Original Article

Journal of INTERNAL MEDICINE

doi: 10.1111/j.1365-2796.2010.02259.x

Combined analyses and extended follow-up of two randomized controlled homocysteine-lowering B-vitamin trials

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Abstract. Ebbing M., Bønaa K.H., Arnesen E., Ueland P.M., Nordrehaug J.E., Rasmussen K., Njølstad I., Nilsen D.W., Refsum H., Tverdal A., Vollset S.E., Schirmer H., Bleie Ø., Steigen T., Midttun Ø., Fredriksen Å., Pedersen E.R., Nygård O. (From the Departments of [1]Heart Disease, Haukeland University Hospital, Bergen; Heart Disease, University Hospital of North Norway; Department of Community Medicine, University of Tromsø, Tromsø; Institute of Medicine, University of Bergen, Bergen; Department of Clinical Medicine, University of Tromsø, Tromsø; Department of Cardiology, Stavanger University Hospital, Stavanger; Institute of Basic Medical Sciences, University of Oslo, Oslo, Norway; Department of Physiology, Anatomy & Genetics, University of Oxford, Oxford, UK; Division of Epidemiology, the Norwegian Institute of Public Health, Oslo; Department of Public Health and Primary Health Care, University of Bergen; Bevital AS, Bergen; Norway) Combined Analyses and Extended Follow-Up of Two Randomized Controlled Homocysteine-Lowering B-Vitamin Trials. *J Intern Med* 2010; **268**: 367–382.

Objectives. In the Norwegian Vitamin Trial and the Western Norway B Vitamin Intervention Trial, patients were randomly assigned to homocysteine-lowering B-vitamins or no such treatment. We investigated their effects on cardiovascular outcomes in the trial populations combined, during the trials and during an extended follow-up, and performed exploratory analyses to determine the usefulness of homocysteine as a predictor of cardiovascular outcomes.

Design. Pooling of data from two randomized controlled trials (1998–2005) with extended post-trial observational follow-up until 1 January 2008.

Setting. Thirty-six hospitals in Norway.

Subjects. 6837 patients with ischaemic heart disease.

Interventions. One capsule per day containing folic acid (0.8 mg) plus vitamin B12 (0.4 mg) and vitamin B6 (40 mg), or folic acid plus vitamin B12, or vitamin B6 alone or placebo.

Main outcome measures. Major adverse cardiovascular events (MACEs; cardiovascular death, acute myocardial infarction or stroke) during the trials and cardiovascular mortality during the extended follow-up.

Results. Folic acid plus vitamin B12 treatment lowered homocysteine levels by 25% but did not influence MACE incidence (hazard ratio, 1.07; 95% CI, 0.95–1.21) during 39 months of follow-up, or cardiovascular mortality (hazard ratio, 1.12; 95% CI, 0.95–1.31) during 78 months of follow-up, when compared to no such treatment. Baseline homocysteine level was not independently associated with study outcomes. However, homocysteine concentration measured after 1–2 months of folic acid plus vitamin B12 treatment was a strong predictor of MACEs.

Conclusion. We found no short- or long-term benefit of folic acid plus vitamin B12 on cardiovascular outcomes in patients with ischaemic heart disease. Our data suggest that cardiovascular risk prediction by plasma total homocysteine concentration may be confined to the homocysteine fraction that does not respond to B-vitamins.

Keywords: cardiovascular disease, folic acid, homocysteine, randomized controlled trial, vitamin B12.

Abbreviations: CABG, coronary artery bypass graft surgery; CAD, coronary artery disease; CVD, cardiovascular disease; Hcy, homocysteine; HR, hazard ratio;

MACE, major adverse cardiovascular event; MI, myocardial infarction; *MTHFR*, 5,10-methylenetetrahydrofolate reductase; NORVIT, the Norwegian Vitamin Trial; PCI, percutaneous coronary intervention; tHcy, total homocysteine; WENBIT, the Western Norway B Vitamin Intervention Trial.

Introduction

Observational studies during the 1980s and 1990s demonstrated that the plasma concentration of total homocysteine (tHcy) is associated with cardiovascular disease (CVD) [1]. In cohort studies from Norway, plasma tHcy concentration was an independent predictor of myocardial infarction (MI) in the general population [2], and a strong predictor of all-cause mortality in patients with angiographically confirmed coronary artery disease (CAD) [3]. Several experimental studies have demonstrated biologically plausible mechanisms by which homocysteine (Hcy) may promote thromboembolism and atherogenesis [1]. Hcy levels are inversely related to plasma/serum concentrations of the B-vitamins folate and cobalamin (vitamin B12) [4, 5]. In addition, case–control studies have demonstrated inverse associations between circulating vitamin B6 levels and risk of CAD [6] or CVD [7], independent of Hcy levels. Furthermore, an inverse relationship between the intake of vitamin B6 and risk of CAD was shown in a large cohort study [8]. Because of these results, a series of Hcy-lowering randomized controlled trials using folic acid (the synthetic form of folate) alone or in combination with vitamin B12 and/or B6 were initiated during the late 1990s in patients with cardiovascular or chronic kidney disease [9].

In 1998, mandatory folic acid fortification of cereal grains was implemented in the USA and Canada, primarily to prevent neural tube defects, but there was also a hope that increased folate levels, and thereby lowered plasma tHcy concentrations, would prevent CVD in the general population [10, 11]. More than 50 countries have subsequently implemented fortification, and by 2007 one-third of the global population had access to folic acid-fortified wheat flour [12]. However, folic acid-based Hcy-lowering treatment has not proven beneficial in large trials, whether conducted in patients at high risk of or with established CVD [13, 14], with prior stroke [15], ischaemic heart disease [16–19] or chronic kidney disease [20] or in patients with renal transplants [21]. A recent systematic review of Hcy-lowering trials in people with or without pre-existing CVD (n = 24 210) demonstrated pooled risk ratios of 1.03, 0.89 and 1.00 for the outcomes of MI, stroke or death by any cause, respectively, with the 95% confidence intervals (CIs) including the value of one [22].

The 5,10-methylenetetrahydrofolate reductase (*MTHFR*) 677 C—>T single-nucleotide polymorphism is a major determinant of plasma tHcy concentration. The *MTHFR* 677C—>T polymorphism, encoding for an enzyme with less catalytic activity, leads to higher plasma tHcy concentrations, especially in conditions with low serum folate levels [23, 24]. Thus, the presence or absence of the T allele can be considered a random allocation into groups with life-long differences in plasma tHcy levels. Two meta-analyses of studies of CVD incidence across the *MTHFR* 677 genotypes up to 2001 supported the hypothesis that Hcy may be causally related to CVD [23, 24].

Two of the aforementioned trials, the Norwegian Vitamin (NORVIT) Trial [17] and the Western Norway B Vitamin Intervention Trial (WENBIT) [18], used identical B-vitamin intervention and were conducted in patients with ischaemic heart disease in Norway, where there is no folic acid fortification of foods. The objective of this analysis was to assess the short- and long-term effects of the intervention on cardiovascular outcomes in these two trial populations combined. We also performed analyses of cardiovascular outcomes in subgroups defined by patient baseline characteristics including the *MTHFR* 677C—>T polymorphism, and exploratory analyses of baseline and follow-up plasma tHcy concentration as predictor of outcomes.

Materials and methods

Study design and setting

Here, we present the combined results from two randomized, double-blind, placebo-controlled clinical trials conducted in Norway from 1998 to 2005, NORVIT [17] and WENBIT [18], as well as data on cardiovascular mortality from the extended follow-up of these study populations until 1 January 2008. Details and primary results of the two separate trials have been published previously [17, 18]. The preplanned pooling of data was appropriate as the two trials included similar

patients, used identical study design and intervention, had similar follow-up routines and used the same core laboratory for study-related blood analyses.

In brief, the aim of both trials was to assess whether Hcy-lowering treatment with folic acid plus vitamin B12, or treatment with vitamin B6, could reduce cardiovascular morbidity and mortality in patients who had survived an acute MI [17] or had undergone coronary angiography for suspected CAD or aortic valve stenosis [18]. Study protocols were in accordance with the principles of the Declaration of Helsinki, and all participants gave written informed consent. The study funders had no role in the design, conduct or reporting of the trials.

Participants were randomly assigned to receive a capsule with one of the following compositions: (1) folic acid (0.8 mg per day) plus vitamin B12 (cyanocobalamin; 0.4 mg per day) and vitamin B6 (pyridoxine hydrochloride; 40 mg per day); (2) folic acid (0.8 mg per day) plus vitamin B12 (0.4 mg per day); (3) vitamin B6 alone (40 mg per day); or (4) placebo. Treatment with folic acid was expected to lower plasma tHcy concentration by 23–28% [1]. Vitamin B12 was added to folic acid primarily to prevent possible masking of vitamin B12 deficiency by folic acid but also to further lower tHcy levels by 3–10% [1]. Participants assigned to folic acid plus vitamin B12 received an extra capsule with a loading dose of 5 mg of folic acid per day during the first 2 weeks after randomization. All participants were requested to abstain from taking over-the-counter supplements containing B-vitamins. Otherwise, they were given conventional medical treatment and underwent myocardial revascularization procedures and/or valve surgery at the discretion of the treating physician.

In both trials, clinical information and blood samples were obtained at baseline, at the follow-up visit 1–2 months after randomization and at a final study visit. Compliance was judged by capsule counts and interviews. Study-related analyses of circulating B-vitamin and homocysteine levels and genotyping of the *MTHFR* (NCBI Entrez Gene 4524) 677C—>T polymorphism were performed at Bevital AS, Bergen, Norway, using published methods [25–29].

NORVIT was terminated in March 2004, and WENBIT in October 2005. When the primary results became available, participants were informed by letter that there was no apparent health benefit from the B-vitamin intervention and that such vitamin supplementation was not recommended as secondary prevention for patients with ischaemic heart disease.

The post-trial observational follow-up did not require any further patient contribution. This study was approved by the Regional Committee for Medical and Health Research Ethics and the Norwegian Directorate of Health, and the data handling procedures were approved by the Data Inspectorate. The study is registered with ClinicalTrials, identifier: NCT00671346.

Definition and ascertainment of outcomes

For the current analysis, the primary outcome during trials was major adverse cardiovascular events (MACEs) defined as a composite of cardiovascular death, nonfatal acute MI (except procedure-related MI) and nonfatal stroke. Secondary outcomes were fatal and nonfatal acute MI (procedure-related MI included), fatal and nonfatal stroke, acute hospitalization for angina pectoris, and percutaneous coronary intervention (PCI) or coronary artery bypass graft surgery (CABG). The outcome during the extended follow-up from randomization until 1 January 2008 was cardiovascular mortality.

Clinical outcomes during the trials were adjudicated by the end-points committees blinded for treatment allocation [17, 18]. PCI or CABG performed <6 months following the qualifying acute MI at inclusion in NORVIT, or determined by baseline angiography in WENBIT, were not included in the secondary outcome. Data on cause-specific mortality by 31 December 2007 were obtained by linking individuals' unique 11-digit personal identification numbers to the Cause of Death Registry kept by Statistics Norway [30]. A subject was considered to have died as a result of CVD if the underlying cause of death was coded as *International Statistical Classification of Diseases, 10th Revision (ICD-10)*, codes I00 to I99, or code R96.

Statistical analysis

We present symmetrically distributed continuous variables as mean (SD) and skewed continuous variables as median (25–75 percentiles). Differences between groups were tested with chi-squared test for categorical variables and parametric or nonparametric methods for continuous variables, as appropriate. Pearson partial correlations were used to explore the relationship between baseline levels of plasma tHcy and serum/plasma B-vitamins or serum creatinine,

after logarithmic transformation of the skewed variables, and with adjustment for possible confounders.

Analyses of treatment effects were conducted according to the intention to treat principle. Survival curves were constructed using the Kaplan–Meier method, and the differences in survival between the groups were analysed by the log-rank test. We obtained estimates of the hazard ratios (HRs) and 95% CIs using Cox proportional hazards regression, stratified by trial, with separate analyses for the groups assigned to folic acid plus vitamin B12 treatment (folic acid groups) versus no folic acid/vitamin B12 (nonfolic acid groups), and for the groups assigned to vitamin B6 treatment (vitamin B6 groups) versus no vitamin B6 (nonvitamin B6 groups), according to the two-by-two factorial design. There was no interaction effect between folic acid plus vitamin B12 treatment and vitamin B6 treatment, with respect to the primary outcomes (*P* for interaction ≥0.60). Proportional hazards assumptions were tested by Stata's estat phtest procedure based on Schoenfeld residuals [31]; we found no evidence of nonproportionality.

We assessed effect modifications of B-vitamin treatment and subgroup indicators, by including the relevant interaction terms in the main effects model. Six predefined participant baseline characteristics (two levels each: trial, age, sex, smoking, plasma tHcy concentration and *MTHFR* 677 genotype) were examined for MACEs during the in-trial follow-up and for cardiovascular mortality during the extended follow-up. Of the resulting 24 comparisons, there was a 70.8% probability that one or more statistically significant *P* value would appear based on chance alone.

To explore the predictive value of plasma tHcy level at baseline or at the follow-up visit 1–2 months after randomization to folic acid plus vitamin B12, we included participants in the folic acid groups with tHcy measurements at both time-points. Amongst these, we used Cox proportional hazards regression, stratified by trial, with plasma tHcy concentration at baseline or at first follow-up as exposure variable, and survival time to first MACE (after the follow-up visit) or to death from CVD as outcome, unadjusted and adjusted for vitamin B6 treatment and possible confounders.

A two-sided statistical significance level of 0.05 was applied throughout, and the reported *P* values were not adjusted for multiple comparisons. We used the statistical software packages SPSS version 15.0 (SPSS Inc., Chicago, IL, USA) and Stata version 10 (StataCorp LP, College Station, TX, USA).

Results

Patients

A total of 6837 individuals were included in the combined analyses. The numbers of participants in the two trials and post-trial follow-up until 1 January 2008 are shown in Fig. 1. At the end of the in-trial follow-up, 6341 (92.7%) participants were alive. Sixteen participants had emigrated during the trials, and 64 declined consent to post-trial follow-up; these 80 participants were censored at the date of their last in-trial study visit. Thus, 6261 (98.7% of those who were alive at the end of the trial) participants were included in the post-trial follow-up. Median (25–75 percentiles) duration of extended follow-up until 1 January 2008 was 78 (61–90) months, including a median (25–75 percentiles) of 39 (31–42) months of in-trial follow-up.

Baseline demographics, clinical and laboratory characteristics, risk factors and concomitant medication are shown in Table 1. Mean (SD) age was 62.3 (11.0) years, 76.5% of participants were men. A total of 39.2% of participants were currents smokers, 36.6% were treated for hypertension and 10.6% had diagnosed diabetes mellitus at trial entry. After baseline acute MI or coronary angiography, participants received the following medications: aspirin, 89.4%; lipid-lowering agents, 84.7%; beta blockers, 85.1%; angiotensin-converting enzyme inhibitors and/or angiotensin-2 receptor blockers, 33.9%.

Baseline homocysteine levels

Median (25–75 percentiles) baseline plasma tHcy concentration was 11.1 (9.1–13.7) μmol L^{-1}. A total of 1179 (17.3%) participants had hyperhomocysteinaemia (currently defined as plasma tHcy ≥15 μmol L^{-1} [32]) (Table 1). These patients were older, had higher serum total cholesterol and creatinine levels, were more often smokers and were more often included after an acute MI (data not shown), all $P < 0.001$.

The frequency of the 677 T allele in the *MTHFR* gene was 28.9%, and 8.2% of individuals were homozygous for the TT genotype (Table 1). Amongst individuals with the CC or CT genotype, median (25–75 percentiles) baseline plasma tHcy concentration was 11.0 (9.1–13.4) μmol L^{-1}, whereas amongst those

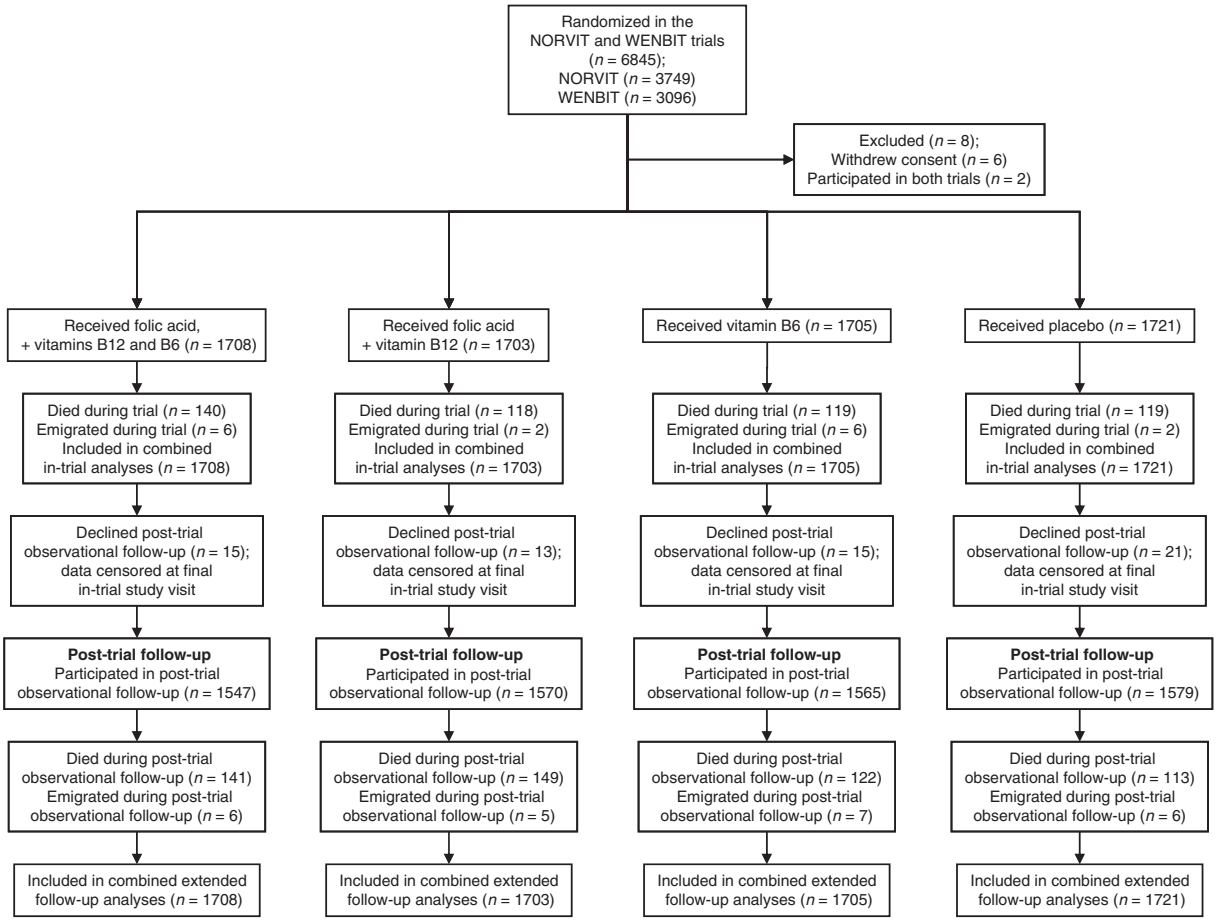

Fig. 1 *Flow of Participants in the NORVIT and WENBIT Trials and Post-trial Observational Follow-up. NORVIT, Norwegian Vitamin Trial; WENBIT, Western Norway B Vitamin Intervention Trial.*

with the TT genotype, the concentration was 13.1 (10.4–17.2) μmol L^{-1} ($P < 0.001$).

After adjustment for age, sex, *MTHFR* 677C—>T polymorphism, prior MI, PCI, CABG, carotid stenosis, transient ischaemic attack or stroke, current smoking, hypertension, obesity, diabetes mellitus and the clinical presentation of the ischaemic heart disease (acute MI, unstable angina or stable angina) at trial entry, baseline plasma tHcy concentration correlated with serum levels of folate ($r = -0.33$, $P < 0.001$), creatinine ($r = 0.27$, $P < 0.001$) and cobalamin ($r = -0.20$, $P < 0.001$).

Compliance and homocysteine-lowering effects

Almost 85% of participants took at least 80% of the study capsules throughout the in-trial follow-up. The high compliance was corroborated by follow-up measurements of B-vitamin and tHcy concentrations in serum/plasma (Table 2). In the folic acid groups, plasma tHcy level was lowered from a median of 11.1–8.3 μmol L^{-1} (25%; $P < 0.001$) after 1–2 months of follow-up. Amongst patients with baseline hyperhomocysteinaemia, treatment with folic acid plus vitamin B12 lowered plasma tHcy concentration from a median of 17.7–11.2 μmol L^{-1} (37%; $P < 0.001$), whereas amongst patients without hyperhomocysteinaemia, this treatment lowered plasma tHcy from a median of 10.4–8.0 μmol L^{-1} (23%; $P < 0.001$).

Cardiovascular outcomes

Table 3 shows the numbers and rates per 1000 person-years for the primary and secondary outcomes during the trials, and for cardiovascular mortality

Table 1 *Baseline characteristics and use of concomitant medications*[a]

Characteristics	Intervention group			
	Folic Acid + Vitamins B12 and B6 (n = 1708)	Folic Acid + Vitamin B12 (n = 1703)	Vitamin B6 (n = 1705)	Placebo (n = 1721)
Included in NORVIT, No. (%)	937 (54.9)	935 (54.9)	934 (54.8)	943 (54.8)
Included in WENBIT, No. (%)	771 (45.1)	768 (45.1)	771 (45.2)	778 (45.2)
Age, mean ± SD, y	62.7 ± 11.2	62.3 ± 10.9	62.0 ± 10.9	62.3 ± 10.7
Male sex, No. (%)	1310 (76.7)	1313 (77.1)	1304 (76.5)	1300 (75.5)
Body mass index, mean ± SD[b]	26.6 ± 3.9	26.5 ± 3.8	26.5 ± 3.7	26.7 ± 3.8
Blood pressure, mean ± SD, mm Hg				
Systolic	132 ± 22	133 ± 22	132 ± 22	132 ± 22
Diastolic	77 ± 13	76 ± 12	76 ± 13	76 ± 13
Serum total cholesterol, mean ± SD, mmol L^{-1}	5.5 ± 1.2	5.5 ± 1.3	5.5 ± 1.3	5.4 ± 1.3
Creatinine, median (25–75 percentiles), μmol L^{-1}	89 (79-99)	89 (79-99)	88 (78-99)	89 (79-99)
tHcy ≥ 15.0 μmol L^{-1}, No./Total No. (%)	299/1706 (17.5)	283/1697 (16.7)	305/1700 (17.9)	292/1711 (17.1)
MTHFR 677 genotype, No./Total No. (%)				
CC	806/1627 (49.5)	862/1627 (53.0)	810/1636 (49.5)	816/1643 (49.7)
CT	677/1627 (41.6)	636/1627 (39.1)	699/1636 (42.7)	692/1643 (42.1)
TT	144/1627 (8.9)	129/1627 (7.9)	127/1636 (7.8)	135/1643 (8.2)
Vitamin supplements, No. (%)[c]	401 (23.5)	398 (23.4)	390 (22.9)	392 (22.8)
CVD history, No./Total No. (%)				
MI	463/1692 (27.4)	477/1689 (28.2)	480/1696 (28.3)	486/1711 (28.4)
PCI	196/1707 (11.5)	200/1703 (11.7)	205/1705 (12.0)	215/1721 (12.5)
CABG	168/1708 (9.8)	127/1703 (7.5)	147/1705 (8.6)	150/1721 (8.7)
Carotid artery stenosis, TIA or stroke	102/1700 (6.0)	85/1695 (5.0)	88/1697 (5.2)	74/1713 (4.3)
Smoking, No./Total No. (%)				
Never	488/1706 (28.6)	514/1700 (30.2)	449/1702 (26.4)	487/1715 (28.4)
Ex[d]	553/1706 (32.4)	565/1700 (33.2)	538/1702 (31.6)	552/1715 (32.2)
Current	665/1706 (39.0)	621/1700 (36.5)	715/1702 (42.0)	676/1715 (39.4)
Clinical presentation at trial entry, No. (%)				
Acute MI	1016 (59.5)	1013 (59.5)	1018 (59.7)	1025 (59.6)
Unstable angina	31 (1.8)	39 (2.3)	35 (2.1)	32 (1.9)
Stable angina	644 (37.7)	645 (37.9)	646 (37.9)	649 (37.7)
Aortic valve stenosis	17 (1.0)	6 (0.4)	6 (0.4)	15 (0.9)
Concomitant disease, No./Total No. (%)				
Hypertension[e]	627/1699 (36.9)	605/1687 (35.9)	615/1693 (36.3)	643/1717 (37.4)
Obesity[f]	278/1707 (16.3)	271/1700 (15.9)	283/1702 (16.6)	308/1718 (17.9)
Diabetes mellitus[g]	187/1696 (11.0)	175/1693 (10.3)	163/1700 (9.6)	199/1719 (11.6)
Concomitant medication, No./Total No. (%)				
Acetylsalicylic acid	1444/1645 (87.8)	1486/1648 (90.2)	1458/1623 (89.8)	1485/1656 (89.7)

Table 1 (*Continued*)

Characteristics	Intervention group: Folic Acid + Vitamins B12 and B6 (n = 1708)	Folic Acid + Vitamin B12 (n = 1703)	Vitamin B6 (n = 1705)	Placebo (n = 1721)
Warfarin	163/1638 (10.0)	124/1640 (7.6)	133/1622 (8.2)	136/1656 (8.2)
Lipid-lowering drugs	1370/1643 (83.4)	1389/1644 (84.5)	1398/1622 (86.2)	1401/1651 (84.9)
β-Blockers	1394/1643 (84.8)	1422/1647 (86.3)	1376/1623 (84.8)	1397/1658 (84.3)
Calcium antagonists	261/1637 (15.9)	244/1639 (14.9)	241/1617 (14.9)	250/1655 (15.1)
ACE inhibitors/ARBs	549/1636 (33.6)	556/1639 (33.9)	537/1623 (33.1)	582/1654 (35.2)
Diuretics	295/1638 (18.0)	262/1638 (16.0)	269/1622 (16.6)	292/1655 (17.6)

Abbreviations: ACE, angiotensin-converting enzyme; ARB, angiotensin II receptor blocker; CABG, coronary artery bypass graft surgery; CVD, cardiovascular disease; MI, myocardial infarction; *MTHFR*, 5,10-methylenetetrahydrofolate reductase; NORVIT, the Norwegian Vitamin Trial; PCI; percutaneous coronary intervention; tHcy, total homocysteine; TIA, transient ischaemic attack; WENBIT, the Western Norway B Vitamin Intervention Trial.
[a]Because of rounding, percentages may not total 100.
[b]Body mass index was calculated as weight in kilograms divided by height in metres squared.
[c]Regularly taking any over-the-counter vitamin supplements at trial entry.
[d]Quit smoking > 1 month before trial entry.
[e]Medically treated hypertension.
[f]Body mass index ≥ 30.
[g]Includes diabetes mellitus types 1 and 2.

during the extended follow-up, with HRs and 95% CIs for the comparison between folic acid versus nonfolic acid groups and for the comparison between vitamin B6 versus nonvitamin B6 groups. Figure 2 shows Kaplan–Meier plots for MACEs during the trials (Panels A and B) and for cardiovascular mortality during the extended follow-up (Panels C and D).

During the in-trial follow-up, 531 (15.6%) participants who received folic acid plus vitamin B12 versus 503 (14.7%) of those who did not receive this treatment experienced a MACE (HR, 1.07; 95% CI, 0.95–1.21; P = 0.28). In the vitamin B6 groups, a total of 524 (15.4%) participants experienced a MACE versus 510 (14.9%) participants in the nonvitamin B6 groups (HR, 1.04; 95% CI, 0.92–1.18; P = 0.53) (Table 3 and Fig. 2, panels A and B). There were no statistically significant differences in the secondary outcomes of acute MI, stroke, acute hospitalization for angina pectoris, PCI or CABG between the folic acid and nonfolic acid groups, or between the vitamin B6 and nonvitamin B6 groups (Table 3).

During the extended follow-up, 317 (9.3%) participants in the folic acid groups versus 287 (8.4%) participants in the nonfolic acid groups died from CVD (HR, 1.12; 95% CI 0.95–1.31; P = 0.18). Long-term cardiovascular mortality was also similar in the vitamin B6 and nonvitamin B6 groups with 308 (9.0%) vs. 296 (8.6%) cardiovascular deaths (HR, 1.06; 95% CI, 0.90–1.24; P = 0.51) (Table 3 and Fig. 2, panels C and D). There were no differences in coronary mortality, stroke mortality or other CVD mortality between the folic acid and nonfolic acid groups, or between the vitamin B6 and nonvitamin B6 groups (data not shown).

When restricting analyses to participants who took study capsules for more than 6 months following randomization (n = 6218, 90.9% of all participants), the results remained unchanged.

Subgroups

Figure 3 shows results for MACEs during the trials and for cardiovascular mortality during the extended follow-up, with respect to folic acid plus vitamin B12 treatment, or vitamin B6 treatment, in patient subgroups determined by baseline characteristics. There was no evidence of effect modification of folic acid plus vitamin B12 treatment, or of vitamin B6 treatment, by trial, age below or above the median (62.5 years), sex or current smoking. However, in patients with hyperhomocysteinaemia, treatment with folic acid plus vitamin B12 was

Table 2 *Circulating B-vitamins and total homocysteine during trials*

Measurements	Intervention group				P^a
	Folic Acid + Vitamins B12 and B6 (n = 1708)	Folic Acid + Vitamin B12 (n = 1703)	Vitamin B6 (n = 1705)	Placebo (n = 1721)	
Serum folate, nmol L^{-1}					
Baseline (n = 6773)	8.9 (6.5–13.0)	8.8 (6.4–12.4)	8.7 (6.4–12.7)	8.8 (6.5–12.8)	0.56
1–2 months (n = 6126)	57.3 (42.0–75.0)	67.4 (50.7–84.1)	6.9 (5.3–9.3)	8.9 (6.7–12.4)	<0.001
Final study visit[b] (n = 5567)	57.8 (36.7–77.2)	66.4 (41.2–85.5)	8.4 (6.2–12.2)	10.2 (7.3–14.7)	<0.001
Serum cobalamin, pmol L^{-1}					
Baseline (n = 6749)	352 (273–440)	352 (269–448)	352 (273–441)	347 (269–443)	0.73
1–2 months (n = 6138)	511 (414–625)	504 (407–621)	366 (287–453)	355 (280–448)	<0.001
Final study visit[b] (n = 5578)	562 (443–694)	561 (444–698)	343 (274–435)	349 (273–435)	<0.001
Plasma pyridoxal 5′ phosphate, nmol L^{-1}					
Baseline (n = 6722)	33 (23–48)	32 (23–45)	33 (23–48)	32 (23–45)	0.07
1–2 months (n = 6028)	354 (265–438)	38 (28–52)	361 (276–451)	38 (27–53)	<0.001
Final study visit[b] (n = 5496)	306 (150–405)	37 (27–53)	305 (133–412)	38 (28–54)	<0.001
Plasma total homocysteine, μmol L^{-1}					
Baseline (n = 6814)	11.0 (9.2–13.7)	11.1 (9.2–13.6)	11.0 (9.1–13.7)	11.2 (9.2–13.8)	0.75
1–2 months (n = 6142)	8.3 (7.0–9.9)	8.4 (7.2–10.0)	11.2 (9.4–13.8)	11.5 (9.5–14.1)	<0.001
Final study visit[b] (n = 5582)	8.1 (6.9–9.8)	8.3 (7.0–10.1)	11.2 (9.2–13.6)	11.2 (9.2–13.9)	<0.001

Values are expressed as median (25–75 percentiles).
[a]Kruskal–Wallis test of difference across the four intervention groups.
[b]The final study visit occurred after median (25–75 percentiles) 39 (31–42) months of in-trial follow-up.

associated with increased risk of in-trial MACEs, and of long-term cardiovascular mortality (P for interaction = 0.01 and 0.03, respectively). We could not demonstrate any effect modification by *MTHFR* 677 genotype (Fig. 3).

Homocysteine as predictor of outcomes

Table 4 shows the results from the exploratory analyses of associations between plasma tHcy concentration measured at baseline or at the follow-up visit 1–2 months later, and risk of in-trial MACEs and long-term cardiovascular death, amongst participants assigned to folic acid plus vitamin B12 treatment. Baseline plasma tHcy concentration was a significant predictor of these outcomes in univariate analyses, but not after adjustment for significant confounders (age, sex, serum creatinine, prior MI, PCI, CABG, carotid stenosis, transient ischaemic attack or stroke, current smoking, hypertension, obesity, diabetes mellitus and the clinical presentation of the ischaemic heart disease at trial entry). Notably, plasma tHcy concentration measured at the follow-up visit after 1–2 months of folic acid plus vitamin B12 treatment was significantly associated with increased risk of in-trial MACEs (HR for every 5-μmol L^{-1} increment, 1.31; 95% CI, 1.09–1.56; P = 0.004), but not with long-term cardiovascular death (HR, 1.20; 95% CI 0.96–1.50; P = 0.11), after adjustment for the aforementioned confounders (Table 4). To further explore this, we performed separate analyses in participants with (responders) and without (nonresponders) reduction in plasma tHcy concentration during the first 1–2 months of treatment with folic acid plus vitamin B12. The association between plasma tHcy concentration measured after 1–2 months of treatment and increased risk of subsequent in-trial MACEs appeared to be especially strong amongst the nonresponders (HR for every 5-μmol L^{-1} increment, 1.62; 95% CI, 1.14–2.31; P = 0.007) (Table 4). However, there was no interaction effect between the responders and nonresponders (P for interaction = 0.25).

Table 3 *Cardiovascular outcomes and Cox proportional hazards*

	No. of Cases[a] (Rate per 1000 Person-Year)				Hazard Ratio (95% CI)[b]		Hazard Ratio (95% CI)[b]	
Events	Folic Acid + Vitamins B12and B6 (n = 1708)	Folic Acid + Vitamin B12 (n = 1703)	Vitamin B6 (n = 1705)	Placebo (n = 1721)	Folic Acid vs. NonFolic Acid Groups	P	Vitamin B6 vs. NonVitamin B6 Groups	P
MACE[c]	268 (55.9)	263 (55.1)	256 (53.3)	247 (50.2)	1.07 (0.95–1.21)	0.28	1.04 (0.92–1.18)	0.53
Acute MI[d]	255 (53.3)	235 (48.8)	231 (47.9)	226 (46.0)	1.08 (0.95–1.23)	0.22	1.07 (0.94–1.22)	0.30
Stroke[e]	35 (6.8)	53 (10.4)	43 (8.4)	50 (9.6)	0.95 (0.71–1.27)	0.73	0.76 (0.57–1.02)	0.07
Acute hospitalization for angina[f]	209 (44.4)	218 (46.2)	208 (43.9)	241 (51.3)	0.95 (0.83–1.08)	0.44	0.91 (0.79–1.03)	0.14
Myocardial revascularization procedures[g]	263 (62.2)	269 (63.5)	272 (64.4)	261 (60.3)	1.01 (0.89–1.13)	0.93	1.02 (0.91–1.15)	0.70
Cardiovascular death during extended follow-up[h]	163 (15.8)	154 (14.7)	145 (13.9)	142 (13.4)	1.12 (0.95–1.31)	0.18	1.06 (0.90–1.24)	0.51

Abbreviations: CI, confidence interval; MACE, major adverse cardiovascular event; MI, myocardial infarction;
[a]The first event of the actual category for each participant.
[b]Hazard ratios by Cox proportional hazards regression, stratified by trial, unadjusted.
[c]The primary outcome during in-trial follow-up was major adverse cardiovascular events as a composite of cardiovascular death, nonfatal acute myocardial infarction and of nonfatal stroke.
[d]Fatal and nonfatal acute myocardial infarction, including procedure-related acute myocardial infarction.
[e]Fatal and nonfatal stroke.
[f]Unstable or acute angina pectoris.
[g]Percutaneus coronary intervention or coronary artery bypass grafting performed > 6 months after the index myocardial infarction in the Norwegian Vitamin Trial, or after baseline procedures in the Western Norway B Vitamin Intervention Trial.
[h]Deaths classified with *International Statistical Classification of Diseases, 10th Revision (ICD-10)*, codes I00 to I99, or code R96 as underlying cause of death.

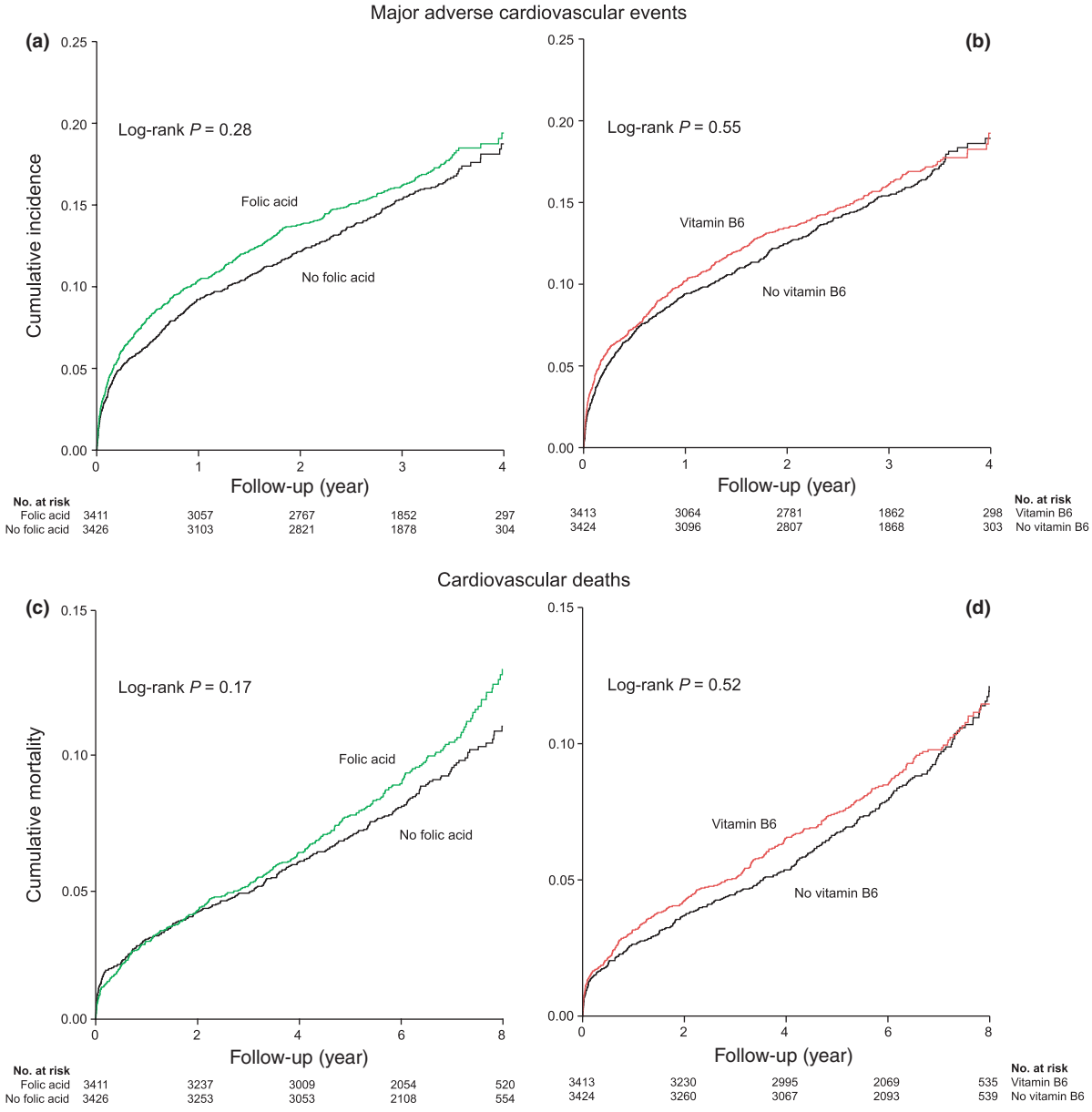

Fig. 2 *Kaplan–Meier Curves for Major Adverse Cardiovascular Events and for Cardiovascular Mortality. The primary outcome during trials was major adverse cardiovascular events (a composite of cardiovascular death, acute myocardial infarction and stroke) (Panels a and b). The outcome during extended follow-up from randomization until 1 January 2008 was cardiovascular mortality (Panels c and d). The comparisons were between folic acid and nonfolic acid groups (Panels a and c), and between vitamin B6 and nonvitamin B6 groups (Panels b and d).*

MTHFR 677 C—>T polymorphism

The *MTHFR* 677 C—>T polymorphism was not a predictor of in-trial MACEs or long-term cardiovascular mortality in the study population, without or with adjustment for folic acid plus vitamin B12 treatment (data not shown).

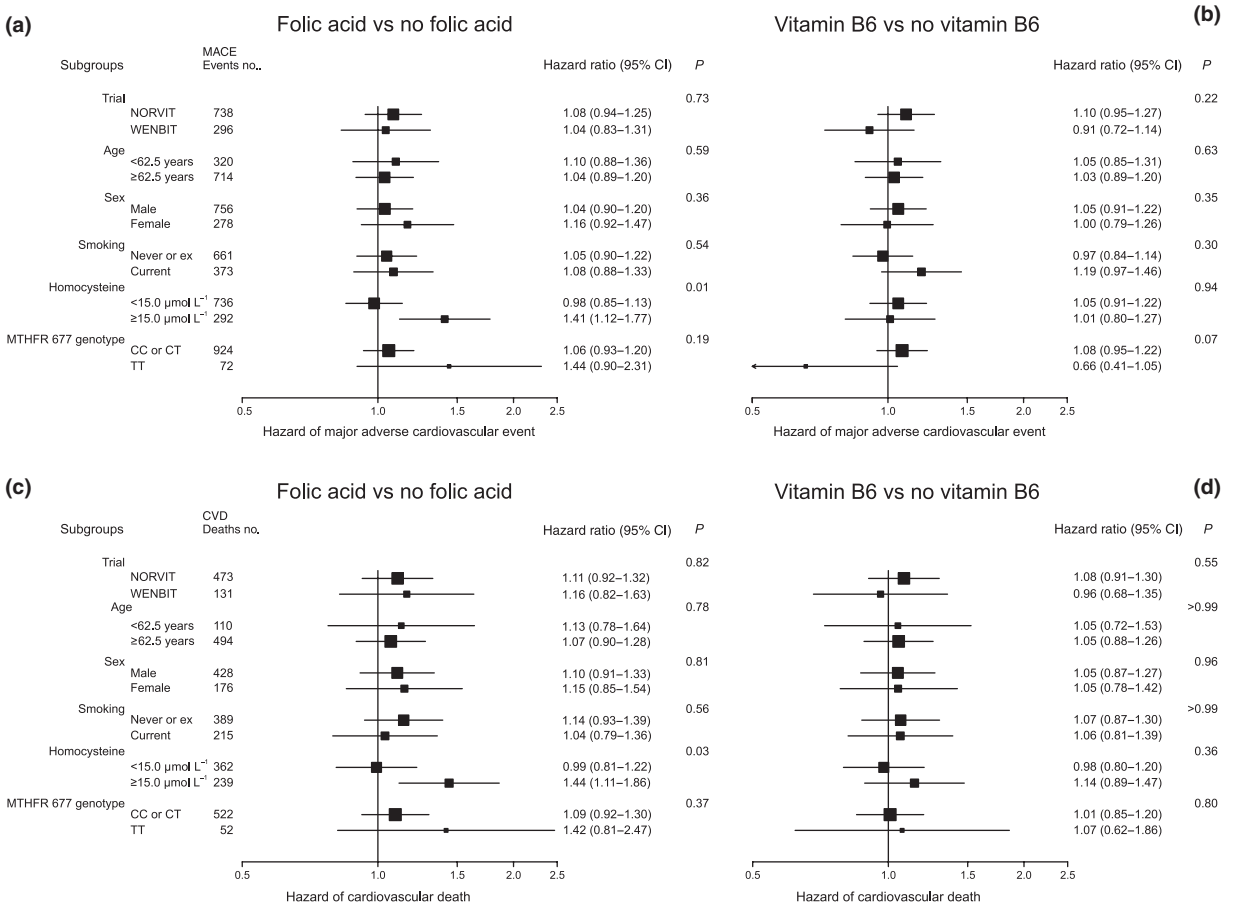

Fig. 3. *Hazard Ratios for Major Adverse Cardiovascular Events and for Cardiovascular Mortality in Patient Subgroups. The primary outcome during trials was major adverse cardiovascular events (a composite of cardiovascular death, acute myocardial infarction and stroke). The outcome during extended follow-up from randomization until 1 January 2008 was cardiovascular mortality. The comparisons were between folic acid versus no folic acid treatment (Panels a and c), and between vitamin B6 versus no vitamin B6 treatment (Panels b and d) in patient subgroups. Squares with horizontal lines are hazard ratios and the corresponding 95% confidence intervals.*

Discussion

In this combined analysis and prolonged follow-up of two large, similar trials amongst 6837 patients with ischaemic heart disease, we found no overall effects on the risk of in-trial MACEs or long-term cardiovascular mortality of treatment with folic acid plus vitamin B12 for a median of 39 months. This was despite a substantial, quick and long-lasting Hcy-lowering effect during the intervention. Amongst patients with baseline hyperhomocysteinaemia, the folic acid plus vitamin B12 treatment appeared to have harmful effects. Otherwise, there were no effects in subgroups defined by baseline characteristics. We found no separate clinical effects of vitamin B6. Amongst participants randomly allocated to folic acid plus vitamin B12, the lowered plasma tHcy concentration measured after 1–2 months of intervention (but not the tHcy concentration measured at baseline) showed a strong, independent association with increased risk of subsequent MACEs.

Major strengths of this analysis include the double-blind randomized design, the large number of participants and events and the longitudinal measurements of levels of plasma tHcy and serum/plasma B-vitamins at baseline and at two time-points during the in-

Table 4 *Plasma total homocysteine as predictor of outcomes in folic acid groups*[a]

Outcome	Time-point for plasma tHcy measurement	Participants in Folic Acid Groups	Unadjusted[b] No. of events/ no. of participants	Unadjusted[b] HR (95% CI) per 5 μmol L^{-1} increment of plasma tHcy	Unadjusted[b] *P*	Adjusted[c] No. of events/no. of participants	Adjusted[c] HR (95% CI) per 5 μmol L^{-1} increment of plasma tHcy	Adjusted[c] *P*
MACE	Baseline	All	324/2991[d]	1.30 (1.20–1.42)	<0.001	306/2914[e]	1.05 (0.93–1.18)	0.41
	1–2 months	All	324/2991[d]	1.78 (1.58–2.02)	<0.001	306/2914[e]	1.31 (1.09–1.56)	0.004
		Responders[f]	282/2660[d]	1.85 (1.60–2.15)	<0.001	267/2594[e]	1.25 (0.99–1.58)	0.06
		Nonresponders[f]	42/331[d]	1.78 (1.38–2.30)	<0.001	39/320[e]	1.62 (1.14–2.31)	0.007
Cardiovascular death	Baseline	All	240/3100	1.50 (1.37–1.63)	<0.001	226/3011[g]	1.05 (0.93–1.19)	0.44
	1–2 months	All	240/3100	1.99 (1.76–2.26)	<0.001	226/3011[g]	1.20 (0.96–1.50)	0.11
		Responders[f]	208/2755	2.15 (1.87–2.47)	<0.001	195/2680[g]	1.18 (0.90–1.54)	0.24
		Nonresponders[f]	32/345	1.62 (1.19–2.21)	0.002	31/331[g]	1.17 (0.75–1.83)	0.50

Abbreviations: CI, confidence interval; HR, hazard ratio; MACE, major adverse cardiovascular event; tHcy, total homocysteine.

[a]Analyses were restricted to participants in the folic acid groups with plasma tHcy measurements at baseline and at the first follow-up visit which occurred 1–2 months after randomization (n = 3100).

[b]HRs were estimated by Cox proportional hazards regression, stratified by trial, unadjusted.

[c]HRs were estimated by Cox proportional hazards regression, stratified by trial, adjusted for vitamin B_6 treatment, age, sex, serum creatinine, prior myocardial infarction, prior percutaneous coronary intervention, prior coronary artery bypass graft surgery, prior carotid stenosis, transient ischaemic attack or stroke, baseline smoking, indication for trial entry, hypertension, obesity and diabetes mellitus.

[d]Participants who experienced a MACE between the two plasma tHcy measurements (n = 109) were excluded from this analysis.

[e]A total of 77 participants had missing values for one or several of the variables that were adjusted for.

[f]Responders and nonresponders were defined as participants with or without reduction in plasma tHcy concentration during the first 1–2 months of folic acid plus vitamin B12 treatment.

[g]A total of 89 participants had missing values for one or several of the variables that were adjusted for.

trial follow-up. Also, we obtained data regarding the *MTHFR* 677 C—>T polymorphism in 95.6% of participants. Because there is no folic acid fortification of foods in Norway, and the use of over-the-counter vitamins at trial entry was modest, the Hcy-lowering effect of the folic acid plus vitamin B12 intervention was substantial.

Loss to long-term follow-up for cardiovascular mortality was minimal (1.3%), and ascertainment of the cause of death was almost complete as a result of linkage to the population-based Cause of Death Registry [30]. A possible limitation is that we did not obtain data on nonfatal cardiovascular events during post-trial follow-up. However, given the absence of effects on long-term coronary, stroke or other cardiovascular mortality, significant effects on post-trial nonfatal cardiovascular events are unlikely.

Since the initiation of NORVIT and WENBIT, further evidence of the association between Hcy and CVD has emerged. As in patients with stable CAD [3, 33], Hcy level also predicts adverse outcomes in patients with acute coronary syndromes [34, 35]. A recent meta-analysis of cohort studies in healthy populations concluded that for each 5 μmol L^{-1} increment in tHcy level, the risk of coronary events increases by 18%, independently of traditional CAD risk factors [36]. Also, a later large case–control study conducted in a Swedish population without folic acid fortification demonstrated that plasma tHcy concentration measured at baseline was strongly and independently associated with risk of MI after 13 years of follow-up [37].

The evidence up to 2001 from Mendelian randomization studies pointed towards Hcy being causally related to CVD [23, 24]. However, a later meta-analysis of studies up to 2004 found no association between the *MTHFR* 677C—>T polymorphism and CAD in European, North-American or Australian populations [38]. Furthermore, a recent large study amongst healthy US women found no association between TT genotype and CVD after 10 years of follow-up [39]. In line with this, we found the frequency of the T allele and individuals with the TT genotype to be similar to the frequencies in a large sample of the general Norwegian population [40], and this polymorphism was not associated with cardiovascular outcomes.

Elevated plasma tHcy concentration could result from deficiencies of folate, vitamin B12 or B6, or from impaired functions of the enzymes involved B-vitamin and/or Hcy metabolism [41] but may also be associated with a variety of factors that do not reflect B-vitamin status. The latter include several risk factors for CVD such as smoking, low level of physical activity, high blood pressure, high total cholesterol level, impaired renal function and cellular immune activation [42–44]. An intriguing finding in the present study is that plasma tHcy concentration after 1–2 months of folic acid plus vitamin B12 treatment (i.e. the B-vitamin nonresponsive fraction of plasma tHcy) was a strong, independent predictor of risk of subsequent in-trial MACEs. Thus, the remaining fraction of plasma tHcy may reflect levels of cardiovascular risk factors that are not linked to one-carbon metabolism. This is also in line with our main finding that lowering plasma tHcy concentration with folic acid plus vitamin B12 did not lower the risk of cardiovascular outcomes.

The administration of pharmacological doses of B-vitamins could influence several biological systems, in addition to Hcy remetylathion [41, 45]. In the current study population, folic acid plus vitamin B12 treatment was associated with increased risk of cancer incidence, cancer mortality and all-cause mortality [46]. The increased risk of cardiovascular outcomes by folic acid plus vitamin B12 amongst patients with baseline hyperhomocysteinaemia could be a chance finding. However, it is possible that this intervention, which may promote DNA synthesis and cell proliferation, enhances neointimal proliferation in high-risk individuals with established atherosclerosis [41, 45], leading to accelerated disease progression.

We found no separate effect of the vitamin B6 intervention on cardiovascular outcomes. This is in line with the results of recent observational studies demonstrating that low circulating levels of vitamin B6 may be a consequence of inflammation accompanying CAD and/or smoking, rather than being causally related to CAD [47].

In conclusion, combined analyses and extended follow-up of two trials conducted amongst almost 7000 patients with ischaemic heart disease revealed no protective effect of Hcy-lowering intervention with folic acid plus vitamin B12 on in-trial MACEs or long-term cardiovascular mortality. Amongst patients who received folic acid plus vitamin B12, plasma tHcy concentration measured after 1–2 months of follow-up (as opposed to the concentration measured at baseline) was independently associated with risk of

in-trial MACEs. This suggests that cardiovascular risk prediction by plasma tHcy concentration is mainly confined to the B-vitamin nonresponsive Hcy fraction. Our findings are consistent with the lack of effect of Hcy-lowering B-vitamin intervention demonstrated in large randomized controlled trials to date [13–22], and therefore B-vitamins should not be recommended for patients with CVD to lower their Hcy levels.

Conflicts of interest

The authors have no conflicts of interest to declare.

Acknowledgements

We thank the recruiting physicians, study nurses, laboratory personnel and other co-workers. We also thank all 6837 participants for their time and motivation.

The NORVIT and WENBIT trials were supported by: the Advanced Research Program and Research Council of Norway; the Norwegian Foundation for Health and Rehabilitation; the Norwegian Council on Cardiovascular Disease; the Norwegian Heart and Lung Patient Organisation; the Norwegian Red Cross; the Northern Norway Regional Health Authority; the Western Norway Regional Health Authority; the Norwegian Ministry of Health and Care Services; the University of Tromsø, Tromsø; the University of Bergen, Bergen; the Department of Heart Disease at Haukeland University Hospital, Bergen; the Foundation to Promote Research into Functional Vitamin B12 Deficiency, Bergen; Norway; an unrestricted private donation; and Alpharma Inc, Copenhagen; Denmark.

The post-trial follow-up was supported by: the Norwegian Foundation for Health and Rehabilitation; the Department of Heart Disease at Haukeland University Hospital, Bergen; the University of Tromsø, Tromsø; the University of Bergen, Bergen; and the Foundation to Promote Research into Functional Vitamin B12 Deficiency, Bergen; Norway.

The main funders of the study were nonprofit organizations with no participating role in the trials. Alpharma Inc provided the study capsules, generated the randomization sequence and concealed the randomization code, free of charge and provided a limited grant to finance the initial phase of the trials. However, the company had no role in the design and conduct of the trials or the current study, or in the collection, management, analysis and interpretation of the data, the decision to publish, or the preparation, review or approval of the manuscript. Dr Ebbing had full access to all data at the end of the study and had the final responsibility for the decision to submit for publication.

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Clinical trial registration information

Clinicaltrials.gov Identifier: NCT00671346.

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