reactant; the production of CRP is stimulated by interleukin (IL)-6 effects on the liver. CRP represents an overall marker of the inflammation and is extensively investigated and characterized [45]. CD40L is produced by many cell types, but is excreted mainly from platelets and is related to unstable coronary artery disease [46]. Soluble CD40L (sCD40L) may therefore reflect the role of activated platelets in inflammation [47]. Neopterin is a pteridine which increases during activation of the cellular part of inflammation, and is released from activated monocytes through stimulation by interferon gamma (IFN-γ) from T-lymphocytes [48]. Neopterin has been related to the extent [49], complexity [50] and progression [51] of the atherosclerotic disease. Furthermore, neopterin is strongly related to the homocysteine homeostasis [52].

The thiol group of homocysteine may undergo auto-oxidation in plasma to generate reactive oxygen species (ROS), and thereby induce (endothelial) cell injury through a mechanism involving oxidative stress [53]. The results are conflicting, and one study demonstrates that homocysteine does not significantly increase the production of ROS and may, as opposed to pro-oxidant characteristics, display antioxidant effects [54].

In two recent reports, homocysteine was shown to reduce the concentration of HDL-cholesterol in plasma by inhibiting the hepatic synthesis of apoA-I, the main HDL apolipoprotein [55,56]. Since a low concentration of HDL-cholesterol predicts development cardiovascular disease [57], this mechanism possibly links homocysteine level to the development of atherosclerosis.

In patients with homocystinuria, tissue samples showed marked neointima proliferation [15]. It has been reported that elevated homocysteine levels stimulate proliferation of vascular smooth muscle cells [58] and increase collagen deposition in vessel walls [59]. These

effects of homocysteine are of potential importance, not only for promoting atherosclerosis, but also in relation to restenosis after coronary artery intervention [60,61].

Treatment of elevated homocysteine levels

The idea of treating elevated homocysteine was pioneered in patients with homocystinuria due to cystathionine synthase deficiency. Treatment of homocystinuria with pyridoxine decreased the plasma tHcy concentration and urinary excretion of methionine and homocystine [62], and adding betaine treatment substantially decreased plasma tHcy levels in pyridoxine non-responders [63]. Later, when epidemiological studies showed association between moderate hyperhomocysteinemia and CVD, B-vitamin treatment to lower tHcy was introduced with the aim of reducing risk of CVD and its complications [64]. A meta-analysis of randomised trials demonstrated a 23 % tHcy-lowering effect of a daily folic acid dose of 0.8 mg with an additional 7 % reduction of tHcy by adding cyanocobalamin 0.4 mg, and no further reduction by adding vitamin B6 [65].

AIMS OF THE STUDIES

The aims of these studies were to investigate effects of homocysteine-lowering B-vitamin therapy, as administered in the Western Norway B-vitamin Intervention Trial (WENBIT), on patients with established coronary artery disease. The specific objects of this work were:

1. To evaluate acute and long-term effects of the B-vitamin intervention on tHcy and related metabolites, in the basal state and after repeated methionine loading.
2. To investigate the association between plasma betaine and tHcy before and after methionine loading and the effect of long-term B-vitamin treatment on these associations.
3. To investigate the effects of tHcy-lowering B-vitamins on inflammatory markers associated with atherosclerosis.
4. To evaluate the long-term effect of tHcy-lowering B-vitamin therapy on coronary vascular function.

SUMMARY OF RESULTS

Paper 1. Changes in basal and postmethionine load concentrations of total homocysteine and cystathionine after B vitamin intervention.

Am J Clin Nutr 2004;80(3):641-8.

This study (WENBIT 90 sub-study) is based on the first 90 patients recruited to the WENBIT study at Haukeland University Hospital in the period April 1999 to September 1999. The WENBIT 90 sub-study was designed to investigate in details the biochemical associations and responses of B-vitamin therapy. Patients, aged 38 – 80 years (21 women and 69 men), were randomised to daily oral treatment with A) folic acid (0.8 mg)/vitamin B12 (0.4 mg)/vitamin B6 (40 mg), B) folic acid/B12, C) B6 alone or D) placebo. For the first two weeks, groups A and B received additional folic acid 5 mg/day. Blood sampling was done at baseline, after 3 days, 2 weeks, 1, 3, 6 months and later at 12 months of B-vitamin intervention. An oral methionine loading test (0.1 g/kg body weight) was done at baseline and after 3 months. Post-methionine load blood samples were drawn 4 hours after methionine intake.

We found that mean plasma folate concentration significantly increased from 8.1 nmol/L to a steady state of 57.0 nmol/L in subjects randomised to treatment with folic acid 0.8 mg/day (with additional folic acid 5 mg/day for the first two weeks). Interestingly, plasma cobalamin was significantly increased already at day three in the groups treated with oral vitamin B12 0.4 mg/day (from 374 pmol/L to 448pmol/L), and a moderate additional increase was observed during the next six months (to 546 pmol/L). Following treatment with vitamin B6 40 mg/day, concentrations of PLP was increased 10-fold within three days, and PLP remained at this level during the observation period.

The main results of this study were documentation of an immediate tHcy decrease following supplementation with folic acid/vitamin B12. A significant reduction was observed at three days and a maximum reduction of 31% within two weeks. Also, the effect was maintained for at least six months despite the lower long-term dose. Vitamin B6 had no effect on tHcy, but reduced cystathionine by 31 %. PML tHcy increased almost 3-times and PML cystathionine 9-times. After 3 months of treatment, PML tHcy was significantly reduced both by folic acid/vitamin B12 (22 %) and vitamin B6 (10 %), with the greatest reduction achieved by the combination of these vitamins (27 %). Vitamin B6 treatment for three months reduced PML cystathionine by 42% compared to baseline, and almost completely suppressed high PML cystathionine concentrations.

Paper 2. Betaine as a determinant of postmethionine load total plasma homocysteine before and after B-vitamin supplementation.

Arterioscler Thromb Vasc Biol 2004;24(2):301-7.

Based on the patients in the WENBIT-90 sub-study, we studied betaine and its relation to homocysteine metabolism. This was done after methionine loading, a situation that maximally challenges the methionine-homocysteine pathways.

We found that plasma betaine level increased transiently after methionine loading and is remarkably stable over time. Our study confirmed other reports showing higher betaine levels for men compared with women. The main finding was a strong inverse association between PML betaine and PML increase in tHcy, an association that was essentially abolished in patients receiving folic acid/vitamin B12.

Paper 3. Homocysteine-lowering therapy does not affect inflammatory markers of atherosclerosis in patients with stable coronary artery disease.

J Intern Med 2007; 262:244-253.

This study is also based on the WENBIT-90 sub-study. The current investigation was designed to assess the effects of homocysteine-lowering B-vitamins on inflammatory markers associated with atherosclerosis. We measured the biomarkers C-reactive protein (CRP) (and interleukin (IL)-6 which stimulates the production of CRP), CD40L and neopterin, since these markers represent three different lines in the inflammation cascade related to atherogenesis. CRP represents an overall marker of the inflammatory cascade and is extensively investigated and characterised. CD40L is produced by many cell types, but is excreted mainly from platelets and is related to unstable coronary artery disease. Soluble CD40L (sCD40L) may therefore reflect the role of activated platelets in inflammation. Neopterin is a pteridine, which reflects activation of the cellular part of inflammation, and is released from activated monocytes through stimulation by interferon gamma from T-lymphocytes. Neopterin is related to the extent, complexity and progression of the atherosclerotic disease. Furthermore, neopterin is strongly related to the homocysteine homeostasis.

Both tHcy and neopterin were significantly associated with creatinine, age and gender. After adjustment for GFR, tHcy was still significantly correlated with neopterin (r=0.38,

p<0.001). Despite the fact that plasma tHcy was lowered 33% by folate 0.8 mg/vitamin B12 0.4 mg, and that a high dose of vitamin B6 (40 mg) was given, no significant changes in the neopterin, sCD40L, IL-6 or CRP were observed.

Paper 4. Folic acid intervention increases coronary blood flow in patients with stable coronary artery disease.

Submitted.

The objective of this substudy of WENBIT, was to evaluate the long-term effect of homocysteine-lowering B-vitamin therapy on coronary vascular function. The study population was 40 patients with established stable CAD receiving conventional medical therapy, including statins, and with no selection according to tHcy levels at baseline.

After successful treatment of at least one significant coronary stenosis, a non-intervened coronary artery was used for coronary function testing (study vessel). Patients were followed with repeated testing after nine months, and 35 patients returned for a third testing after two years of vitamin treatment.

Our study demonstrated that two years of treatment with moderate doses of folic acid and vitamin B12 improved basal coronary blood flow and blood flow at maximum hyperaemia induced by adenosine, reflecting improved coronary vascular function. In contrast, folic acid/vitamin B12 did not improve coronary endothelial function as measured by flow-induced change in coronary vessel diameter or change in flow following stimulation with acetylcholine. Vitamin B6 treatment did not change any of the vascular function variables.

DISCUSSION

General introduction

Although epidemiologic and clinical data indicate that hyperhomocysteinemia is associated with CVD [33,34,36-38], exact mechanisms relating homocysteine with atherosclerosis development have not been determined [21,33,66]. Notably, some data suggest that important determinants of the tHcy level, such as folate and vitamin B6, may be associated with CVD and vascular function independently of the tHcy level [67-70]. These B-vitamins influence other metabolic pathways in addition to homocysteine, vitamin B6 alone acts as a co-factor of more than hundred different reactions [71]. Based on these observations, several randomised clinical trials with homocysteine-lowering B-vitamins have been initiated in order to investigate whether homocysteine is causally related to atherosclerosis [72].

WENBIT is a prospective, randomised double-blind placebo controlled secondary prevention study on the clinical effects of B-vitamin treatment in adults having undergone coronary angiography for suspected CAD or aortic valve stenosis. The studies presented in this thesis, are sub-studies of WENBIT, designed to assess effects of homocysteine-lowering therapy from a mechanistic point of view.

Materials and methods

Population

All patients had to be eligible in WENBIT; men and women aged >18 years undergoing coronary angiography for suspected CAD and/or aortic valve stenosis at Haukeland University Hospital were included. Exclusion criteria were inability to follow-up, participation in other trials, known alcohol abuse, serious mental illness or cancer. Patient data were collected from patient-administered questionnaires, and a full routine medical examination was done at baseline before starting vitamin therapy.

The current thesis present data based on two sub-groups of patients from WENBIT:

WENBIT 90. The group, WENBIT 90, is confined to the first 90 consecutive patients who were recruited at start of the WENBIT study, in the period of April 1999 to September 1999. Patients underwent frequent follow-up during the initial 6 months of the intervention, and the study was designed to investigate mechanisms and biochemical effects of provided treatment.

Follow-up was not complete. One patient died, 2 patients withdrew their consent. A total of 81 patients (90%) attended at the second methionine loading at three months, 83 (92%) patients attended the visit at 6 months and 74 patients (82%) attended all six visits.

Patients were all Caucasian, 21 female and 69 male, with median age 62 (range 38-80) years. Major characteristics of the patients are given in **Table 1**. Coronary angiography was performed three days after inclusion.

Non-fasting (basal) blood samples were collected at baseline, after 3 days, 2 weeks, 1, 3 and 6 months 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 months. Post-methionine load blood samples were drawn 4 hours after methionine intake

TABLE 1 Characteristics of the study population at baseline, WENBIT 90 (paper 1-3)

	*Total group** (n=90)	*Treatment groups**				*P-values*†
		Folic acid/B12/B6 (n=22)	*Folic acid/B12* (n=23)	*B6* (n=21)	*Placebo* (n=24)	
Age (years)	62 (54-68)	64 (55-70)	59 (52-65)	61 (57-71)	64 (53-71)	0.4
Women, n (%)	21 (23)	6 (27)	3 (13)	7 (33)	5 (21)	0.4
Creatinine (µmol/L)	94 (87-101)	91 (83-98)	94 (89-101)	90 (82-98)	98 (91-114)	0.07
GFR (ml/min/1.73 m^2)	73 (64-79)	75 (65-82)	73 (67-80)	72 (64-80)	74 (58-76)	0.5
Total cholesterol (mmol/L)	5.3 (4.8-6.1)	5.5 (4.7-6.2)	5.3 (4.9-5.7)	5.4 (4.8-6.4)	5.2 (4.2-7.0)	0.9
LDL cholesterol (mmol/L)	3.3 (2.7-4.0)	3.6 (2.7-4.0)	3.3 (2.8-3.9)	3.4 (2.4-4.4)	3.3 (2.3-4.8)	0.9
Statin use, n (%)	65 (72)	14 (64)	20 (87)	13 (62)	18 (75)	0.2
tHcy (µmol/L)	11.0 (9.3-12.9)	9.9 (9.0-11.9)	12.0 (10.3-13.1)	11.3 (8.8-12.1)	11.4 (9.9-15.8)	0.05
Plasma folate (nmol/L)	8.2 (6.1-11.1)	8.3 (6.1-10.9)	7.7 (5.6-11.3)	8.2 (6.6-10.7)	9.0 (5.8-11.8)	0.9
Cobalamin (pmol/L)	369 (311-431)	361 (321-402)	400 (316-469)	361 (313-463)	349 (270-400)	0.2
Vitamin B6 (PLP) (nmol/L)	23.0 (17.7-37.1)	21.5 (16,5-33.5)	27.9 (19.3-44.6)	21.8 (15.0-39.7)	24.3 (16.1-39.5)	0.6

*Data are presented as median (25^{th} – 75^{th} percentiles) or numbers (%).†Kruskal Wallis Test between treatment groups. GFR = estimated glomerular filtration rate; LDL = low-density lipoprotein; B6 = vitamin B6; PLP = pyridoxal 5-phosphate.

Invasive sub-study of vascular function. Patients with stable CAD scheduled for elective percutaneous coronary intervention were eligible. Additional exclusion criteria were predicted high risk for procedural complications, severe chronic obstructive pulmonary disease, pulmonary hypertension, significant valvular disease, glaucoma, poorly regulated diabetes, or use of systemic corticosteroids. There should be no indication for starting vasoactive medical therapy at the time of inclusion. All patients were treated with statins for at least two months prior to inclusion.

A total of 40 patients (8 female and 32 male) with median age 57 (range 39-74) years were enrolled. Key baseline demographic and clinical characteristics and medical treatment

are given in **Table 2**. Mean (SD) serum folate was 12.2 (6.5) nmol/L, cobalamin 381 (129) pmol/L, plasma pyridoxal phosphate (PLP) 43.3 (25.0) nmol/L and tHcy 10.7 (2.9) µmol/L.

TABLE 2 Characteristics of the study population at baseline, coronary vascular function (paper 4)

	*Total group** (n=40)	*Treatment groups**					
		Folic acid/B12 (n=20)	Non-folic acid/B12 (n=20)	*P-values*†	B6 (n=20)	Non-B6 (n=20)	*P-values*†
Age, years	57.8 (9.0)	57.4 (10.4)	58.2 (7.7)	0.78	56.3 (6.3)	59.4 (11.1)	0.28
Women, n (%)	8 (20)	3 (15)	5 (25)	0.44	3 (15)	5 (25)	0.44
Current smokers, n (%)	13 (33)	7 (35)	6 (30)	0.50	9 (45)	4 (20)	0.04
BMI, kg/m^2	26.7 (2.8)	26.6 (2.5)	26.8 (3.1)	0.89	26.0 (2.1)	27.4 (3.2)	0.11
Waist, cm	94.2 (9.1)	93.8 (7.6)	94.7 (10.6)	0.79	91.7 (5.4)	96.9 (11.4)	0.08
Systolic blood pressure, mmHg	146 (25.4)	144 (20.4)	147 (29.9)	0.67	143 (17.4)	149 (31.6)	0.46
Diastolic blood pressure, mmHg	80 (11.8)	81 (10.7)	79 (13.0)	0.63	83 (9.3)	77 (13.4)	0.10
Creatinine, µmol/L	88 (9.9)	89 (11.1))	87 (8.9)	0.67	88 (11.2)	87 (8.7)	0.74
GFR, mL/min/1.73m^2	76 (12.3)	76 (12.9)	77 (11.9)	0.95	79 (13.7)	74 (10.6)	0.26
LDL-cholesterol, mmol/L	2.9 (0.7)	2.9 (0.5)	2.9 (0.9)	0.95	2.8 (0.6)	3.0 (0.9)	0.36
CRP, mg/L	1.6 (0.9-2.7)	1.6 (0.9-2.7)	1.5 (0.9-2.7)	0.82	1.7 (1.0-2.5)	1.5 (0.7-2.8)	0.72

*Mean (SD) or numbers (%), except CRP which is given in median (25-75th percentile). †Comparison of continuous variables between groups by ANOVA, except for CRP in which is comparison is done by Kruskal-Wallis Test. Comparison of proportions between groups by Chi-Square Test. BMI = body mass index, GFR = estimated glomerular filtration ratio, LDL-cholesterol = low density lipoprotein cholesterol, CRP = C-reactive protein.

Intervention

Using a 2x2 factorial block design, patients were randomised into 4 groups of daily oral treatment with A) folic acid (0.8 mg)/vitamin B12 (0.4 mg)/vitamin B6 (40 mg), B) folic acid/B12, C) B6 alone or D) placebo. This design, allow us to discriminate the effects of folic acid/vitamin B12 and vitamin B6. For the first two weeks, groups A and B received additional folic acid 5 mg/day to secure rapid initial effect. Packages of trial capsules were prepared and given serial number in random order in blocks of 20 by Alpharma A/S (Copenhagen, Denmark).

Procedures and measurements

Blood collection, storage and analyses. Blood sampling was done by study personal to ensure adequate handling. The concentration of homocysteine increases within hours when stored in room temperature or if samples are not separated from blood cells [11]. Our measurements of tHcy and metabolites were done in EDTA-plasma, samples were placed on ice, centrifuged within 30 min and stored at -80 ºC until further analysed. Analyses of vitamins, tHcy and related metabolites were done at the laboratories of Locus for Homocysteine and Related Vitamins. Routine blood analyses, including haematological

parameters, renal function markers and lipid related factors, were analysed at the Central laboratory of the Haukeland University Hospital. Inflammatory parameters were measured with commercially available immunoassays [73].

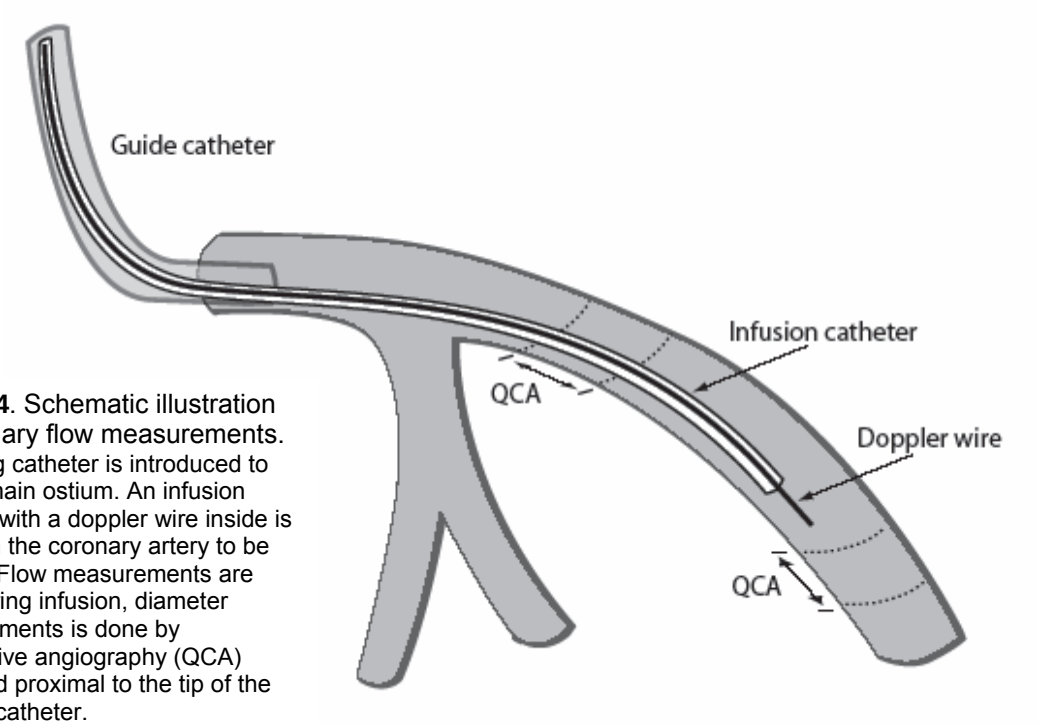

Figure 4. Schematic illustration of coronary flow measurements. A guiding catheter is introduced to the left main ostium. An infusion catheter with a doppler wire inside is placed in the coronary artery to be studied. Flow measurements are done during infusion, diameter measurements is done by quantitative angiography (QCA) distal and proximal to the tip of the infusion catheter.

Assessment of coronary vascular function. Measurements were done during consecutive intracoronary administration of saline, acetylcholine, adenosine and nitroglycerin. A Doppler guide wire was placed in a non-branching segment of the study vessel through the inner lumen of a 2.9-French coronary infusion catheter (UltraFuse-X) ending 1 cm distal to the catheter tip (**Figure 4**).

Infusions through the UltraFuse-X catheter were done as follows in seven steps: 1) Saline 0.9% for three minutes, 2-4) incremental dosage (0.72 µg/min, 7.2 µg/min and 36.0 µg/min) of acetylcholine for three minutes and 20 seconds each (estimated transit time of 20 seconds), 5) saline 0.9% for approximately five minutes until return to basal flow, 6) adenosine at a dose rate of 2.4 mg/min for 3 min and 20 sec., and finally the infusion line was flushed with saline, and 7) a 0.2 mg bolus of nitroglycerin was given. Average peak flow velocity (APV) was continuously recorded (**Figure 5**). At the end of each infusion step, an angiogram was done in the same position and angle. A coronary artery segment of 10 mm, 2-3 mm distal to the Doppler wire, was used for mean diameter measurement by digitalized

quantitative coronary angiography (QCA) with the contrast-filled catheter as reference for calibration. Coronary blood flow (CBF) was calculated by use of APV and vessel diameter (CBF = πr2(½APV)) [74]. Flow mediated dilatation (FMD) was calculated comparing mean diameter in a 10 mm segment of the study vessel proximal to the infusion catheter tip during saline infusion and during hyperaemia induced by adenosine infusion.

Coronary vascular function was assessed by CBF measurements at each step and proximal coronary FMD at maximum hyperaemic flow. Also, as a measure of endothelial function, response to acetylcholine infusion was calculated as percent increase in CBF at each dose of acetylcholine compared to baseline CBF.

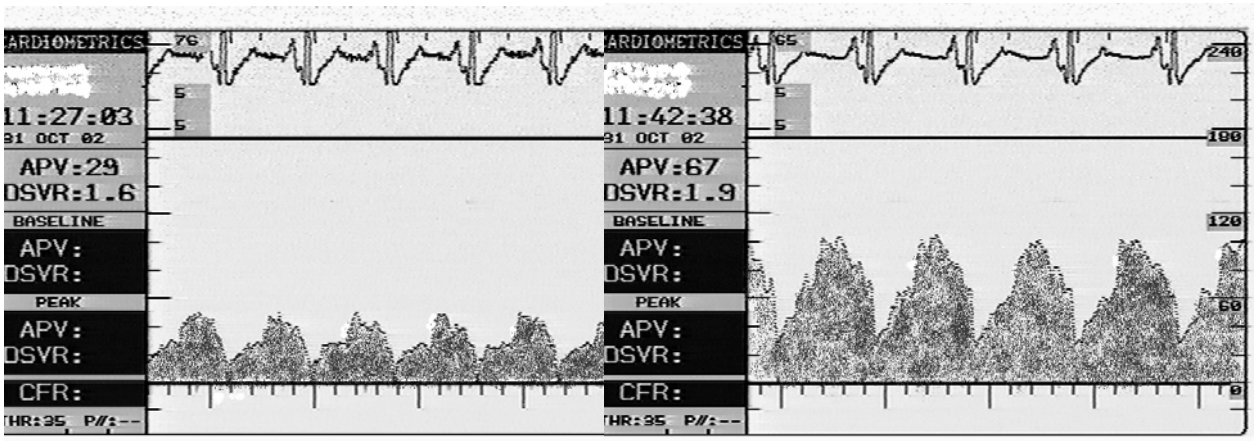

Figure 5. Doppler measurements. Average peak velocity (APV) at basal conditions (left), and at maximal hyperaemia (right).

Statistical methods

Summary measures for continuous variables are reported as means (SD) and categorical variables as proportions (%). ANOVA was used for comparison of continuous variables between treatment groups and Chi-Square Test for comparison of proportions. Skewed data, like plasma concentration of tHcy and metabolites, and even more for inflammatory markers, are presented as medians with ranges and comparison between groups is done with Kruskal-Wallis Test or Mann-Whitney U-test. Alternatively, logarithmic transformation is done.

Associations were assessed by Pearson correlation coefficients, Spearman rank correlation and linear regression. Paired sample t-test or Wilcoxon signed rank test were used for comparison within groups, and repeated measures ANOVA for comparison between groups, over time. Statistical package SPSS 11.0 – 13.0 was used. In paper 4, differences in time trends of flow (change during follow-up) by treatment were analysed by a linear mixed-

effect model with random intercept on subject level (S-PLUS 7.0 for Windows). A two-tailed $P < 0.05$ was considered statistical significant.

Ethics

Written informed consent was obtained from all patients. Study protocol was in accordance with the principles of the Declaration of Helsinki and approved by the Regional Committee for Medical Research Ethics. The study medication was approved by the Norwegian Medicines Agency and the data registration was approved by the Data Inspectorate.

Results and relation to other studies

Paper 1

In WENBIT, a relatively low daily folic acid dose of 0.8 mg was chosen for long-term treatment. To ensure rapid effect, our regimen also includes a high additional folic acid dose of 5 mg/day given for the first two weeks. This loading dose caused an immediate tHcy response with a significant reduction at three days, and a maximum reduction of 31% was reached within two weeks. Also, the effect was maintained for at least six months despite the low long-term dose. The dose required for effective and safe tHcy reduction during long-term supplementation for years has been debated, and there are safety concerns about daily doses above 1 mg, since high doses may lead to free folic acid in serum [75]. A high folic acid dose of 2 – 5 mg is used in many ongoing trials [76], probably because such a dose may have additional tHcy reducing effects in some patients, such as those with renal impairment [77]. We could demonstrate a significant reduction of tHcy within 3 days, and a long-term effect of the 0.8 mg dose later confirmed effective in meta-analysis [65].

Our study confirms previous reports that treatment with B6 had no significant effect on basal tHcy [64,78] but caused a significant reduction in PML tHcy [79,80]. In our patients, the vitamin B6 effect on PML tHcy was independent of and added to the effect of the folic acid/B12 combination. The most striking effect of the B6 supplementation was a rapid and pronounced reduction in the plasma cystathionine levels. Vitamin B6 treatment eliminated high levels of cystathionine after methionine loading with no change in PML methionine. Our findings are in agreement with published data showing that cystathionine levels are elevated in patients with B vitamin deficiency [81,82], that a triple combination with folic acid, B12 and B6 lowers basal cystathionine concentrations in elderly people [78], and that vitamin B6 alone lowers basal as well as PML cystathionine levels in both healthy and vitamin B6 deficient [83].

The vitamin B6 effect on cystathionine levels probably reflect an enhanced activity of the cystathionine-γ-lyase which is known to be very sensitive to PLP depletion [83-85]. In our study population, we found that only vitamin B6 treatment influences cystathionine levels, suggesting that cystathionine levels may be a unique marker for identifying subjects that will respond to vitamin B6 intervention.

Paper 2

The median plasma concentration of betaine reported (median 36.9 μmol/L) is similar to earlier reports [86], with a remarkable stable level throughout the observation period, except for a transient increase after methionine loading. Methionine loading, a situation that maximally challenges the methionine-homocysteine pathways, was done at baseline and after 3 months. At baseline we found a strong inverse association between PML betaine and PML increase in tHcy, and somewhat weaker association between basal betaine and PML increase in tHcy. After B-vitamin treatment, PML increase in tHcy was reduced, its inverse relation to betaine weakened and was essentially abolished in patients receiving folic acid/vitamin B12. Our data is in concordance with a betaine supplementation study, which show a reduced PML tHcy after oral betaine supplementation [87].

These data indicate that betaine through the BHMT reaction buffers and tends to reduce the homocysteine response during methionine excess. This reaction, which conserves the homocysteine carbon backbone, may be beneficial under conditions of limiting supply of folate required for the methionine synthase reaction. These findings emphasize the complementary relationship between betaine and folate metabolism. Furthermore, because PML tHcy confers increased risk for occlusive vascular disease independent of fasting tHcy [88], betaine and related metabolites should be included in future studies of homocysteine and cardiovascular risk.

Paper 3

In the current sub study of WENBIT, we evaluated the effect of B-vitamin intervention on important biomarkers of atherogenesis, including neopterin, sCD40L, IL-6 and CRP. Prior investigations have not only indicated that tHcy is significantly related to many of these processes [38,89], but also indicate that folate and vitamin B6 are associated with CVD, independent from their relation with tHcy [69].

We found that neopterin is related to tHcy, even when renal function is corrected for. In multiple linear regression analyses with neopterin as dependent variable, and tHcy, GFR, LDL-cholesterol, folate, vitamin B6 and vitamin B12 as independent variables, predictors of neopterin were levels of tHcy (β=0.35, $p<0.001$), GFR (β=-0.37, $p<0.001$) and LDL-cholesterol (β=0.34, $p<0.001$). Our observation of a particular strong relation between tHcy and neopterin, a marker of cellular immune response, corroborates results from several previous reports [90,91]. After 6 months of treatment, the strong relation seen at baseline between tHcy and neopterin was no longer present in patients receiving folic acid and vitamin B12. This could suggest that an optimal folate status overrides the influence of immunostimulation on tHcy plasma levels. Our data further indicate that the utility of homocysteine as a predictor of CVD is limited to subjects not taking folic acid/vitamin B12 supplement.

However, despite the fact that plasma tHcy was lowered by 33% by folate 0.8 mg combined with vitamin B12 0.4 mg, and that a high dose of vitamin B6 (40 mg) was given, no significant changes in the evaluated biomarkers, neopterin, sCD40L, IL-6 or CRP, were observed. Failure to reverse inflammatory processes associated with atherosclerosis may partly explain the negative results of B-vitamin intervention in patients with established CVD treated with conventional therapy.

Paper 4

In this trial, we have shown that two years of treatment with folic acid/vitamin B12 improved basal coronary blood flow and blood flow at maximum hyperaemia induced by adenosine. In contrast, folic acid/vitamin B12 did not improve coronary endothelial function as measured by flow-induced change in coronary vessel diameter or change in flow following stimulation with acetylcholine. Vitamin B6 treatment did not change any of the vascular function variables.

Although the number of patients (n=40) may seem limited, this is the largest study on folate therapy and coronary vascular function. Most other studies on endothelial function and B-vitamins, have measured brachial FMD [41]. Only one study on the effect of B-vitamins on coronary flow in patients with CAD has been published [92], with a total of 15 patients randomised to treatment with folic acid (5 mg/d) and vitamin B12 (0.4 mg/d) or placebo for six months. In that study, B-vitamin treatment was associated with a significant improvement in acetylcholine-induced coronary blood flow. It is noteworthy that these patients had relatively high baseline tHcy (17.9 μmol/L), few patients received statin therapy, and a high

dose of folic acid was used. In contrast, we found an effect of folic acid/vitamin B12 on basal and maximal hyperaemic coronary blood flow, but no effect on acetylcholine stimulated flow.

The reported relations between folate or tHcy levels and brachial endothelial-dependent FMD are somewhat conflicting [93]. However, most studies suggest that a rapid increase in plasma tHcy levels, as observed during methionine or homocysteine loading [41,93,94], or after a protein rich meal [95], impairs brachial endothelial function, while high doses of folic acid improve endothelial function, possibly partly independent of its homocysteine-lowering effect[41,96]. The mechanism by which homocysteine impairs endothelial function may involve homocysteine-induced reduction of intracellular tetrahydrobiopterin, thereby causing eNOS-uncoupling [97]. Folic acid, through its circulating form, 5-methyltetrahydrofolate, is believed to enhance regeneration of tetrahydrobiopterin and improve eNOS-coupling and thereby improve endothelial function independently of homocysteine [98,99]. Recent data from an isolated rat heart model supports our findings of increased coronary flow by folic acid treatment, and suggests a mechanistic role of NO [100].

Whereas NO is an established regulator of vascular tone, there is some evidence that endothelium-derived hyperpolarizing factor (EDHF) plays a major role in regulating microcirculation [101]. In renal microcirculation of rats, EDHF-mediated vasodilatation is impaired during methionine loading and partly restored by 5-methyltetrahydrofolate [102]. This suggests an additional mechanism by which folic acid therapy in our patients has improved vascular tone and microcirculation, since resting coronary flow (CBF-basal) and maximal hyperaemia (CBF-ado) largely depend on microvascular dilatation in non obstructive coronary vessels [103]. A beneficial effect on microvascular flow, together with reduced arterial stiffness [104], may explain a reduced frequency of electrocardiographic changes at exercise tests [105] and reduction in blood pressure [106] observed after treatment with folic acid.

If long-term treatment with folic acid improves vascular function, this may have important clinical implications. and explain a reduction in stroke mortality rate observed in North America following folic acid fortification [107] and a reduction in stroke events in the HOPE-2 study [108]. Prior studies have demonstrated that even minor reductions in blood pressure are associated with significant lower risk of stroke, but with less effect on ischemic heart disease [109]. Thus, potential treatment effects of folic acid related to a reduction in blood pressure, may act differently upon risk of stroke and CAD [19,109]. Notably, folic acid/B12 therapy has not been associated with reduced risk of coronary events in any of the published randomised trials with B-vitamin intervention in patients with established CVD [110,111].

MAIN CONCLUSIONS

1. The folic acid/vitamin B12 combination used in WENBIT provides a rapid, substantial and long-term tHcy lowering effect and significantly reduces PML tHcy.
2. Vitamin B6 does not influence basal tHcy, but reduces PML tHcy.
3. Vitamin B6 treatment markedly reduces basal and PML cystathionine levels.
4. Folic acid and betaine are interchangeable methyl donors in homocysteine metabolism.
5. Treatment with folic acid/vitamin B12 or vitamin B6 has no detectable effect on levels of neopterin, sCD40L, IL 6 or CRP, inflammatory markers, which are related to atherosclerosis.
6. Treatment with moderate doses of folic acid in combination with vitamin B12 is associated with a significant increase in both basal and in adenosine-stimulated maximal coronary blood flow, reflecting improved vascular function.

FURTHER PERSPECTIVES

Numerous studies demonstrate that hyperhomocysteinemia is related to an increased risk of the existence, extent, progression and prognosis of CVD [33,34]. In contrast, in recent published trials, no significant effect on the primary outcome was documented in patients with stroke (VISP) [111], acute myocardial infarction (NORVIT) [110], CVD (HOPE-2) [108] or chronic renal failure (ASFAST) [112]. A nearly significant increase of myocardial infarction was demonstrated in the NORVIT trial (in the group receiving both folic acid and vitamin B6) [110], whereas a significant reduction in stroke was demonstrated in HOPE-2 [108]. The latter finding is in accordance with a reduction in stroke mortality rate observed in North America following folic acid fortification [107].

We have recently presented preliminary clinical data from WENBIT (ESC -07, M.Ebbing), which do not support a secondary preventive effect of B-vitamins in a population with CVD. Both the VISP and the ASFAST trials, and possibly WENBIT, were underpowered to detect clinical effects of the intervention of a magnitude similar to what might have been expected from the most recent epidemiological data [111,112]. Thus, the results from these clinical trials still leave uncertainty of the possible role of homocysteine in the progression of atherosclerotic disease and further trials (WACS, SEARCH, PACIFIC, CHOAS-2, VITATOPS) must be completed.

In addition, two randomised trials evaluating restenosis after PCI showed conflicting results [60,61]. WENBIT will provide data on B-vitamins and restenosis after coronary stenting to further elucidate this question.

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Paper I

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

Øyvind Bleie, Helga Refsum, Per Magne Ueland, Stein Emil Vollset, Anne Berit Guttormsen, Ebba Nexo, Jørn Schneede, Jan Erik Nordrehaug, and Ottar Nygård

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.

[1] From the Institute of Medicine (ØB, JEN, and ON), Department of Pharmacology (HR and ABG), and Locus for Homocysteine and Related Vitamins (PMU, SEV, JS, and ON), University of Bergen, Norway; the Department of Pharmacology, University of Oxford, United Kingdom (HR); and the Department of Clinical Biochemistry, AKH, Aarhus University Hospital, Aarhus, Denmark (EN).

[2] Supported by the Biomed project "Homocysteine Determination in Laboratory Diagnostics" (BMH4-98-3549), Alpharma A/S, The Norwegian Foundation for Health and Rehabilitation, The Norwegian Heart and Lung Association, the Advanced Research Program (Norway), The Norwegian Research Council, and the Regional Committee of Health Region West, Norway. Alpharma A/S, Copenhagen, Denmark, provided the study medication free of charge.

[3] Address reprint requests to Ø Bleie, Department of Heart Disease, Haukeland University Hospital, N-5021 Bergen, Norway. E-mail: oyvind.bleie@haukeland.no.

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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 $\pm$ 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

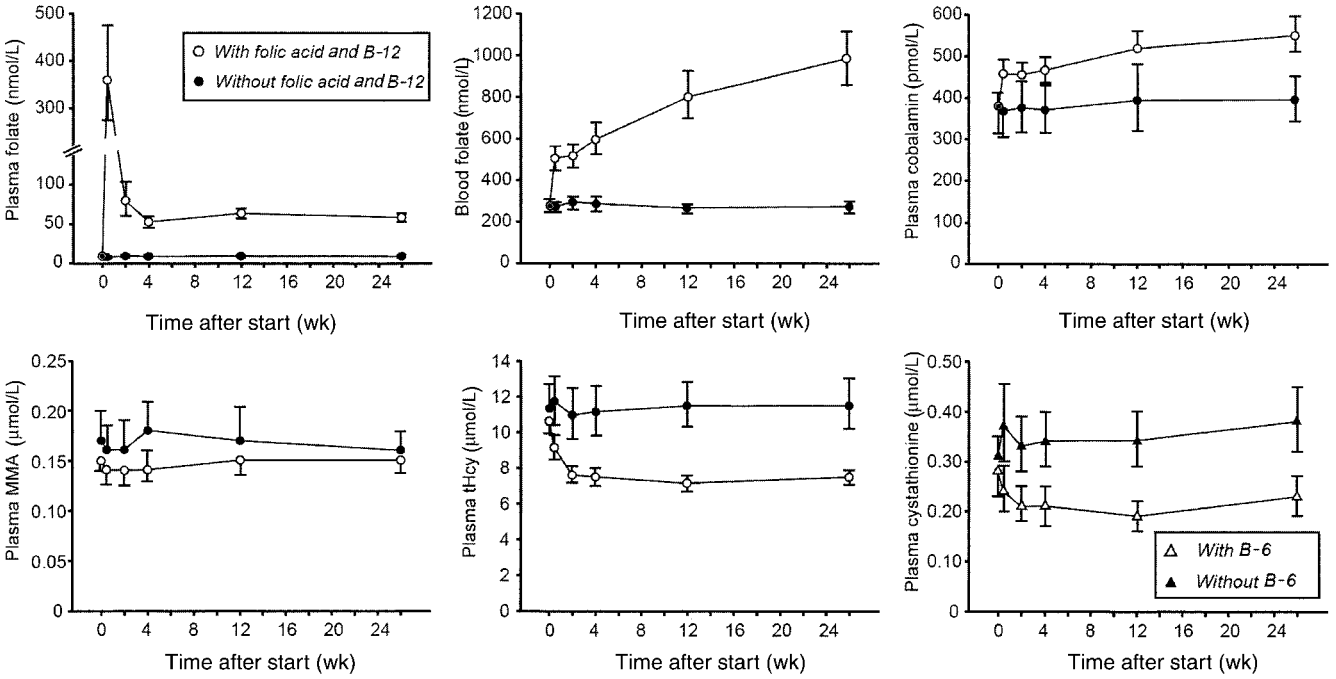

FIGURE 1. Geometric mean changes in vitamin and metabolite concentrations during 6 mo of B vitamin supplementation. Error bars represent 95% CIs. Only patients attending all 6 sessions are included in these analyzes ($n = 74$). The 2 patient groups with and without the actual vitamins were combined in 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

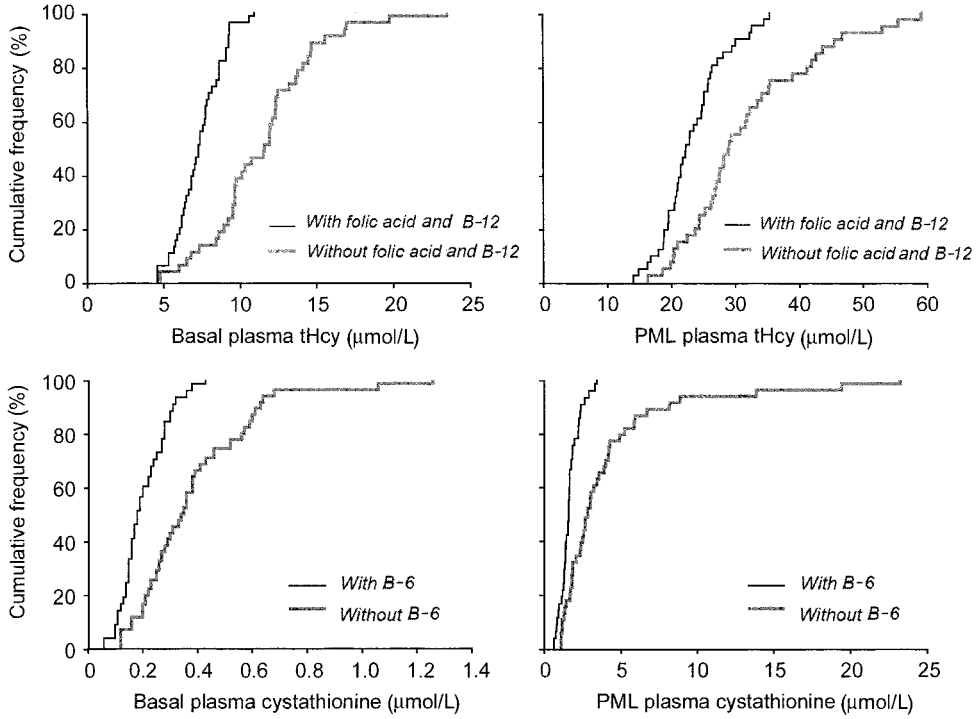

FIGURE 2. Cumulative frequency plots of plasma total homocysteine (tHcy) and plasma cystathionine after 3 mo of B vitamin intervention ($n = 81$). The effectiveness of folic acid and vitamin B-12 ($n = 41$) in reducing both high and low basal plasma and postmethionine load (PML) tHcy compared with vitamin 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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ØB participated in the design of the study and recruitment of patients, supervised blood processing, participated in statistical analyses and interpretation, and was responsible for writing the article. HR participated in the design of the study, supervised blood processing, participated in the interpretation of data, and provided significant input for writing the article. PMU participated in the design of the study and in the interpretation of data and provided significant input for writing the article. SEV participated in the design of the study, in the statistical analyses, and in the interpretation of data and provided input for writing the article. ABG developed the cystathionine analyses and contributed in interpretation of these data. EN was responsible for plans for and performance of vitamin B-6 analysis and provided input for the article. JS supervised blood processing and contributed in interpretation of data. JEN participated in the design and supervision of the study and provided input for writing the article. ON was responsible for the design of the study and recruitment of patients, supervised blood processing, participated in statistical analyses and data interpretation, and provided significant input for writing the article. There is no financial conflict of interest in relation to this study.

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Paper II

Betaine as a Determinant of Postmethionine Load Total Plasma Homocysteine Before and After B-Vitamin Supplementation

Pål I. Holm, Øyvind Bleie, Per M. Ueland, Ernst A. Lien, Helga Refsum, Jan E. Nordrehaug, Ottar Nygård

Objective—Betaine is a substrate in the betaine–homocysteine methyltransferase reaction, converting homocysteine to methionine. There are only sparse data on plasma betaine as a determinant of the plasma total homocysteine (tHcy) concentration.

Methods and Results—Ninety patients undergoing coronary angiography were randomized into 4 groups administered oral: (1) folic acid (0.8 mg), vitamin B12 (0.4 mg), and vitamin B6 (40 mg); (2) folic acid and vitamin B12; (3) vitamin B6 alone; or (4) placebo. Nonfasting blood samples were collected at baseline and 3, 14, and 28 days and 3, 6, and 12 months after treatment start. A 4-hour methionine-loading test (0.1 g/kg) was performed at baseline and after 3 months. At baseline, median (interquartile range) plasma betaine was 36.9 μmol/L (range: 30.3 to 46.8) and was increased by 15% after methionine loading. The postmethionine load (PML) increase in tHcy was inversely related to plasma betaine ($\beta=-0.29$, $P=0.02$) and even more strongly to PML betaine ($\beta=-0.47$, $P<0.001$). After 3 months of intervention, the relation between the PML increase in tHcy and PML betaine was weakened ($\beta=-0.33$, $P=0.007$).

Conclusions—Plasma betaine is a strong determinant of the PML increase in tHcy in subjects not supplemented with B-vitamins. **(*Arterioscler Thromb Vasc Biol.* 2004;24:301-307.)**

Key Words: betaine ■ homocysteine ■ folate ■ vitamin B12 ■ vitamin B6

Homocysteine is an established risk factor for occlusive vascular disease.[1] High plasma concentration is also associated with impaired cognitive function, Alzheimer disease, adverse pregnancy outcomes, and congenital malformations, particularly neural tube defects.[2]

The plasma concentration of total homocysteine (tHcy) is determined by a variety of physiological, lifestyle, and genetic factors and disease states.[3] Among these, renal function, folate, and cobalamin status are particularly influential.[4] The vitamin effects are explained by the functions of 5-methyltetrahydrofolate as substrate and cobalamin as cofactor in the ubiquitous methionine synthase reaction, which catalyzes the remethylation of homocysteine to methionine.[5] Vitamin B6, however, has no[6,7] or a moderate[8,9] effect on plasma tHcy.

Methionine loading, which involves measurement of tHcy after a standard oral methionine dose, was originally designed to reveal defects in the vitamin B6-dependent transsulfuration pathway.[10] Plasma postmethionine load (PML) tHcy has since been associated with cardiovascular risk independent of basal tHcy.[11] Folate, as well as vitamin B6 status,[9,11] are significant predictors of PML tHcy. These vitamin effects in humans are at variance with the idea, based on enzymological data,[5] animal,[12] and human studies,[6] that PML tHcy is determined by the activity of vitamin B6-dependent enzyme, cystathionine β-synthase, whereas the folate-dependent homocysteine remethylation regulates basal tHcy.

Betaine (trimethylglycine) is obtained in small amounts through the diet or is generated in liver and kidney from choline through the sequential action of choline oxidase (EC 1.1.3.17) and betaine-aldehyde dehydrogenase (EC 1.2.1.8).[5,13] Betaine serves as methyl donor of the zinc metalloenzyme, betaine-homocysteine methyltransferase (BHMT; EC 2.1.1.5). This enzyme is mainly confined to the liver and kidney and catalyzes an alternative route of homocysteine remethylation to methionine[5] (Figure 1). The role of betaine as a methyl donor explains the observations that betaine supplementation reduces tHcy in homocystinurics[14] and in healthy individuals.[15–18] Betaine also attenuates the tHcy increase after methionine loading in healthy subjects[16,18] and in cardiovascular[19] and renal patients.[20] However, except for a recent Canadian study reporting on an inverse relation between fasting tHcy and betaine in cardiovascular patients,[21] no study has addressed the question of the role of endogenous betaine as a determinant of basal and PML tHcy.

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From Locus for Homocysteine and Related Vitamins (P.I.H., Ø.B., P.M.U., E.A.L., O.N.), Institute of Medicine (Ø.B., J.E.N., O.N.), University of Bergen, Norway, and the Department of Pharmacology (H.R.), University of Oxford, United Kingdom.

Correspondence to Per Magne Ueland, LOCUS for Homocysteine and Related Vitamins, Department of Pharmacology, University of Bergen, N-5021 Bergen, Norway. E-mail per.ueland@ikb.uib.no

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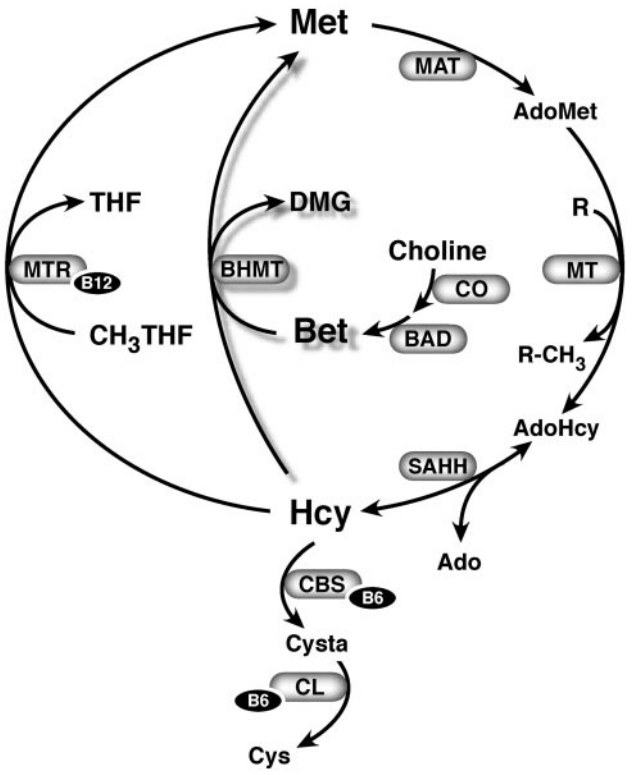

Figure 1. Betaine metabolism, homocysteine remethylation, and transsulfuration. Ado indicates adenosine; AdoHcy, S-adenosylhomocysteine; AdoMet, S-adenosylmethionine; BAD, betaine aldehyde dehydrogenase; Bet, betaine; BHMT, betaine-homocysteine methyltransferase; CBS, cystathionine β-synthase; CL, cystathionine lyase; CH3THF, methyltetrahydrofolate; CO, choline oxidase; DMG, dimethylglycine; Cys, cysteine; Cysta, cystathionine; Hcy, homocysteine; MAT, methionine adenosyltransferase; Met, methionine; MT, methyltransferase; MTR, methionine synthase; SAHH, S-adenosylhomocysteine hydrolase; THF, tetrahydrofolate.

Using a newly developed liquid chromatography–tandem mass spectrometry method,[22] we investigated the association between plasma betaine and tHcy before and after methionine loading in 90 subjects. The subjects were enrolled in the Western Norway B-Vitamin Intervention Trial (WENBIT) and followed-up for 1 year of B-vitamin intervention as implemented in this trial.

Methods

Subjects

We investigated 90 consecutive patients enrolled in the WENBIT, an ongoing study on the effects of tHcy-lowering therapy on mortality and cardiovascular events in 3000 patients. Eligible subjects were adult patients (older than 18 years) undergoing coronary angiography for suspected coronary artery disease or aortic valvular stenosis. Exclusion criteria were malignant disease, alcohol abuse, mental illness, patient unwillingness to participate in long-term follow-up, and participation in other studies. The subjects were recruited at Haukeland University Hospital from April 1999 to September 1999. Informed consent was obtained from all patients. The study protocol was approved by the regional ethics committee and Norwegian Medicines Agency.

Protocol

Recruited patients were randomized into 4 groups in a 2×2 factorial block design. Group FB (n=22) was administered daily folic acid (0.8 mg), vitamin B12 (cyanocobalamin, 0.4 mg), and vitamin B6 (pyridoxine, 40 mg). Group F (n=23) was administered folic acid and vitamin B12. Group B (n=21) was administered vitamin B6. Group P (n=24) was administered placebo. For the first 2 weeks, the groups (FB and F) receiving folic acid were administered an additional loading dose of folic acid (5 mg/d). Packages of trial tablets were prepared and given serial number in random order in blocks of 20 by Alpharma A/S (Copenhagen, Denmark). Compliance monitoring was accomplished by tablet counting and by determination of vitamin B concentrations in plasma.

The subjects underwent a full routine medical examination at baseline before coronary angiography. Coronary angiography was performed 3 days after starting vitamin therapy.

Blood Collection and Biochemical Analyses

Nonfasting (basal) blood samples were collected at baseline, after 3 days, at 2 and 4 weeks, and at 3, 6, and 12 months of B-vitamin intervention. A methionine loading test (0.1 g/kg body weight) was performed at baseline and after 3 months. PML blood samples were drawn at 4 hours after loading. EDTA blood samples were immediately placed on ice, centrifuged within 30 minutes, and EDTA plasma stored at −80°C until analysis.

Routine blood analyses, including serum creatinine, were analyzed at the Central Laboratory of Haukeland University Hospital on Technicon Chem 1 (Bayer, Leverkusen, Germany). The tHcy,[23] betaine, choline, di-MethylGlyoxime (DMG),[22] cobalamin[24] plasma folate,[25] and pyridoxal phosphate[26] were analysed by published methods.

Statistics

Because of skewed distribution of values for tHcy and some metabolites, data are presented as median values with interquartile ranges. Associations between variables were evaluated by Spearman rank correlation and multiple linear regression analyses. Variables considered likely to influence the outcome parameters were included in the multivariate models. Mann-Whitney *U* test or Kruskal-Wallis test was used for between-group comparisons of continuous variables, and Wilcoxon signed rank test was performed for comparison within groups at baseline and after 3 months. Data were analyzed using SPSS 11.0 (SPSS Inc.).

Results

Subject Characteristics and Blood Indices at Baseline

A total of 90 patients were included. Their median age was 62 years (range 38 to 80); 77% were males. Blood indices at baseline for the whole study group are given in Table 1. Median betaine was 36.9 μmol/L and median PML betaine was 44.5 μmol/L. One patient (in group P) had plasma cobalamin (117 pmol/L) below the lower reference limit (200 pmol/L), along with elevated MMA (0.77 μmol/L), and was considered cobalamin-deficient. Plasma folate was below the lower reference limit (4.5 nmol/L) in 10 patients.

Table 1 also shows the expected marked increase in tHcy and also a moderate increase in median betaine (15%; $P<0.001$), choline (7%; $P=0.001$), and DMG (18%; $P<0.001$) after methionine loading.

The concentrations of basal and PLM betaine in particular, but also of choline and creatinine, were markedly higher in males than in females (Table 1). No parameters differed significantly according to treatment group, except basal tHcy (18% difference; $P=0.04$) and basal choline (15%; $P=0.01$) and PML choline (21%; $P=0.04$) (data not shown). Details on plasma pyridoxal phosphate[26] and cobalamin[27] in this population have been given in separate publications.

TABLE 1. Subject Characteristics and Blood Indices at Baseline According to Gender*

	Total n=90	Men n=69	Women n=21	*P* Value†
tHcy, μmol/L	11.0 (9.3–12.9)	11.4 (9.8–13.0)	9.6 (7.7–12.9)	0.09
PML tHcy, μmol/L	31.3 (27.0–37.6)	30.6 (27.1–36.0)	32.1 (24.3–39.0)	0.9
Betaine, μmol/L	36.9 (30.3–46.8)	39.2 (33.2–48.0)	27.9 (22.8–33.0)	<0.001
PML betaine, μmol/L	44.5 (34.1–53.9)	47.2 (40.1–58.3)	29.3 (27.0–38.8)	<0.001
Choline, μmol/L	9.0 (7.7–10.7)	9.4 (7.9–11.0)	8.0 (7.3–8.9)	0.008
PML choline, μmol/L	10.3 (8.3–11.4)	10.4 (8.4–12.0)	9.6 (7.0–11.0)	0.02
DMG, μmol/L	3.64 (3.17–4.58)	3.67 (3.27–4.63)	3.32 (2.79–4.10)	0.1
PML DMG, μmol/L	4.54 (3.58–5.44)	4.58 (3.72–5.42)	4.17 (3.42–6.11)	0.4
Age, y	62 (54–68)	62 (55–68)	62 (53–70)	1.0
Creatinine, μmol/L	94 (87–101)	96 (89–103)	80 (73–93)	<0.001
Folate, nmol/L	8.2 (6.1–11.1)	7.8 (6.1–10.9)	8.8 (6.6–12.2)	0.4
Cobalamin, pmol/L	369 (311–481)	373 (319–437)	355 (297–425)	0.6
Vitamin B6‡, nmol/L	23.0 (17.7–37.1)	22.9 (15.7–37.0)	23.0 (18.3–39.7)	0.5

*Data are given as medians with interquartile ranges (25th–75th percentiles) in parentheses.
†Mann-Whitney *U* test.
‡Pyridoxal phosphate.
tHcy indicates total homocysteine; PML, postmethionine load; DMG, dimethylglycine.

Simple Correlations at Baseline

Simple correlations at baseline are shown in Table 2. Baseline tHcy was positively related to age, creatinine, and basal DMG, and were inversely related to plasma folate and cobalamin.

The increase in tHcy after methionine loading (ΔPML tHcy) was not related to the levels of B-vitamins. The ΔPML tHcy showed a strong inverse association with PML betaine ($r=-0.37$, $P<0.001$) and a moderate association with basal betaine ($r=-0.23$, $P=0.03$), as also depicted in Figure 2.

The concentrations of betaine, choline, and DMG, measured before and after loading (PML), were significantly and positively correlated (Table 2; data for choline and DMG not shown).

Determinants of Betaine, Choline, and Dimethylglycine

We assessed age, gender, creatinine, and the B-vitamins as predictors of basal betaine, choline, and DMG at baseline by multiple linear regression (Table 3). Betaine showed a strong relation to sex, whereas choline in particular, but also DMG, was positively related to creatinine. The metabolites were not significantly related to any B vitamin, ie, folate, cobalamin, or vitamin B6 (Table 3). Betaine, choline, and DMG measured after methionine loading showed similar relations to gender, age, creatinine, and the B-vitamins as observed for the basal concentrations (data not shown).

Betaine as a Determinant of PML tHcy

The ΔPML tHcy was inversely associated with basal betaine (model I), PML betaine (model II) (Table 3), and the PML increase in betaine ($\beta=-0.27$, $P=0.02$) by multiple linear regression. PML betaine was a stronger predictor than basal betaine of ΔPML tHcy. The regression models also included age, sex, creatinine, and the B-vitamins, and these factors were not significantly related to ΔPML tHcy (Table 3). Additional adjustment for basal or PML choline and basal or PML DMG in the respective models had no effect (data not shown).

Betaine, Choline, and DMG According to Duration and Type of Intervention

Figure 3 depicts plasma concentrations for choline, betaine, and DMG measured at intervals from baseline to 12 months. PML concentrations at baseline and after 3 months are included. The levels for betaine in particular, but also for choline and DMG, were higher in men than in women

TABLE 2. Spearman Correlations at Baseline

	Age	Creatinine	ΔPML tHcy	Betaine	PML betaine	Choline	DMG	Folate	Cobalamin	Vitamin B6
tHcy	0.50†	0.44†	0.40†	0.07	0.07	0.18	0.32*	−0.21*	−0.21*	−0.08
ΔPML tHcy	0.14	0.08		−0.23*	−0.37†	0.01	0.03	−0.06	−0.13	0.02
Betaine	0.13	0.07			0.79†	0.49†	0.42†	−0.06	0.02	−0.05
PML betaine	0.15	0.18				0.34*	0.33*	−0.08	0.11	0.00
Choline	0.17	0.28*					0.50†	0.05	−0.12	−0.19
DMG	0.28*	0.15						−0.14	−0.1	−0.09

*$P<0.05$.
†$P<0.001$.
tHcy indicates total homocysteine; ΔPML, postmethionine load increase; DMG, dimethylglycine.

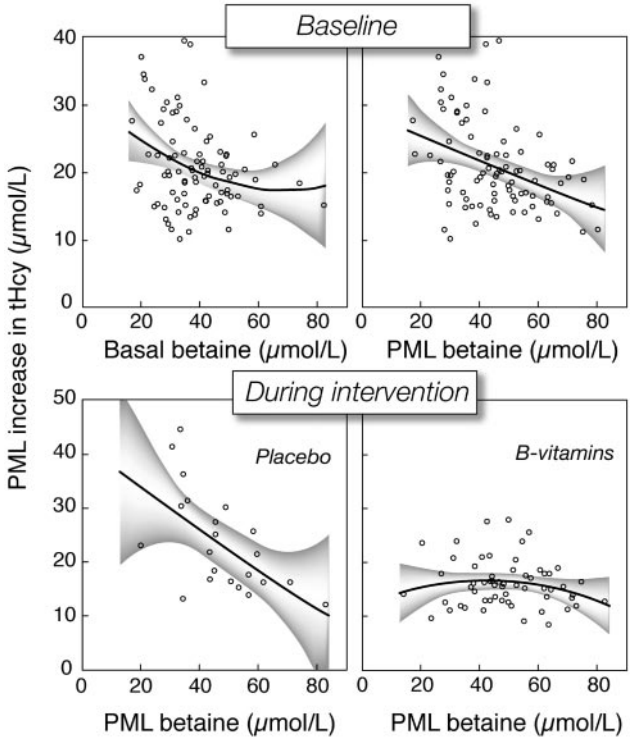

Figure 2. The relation between plasma betaine and the PML increase in plasma tHcy at baseline and after 3 months of B-vitamin supplementation. The upper panels show the relations between betaine (basal and PML) and PML increase in plasma tHcy before intervention. The lower left panel shows PML betaine versus PML increase in tHcy in 22 subjects (group P) administered placebo and the lower right panel shows this relationship in the remaining 68 subjects (groups FB, F, and B) treated with B-vitamins. The curves were obtained by quadratic regression, and the shaded areas represent the 95% confidence interval of mean regression line.

throughout the intervention period and showed a transient increase after methionine loading (Figure 3).

We compared the basal and PML concentrations of betaine, choline, and DMG at 3 months according to the treatment groups FB, F, B, and P. The metabolite concentrations, except PML choline ($P=0.03$), were not significantly different between the groups ($P>0.4$) (data not shown).

B-Vitamin Supplementation and the Betaine–PML tHcy Relationship

After 3 months of intervention, plasma cobalamin and plasma folate were higher than 277 pmol/L and 25 nmol/L, respectively, in all patients in groups FB and F, and pyridoxal phosphate was higher than 142 nmol/L in groups FB and B. No significant changes in median levels of these vitamins occurred in the nonsupplemented groups.

ΔPML tHcy (μmol/L; median [interquartile range]) decreased in the groups FB (from 19.4 [15.5 to 22.7] to 14.7 [11.6 to 17.2], $P=0.004$), F (from 19.7 [16.6 to 24.6] to 15.8 [12.9 to 18.9], $P=0.002$), and B (from 19.3 [15.3 to 23.6] to 16.3 [13.5 to 17.9], $P=0.005$) given B-vitamins. ΔPML tHcy did not change in the placebo group P (from 20.9 [17.0 to 30.0] to 21.8 [16.3 to 30.2], $P=0.5$).

After 3 months of intervention, the inverse association between ΔPML tHcy and PML betaine in the subjects receiving B-vitamins was essentially abolished but was still present in the subgroup (P) receiving placebo, as depicted in Figure 2. Multiple linear regression analyses of the association between ΔPML tHcy and PML betaine in the separate intervention groups demonstrated that the association was absent in the groups FB and F administered folic acid ($P>0.6$), showed a trend in the group B administered vitamin B6 ($P=0.09$), and was still strong and significant in the group P administered no B-vitamins ($\beta=-0.66$, $P=0.02$). All these models were adjusted for age, sex, creatinine, cobalamin, folate, and vitamin B6.

Predictors of ΔPML tHcy at 3 months in the whole study group were assessed by multiple regression (Table I, available online at http://atvb.ahajournals.org). The model contained the B-vitamins, in addition to creatinine, age, and sex.

TABLE 3. Determinants at Baseline of Basal Betaine, Choline, Dimethylglycine, and the increase in tHcy After Methionine Loading by Multiple Linear Regression*

Dependent Variable	Independent Variables	β	P
Basal betaine	Age	0.12	0.06
	Sex	−0.48	<0.001
	Creatinine	−0.17	0.1
	Folate	0.14	0.2
	Cobalamin	−0.09	0.4
	Vitamin B6	−0.005	0.9
Basal choline	Age	0.08	0.4
	Sex	−0.12	0.2
	Creatinine	0.37	0.001
	Folate	0.11	0.3
	Cobalamin	−0.15	0.2
	Vitamin B6	−0.08	0.5
Basal DMG	Age	0.16	0.2
	Sex	−0.07	0.5
	Creatinine	0.24	0.04
	Folate	−0.08	0.5
	Cobalamin	−0.17	0.1
	Vitamin B6	0.10	0.4
tHcy increase (Model I)	Age	0.19	0.1
	Sex	0.02	0.9
	Creatinine	0.02	0.8
	Folate	−0.13	0.3
	Cobalamin	0.06	0.6
	Vitamin B6	−0.08	0.5
	Basal betaine	−0.29	0.02
tHcy increase (Model II)	Age	0.20	0.07
	Sex	−0.10	0.4
	Creatinine	0.06	0.6
	Folate	−0.17	0.1
	Cobalamin	0.09	0.4
	Vitamin B6	−0.02	0.9
	PML betaine	−0.47	<0.001

*All variables are included in each of the models.
DMG indicates dimethylglycine; tHcy, total homocysteine.

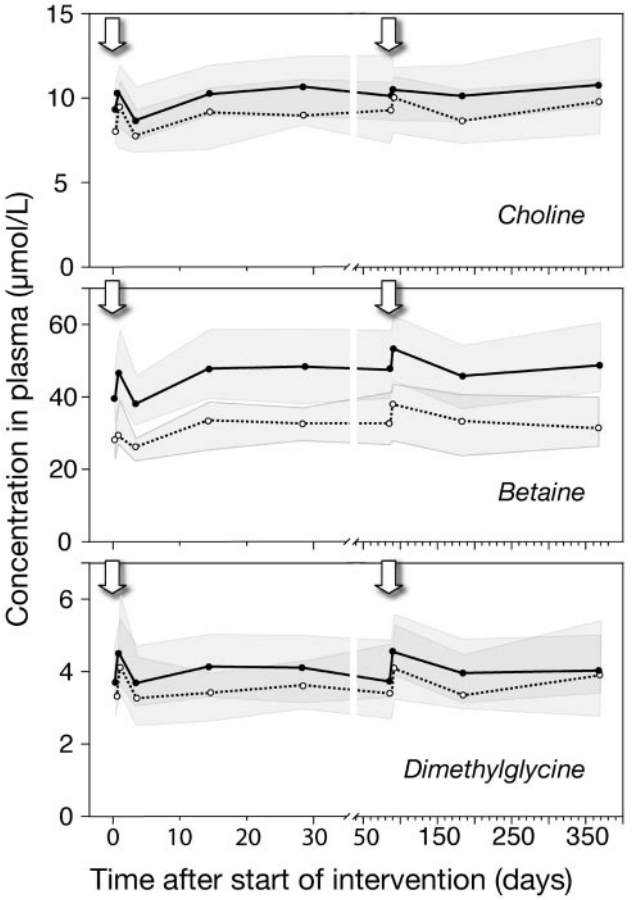

Figure 3. Plasma concentration of choline, betaine, and dimethylglycine during B-vitamin supplementation and after methionine loading. The metabolite concentrations are given separately for men (filled circles) and women (open circles) according to time after start of intervention. Arrows indicate methionine loading, which was performed on day 0 and on day 84, and the values obtained 4 hours after loading are included in the graph. Data are given as medians and the shaded areas indicate the 25 to 75 percentiles.

PML betaine was strongly associated with ΔPML tHcy, but the association was weaker ($\beta=-0.33$, $P=0.007$) than before treatment ($\beta=-0.47$, $P<0.001$; Table 3). Plasma folate and, to a lesser extent, vitamin B6 were also significant predictors.

Substitution of B-vitamin concentration with treatment category in the regression model essentially did not change the association between ΔPML tHcy and PML betaine (data not shown).

Discussion

We present data on the relation between betaine and plasma tHcy in 90 patients with cardiovascular disease before and during long-term intervention with folate, cobalamin, and vitamin B6, as implemented in the WENBIT. We demonstrate, for the first time to our knowledge, that the increase in tHcy after methionine loading (ΔPML tHcy) was determined by PML betaine. Basal betaine was a weaker predictor. In 68 subjects supplemented with B-vitamins for 3 months, ΔPML tHcy was reduced, and its inverse relation to betaine was weakened, particularly in the subgroups administered folic acid.

Study Design

The strength of the study is its longitudinal, prospective design, which allows comparison of betaine effects before and after B-vitamin intervention in the same individuals. It was clearly shown that in the combined group treated with B-vitamins, the betaine-PML-tHcy association was essentially abolished, but the small sample size provided limited statistical power to delineate the effects of individual B-vitamins.

We measured PML tHcy 4 hours after methionine ingestion. This short interval was chosen for logistic reasons. A recent study demonstrated that at this time point, PML tHcy has higher within-subject and between-subject coefficients of variation (CV) than the PML tHcy measured at 6 or 8 hours after methionine intake, possibly because of variable methionine absorption a short time after loading.[10] Furthermore, we observed that betaine showed a stronger association with ΔPML tHcy than with PML tHcy. However, analytical CV increases when PML tHcy is adjusted for basal tHcy to obtain ΔPML tHcy, because the variance has the property of being additive.[10] Thus, increased biological and analytical variations may actually lead to underestimation of the "true" association between betaine status and PML tHcy.

Concentrations of Betaine, Choline, and Dimethylglycine

The median plasma concentration of betaine reported here (≈37 μmol/L; Table 1) is similar to the concentration previously reported by us in healthy blood donors[22] and by others.[21,28] We observed a strong relation between betaine and gender, with higher levels in males than in females (Table 1, Figure 3), which has also been reported by others.[29] The presence of consensus sites for steroid hormones, including estrogen and androgen binding sites, in the human betaine homocysteine methyltransferase gene[30] may represent a molecular basis of sex steroid effects on plasma betaine.

We observed a median concentration of plasma choline (9 μmol/L) that is equal to the concentration (≈10 μmol/L) previously measured in healthy subjects[22,31,32] The median plasma DMG (3.6 μmol/L) is somewhat higher than the DMG levels (≈2 to 3 μmol/L) previously reported by us and others.[22,28,33] Slightly elevated plasma DMG may be related to the possibility that the cardiovascular patients have moderately impaired renal function, which is known to affect the DMG concentration.[33] Conceivably, methodological difference may also account for different metabolite (DMG) levels between various study populations.

Human Studies on Betaine and tHcy

The strong inverse association between ΔPML tHcy and plasma betaine, particularly PML betaine, in subjects not receiving B-vitamin supplementation is the most important result of the present study. The data are in agreement with consistent observations that betaine supplementation reduces PML tHcy and also fasting tHcy in healthy subjects[16,18] and tHcy in renal patients[20] and in homocystinurics.[34–36] However, our data (Table 2) do not confirm a recent observation of a significant inverse relation between (basal) plasma betaine and tHcy in 122 Canadian cardiovascular patients.[21]

The apparent discrepancy may be related to different size, B-vitamin status, or renal function of the two study populations. We are currently addressing the possible relation between betaine and fasting tHcy in large population-based studies.

Mechanisms and Role of Betaine in Homocysteine Remethylation

Methionine loading caused a 3-fold increase in tHcy, a 15% increase in plasma betaine and DMG, and also a significant increase in choline (Table 1, Figure 3). The choline response may reflect enhanced supply of choline for the synthesis of betaine or a choline-sparing effect from superfluous methionine. The elevation of plasma betaine and DMG levels, however, indicates an increased flux through the BHMT pathway, which is in accordance with the observation that ΔPML tHcy is strongly and inversely related to PML betaine and also to PML increase in betaine. Thus, increased metabolic flux through the choline-betaine-DMG pathway may occur in response to high levels of homocysteine and/or methionine.

After treatment with combinations of folic acid, vitamin B12, and vitamin B6 for 3 months, the relation between PML betaine and ΔPML tHcy was weakened in the whole study group and was abolished in the subgroups administered folic acid. This indicates that enhancement of folate-dependent remethylation catalyzed by methionine synthase downregulates the BHMT reactions and suggests a cross-talk between these two pathways.

The significance of betaine in homocysteine homeostasis is not clear, but the view prevails that the BHMT mainly functions to conserve homocysteine under conditions of methionine deficiency.[5] This assumption is based on animal experiments demonstrating a dramatic induction of BHMT during methionine deficiency in combination with excess dietary choline.[5,30,37–39]

Our observation of an inverse relation between betaine and ΔPML tHcy indicates an additional function of the BHMT pathway, ie, reduction of the homocysteine increase after methionine intake. This idea is difficult to reconcile with the fact that BHMT is inhibited by S-adenosylmethionine[5] but gains some support from experiments in rats, demonstrating a moderate net increase in BHMT activity during methionine excess.[5] Suppression of high homocysteine under conditions of excess methionine is thus obtained together with salvage of homocysteine carbon backbone. This metabolic effect contrasts to the result of the vitamin B6-dependent cystathionine-β-synthase reaction, which irreversibly directs superfluous homocysteine into the transsulfuration pathway.

Implications and Conclusion

The data presented here indicate that betaine through the BHMT reaction buffers and tends to reduce homocysteine during methionine excess. This reaction, which conserves the homocysteine carbon backbone, may be beneficial under conditions of limiting supply of folate required for enhancement of the methionine synthase reaction. These findings emphasize the complementary relationship between betaine and folate metabolism and motivate population-based studies on betaine status in pathologies related to folate deficiency and homocysteine status.[2] Furthermore, because PML tHcy confers increased risk for occlusive vascular disease independent of fasting tHcy,[11] betaine and related metabolites should be included in future studies of homocysteine and cardiovascular risk. Because basal betaine showed a significant relation to ΔPML tHcy as well, the role of betaine could be investigated in epidemiological studies based on biobanks, where PML samples may not be available.

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Paper III

Original Article

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Homocysteine-lowering therapy does not affect inflammatory markers of atherosclerosis in patients with stable coronary artery disease

Ø. Bleie[1,2], A. G. Semb[3], H. Grundt[2,4], J. E. Nordrehaug[1,2], S. E. Vollset[5], P. M. Ueland[6], D. W. T. Nilsen[2,4], A. M. Bakken[7], H. Refsum[8,9] & O. K. Nygård[1,2]

From the [1]Department of Heart Disease, Haukeland University Hospital, Bergen, Norway; [2]Institute of Medicine, University of Bergen, Bergen, Norway; [3]Department of Medicine, Diakonhjemmet Hospital, Oslo, Norway; [4]Department of Medicine, Stavanger University Hospital, Stavanger, Norway; [5]Department of Public Health and Primary Health Care; [6]Section for Pharmacology, Institute of Medicine, University of Bergen, Bergen, Norway; [7]Laboratory of Clinical Biochemistry, Haukeland University Hospital, Bergen, Norway; [8]Department of Nutrition, Institute of Basic Medical Sciences, University of Oslo, Oslo, Norway; and [9]Oxford Centre for Gene Function, Department of Physiology, Anatomy & Genetics, University of Oxford, Oxford, UK

Abstract. Bleie Ø, Semb AG, Grundt H, Nordrehaug JE, Vollset SE, Ueland PM, Nilsen DWT, Bakken AM, Refsum H, Nygård OK (Haukeland University Hospital, Bergen; University of Bergen, Bergen; Diakonhjemmet Hospital, Oslo; Stavanger University Hospital, Stavanger; and Institute of Basic Medical Sciences, University of Oslo, Oslo; Norway; and University of Oxford, Oxford, UK). Homocysteine-lowering therapy does not affect inflammatory markers of atherosclerosis in patients with stable coronary artery disease. *J Intern Med* 2007; **262:** 244–253.

Objectives. A high level of total homocysteine (tHcy) is a risk marker for cardiovascular disease (CVD), and is related to inflammation. We wanted to test the effect of homocysteine-lowering B-vitamin therapy, as used in the Western Norway B-vitamin Intervention Trial (WENBIT), on inflammatory markers associated with atherosclerosis.

Design. Single centre, prospective double-blind clinical interventional study, randomised in a 2×2 factorial design.

Subjects and methods. Ninety patients (21 female) with suspected coronary artery disease (CAD), aged 38–80 years, were blindly randomised into one of four groups of daily oral treatment with (A) folic acid (0.8 mg)/vitamin B12 (0.4 mg)/vitamin B6 (40 mg), (B) folic acid/vitamin B12, (C) vitamin B6 alone or (D) placebo. Blood samples were collected before and after 6 months of treatment.

Results. Before intervention, median levels of the analytes were: tHcy 11.0 μmol L^{-1}, neopterin 8.1 nmol L^{-1}, soluble CD40 ligand (sCD40L) 3.9 ng mL^{-1}, interleukin (IL)-6 1.9 pg mL^{-1}, C-reactive protein (CRP) 1.9 mg L^{-1} and low-density lipoprotein (LDL) cholesterol 3.3 mmol L^{-1}. tHcy was significantly associated with neopterin ($r = 0.49$, $P < 0.001$) and with IL-6 ($r = 0.29$, $P = 0.01$), but not with CRP or sCD40L. Neither treatment with folic acid/B12 nor with B6 induced significant changes in any of these inflammatory biomarkers ($P \geq 0.14$). In patients receiving folic acid/B12 (groups A and B), tHcy was reduced with 33% ($P < 0.001$).

Conclusions. In patients with stable CAD, homocysteine-lowering therapy with B-vitamins does not affect levels of inflammatory markers associated with atherogenesis. Failure to reverse inflammatory processes, may partly explain the negative results in clinical secondary B-vitamin intervention trials.

Keywords: atherosclerosis, B-vitamins, homocysteine, inflammation, neopterin, soluble CD40 ligand.

Introduction

Numerous studies demonstrate that hyperhomocysteinaemia is related to an increased risk of the existence, extent and progression and of prognosis of cardiovascular disease (CVD) [1, 2]. Recent data also indicate that homocysteine is associated with several important components of atherogenesis, including endothelial dysfunction, platelet and immune activation and inflammation [3–5].

Based on these observations, several randomised clinical trials with homocysteine-lowering B-vitamins have been initiated in order to investigate whether homocysteine is causally related to atherosclerosis [6]. In published trials, no significant effect on the primary outcome was documented in patients with stroke (VISP) [7], acute myocardial infarction (NORVIT) [8], CVD (HOPE-2) [9] or chronic renal failure (ASFAST) [10]. A nearly significant increase of myocardial infarction was demonstrated in the NORVIT trial (in the group receiving both folic acid and vitamin B6) [8], whereas a significant reduction in stroke was demonstrated in HOPE-2 [9]. Both the VISP and the ASFAST trials were underpowered to detect clinical effects of the intervention of a magnitude similar to what might have been expected from the epidemiological data [7, 10]. In addition, two randomized trials evaluating restenosis after PCI showed conflicting results [11, 12]. Thus, the results from these clinical trials still leave uncertainty of the possible role of homocysteine in the progression of atherosclerotic disease. It is therefore important to investigate the metabolic effects of B-vitamin supplementation to understand why intervention may fail despite of epidemiological evidence.

Inflammation and persistent immune activation are hallmarks of the atherosclerotic process [13]. The markers C-reactive protein (CRP), CD40 ligand (CD40L) and neopterin represent different inflammatory pathways related to atherogenesis. CRP is an acute phase reactant; the production of CRP is stimulated by interleukin (IL)-6 effects on the liver. CRP represents an overall marker of the inflammation and is extensively investigated and characterized [14]. CD40L is produced by many cell types, but is excreted mainly from platelets and is related to unstable coronary artery disease (CAD) [15]. Soluble CD40L (sCD40L) may therefore reflect the role of activated platelets in inflammation [16]. Neopterin is a pteridine which reflects activation of the cellular part of inflammation, and is released from activated monocytes through stimulation by interferon (IFN)-γ from T lymphocytes [17]. Neopterin has been related to the extent [18], complexity [19] and progression [20] of the atherosclerotic disease. Furthermore, neopterin is strongly related to the homocysteine homeostasis [21].

The current investigation was designed to assess the effects of homocysteine-lowering B-vitamins on inflammatory markers associated with atherosclerosis. The investigation is a substudy of the Western Norway B-vitamin Intervention Trial (WENBIT), a randomised placebo-controlled clinical trial in patients with CAD.

Subjects and methods

Patients, recruitment and study design

Western Norway B-vitamin Intervention Trial is a prospective randomised double-blind study on the clinical effects of homocysteine-lowering therapy in 3098 adult patients undergoing coronary angiography for suspected CAD. Using a 2×2 factorial design, we could simultaneously assess the effect of the combination of folic acid/vitamin B12 versus no folic acid/vitamin B12 and separately vitamin B6 versus no vitamin B6. Patients were accordingly randomised into four groups – Group A: folic acid (0.8 mg), vitamin B12 (cyanocobalamin 0.4 mg) and vitamin B6 (pyridoxine 40 mg); Group B: folic acid and vitamin B12; Group C: vitamin B6 or Group D: placebo. For the first 2 weeks, Group A and Group B received an additional loading dose of folic acid (5 mg day^{-1}). Packages of trial capsules were prepared and randomised in blocks of 20 by Alpharma A/S (Copenhagen, Denmark).

The current study is confined to a subgroup of 90 consecutive patients who were recruited at start of

the WENBIT study, in the period of April 1999 to September 1999. These patients underwent more extensive follow up during the initial 6 months of the intervention, a follow up designed to investigate mechanisms and biochemical effects of provided treatment. One patient died, two patients withdrew their consent. A total of 83 patients attended the visit at 6 months. This subpopulation of WENBIT has been characterized in detail previously [22]. The study protocol was approved by the Regional ethics committee and by the Norwegian Medicines Agency. Written informed consent was obtained from all patients.

Blood collection and biochemical analyses

In the present study, we used nonfasting blood samples collected at baseline and after 6 months of B-vitamin intervention. EDTA blood samples for analysis of B-vitamins, total homocysteine (tHcy) and metabolites were immediately placed on ice and centrifuged within 30 min. Plasma and serum were stored at −80 °C until further analysis.

Routine blood analyses, including haematological parameters, renal function markers and lipid-related factors, were analysed at Laboratory of Clinical Biochemistry, Haukeland University Hospital, using Technicon Chem 1® (Bayer, Leverkusen, Germany) and CELL-DYN® 4000 (Abbot, Abbott Park, IL, USA) platforms. tHcy and MMA were analysed using a modification of a gas chromatography method based on ethylchloroformate derivatization [23]. Plasma folate [24] and cobalamin [25] were analysed by published methods. Results on vitamin B6 (pyridoxal phosphate, PLP) have previously been presented [26].

Commercially available enzyme immunoassays were used to determine serum levels of sCD40L [intra-assay coefficient of variation (CV) 6.8%; Bender Medsystems, Vienna, Austria] and serum neopterin (CV 3.6–6.8%; IBL, Hamburg, Germany). Analyses were performed according to the manufacturer's instructions and in duplicates on the same microtitre plate. IL-6 was analysed in duplicates by ELISA technique (CV 6.9–7.8%; R&D Systems, Abingdon, UK). CRP was determined in serum by an ultra sensitive immunoassay applying the Behring nephelometer II system (CV 8.1–11.4%; N Latex CRP mono, Behring Diagnostics, Germany).

Glomerular filtration rate (GFR) was calculated according to the 4-variable Modification of Diet in Renal Disease Equation [27], estimating GFR from creatinine, age, gender and ethnicity.

Statistical analysis

Because of the skewed distribution of vitamins and metabolites, continuous variables are reported as medians with interquartile range or geometric mean with reference range. Categorical variables are presented as numbers and proportions. Associations were assessed by Spearman rank correlation and linear regression. Kruskal–Wallis test was used for comparison of continuous variables between treatment groups. Wilcoxon signed rank test was used for comparison within groups over time. Effect of folic acid/B12 or vitamin B6 over time was also studied by repeated-measures ANOVA according to the 2×2 study design, using log-transformed numbers. Power calculations by PASS 2005 (NCSS, Kaysville, UT, USA) and NQUERY ADVISOR 6.0 (Statistical Solutions, Cork, Ireland) were based on a sample volume of 90 patients, a loss to follow up <10% and a significance level of 0.05. We calculated a power of 95% to detect a change of 20% in neopterin level by treatment (patients divided into two groups; folate and non folate or vitamin B6 and non-B6) using Wilcoxon signed rank test. In the univariate two-group repeated measure analysis, a power of 99% to detect a change in neopterin level of 20% by treatment during 6 months was calculated. Data were analysed using SPSS 13.0 (SPSS Inc., Chicago, IL, USA).

Results

Subject characteristics at baseline

A total of 90 patients, all Caucasian, 21 female and 69 male, with median age 62 years (range: 38–80) were included. Major characteristics of the patients are given in Table 1. Except for tHcy ($P = 0.05$),

no significant differences for these variables were found between the four groups. Additional demographic data, including smoking, diabetes, prior myocardial infarction, prior revascularization, body mass index, blood pressure, details about cholesterol, creatinine and vitamin indices, have been presented earlier [22].

Median levels (25–75th percentiles) of key biomarkers was: tHcy 11.0 (9.3–12.9) μmol L^{-1}, neopterin 8.1 (5.4–10.6) nmol L^{-1}, sCD40L 3.9 (2.3–5.6) ng mL^{-1}, IL-6 1.9 (1.1–3.2) pg mL^{-1}, CRP 1.9 (0.9–3.7) mg L^{-1} and LDL cholesterol 3.3 (2.7–4.0) mmol L^{-1}. Baseline levels of inflammatory markers, except for IL-6, did not differ significantly between treatment groups (Table 2).

Association between tHcy or vitamins and inflammatory biomarkers at baseline

The univariate association between tHcy or vitamins and biomarkers at baseline are shown in Table 3. In addition to its well-known relations with age, creatinine, folate and cobalamin, baseline tHcy was positively associated with IL-6, and neopterin. No association was found between tHcy and LDL cholesterol. Amongst the B-vitamins, plasma folate was not associated with any of the biomarkers. Plasma cobalamin was positively related with sCD40L, whereas vitamin B6 (PLP) showed an inverse relation with IL-6. As expected, we observed significant associations between the different inflammatory markers. Both tHcy and neopterin were significantly associated with creatinine, age and gender. After adjustment for GFR, tHcy was still significantly correlated with neopterin ($r = 0.38$, $P < 0.001$), but not IL-6 ($r = 0.13$, $P = 0.23$).

In order to evaluate the association, we carried out multiple linear regression analyses with each of the various inflammatory markers as dependent variables, and tHcy, GFR, LDL cholesterol, folate, vitamin B6 and vitamin B12 as independent variables (all independent variables in each of the analyses). Predictors of neopterin were levels of tHcy ($\beta = 0.35$, $P < 0.001$), GFR ($\beta = -0.37$, $P < 0.001$) and LDL cholesterol ($\beta = 0.34$, $P < 0.001$). sCD40L was borderline associated with GFR ($\beta = 0.25$, $P = 0.05$). No such associations with CRP or IL-6 were found in this model.

Table 1 Characteristics of the study population at baseline

		Treatment groups[a]				
	Total group[a] ($n = 90$)	Folic acid/B12/B6 ($n = 22$)	Folic acid/B12 ($n = 23$)	B6 ($n = 21$)	Placebo ($n = 24$)	*P*-value[b]
Age (years)	62 (54–68)	64 (55–70)	59 (52–65)	61 (57–71)	64 (53–71)	0.4
Women, *n* (%)	21 (23)	6 (27)	3 (13)	7 (33)	5 (21)	0.4
Creatinine (μmol L^{-1})	94 (87–101)	91 (83–98)	94 (89–101)	90 (82–98)	98 (91–114)	0.07
GFR (mL min/1.73 m^2)	73 (64–79)	75 (65–82)	73 (67–80)	72 (64–80)	74 (58–76)	0.5
Total cholesterol (mmol L^{-1})	5.3 (4.8–6.1)	5.5 (4.7–6.2)	5.3 (4.9–5.7)	5.4 (4.8–6.4)	5.2 (4.2–7.0)	0.9
LDL cholesterol (mmol L^{-1})	3.3 (2.7–4.0)	3.6 (2.7–4.0)	3.3 (2.8–3.9)	3.4 (2.4–4.4)	3.3 (2.3–4.8)	0.9
Statin use, *n* (%)	65 (72)	14 (64)	20 (87)	13 (62)	18 (75)	0.2
tHcy (μmol L^{-1})	11.0 (9.3–12.9)	9.9 (9.0–11.9)	12.0 (10.3–13.1)	11.3 (8.8–12.1)	11.4 (9.9–15.8)	0.05
Plasma folate (nmol L^{-1})	8.2 (6.1–11.1)	8.3 (6.1–10.9)	7.7 (5.6–11.3)	8.2 (6.6–10.7)	9.0 (5.8–11.8)	0.9
Cobalamin (pmol L^{-1})	369 (311–431)	361 (321–402)	400 (316–469)	361 (313–463)	349 (270–400)	0.2
Vitamin B6 (PLP, nmol L^{-1})	23.0 (17.7–37.1)	21.5 (16.5–33.5)	27.9 (19.3–44.6)	21.8 (15.0–39.7)	24.3 (16.1–39.5)	0.6

[a]Data are presented as median (25–75th percentiles) or numbers (%).
[b]Kruskal–Wallis test between treatment groups.
GFR, estimated glomerular filtration rate; LDL, low-density lipoprotein; B6, vitamin B6; PLP, pyridoxal 5-phosphate.

Table 2 Values of inflammatory markers at baseline and after 6 months

		Treatment groups				
	Total group	Folic acid/B12/B6 (A)	Folic acid/B12 (B)	B6 (C)	Placebo (D)	*P*-value[a]
Neopterin (nmol L^{-1})						
n	83	22	21	19	21	
Baseline	7.6 (7.0–8.2)	7.1 (6.1–8.2)	7.9 (6.8–9.3)	6.8 (5.8–8.0)	8.6 (7.1–10.4)	ns
6 months	7.7 (7.1–8.3)	8.1 (7.0–9.3)	7.3 (6.2–8.6)	7.0 (6.2–8.0)	8.3 (6.8–10.1)	ns
P-value[b]	ns	ns	ns	ns	ns	
sCD40L (ng mL^{-1})						
n	81	22	21	19	19	
Baseline	3.6 (3.1–4.1)	3.9 (2.9–5.3)	3.8 (3.0–4.8)	3.5 (2.5–5.0)	3.1 (2.5–3.9)	ns
6 months	3.2 (2.6–4.0)	3.3 (2.1–5.2)	3.6 (2.5–5.3)	3.7 (2.4–5.7)	2.4 (1.4–4.0)	ns
P-value[b]	ns	ns	ns	ns	ns	
IL-6 (pg mL^{-1})						
n	83	22	21	19	21	
Baseline	1.8 (1.5–2.1)	1.5 (1.0–2.0)	1.5 (1.1–2.1)	2.8 (2.1–3.8)	1.8 (1.4–2.5)	0.02
6 months	1.7 (1.5–1.9)	1.6 (1.2–2.2)	1.4 (1.1–1.7)	2.0 (1.5–2.6)	1.8 (1.4–2.3)	ns
P-value[b]	ns	ns	ns	ns	ns	
CRP (mg L^{-1})						
n	83	22	21	19	21	
Baseline	1.8 (1.4–2.3)	1.5 (1.0–2.2)	1.9 (1.3–2.8)	2.4 (1.4–4.1)	1.5 (0.8–2.8)	ns
6 months	1.9 (1.5–2.5)	1.5 (1.0–2.2)	2.4 (1.5–3.8)	2.0 (1.2–3.3)	1.9 (1.0–3.6)	ns
P-value[b]	ns	ns	ns	ns	ns	

[a]Kruskal–Wallis test between treatment groups.
[b]Wilcoxon signed rank test between baseline and 6 months. Data are presented as geometric mean in natural units and geometric reference range in parentheses (antilog: mean log ± 1.96 SEM).
B12, vitamin B12; B6, vitamin B6; sCD40L, soluble CD40 ligand; IL-6, interleukin 6; CRP, C-reactive protein.

Table 3 Spearman correlations at baseline

	Age	Creatinine	GFR	LDL-chol	Neopterin	sCD40L	IL-6	CRP	Folate	Cobalamin	Vitamin 6
tHcy	0.50†	0.44†	−0.34†	−0.09	0.49†	−0.10	0.29†	0.16	−0.21*	−0.21*	−0.02
LDL-chol	−0.13	0.00	−0.03		0.11	0.01	−0.10	0.01	−0.04	0.08	0.09
Neopterin	0.36†	0.41†	−0.45†			0.08	0.30†	0.23*	−0.09	0.07	−0.05
sCD40L	−0.09	−0.15	0.20				0.07	0.01	0.03	0.29†	0.05
IL-6	0.32†	0.07	−0.13					0.41†	−0.09	−0.13	−0.24*
CRP	0.11	0.05	−0.01						−0.09	−0.07	−0.05

*$P < 0.05$; † $P < 0.01$.
GFR, estimated glomerular filtration rate; LDL-chol, low-density lipoprotein cholesterol; sCD40L, soluble CD40 ligand; IL-6, interleukin 6; CRP, C-reactive protein; tHcy, total homocysteine.

Effect of treatment

Combination of folic acid and vitamin B12 caused a significant increase in plasma folate and plasma cobalamin, and a 33% reduction in plasma tHcy (from median 11.0 μmol L^{-1} to median 7.4 μmol L^{-1}, $P < 0.001$; Fig. 1). Neither folic acid/vitamin B12 nor vitamin B6 significantly influenced the levels of the inflammatory markers (Table 2 and Fig. 1). Analysing the effect of folic acid/B12 (combined Groups A + B)

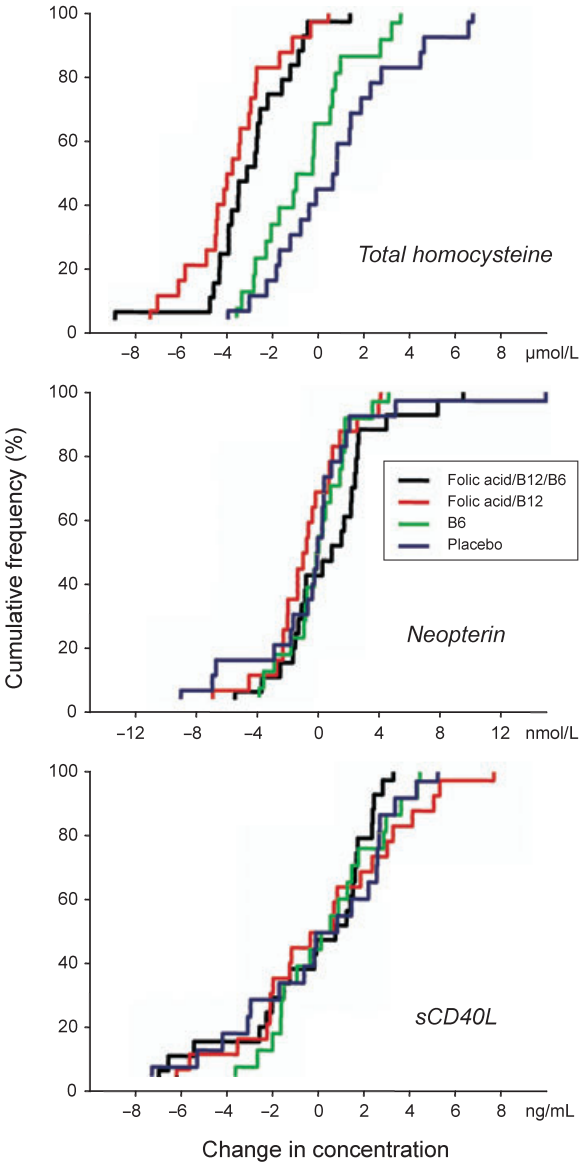

Fig. 1 Cumulative frequency plots of change in concentration between baseline and 6 months of plasma total homocysteine (tHcy; $n = 83$; upper panel), serum neopterin ($n = 83$; middle panel) and serum soluble CD40 ligand (sCD40L; $n = 81$; bottom panel), presented in four treatment groups.

between baseline and 6 months, reveal no significant effect on neopterin ($P = 0.78$), sCD40L ($P = 0.67$), IL-6 ($P = 0.96$) or on CRP ($P = 0.83$). Testing the effect of vitamin B6 (A + C), show a similar negative result; neopterin ($P = 0.25$), sCD40L ($P = 0.45$), IL-6 ($P = 0.50$) and CRP ($P = 0.33$). Tested by repeated measures ANOVA, no effect of folic acid/B12 (Groups A + B versus C + D) was observed on neopterin ($P = 0.76$), sCD40L ($P = 0.99$), IL-6 ($P = 0.34$) or on CRP ($P = 0.83$). Likewise, no effect of vitamin B6 (Groups A + C versus B + D) was observed on neopterin ($P = 0.07$), sCD40L ($P = 0.77$), IL-6 ($P = 0.79$) or on CRP ($P = 0.22$).

The strong relation seen at baseline between tHcy and neopterin in multiple linear regression analyses, was relatively unchanged after 6 months in those subjects receiving vitamin B6 alone or placebo ($\beta = 0.43$, $P = 0.003$). In patients receiving folic acid and vitamin B12, this relation was no longer present ($\beta = -0.05$, $P = 0.8$).

During the observation period, 19 patients started or intensified statin therapy (five patients in Group A, five in Group B, four in Group C and five in Group D) and two patients reduced the statin dose (in Groups C and D). Mean increase in statin dose was equally distributed between Groups A–D (2.8, 2.7, 2.5 and 3.3 mg, respectively). As a result, median LDL cholesterol was reduced by 0.4 mmol L^{-1} ($P = 0.001$) without significant difference in LDL cholesterol between treatment groups ($P > 0.9$). In a multiple linear regression model adjusting for tHcy, GFR, folate, vitamin B6 and vitamin B12, inflammatory markers neopterin, sCD40L, IL-6 or CRP at 6 months were not associated with level of LDL cholesterol ($P \geq 0.1$) or change in LDL cholesterol ($P > 0.3$).

Discussion

In the current substudy of WENBIT, we evaluated the effect of B-vitamin intervention on important biomarkers of atherogenesis. Prior investigations have not only indicated that tHcy is significantly related to many of these processes [5, 28], but also indicate that folate and vitamin B6 are associated with CVD, independent from their relation with tHcy [29]. However, despite the fact that plasma tHcy was lowered by 33% by folate 0.8 mg combined with vitamin B12 0.4 mg, and that a high dose of vitamin B6 (40 mg)

was given, no significant changes in the evaluated biomarkers neopterin, sCD40L, IL-6 or CRP were observed.

Statins is known to have immunomodulatory effects [16], and 72% of patients were on statin therapy at baseline (Table 1). Statin therapy was intensified during the study period, but no differences were observed in LDL cholesterol between groups at follow up. In theory, change in statin therapy and change in level of cholesterol may interfere with the inflammatory markers and even mask an effect of B-vitamin therapy on inflammation. This is, however unlikely, because the LDL reduction was modest and uniform between groups. Moreover, LDL cholesterol at 6 months was not associated with any of the inflammatory markers.

Because a moderate dose of folic acid was provided, we may not exclude that higher doses might have provoked different effects. However, folic acid/vitamin B12 therapy was associated with a marked 33% reduction in tHcy after 6 months. A similar reduction (27%) was found in NORVIT applying an identical regimen [8]. Despite higher doses of folic acid, less tHcy reductions (18–19%) were achieved in other clinical trials. This may partly be due to voluntary intake of B-vitamin supplements [10] or mandatory [7, 9] folate fortification programmes. It is noteworthy that our study is performed in a nonfortified population.

Our observation of a particular strong relation between tHcy and the marker of cellular immune response, neopterin, corroborates results from several previous reports [30, 31]. Neopterin is produced by activated macrophages and dendritic cells in response to stimulation by IFN-γ released from T lymphocytes, which are present at all stages of atherogenesis [17]. IFN-γ also inhibits collagen synthesis in vascular smooth muscle cells and increases collagen degradation by stimulating release of metalloproteinases from macrophages [13]. Elevated levels of neopterin (8.8 nmol L^{-1} compared with 6.9 nmol L^{-1}) [20] have been related to atherosclerotic progression and clinical events, possibly reflecting plaque destabilization associated with cell-mediated (Th1) immune response [5].

It has been suggested that stimulation and proliferation of immune cells may facilitate the production of reactive oxygen species (ROS), which may increase the demand of B-vitamins [5]. Although this was supported by the observation of a significant association between folate status and neopterin in patients with vascular disease [32], folate therapy caused only a minor and nonsignificant reduction of neopterin in the same study [32]. Stimulated human peripheral blood mononuclear cells (PBMCs) may respond with a parallel increase in both neopterin and homocysteine production, indicating that an increased homocysteine level is a direct cause of the Th1 immune response [5].

The most important finding from our study is the lack of effect of B-vitamins on the inflammatory markers, including neopterin, measured at follow up, indicating that vitamin B treatment may not reverse cellular-mediated inflammation involved in atherosclerosis. This is supported by observations on neopterin after B-vitamin treatment in demented patients [33]. Moreover, we found the association between neopterin and homocysteine at baseline was only modestly attenuated when we controlled for GFR, indicating mechanisms for elevated levels of both neopterin and homocysteine independent of renal function. In patients treated with folic acid/vitamin B12, there were no longer an association between homocysteine and neopterin. This suggests that an optimal folate status override the influence of immunostimulation on tHcy plasma levels. Our data further indicate that the utility of homocysteine as a predictor of CVD is limited to subjects not taking folic acid/vitamin B12 supplement.

CD40 ligand is structurally related to tumour necrosis factor (TNF)-α, and the activity of the TNF-α system has recently been shown to be significantly associated with homocysteine concentration [15, 34]. Both transmembrane bound and soluble CD40L, predominantly released from activated platelets, may interact with CD40 resulting in various immunomodulatory or inflammatory responses involved in arthrosclerosis development, progression and plaque destabilization [15, 16]. We found no association between tHcy and

sCD40L in this study, and treatment with B-vitamins did not significantly influence concentrations of sCD40L. Similar results have been observed in patients with cerebrovascular disease [35], although these patients had lower levels of sCD40L compared with our patients (sCD40L 0.55 ng mL^{-1} vs. 3.9 ng mL^{-1}, respectively). Thus, tHcy or B-vitamins may not be an important determinants of platelet activation in CAD patients treated with established medical therapy.

Previous investigations have shown that increasing levels of homocysteine in cell cultures may affect monocytes [36] and endothelial cells [37] to promote IL-6 production. IL-6 is the principal procoagulant cytokine, stimulating increased blood levels of fibrinogen, plasminogen activator inhibitor type 1 and CRP [38], and may promote the atherogenesis [13]. A significant association between tHcy and IL-6 has previously been reported in elderly with diabetes [39], but not in patients with CAD or peripheral artery disease [30]. We found that IL-6 was significantly related to tHcy at baseline, but CRP was not related to tHcy or the B-vitamins. IL-6 and CRP were both unaffected by the B-vitamin treatment, in line with previous results in patients with cerebrovascular disease [35]. Our population had relatively low baseline levels of CRP, possibly reflecting that this cohort comprised medically well treated patients with stable CAD.

Conclusions

Elevation of plasma tHcy is related to cardiovascular events and linked to inflammation in CVD patients not supplemented with B-vitamins. Secondary tHcy-lowering B-vitamin intervention trials have so far demonstrated no significant reduction CVD events [40]. Our study demonstrated that treatment with folic acid/vitamin B12 or vitamin B6 had no detectable effects on levels of neopterin, sCD40L, IL-6 or CRP. Failure to reverse inflammatory processes associated with atherosclerosis may partly explain the negative results of B-vitamin intervention in patients with established CVD treated with conventional therapy.

Conflict of interest statement

P. M. Ueland reports having received consulting fees from Nycomed and is a member of the steering board of both the nonprofit Foundation to Promote Research into Functional Vitamin B12 Deficiency and Bevital, a company owned by the foundation. A PTC application [62924 (52365)] for a patent entitled 'Determination of folate in fresh and stored serum or plasma as paraaminobenzoylglutamate' was filed on 3 March 2005; P. M. Ueland is listed as one of the inventors. The patent is owned by Bevital.

No other potential conflict of interest relevant to this article was reported.

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Correspondence: Øyvind Bleie, Department of Heart Disease, Haukeland University Hospital, N-5021 Bergen, Norway.
(fax: +47 55 97 51 50; e-mail: oyvind.bleie@helse-bergen.no).

Paper IV

Folic Acid Intervention Increases Coronary Blood Flow in Patients with Stable Coronary Artery Disease

Bleie Ø, MD[1,2], Strand E, MSc[1], Ueland PM, MD PhD[3], Vollset SE, MD PhD[4], Refsum H, MD PhD[6,7], Igland J, MSc[5], Nordrehaug JE, MD PhD[1,2], Nygård O, MD PhD[1,2]

[1]Department of Heart Disease, Haukeland University Hospital, Bergen, [2]Institute of medicine, [3]Section of Pharmacology, Institute of medicine, [4]Department of Public Health and Primary Health Care, [5]Unifob Health, University of Bergen, [6]Department of Nutrition, Institute of Basic Medical Sciences, University of Oslo, Norway, and [7]Department of Physiology, Anatomy & Genetics, University of Oxford, UK

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Corresponding author:
Øyvind Bleie,
Department of Heart Disease
Haukeland University Hospital
N-5021 BERGEN, NORWAY
Telephone: +47 55 97 50 00
Fax: +47 55 97 51 50
E-mail: oyvind.bleie@helse-bergen.no

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Key words: coronary disease, blood flow, microcirculation, homocysteine, folic acid

Abstract

Purpose. We examined the effects of B-vitamin therapy on coronary flow and vascular function in patients with established coronary artery disease (CAD).

Methods and results. Forty patients with CAD and on statin treatment, recruited into the Western Norway B-Vitamin Intervention Trial (WENBIT), were randomly assigned to daily oral treatment with 0.8 mg folic acid and 0.4 mg vitamin B12 or placebo, and 40 mg vitamin B6 or placebo, using a 2 x 2 factorial design. At baseline, and after 9 and 24 months, coronary blood flow were assessed by coronary angiography and doppler flow-wire measurements during intra-coronary infusion of saline (basal), incremental (0.72 μg/min, 7.2 μg/min and 36.0 μg/min) doses of acetylcholine, 2.4 mg/min adenosine and nitroglycerin.

At baseline, mean (SD) age was 57.8 (9.0) years (eight females), serum LDL-cholesterol 2.9 (0.7) mmol/L, creatinine 88 (9.9) μmol/L, folate 12.2 (6.5) nmol/L and plasma total homocysteine 10.7 (2.9) μmol/L ($p \geq 0.4$ between treatment groups). We found a significant increase in basal ($p < 0.02$) and adenosine-induced ($p < 0.05$) coronary blood flow in subjects who received folic acid/vitamin B12 compared to placebo or vitamin B6 alone for 24 months. Folic acid/vitamin B12 or vitamin B6 treatment did not change endothelial-dependent response following acetylcholine infusion or flow-dependent proximal dilatation in response to adenosine-induced maximal hyperemia ($p \geq 0.45$).

Conclusion. Treatment with a combination of folic acid and vitamin B12 increased basal and adenosine-induced maximal coronary blood flow, reflecting improved vascular function. The potential beneficial effect of folic acid therapy on vascular function should be furthered studied.

INTRODUCTION

Numerous studies suggest that the serum or plasma concentration of total homocysteine (tHcy) is an independent risk factor for cardiovascular disease (CVD) [1,2]. The mechanisms of this association are not fully understood [3,4]. To date, randomized trials on secondary prevention of CVD have shown no benefit on risk of cardiac events or mortality by homocysteine-lowering folic acid supplementation [5]. Still, high levels of tHcy is associated with arterial endothelial dysfunction [6] and even postprandial increments in tHcy are found to impair endothelial function [7].

Intact endothelium is important for the maintenance of vascular integrity and regulates vasomotor tone through release of nitric oxide to meet increased blood flow demands during physical strain. Endothelial dysfunction is an early marker of atherosclerotic disease, it is involved in the pathogenesis, and is associated with future cardiac events [8,9]. Various treatment strategies have been shown to improve endothelial function and cardiovascular prognosis, including statins, angiotensin converting enzyme inhibitors and exercise [8].

High doses of folic acid given to patients with CVD improve endothelial function measured as flow-mediated dilatation (FMD) in forearm arteries in some, but not in all studies [10]. However, low dose (0.4 mg/d) folate therapy seems to be ineffective [11,12]. Typically, the duration of the studies was for a few weeks to months [10].

So far, only one study has evaluated the effect of folic acid and vitamin B12 on coronary endothelial function in patients with coronary artery disease (CAD) [13], and no study investigated vitamin B6 alone. The objective of this substudy of the Western Norway B-vitamin Intervention Trial (WENBIT) was to evaluate the long-term effect of homocysteine-lowering B-vitamin therapy on coronary vascular function. The study population was patients with established stable CAD receiving contemporary medical therapy, including statins, and with no selection according to tHcy levels at baseline.

SUBJECTS AND METHODS

Patients, recruitment and study design

The current study is a single center substudy of WENBIT, a prospective randomized double-blind study on the clinical effects of homocysteine-lowering therapy in 3090 adult patients undergoing coronary angiography for suspected CAD. Recruitment to WENBIT was started in 1999 and completed in March 2004. Using a 2 x 2 factorial design, we could simultaneously assess the effect of the combination of folic acid/vitamin B12 versus no folic acid/vitamin B12 and separately vitamin B6 versus no vitamin B6. Patients were randomized into four groups: Group A, folic acid (0.8 mg), vitamin B12 (cyanocobalamin 0.4 mg) and vitamin B6 (pyridoxine 40 mg); group B, folic acid and vitamin B12; group C, vitamin B6; group D, placebo. For the first two weeks, group A and group B received an additional loading dose of folic acid (5 mg/day). Packages of trial capsules were prepared and randomized in blocks of 20 by Alpharma A/S (Copenhagen, Denmark).

In this substudy of coronary vascular function, patients with stable CAD scheduled for elective percutaneous coronary intervention were eligible. Exclusion criteria were malignant disease, alcohol abuse, mental illness, reluctance or incapability to long-term follow-up. Other exclusion criteria were predicted high risk for procedural complications, severe chronic obstructive pulmonary disease, pulmonary hypertension, significant valvular disease, glaucoma, poorly regulated diabetes, or use of systemic corticosteroids. Furthermore, blood pressure should be well regulated and there should be no indication for starting angiotensin converting enzyme inhibitor or calcium blocker therapy at the time of inclusion. All patients were treated with statins for at least two months prior to inclusion. Long-acting nitrates were not allowed the last week before the testing procedures.

After successful treatment of at least one significant coronary stenosis, a non-intervened coronary artery (belonging to the left anterior descending artery or circumflex

artery) was used for baseline coronary function testing (study vessel). Forty patients were followed with repeated testing after nine months, and 35 patients returned for a third testing after two years of vitamin treatment. Four patients did not wish to follow the two year per protocol catheterization, and in one patient, the vascular function testing was not successfully performed at the two year visit due to technical problems. No procedure related complications occurred.

The study protocol was approved by the Regional ethics committee, and the medication was approved by the Norwegian Medicines Agency. Written informed consent was obtained from all patients.

Assessment of coronary vascular function

Measurements were done during consecutive intracoronary administration of saline, acetylcholine, adenosine and nitroglycerin. Acetylcholine induces a vasodilatation mediated by release of nitric oxide (NO) from intact endothelium, counterbalancing its direct effect on smooth muscles in the vessel wall causing vasoconstriction. The response to acetylcholine infusion thereby reflects endothelial function [14]. Adenosine provokes hyperemia mainly by stimulating endothelium-independent dilatation in the microcirculation [15] and was used for assessment of maximum hyperemic flow in coronary arteries. Intracoronary nitroglycerin is a direct precursor of NO, and was used to measure endothelial-independent function.

Procedures

All patients were given heparin at start of the procedure to obtain an activated clotting time of 300 s. Guiding catheters 6-French (Launcher, Medtronic, Minnespolis, MN) were used for cannulation of the left main coronary artery. Before vascular function testing, intracoronary nitrates were not permitted. A doppler guide wire (0.014 inch, FloWire, Volcano, Rancho

Cordova, CA) was placed in a non-branching segment of the study vessel through the inner lumen of a 2.9-French coronary infusion catheter (UltraFuse-X, Boston Scientific, Maple Grove, MN) ending 1 cm distal to the catheter tip. The positions of the infusion catheter and doppler wire were documented by angiography. Infusion through the UltraFuse-X catheter was done at 60 mL/hour (1 mL/min) with a pump delivering high-pressure output (Asena, Alaris, Basingstoke, UK). Infusions were done as follows in seven steps: 1) Saline 0.9% for three minutes, 2-4) incremental dosage (0.72 μg/min, 7.2 μg/min and 36.0 μg/min) of acetylcholine (Miochol-E 10 mg/mL, Novartis Healthcare, Copenhagen, Denmark) for three minutes and 20 seconds each (estimated transit time of 20 seconds), 5) saline 0.9% for approximately five minutes until return to basal flow (see below), 6) adenosine (Adenocor, Sanofi-Synthelabo, Bromma, Sweden) at a dose rate of 2.4 mg/min for 3 min and 20 sec., and finally the infusion line was flushed with saline, and 7) a 0.2 mg bolus of nitroglycerin was given. Average peak flow velocity (APV) was continuously recorded (FloMap, Cardiometrics (Volcano), Rancho Cordova, CA). At the end of each infusion step, an angiogram was done in the same position and angle. A coronary artery segment of 10 mm, 2-3 mm distal to the doppler wire, was used for mean diameter measurement by digitalized quantitative coronary angiography (QCA) (Quantcor QCA V5.0, Pie Medical Imaging, Maastricht, Netherlands) with the contrast-filled catheter as reference for calibration. Coronary blood flow (CBF) was calculated by use of APV and vessel diameter ($CBF = \pi r^2(½APV)$)[16]. FMD was calculated comparing mean diameter in a 10 mm segment (or at least 5 mm in shorter available segment lengths) of the study vessel proximal to the infusion catheter tip during saline infusion and during hyperemia induced by adenosine infusion.

Measurements

Coronary vascular function was assessed by five indices: CBF at basal conditions during saline infusion (CBF-basal), infusion with acetylcholine (CBF-ach), adenosine (CBF-ado) and nitroglycerin (CBF-ntg), and proximal coronary FMD at maximum hyperemic flow (FMD-hyperemia). Also, as a measure of endothelial function, response to acetylcholine infusion was calculated as percent increase in CBF-ach at each dose of acetylcholine compared to CBF-saline. A maximum increase in CBF-ach < 50% is considered representing endothelial dysfunction [9].

At follow-up nine months and two years after inclusion, the same protocol and target segments were used. All invasive studies were performed by the same operator. A dedicated technician was in charge of all off-line measurements.

Blood collection and analyses

Blood samples were collected at baseline, after 9 and 24 months and stored at -80º C until processing. Folate and cobalamin were measured in serum samples, otherwise, sample handling and analyses were done as previously described [17]. Glomerular filtration rate (GFR) was calculated according to the four-variable Modification of Diet in Renal Disease Equation [18].

Statistical analyses

Continuous variables are reported as means (SD) if not otherwise indicated. Skewed variables are presented as median and selected percentiles. Categorical variables are presented as numbers and proportions. Chi-Square test was used for comparison of proportions. ANOVA was used to compare mean levels between groups, and paired-samples t-test was used for comparison within groups over time. Treatment effects of folic acid/B12 or vitamin B6 over time and interaction between treatment groups and acetylcholine dose during follow-up were

studied by repeated-measures ANOVA according to the 2 x 2 study design. Statistical package SPSS 13.0 (SPSS Inc., Chicago, IL) was used. Differences in time trends of flow (change during follow-up) by treatment were analyzed by a linear mixed-effect model with random intercept on subject level (S-PLUS 7.0 for Windows, Insightful Corporation, Seattle, WA). A two-tailed $P < 0.05$ was considered statistical significant.

RESULTS

Subject and baseline characteristics

A total of 40 patients (8 female and 32 male) with median age 57 (range 39-74) years were enrolled. Key baseline demographic and clinical characteristics and medical treatment are given in **Table 1**. Mean (SD) serum folate was 12.2 (6.5) nmol/L, cobalamin 381 (129) pmol/L, plasma pyridoxal phosphate (PLP) 43.3 (25.0) nmol/L and tHcy 10.7 (2.9) μmol/L. No significant differences between folic acid/B12 vs non-folic acid/B12 groups or between vitamin B6 vs non-B6 groups were found with respect to these characteristics.

B-Vitamins and total homocysteine after treatment

In patients randomized to folic acid and vitamin B12 treatment (groups A + B), serum folate concentrations increased significantly to 70.6 (18.6) nmol/L at 9 months ($p<0.001$) and serum cobalamin increased to 622 (215) pmol/L ($p<0.001$). There was a concurrent 34% reduction in plasma tHcy to 7.3 (2.0) μmol/L ($p<0.001$). At 24 months, tHcy remained at the same low level (7.4 (2.0) μmol/L, $p=0.5$). In the patients randomized to vitamin B6 alone or placebo (non-folic acid, groups C + D), no significant change in folate, vitamin B12 and tHcy were observed (all p-values >0.4).

In patients treated with vitamin B6 (groups A + C), plasma PLP increased significantly to 375 (154) nmol/L ($p<0.001$) at 9 months. No significant change in PLP was observed in patients randomized to treatment with folic acid/vitamin B12 alone or placebo (groups B + D, $p=0.8$). Vitamin B6 treatment did not change levels of tHcy, folate or cobalamin (all p-values >0.6).

Basal coronary blood flow before and after B-vitamin treatment

Basal coronary blood flow during saline infusion (CBF-basal) at inclusion was non-significantly higher (mean (SD) 31.8 (11.8) mL/min) in patients allocated to folic acid /B12 compared with patients not randomized to folic acid /B12 (25.7 (7.4) mL/min, p=0.06) (**Figure 1**). During the two years of follow-up, CBF-basal increased among patients treated with folic acid /B12, as apposed to a decrease in patients not receiving folic acid /B12, with a significant difference in time-trends of CBF-basal between the treatment groups (p=0.016) (Figure 1). After two years, CBF-basal was 36.3 (15.0) mL/min and 21.6 (9.2) mL/min in patients treated and not treated with folic acid/B12, respectively (p=0.002). Vitamin B6 treatment was not associated with significant changes in CBF-basal after follow-up.

Coronary blood flow and response to acetylcholine infusion

Maximum achieved coronary blood flow during incremental dosages of acetylcholine (CBF-ach) at inclusion was 59.5 (39.2) mL/min. At the same time, maximum flow response to acetylcholine, measured as percent change in CBF-ach compared to CBF-basal, was median (25 – 75th percentile) 90 (35-162) %. A total of 13 patients (33%) achieved increase in CBF-ach less than 50%. Flow profile during infusion with incremental dosages of acetylcholine did not change after 9 months and two years of treatment with either folic acid/B12 (p=0.9) (**Figure 2**) or vitamin B6 (p=0.7). Likewise, flow response during acetylcholine infusion, as a measure of endothelial-dependent response, did not change significantly after B-vitamin intervention for two years (**Table 2**, all p-values ≥ 0.45).

Maximum hyperemia and flow-dependent response following adenosine infusion

Adenosine infusion was used to achieve maximum hyperemic coronary flow (CBF-ado), mainly due to effects on microcirculation[15]. At study start, there was no difference in CBF-ado between the folic acid/B12 group, mean (SD) 96.8 (39.1) mL/min, and the non-folic acid/B12 group, 90.0 (32.9) mL/min (p=0.6) (Figure 1). Similar to observations with CBF-basal, treatment with folic acid /B12 was associated with a gradual increase in CBF-ado, whereas a decrease in CBF-ado was seen among patients not receiving folic acid /B12. As a result, there was a significant difference in time-trends of CBF-ado according to folic acid/B12 therapy (p=0.049) (Figure 1). At two years, mean (SD) CBF-ado was 121.8 (77.5) mL/min in patients allocated to folic acid/B12 compared to 76.0 (33.1) mL/min in patients not receiving folic acid /B12 (p=0.04). Vitamin B6 had no effect on CBF-ado ($p \geq 0.3$).

FMD-hyperemia, a measurement of endothelial function, was 0.10 (0.23) mm at baseline, with no overall change at 9 months (0.10 (0.21) mm, p=0.9) or 2 years (0.13 (0.39) mm, p=0.6). There was no significant difference in FMD-hyperemia according to folic acid/B12 or vitamin B6 during follow-up (repeated measure ANOVA; p=0.16 and p=0.84 respectively).

Endothelial-independent flow response following nitroglycerin infusion

Coronary blood flow after nitoglycerin administration (CBF-ntg) was 109.1 (34.3) mL/min in the folic acid/B12 group and 94.1 (34.5) mL/min in non-folic acid group at randomization (p=0.18). Although we observed a significant difference in CBF-ntg at two years between patients treated with folic acid/B12 compared to those not treated with folic acid/B12 (133.5 (62.8) and 88.9 (45.0) mL/min respectively (p = 0.02), the difference in time-trends of CBF-ntg did not reach statistically significance (p=0.10) (Figure 1). CBF-ntg was not influenced by vitamin B6 treatment ($p \geq 0.5$).

DISCUSSION

In this randomized controlled trial, we have shown that two years of treatment with folic acid/vitamin B12 improved basal coronary blood flow and blood flow at maximum hyperaemia induced by adenosine. In contrast, folic acid/vitamin B12 did not improve coronary endothelial function as measured by flow-induced change in coronary vessel diameter or change in flow following stimulation with acetylcholine. Vitamin B6 treatment did not change any of the vascular function variables.

Measurements in different target organs

We have investigated parameters of vascular function in coronary vessels. This is in contrast to most other studies on endothelial function and B-vitamins, which have measured brachial FMD [10]. The relation between coronary endothelial function and brachial FMD is uncertain, and, although one study found a significant but weak relation between brachial FMD and coronary response to acetylcholine [19], other studies find no such relation [20,21]. Therefore, caution must be taken when comparing results obtained in different target organs.

Strengths and weaknesses

Intra-coronary measurements in a population with no folate fortification, combined with long-term follow-up, are major strengths of our study. The factorial design allowed us to examine separate effects of folic acid/B12 and vitamin B6 treatment. Although the number of patients may seem limited, this is the largest study on folate therapy and coronary vascular function.

Earlier studies on coronary vascular function

There is only one published study on the effect of B-vitamins on coronary flow in patients with CAD [13]. A total of 15 patients were randomized to treatment with folic acid (5 mg/d) and

vitamin B12 (0.4 mg/d) or placebo for six months. B-vitamin treatment was associated with a significant improvement in acetylcholine-induced coronary blood flow. It is noteworthy that these patients had relatively high baseline tHcy (17.9 μmol/L), few patients received statin therapy, and a high dose of folic acid was used. In contrast, we found an effect of folic acid/vitamin B12 on basal and maximal hyperemic coronary blood flow, but no effect on acetylcholine stimulated flow.

Possible mechanisms

The reported relations between folate or tHcy levels and brachial endothelial-dependent FMD are somewhat conflicting [22]. However, most studies suggest that a rapid increase in plasma tHcy levels, as observed during methionine or homocysteine loading [6,10,22], or after a protein rich meal [7], impairs brachial endothelial function, while high doses of folic acid improve endothelial function, possibly partly independent of its homocysteine-lowering effect[10,23]. The mechanism by which homocysteine impairs endothelial function may involve homocysteine-induced reduction of intracellular tetrahydrobiopterin, thereby causing eNOS-uncoupling [24]. Folic acid, through its circulating form, 5-methyltetrahydrofolate, is believed to enhance regeneration of tetrahydrobiopterin and improve eNOS-coupling and thereby improve endothelial function independently of homocysteine [25,26]. Recent data from an isolated rat heart model supports our findings of increased coronary flow by folic acid treatment, and suggests a mechanistic role of NO [27].

Whereas NO is an established regulator of vascular tone, there is some evidence that endothelium-derived hyperpolarizing factor (EDHF) plays a major role in regulating microcirculation [28]. In renal microcirculation of rats, EDHF-mediated vasodilatation is impaired during methionine loading and partly restored by 5-methyltetrahydrofolate [29]. This suggests an additional mechanism by which folic acid therapy in our patients has improved

vascular tone and microcirculation, since resting coronary flow (CBF-basal) and maximal hyperemia (CBF-ado) largely depend on microvascular dilatation in non obstructive coronary vessels [15]. A beneficial effect on microvascular flow, together with reduced arterial stiffness [30], may explain a reduced frequency of electrocardiographic changes at exercise tests [31] and reduction in blood pressure [32] observed after treatment with folic acid.

Dosage of folic acid

High doses of folic acid 5 – 10 mg/d improve endothelial function as measured by FMD [33]. Likewise, a short-term study (six weeks) in patients with CAD showed no effect on forearm FMD with 0.4 mg/d of folic acid, but a significant improvement with 5 mg daily [11]. In contrast, a study using magnetic resonance imaging revealed that both 0.4 mg/d and 5 mg/d of folic acid for seven weeks improved FMD in brachial artery [30]. The latter study also demonstrated that the low-dose folic acid significantly enhances folate status in the vascular endothelium, with no significant additional increase in vascular tissue 5-methyltetrahydrofolate level in the high-dose group [30]. Thus, our dose of 0.8 mg/d should be adequate to obtain optimal effect on endothelial-dependent response and vascular stiffness.

Clinical trials

If long-term treatment with folic acid improves vascular function, this may have important clinical implications. and explain a reduction in stroke mortality rate observed in North America following folic acid fortification [34] and a reduction in stroke events in the HOPE-2 study [35]. Prior studies have demonstrated that even minor reductions in blood pressure are associated with significant lower risk of stroke, but with less effect on ischemic heart disease [36]. Thus, potential treatment effects of folic acid related to a reduction in blood pressure, may act differently upon risk of stroke and CAD [1,36]. Notably, folic acid/B12 therapy has not been

associated with reduced risk of coronary events in any of the published randomized trials with B-vitamin intervention [37,38].

Vitamin B6

Data on vitamin B6 and vascular function are sparse [39,40], and our study is the first to examine the effect of B6 in the coronary circulation. We observed no effect of vitamin B6 treatment on the vascular indices despite marked increases in plasma concentrations of PLP. Vitamin B6 status is associated with CVD in some observational studies [41,42], but supplementation studies with vitamin B6 have revealed no clinical benefits [37].

Conclusion

In patients with stable CAD, we demonstrate that treatment with moderate doses of folic acid in addition to vitamin B12 is associated with a significant increase in both basal and in adenosine-stimulated maximal coronary blood flow, reflecting improved vascular function. This treatment does however not change endothelial-dependent acetylcholine-induced response or flow-mediated coronary dilatation. Treatment with high doses of vitamin B6 shows no effect on coronary vascular function. The effect of folate therapy on vascular function should be furthered studied.

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Disclosures.

PM Ueland reports having received consulting fees from Nycomed and is a member of the steering board of both the non-profit Foundation to Promote Research into Functional Vitamin B12 Deficiency and Bevital, a company owned by the foundation. A PTC application (62924 [52365]) for a patent entitled “Determination of folate in fresh and stored serum or plasma as paraaminobenzoylglutamate” was filed on March 3, 2005; PM Ueland is listed as one of the inventors. The patent is owned by Bevital.

No other potential conflict of interest relevant to this article was reported

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Figure 1. Coronary blood flow during intra coronary infusion of saline (CBF-basal), maximal hyperemia during infusion of adenosine 2.4 mg/min (CBF-ado) and endothelium-independent flow induced by nitroglycerin (CBF-ntg). The flow indices (mean, SEM) at inclusion, 9 months and 24 months for the folic acid/vitamin B12 group (n = 20, 20, 19) and non-folic acid group (n = 20, 20, 16) are shown.
* P-values for differences in time trends according to folic acid/vitamin B12 treatment and p-value between intervention groups at each study visit byANOVA.

Figure 2. Coronary blood flow during infusion of incremental doses of acetylcholine (CBF-ach) 0.72 µg/min, 7.2 µg/min and 36 µg/min. CBF-ach (mean, SEM) at inclusion, 9 months and 24 months for the folic acid/vitamin B12 group (n = 20, 20, 19) and non-folic acid group (n = 20, 20, 16) are shown. There was no significant treatment effect of folic acid/vitamin B12 during follow-up (p=0.85, repeated measures ANOVA, effect between treatment groups and dosage of Ach).

TABLE 1 Characteristics of the study population at baseline

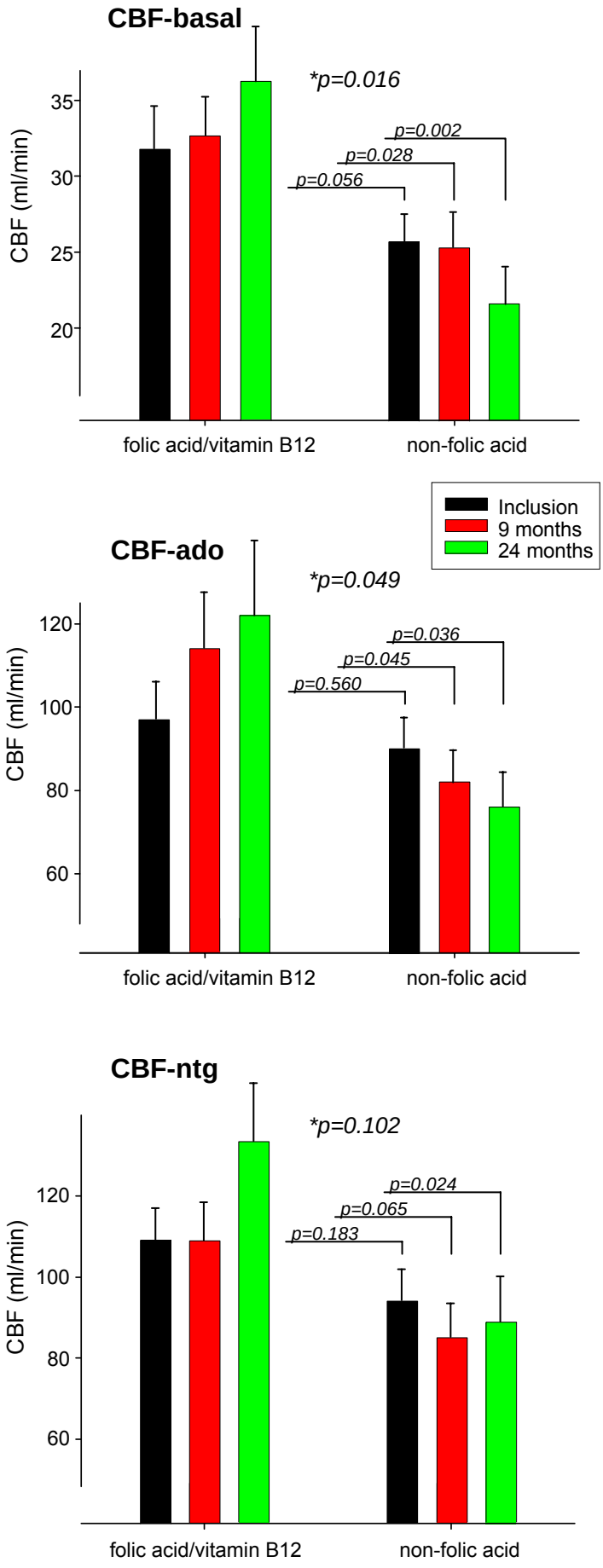
	Total group * (n=40)	*Treatment groups* *					
		Folic acid/B12 (n=20)	Non-folic acid/B12 (n=20)	*P-values*†	B6 (n=20)	Non-B6 (n=20)	*P-values*†
Age, years	57.8 (9.0)	57.4 (10.4)	58.2 (7.7)	0.78	56.3 (6.3)	59.4 (11.1)	0.28
Women, n (%)	8 (20)	3 (15)	5 (25)	0.44	3 (15)	5 (25)	0.44
Current smokers, n (%)	13 (33)	7 (35)	6 (30)	0.50	9 (45)	4 (20)	0.04
Diabetes, n (%)	2 (5)	2 (10)	0 (0)	0.15	0 (0)	2 (10)	0.15
Prior PCI, n (%)	9 (23)	4 (20)	5 (25)	0.71	4 (20)	5 (25)	0.71
Prior AMI, n (%)	6 (15)	2 (10)	4 (20)	0.38	2 (10)	4 (20)	0.38
BMI, kg/m^2	26.7 (2.8)	26.6 (2.5)	26.8 (3.1)	0.89	26.0 (2.1)	27.4 (3.2)	0.11
Waist, cm	94.2 (9.1)	93.8 (7.6)	94.7 (10.6)	0.79	91.7 (5.4)	96.9 (11.4)	0.08
Systolic blood pressure, mmHg	146 (25.4)	144 (20.4)	147 (29.9)	0.67	143 (17.4)	149 (31.6)	0.46
Diastolic blood pressure, mmHg	80 (11.8)	81 (10.7)	79 (13.0)	0.63	83 (9.3)	77 (13.4)	0.10
ACEI, n (%)	14 (35)	7 (35)	7 (35)	1.00	6 (30)	8 (40)	0.51
Beta blockers, n (%)	29 (73)	14 (70)	15 (75)	0.73	13 (65)	16 (80)	0.29
Ca-channel blockers, n (%)	7 (18)	3 (15)	4 (20)	0.68	5 (25)	2 (10)	0.22
Serum Creatinine, µmol/L	88 (9.9)	89 (11.1))	87 (8.9)	0.67	88 (11.2)	87 (8.7)	0.74
GFR, $mL/min/1.73m^2$	76 (12.3)	76 (12.9)	77 (11.9)	0.95	79 (13.7)	74 (10.6)	0.26
Serum LDL-cholesterol, mmol/L	2.9 (0.7)	2.9 (0.5)	2.9 (0.9)	0.95	2.8 (0.6)	3.0 (0.9)	0.36
CRP, mg/L	1.6 (0.9-2.7)	1.6 (0.9-2.7)	1.5 (0.9-2.7)	0.82	1.7 (1.0-2.5)	1.5 (0.7-2.8)	0.72

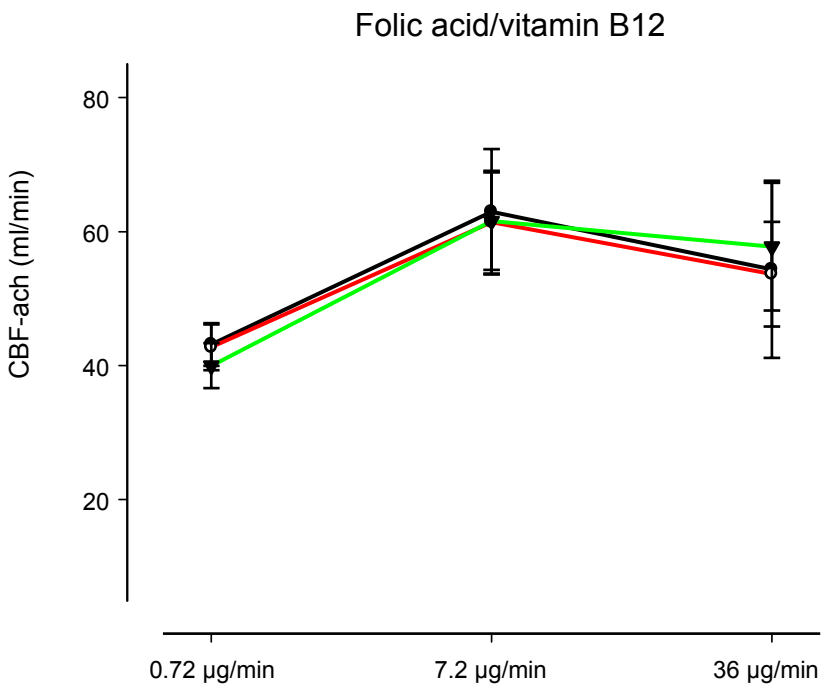
*Mean (SD) or numbers (%), except CRP which is given in median (25-75th percentile). †Comparison of continuous variables between groups by ANOVA, except for CRP in which is comparison is done by Kruskal-Wallis Test. Comparison of proportions between groups by Chi-Square Test. BMI = body mass index, ACEI = angiotensin converting enzyme inhibitor, GFR = estimated glomerular filtration ratio, LDL-cholesterol = low density lipoprotein cholesterol, CRP = C-reactive protein.

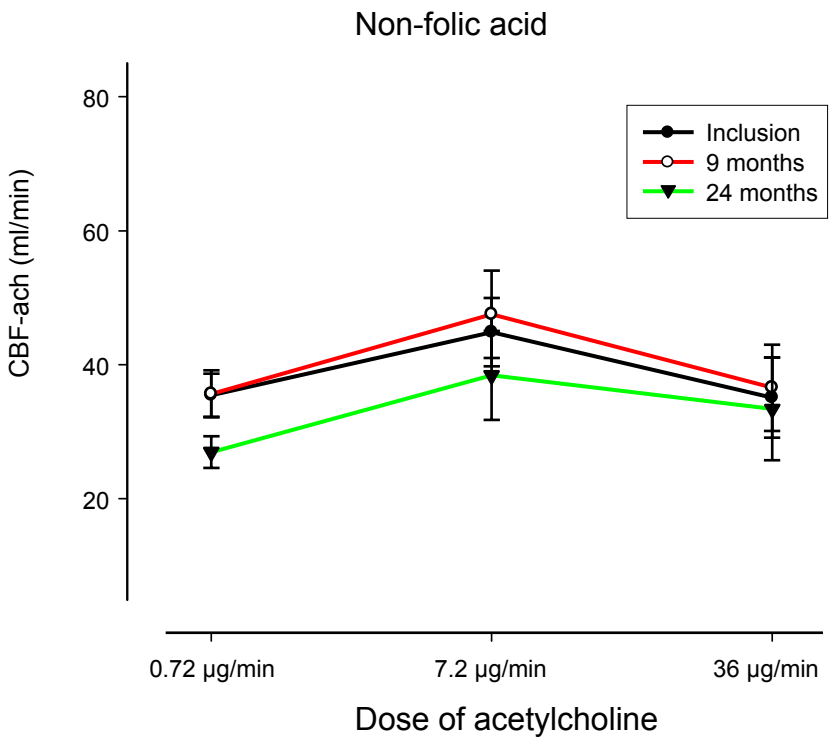
TABLE 2 Coronary blood flow response to acetylcholine infusion at inclusion and after 9 and 24 months of B-vitamin treatment.

			% change in flow compared to baseline (NaCl infusion)*			
Acetylcholine dosage			*Folate*	*Non-Folate*	*B6*	*Non-B6*
0.72 µg/min	Inclusion	(n=40)	39.8 (8.7)	38.1 (9.5)	43.7 (9.8)	34.2 (8.3)
	9 months	(n=40)	34.8 (9.2)	39.5 (8.5)	43.9 (9.7)	30.4 (7.6)
	24 months	(n=35)	14.5 (6.8)	35.5 (13.3)	33.5 (9.5)	14.2 (10.7)
	P-values†		p=0.51		p=0.91	
7.2 µg/min	Inclusion	(n=40)	93.4 (20.1)	76.8 (18.4)	105.6 (21.7)	64.7 (16.0)
	9 months	(n=39)	87.3 (19.9)	83.5 (21.8)	108.7 (23.8)	60.9 (15.0)
	24 months	(n=34)	71.8 (14.1)	80.6 (26.8)	99.2 (19.6)	49.8 (20.0)
	P-values†		p=0.60		p=0.60	
36.0 µg/min	Inclusion	(n=38)	54.7 (27.4)	34.1 (21.4)	55.2 (30.1)	32.5 (16.4)
	9 months	(n=35)	77.3 (26.5)	35.3 (22.6)	74.4 (28.7)	33.6 (16.8)
	24 months	(n=32)	53.5 (18.8)	42.0 (23.5)	54.7 (21.6)	40.4 (19.0)
	P-values†		p=0.58		p=0.45	
	P-values overall‡		p=0.66		p=0.77	

*Values in %, mean (SEM). †Repeated measures ANOVA, effect between groups during 24 months. ‡Repeated measures ANOVA, effect between groups and dosage of Ach during 24 months


CBF-basal
*p=0.016
p=0.002
p=0.028
p=0.056
CBF (ml/min)
35
30
25
20
folic acid/vitamin B12
non-folic acid
Inclusion
9 months
24 months
CBF-ado
*p=0.049
p=0.036
p=0.045
p=0.560
120
100
80
60
folic acid/vitamin B12
non-folic acid
CBF-ntg
*p=0.102
p=0.024
p=0.065
p=0.183
120
100
80
60
folic acid/vitamin B12
non-folic acid


Folic acid/vitamin B12
CBF-ach (ml/min)
80
60
40
20
0.72 µg/min
7.2 µg/min
36 µg/min


Non-folic acid
CBF-ach (ml/min)
80
60
40
20
Inclusion
9 months
24 months
0.72 µg/min
7.2 µg/min
36 µg/min
Dose of acetylcholine