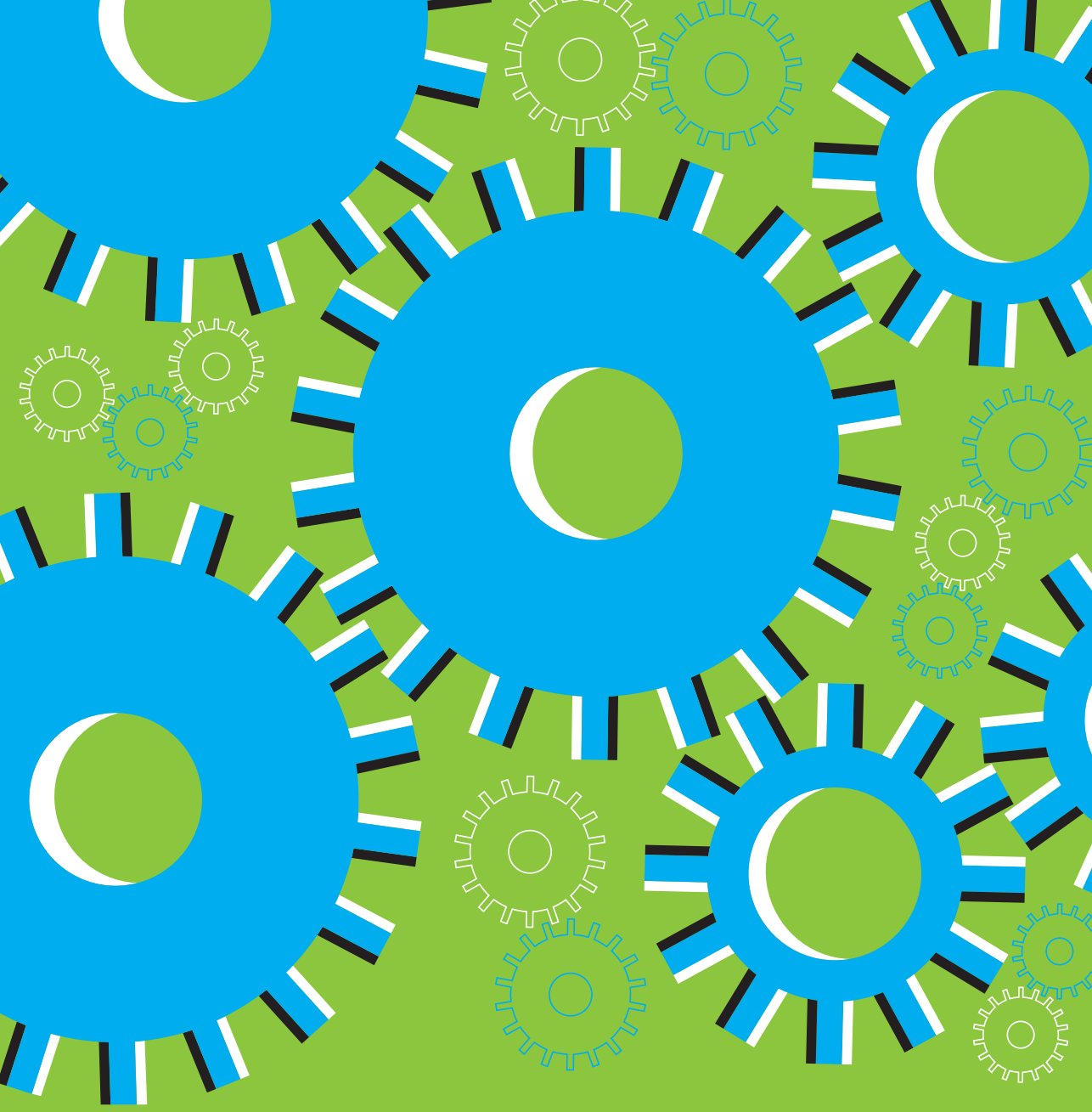

The role of homocysteine in bone

Anke Enneman

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De rol van homocysteine in bot

Anke Enneman

Colophon

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THE ROLE OF HOMOCYSTEINE IN BONE

DE ROL VAN HOMOCYSTEINE IN BOT

Proefschrift

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Dr. M.C. Zillikens

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General introduction and introduction to B-PROOF

Chapter 1.1

General introduction

OSTEOPOROSIS AND FRACTURES

The problem

Osteoporosis is characterized by low bone mass and deterioration of micro-architectural structure [1]. It is a very common disease; in 2007 it was estimated that 1.9 in 1,000 men and 16.1 in 1,000 women had diagnosed osteoporosis in the Netherlands. However, the true number is expected to be 2-3 times higher, since osteoporosis often goes undetected because it causes no symptoms until a fracture occurs [2]. Whether one develops osteoporosis is determined by multiple factors; for instance, high age, female sex, low body weight, smoking, limited physical activity and use of medication such as glucocorticoids are all risk factors [3]. In addition, osteoporosis is highly heritable; bone mineral density (BMD), the parameter obtained by dual-energy X-ray absorptiometry (DXA) scanning to diagnose osteoporosis, has an estimated heritability of 50-85% [4]. Recently, 56 genetic determinants have been identified that influence BMD [5, 6].

Fractures are the most important possible consequence of osteoporosis. They form a major health care burden. For example, in 2005, more than 2 million fractures were reported in the United States only, leading to 17 billion dollars of costs [7]. Moreover, fractures lead to morbidity and mortality. In the Netherlands, a striking 25% of persons who sustained a hip fracture dies within one year [8]. The problem of osteoporosis and fractures is expected to increase over time because of global demographic changes due to improved health; the number of people aged ≥65 years is expected to increase from 506 million in 2008 to 1.3 billion in 2040 [7].

Pathophysiology

Bone tissue needs to be remodeled constantly to maintain its structure and strength. The balance of the activity of osteoblasts, the bone forming cells, and osteoclasts, the bone resorbing cells, is crucial in this process. Human bone mass reaches its peak at young adulthood, around the age of 30. Until that time, bone formation occurs at a higher rate than bone resorption, net leading to bone being accrued. However, with ageing bone resorption starts to outweigh bone formation, leading to a decrease in bone mass and more porous bone. In women, this process is more pronounced after menopause, since levels of estrogen, which stimulates bone formation and inhibits bone resorption, steeply decrease. Whether an indi-

vidual actually develops osteoporosis or not depends on his or her peak bone mass and on the amount of bone loss over time [9].

Diagnosis

The gold standard for diagnosing osteoporosis is DXA, by which BMD can be assessed. This measurement is most often performed and best-validated at the femoral neck and lumbar spine (Figure 1). A 'T-score' is derived from these measurements, reflecting how many standard deviations the BMD differs from the average BMD of a young, healthy adult. A T-score ≤-2.5 is defined as osteoporosis. Osteopenia, the precursor stage of osteoporosis, is diagnosed when the T-score is between -1 and -2.5 [10].

Next to DXA, other techniques have been developed to investigate bone health. Amongst others, quantitative ultrasound (QUS) forms an alternative with several advantages as compared with DXA; it is measured using a portable device which makes use of ultrasonic waves instead of ionizing radiation. Although its measurement can be performed at several sites in the body, the best validated site is the

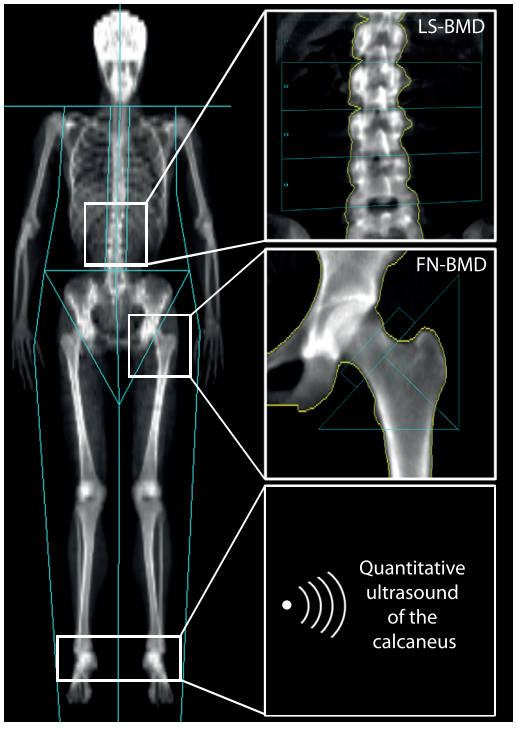

Figure 1. Best-validated and most frequently measured skeletal sites using DXA/QUS.
LS-BMD=lumbar spine bone mineral density, FN-BMD=femoral neck bone mineral density.

calcaneus [11] (Figure 1). Speed of sound and ultrasound attenuation are two parameters which are obtained. These are claimed to reflect bone micro-architecture [12] and are able to predict fracture incidence partly independently of BMD [13].

In addition, the measurement of bone turnover markers has shown to reflect bone remodeling. Bone turnover markers are divided in bone formation and bone resorption markers. Bone formation markers, such as alkaline phosphatase, osteocalcin and propeptides of type I procollagen, are produced by osteoblasts. Bone resorption markers include products from the degradation of type I collagen, such as pyridinoline, deoxypyridinoline and telopeptides of type I collagen, and products that reflect osteoclast activity. In particular, bone turnover markers can be used to monitor anti-osteoporotic treatment efficacy [14], but its use in clinical practice is limited by lack of standardization [15].

Prevention and treatment

Currently, several preventive strategies and treatment options for osteoporosis exist. Adequate calcium and vitamin D intake or sunlight exposure, no smoking nor heavy drinking, and regular (preferably weight-bearing) physical activity are important preventive measures for osteoporosis [9]. For treatment purposes, calcium and vitamin D supplements can be prescribed. In addition, prescription of medicines has been shown to effectively reduce fracture risk [3]. Generally used anti-osteoporotic medications can be roughly divided into inhibitors of bone resorption, such as bisphosphonates, selective estrogen receptor modulators, and antibodies to RANKL (denosumab), and stimulants of bone formation, such as teriparatide and PTH 1-84 [3]. These drugs are available in the form of oral, subcutaneous or intravenous formulations. While these medications are very effective in reducing fracture risk (with an estimated decrease of 50-60% in the risk of vertebral fractures and 20-30% for non-vertebral fractures), they also have some side-effects, e.g. on the gastro-intestinal system, leading to a disappointingly low adherence to the prescribed treatment. For example, it has been shown that approximately half of the patients stops taking their oral anti-osteoporotic medication within one year [3, 16].

HOMOCYSTEINE: A NEW RISK FACTOR FOR OSTEOPOROTIC FRACTURES

Approximately a decade ago, high plasma homocysteine levels have been associated with increased risk of incident osteoporotic fractures [17, 18]. The reason for investigating this was the observation that patients who suffer from homocystinuria, a disease that is often caused by a genetically determined cystathionine β-synthase (CBS) deficiency, also frequently suffer from osteoporosis [19]. Recently, the observed association between homocysteine and fractures was confirmed in a meta-analysis [20]. More details about this association are reported in Chapter 1.2.

Homocysteine is an amino-acid. It is absent in the human diet, but is formed after demethylation of methionine [21, 22], an amino-acid which is essential and is present in our food. Fasting plasma reference values of homocysteine are between 6 and 19 µmol/l, but levels above 15 µmol/l are generally regarded as elevated

Figure 2. Homocysteine metabolism.
(diamond: co-factor, square: enzyme, rectangle: enzymatic product. B_2=riboflavin, B_6=pyridoxine, B_{12}=cobalamin, MTHFR=methylenetetrahydrofolate-reductase, THF=tetrahydrofolate).

[23, 24]. In Figure 2, the commonly accepted methylation cycle is depicted. As can be seen, s-adenosylmethionine (SAM) and s-adenosylhomocysteine (SAH) are two intermediates in the conversion from methionine to homocysteine. A methyl group is released when SAM converts to SAH, and this methyl group can be used to, for example, methylate DNA.

Homocysteine can be broken down via the transsulfuration pathway. For this pathway, the enzyme CBS is needed. Severe hyperhomocysteinemia (>50 µmol/l) or homocystinuria (a state in which homocystine is excreted in urine) can, amongst others, be caused by a mutation in the gene encoding for this enzyme [21]. Moreover, homocysteine can also be reconverted to methionine. In this remethylation pathway, vitamin B_{12} and folate are two important co-factors. It is therefore of no surprise that already two decades ago it has been shown that homocysteine levels are negatively associated with plasma vitamin B_{12} and folate and folate intake [25], and that supplementation with these vitamins is able to lower homocysteine levels [26]. Vitamin B_2 (riboflavin) and vitamin B_6 (pyridoxine) are also co-factors which are of importance in the metabolism of homocysteine. However, it has been shown that supplementation with B_6 complementary to folic acid and vitamin B_{12} supplementation does not have an additional lowering effect on homocysteine levels [26].

Plasma homocysteine levels are also partly genetically determined, as was mentioned in the example of CBS. Genetic determinants can be divided in rare Mendelian mutations detected in family studies, and more commonly present variations such as single nucleotide polymorphisms, which can be identified using genome-wide association studies and which generally have more subtle effects. The most important and well-known genetic polymorphism known to influence homocysteine levels resides in the gene encoding for methylenetetrahydrofolate-reductase (MTHFR). This enzyme catalyzes the conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate. In persons with the MTHFR C677T TT genotype, MTHFR is less active and these persons therefore on average have 2.6 µmol/l higher plasma homocysteine levels than persons having the CC genotype [27]. The homocysteine-increasing effect of the TT genotype is especially pronounced in persons who have low (below median) plasma folate levels [27].

Elevated plasma homocysteine levels have not only been associated with osteoporotic fractures, but also with several other unfavorable health outcomes, such as

cardiovascular disease [28] and cognitive impairment [29]. All these associations are of great interest since plasma homocysteine is an easily modifiable factor. However, whether the association with fractures is causal or not is an important question which remains to be answered.

VITAMIN B_{12}, FOLATE, AND OLDER PERSONS

Vitamin B_{12}

As mentioned above, vitamin B_{12} and folate are important co-factors in the homocysteine metabolism. Vitamin B_{12}, or cobalamin, is an essential vitamin that is present in products of animal origin, such as meat, dairy, fish and eggs. It is a water-soluble vitamin, but it nonetheless can be stored in the human liver. Recommended daily intake of vitamin B_{12} for adults is 2.8 µg in the Netherlands. To make its uptake in the intestine possible, vitamin B_{12} first has to be released from dietary proteins. This is facilitated by gastric acid and pepsin in the stomach. Then, B_{12} can be coupled to Intrinsic Factor (IF), a glycoprotein that is produced in the gastric wall. This complex can subsequently be taken up in the ileum. When IF is not sufficiently produced, vitamin B_{12} cannot be taken up effectively and a vitamin B_{12} deficiency can develop. In addition, in elderly a vitamin B_{12} deficiency can also be caused by atrophic gastritis, which in turn can be associated with Helicobacter pylori infection. Atrophic gastritis can lead to reduced gastric acid production and/or proteolytic activity. It should be noted that a small part (±1%) of vitamin B_{12} can be taken up passively. Therefore, when taking vitamin B_{12} in relatively large amounts, for example in the form of supplements (cyanocobalamin), passive diffusion can importantly contribute to vitamin B_{12} uptake. This way, IF-dependent uptake of vitamin B_{12} can be bypassed by consuming high doses of vitamin B_{12} supplements [30, 31].

Clinically, vitamin B_{12} deficiency can result in pernicious anemia and neurological symptoms, which may be irreversible. Measurement of vitamin B_{12} status can be done in several ways, however, no gold standard has been defined as of yet. Serum levels of total vitamin B_{12} can be measured, and although they are sensitive for detecting clinical deficiency, they do not necessarily represent a functional but subclinical vitamin B_{12} deficiency. To detect a functional deficiency, resulting in suboptimal function of the involved enzymes, measurement of methylmalonic

acid (MMA) or homocysteine is preferred, since both of these markers accumulate in the presence of too low vitamin B_{12} levels. However, both also strongly depend on renal function. Currently, holotranscobalamin is also regarded as an informative marker of B_{12} status, as this is the metabolically available form of vitamin B_{12} [30, 32].

Folate

Folate is an essential, water-soluble B-vitamin. In its natural form, it is mostly found in grains and green leafy vegetables. In addition to the natural folate, also a more stable, synthetic form exists, named folic acid. This is generally used in supplements. Recommended daily folate/folic acid intake in the Netherlands is 300 µg [30]. In addition, the Dutch Health Council recommends to not exceed 1 mg of intake of synthetic folic acid, since excess intake may mask the hematological signs (macrocytosis) of vitamin B_{12} deficiency. A vitamin B_{12}-deficiency may thus remain undetected and untreated, which may lead to ongoing neurological damage. In the Netherlands, women who want to become pregnant are advised to take 400 µg folic acid daily to prevent neural tube defects in the newborn. However, contrary to many other countries, such as the United States and Canada [30], no general mandatory folic acid food fortification exists in the Netherlands. An overview of countries with mandatory folic acid food fortification is presented in Figure 3.

Folate status can be determined by measuring folate levels in serum or in red blood cells. Normal reference values are 8 to 28 nmol/l and 390 to 1560 nmol/l

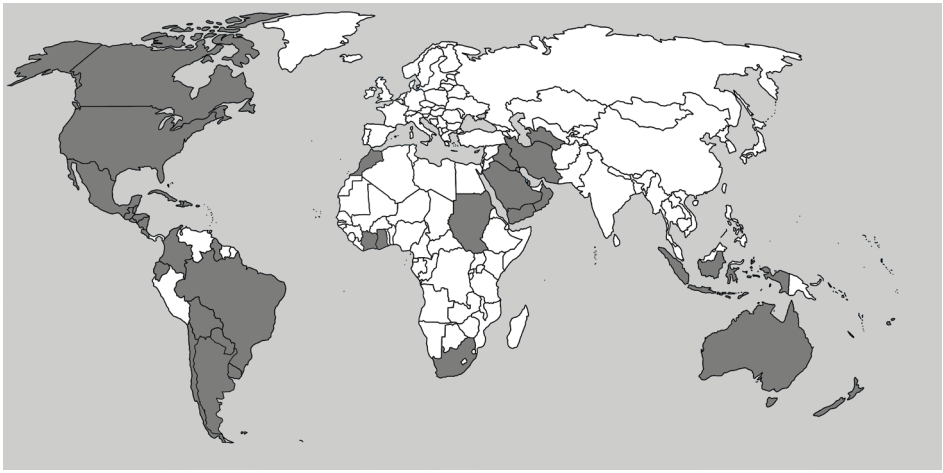

Figure 3. Countries with mandatory folic acid food fortification (shown in dark grey) (***adapted from*** [33]).

[23], respectively. Serum levels reflect short-term folate status, while red blood cell levels represent folate status over a longer term (about 3 months). Deficiency in folate can give rise to impaired DNA-synthesis and consequently to megaloblastic anemia [30].

Especially older persons are prone to develop deficiencies in folate and vitamin B_{12}. Consistently, levels of homocysteine tend to rise with age. Therefore, with regard to lowering homocysteine levels, supplementation with B-vitamins may be crucial, especially in older persons, to normalize homocysteine levels.

AIMS AND OUTLINE OF THIS THESIS

Taken together, osteoporosis and fractures form an important and increasing health care burden, for which preventive and treatment strategies may still be improved. Homocysteine is a modifiable risk factor, however, whether its association with incident osteoporotic fractures is causal is, as yet, undetermined. This thesis contains several studies aiming to investigate the role of homocysteine and a homocysteine-lowering intervention on both clinical and intermediate endpoints related to bone health.

Chapter 1.2 describes the rationale and design of the B-PROOF (B-vitamins for the PRevention Of Osteoporotic Fractures)-study, a trial that was designed to assess the efficacy of vitamin B_{12} and folic acid supplementation in fracture prevention in hyperhomocysteinemic elderly men and women. In this chapter, the actual link between the topics 'bone' and 'homocysteine' will be further explained. Due to its descriptive character, **Chapter 1.2** forms an addition to this introduction.

In **Chapter 2.1**, cross-sectional associations between homocysteine and bone mineral density and bone quality are studied. For this purpose, not only B-PROOF-data, but also data of the Rotterdam Study, which is a large prospective observational cohort study, have been analyzed. In **Chapter 2.2**, Rotterdam Study data were used to assess associations between SAM and SAH, as a measure of methylation capacity, and incident fractures.

The results concerning B-PROOF's main study outcome – osteoporotic fractures – are presented in **Chapter 3.1**. Next, effects of the same intervention on bone min-

eral density and quality parameters (**Chapter 3.2**) and bone turnover markers (**Chapter 3.3**) are investigated.

Chapter 4.1 describes a Mendelian randomization approach, used to investigate the association of a genetic risk score predicting plasma Hcy levels with fractures and bone mineral density within the international GEFOS (GEnetic Factors for OSteoporosis)-consortium and B-PROOF.

In **Chapter 5**, a reflection on all findings that are described in this thesis is presented and finally, a summary is provided in **Chapter 6**.

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Chapter 1.2

Rationale and design of the B-PROOF study, a randomized controlled trial on the effect of supplemental intake of vitamin B_{12} and folic acid on fracture incidence

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ABSTRACT

Background

Osteoporosis is a major health problem, and the economic burden is expected to rise due to an increase in life expectancy throughout the world. Current observational evidence suggests that an elevated homocysteine concentration and poor vitamin B_{12} and folate status are associated with an increased fracture risk. As vitamin B_{12} and folate intake and status play a large role in homocysteine metabolism, it is hypothesized that supplementation with these B-vitamins will reduce fracture incidence in elderly people with an elevated homocysteine concentration.

Methods/Design

The B-PROOF (B-Vitamins for the PRevention Of Osteoporotic Fractures) study is a randomized double-blind placebo-controlled trial. The intervention comprises a period of two years, and includes 2919 subjects, aged 65 years and older, independently living or institutionalized, with an elevated homocysteine concentration (≥12 µmol/L). One group receives daily a tablet with 500 µg vitamin B_{12} and 400 µg folic acid and the other group receives a placebo tablet. In both tablets 15 µg (600 IU) vitamin D_3 is included. The primary outcome of the study is osteoporotic fractures. Measurements are performed at baseline and after two years and cover bone health i.e. bone mineral density and bone turnover markers, physical performance and physical activity including falls, nutritional intake and status, cognitive function, depression, genetics and quality of life. This large multi-center project is carried out by a consortium from the Erasmus MC (Rotterdam, the Netherlands), VUmc (Amsterdam, the Netherlands) and Wageningen University, (Wageningen, the Netherlands), the latter acting as coordinator.

Discussion

To our best knowledge, the B-PROOF study is the first intervention study in which the effect of vitamin B_{12} and folic acid supplementation on osteoporotic fractures is studied in a general elderly population. We expect the first longitudinal results of the B-PROOF intervention in the second semester of 2013. The results of this intervention will provide evidence on the efficacy of vitamin B_{12} and folate supplementation in the prevention of osteoporotic fractures.

Trial Registration

The B-PROOF study is registered with the Netherlands Trial Register (NTR NTR1333) and with ClinicalTrials.gov (NCT00696514).

BACKGROUND

Osteoporosis is a chronic, multifactorial disorder which is characterized by low bone mass and micro architectural deterioration of bone tissue [1]. Its major consequence is fractures, and especially hip fractures are associated with institutionalization and increased mortality. In 2000, approximately 9 million fractures occurred worldwide, leading to a loss of 5.8 million disability adjusted life-years (DALYs) [2]. Due to a rise in life expectancy, the economic burden of osteoporotic fractures in Europe is expected to increase substantially in the coming decades: from €36.3 billion in 2000 to €76.8 billion in 2050 [3].

Pharmacological interventions may prevent 30-60% of fractures in patients with osteoporosis [4]. However, due to the high prevalence of osteoporosis and osteoporotic fractures, attention has been shifted towards preventive lifestyle interventions, such as vitamin D and calcium supplementation and promoting physical activity. Vitamin D and calcium supplementation has been shown to decrease the incidence of hip fractures and other non-vertebral fractures by 23-26% [5]. Increased physical activity is related to higher bone mineral density (BMD), bone structure and elasticity [6, 7] and is suggested to reduce the risk of hip fracture [8].

Besides those well-established factors, it has been shown that elevated homocysteine concentrations and low vitamin B_{12} status are strongly associated with lower bone mass and higher fracture risk in independently living elderly [9-11] and frail elderly [12]. Vitamin B_{12} and folate deficiencies and elevated homocysteine concentrations have been associated with lower BMD [13-18].

An elevated plasma homocysteine concentration (≥15 µmol/L) is prevalent in 30-50% of Dutch people older than 60 years, increases with age [19-21] and is multifactorial; age, sex and lifestyle factors, as well as environmental and genetic factors, nutritional intake of B-vitamins and hormonal factors affect homocysteine status [22]. B-vitamins play a central role in the homocysteine metabolism [23]. Treatment with vitamin B_{12} and folic acid supplements is effective in normalizing homocysteine concentrations [24, 25].

Evidence of a beneficial effect of supplementation with B-vitamins on fracture incidence has been signalled in Japan in elderly hemiplegic patients following stroke [26]. However, the generalizability of these findings is limited, since a highly selective patient population with a high percentage of vitamin D deficiency and a high fracture risk was studied. Moreover, pharmacological doses of folic acid (5 mg/day) and vitamin B_{12} (1.5 mg/day) were given, which may increase the risk of adverse effects.

In vitro studies support the hypothesis of a beneficial effect of vitamin B_{12} supplementation. Vitamin B_{12} has been shown to stimulate osteoblast proliferation and alkaline phosphatase activity [27] and vitamin B_{12} deficiency has been associated with defective functional maturation of osteoblasts [28]. Recent publications indicate a shift to more evidence of osteoclast stimulation by high homocysteine and low vitamin B_{12} concentrations [29-31]. These mechanisms might be interrelated with another, with subsequent interference of homocysteine with collagen cross-linking. Cross-links are important for stability and strength of the collagen network. Interference in cross-link formation would cause an altered bone matrix, further resulting in more fragile bone [32].

Accordingly, these mechanistic studies support the hypothesis of a beneficial effect of homocysteine reduction by B-vitamin supplementation on fracture incidence and related outcome measures. However, it remains unknown whether this relationship is causal as evidence from Randomized Controlled Trials (RCTs) is still limited. It would be most valuable to assess this relationship in a population consisting of generally healthy elderly people as deficiencies of vitamin B_{12} and folate are highly prevalent in this population and lead to elevated homocysteine concentrations.

The primary aim of our current intervention is therefore to assess the efficacy of oral supplementation with vitamin B_{12} and folic acid in the prevention of fractures in Dutch elderly people with elevated homocysteine concentrations. We will address potential pathways and phenotypes leading to fractures, osteoporosis measures, falls and physical performance. We will concurrently address other outcomes associated with elevated homocysteine concentrations, such as cognitive function [33] and cardiovascular disease [34]. The aim of this article is to describe the design of our intervention and to describe the baseline characteristics of the population enrolled.

METHODS/DESIGN

Study design

The B-PROOF study is a randomized, placebo-controlled, double-blind, parallel intervention study. B-PROOF is an acronym for 'B-vitamins for the PRevention Of Osteoporotic Fractures'. This large multi-centre project is carried out in The Netherlands by a consortium from Erasmus MC (EMC, Rotterdam), VU University Medical Center (VUmc, Amsterdam) and Wageningen University (WU, Wageningen), the latter acting as coordinator. The study aimed to include 3000 subjects, aged 65 years and older, with elevated plasma homocysteine concentrations (≥12 µmol/L). The intervention period is 2 years. Participants were randomly allocated in a 1:1 ratio to the intervention group or to the control group. We stratified for study centre, sex, age (65-80 years, >80 years), and homocysteine concentration (12-18 µmol/L, ≥18 µmol/L). The intervention group receives a daily tablet with 500 µg vitamin B_{12} and 400 µg folic acid and the control group receives a daily placebo tablet. Both tablets contain 15 µg (600 IU) of vitamin D_3 to ensure a normal vitamin D status [35]. The intervention and placebo tablets, produced by Orthica, Almere, the Netherlands, are indistinguishable in taste, smell and appearance. The random allocation sequence and randomization were generated and performed using SAS 9.2 by an independent research dietician.

Recruitment took place from August 2008 until March 2011. The B-PROOF study has been registered with the Netherlands Trial Register http://www.trialregister.nl website under identifier NTR 1333 since June 1, 2008 and with ClinicalTrials.gov under identifier NCT00696514 since June 9, 2008. The WU Medical Ethics Committee approved the study protocol, and the Medical Ethics committees of EMC and VUmc gave approval for local feasibility.

Sample size

Sample size calculation was based on the primary outcome measure of the intervention, i.e. osteoporotic fractures. The fracture rate in the non-treated group was estimated to be 5-6% in a period of two years, based on osteoporotic fracture incidence in both independently living and institutionalized elderly. Elderly in the highest quartile of homocysteine concentrations have been shown to have a doubled risk of fracture [11], we expected that the fracture rate in the treated group

would be reduced by 34%. With a power of 80%, a significance level (α) of 0.05, one tail, 1500 participants were required for both intervention and placebo group. To compensate for the expected drop-out rate of 15%, we extended the intervention period with one year for the first 600 participants of the study.

Subjects

Most participants were recruited via the registries of municipalities in the area of the research centres by inviting all inhabitants aged 65 years and older by mail. Furthermore, inhabitants of elderly homes in the area of Rotterdam, Amsterdam and Wageningen were invited to participate, after providing information brochures and information meetings. In addition, elderly who participated in previous studies of the research centres were approached. All participants gave written informed consent before the start of the intervention.

A total of 2919 subjects were included in the intervention (Figure 1). Inclusion and exclusion criteria are listed in Table 1.

Table 1. Inclusion and exclusion criteria for the B-PROOF study.

Inclusion criteria	Exclusion criteria
Men and women, aged 65 years and older	Immobilization: being bedridden or wheelchair bound
Compliance for tablet intake of >85% 4-6 weeks prior to start of the trial	Cancer diagnosis within the last 5 year, except skin cancer as basal cell carcinoma and squamous cell carcinoma
Competent to make own decisions	Serum creatinine level >150 µmol/L
Elevated homocysteine level (≥12 µmol/L and ≤50 µmol/L)	Current or recent (<4 months) use of supplements with very high doses of vitamin B_{12} (intramuscular injections) or folic acid (>300 µg)
	Participation in other intervention studies

Changes to inclusion criteria after trial commencement

The inclusion criteria regarding cut-off values for plasma homocysteine concentrations and age were adapted during the first phase of the intervention. The initial eligibility criterion for plasma homocysteine concentrations has been adjusted from ≥15 µmol/L to ≥12 µmol/L before the start of the study. Extended data analyses (unpublished data), based on Van Meurs et al., 2004, showed that a relation between homocysteine status and fracture incidence is also present at a lower homocysteine concentration (~14 µmol/L). Furthermore, cross-calibration between different local

homocysteine methods used in the current study (Architect Analyser, HPLC and LC-MS) and the methods used in the previous leading studies [9, 11] showed that a homocysteine concentration of 14 µmol/L in these studies corresponded with a concentration of 12 µmol/L when using the current methods.

It was decided to adapt the criterion for age from 70 years and older to 65 years and older after the first year of recruitment, because the association between homocysteine and fractures is also present in people aged 65-70 years [9, 11].

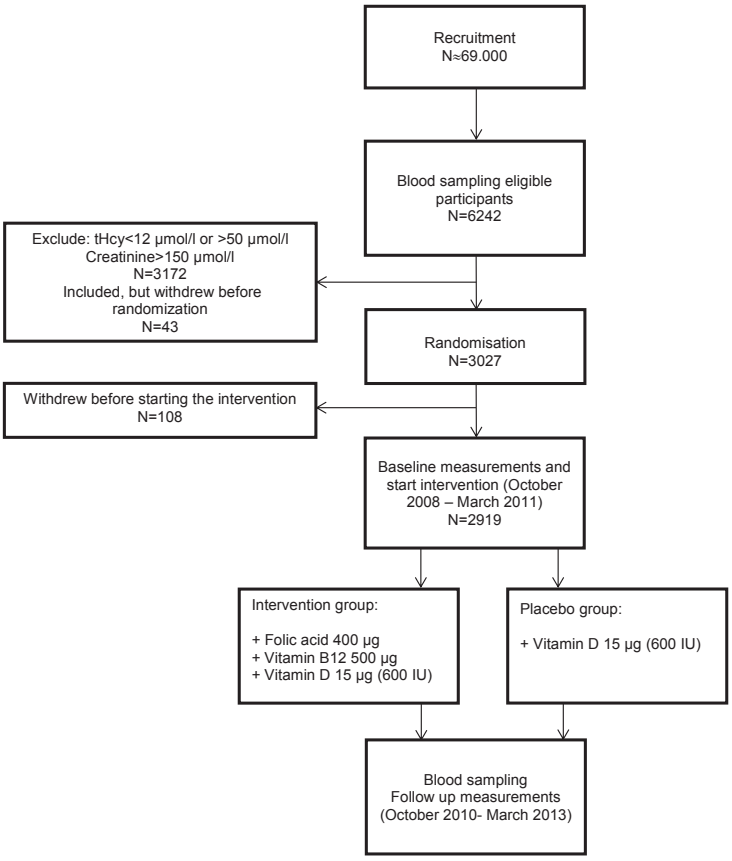

Figure 1. Recruitment and baseline measurements in participants of the B-PROOF study.

Screening and run-in period

Blood samples were obtained from participants in the morning at the research centres or at an external location in the living area of the participants. Participants

were in a fasted state, or had taken a light breakfast. Venous blood was drawn by a skilled nurse to obtain plasma, serum and buffy coats. For homocysteine analysis, a plasma EDTA tube was stored on ice immediately after blood drawing and samples were processed within 4 hours after blood drawing, to prevent a temperature- and time-dependent increase in plasma homocysteine [36]. Plasma homocysteine was measured using the Architect i2000 RS analyser (VUmc, intra assay CV=2%, inter assay CV=4%), HPLC method [37] (WU, intra assay CV=3.1%, inter assay CV=5.9%) and LC-MS/MS (EMC, CV=3.1%). According to a cross-calibration, outcomes of the three centres did not differ significantly. Serum creatinine was measured with the enzymatic colorimetric Roche CREA plus assay (CV=2%). The remaining plasma, serum and buffy coats samples were kept frozen at -80°C until further analysis.

After blood sampling participants started with a six-week run-in period, in which the participants took placebo tablets and were asked to daily fill out their study supplement intake on a research calendar. Subsequently, participants were informed whether they could further participate in the study or not, as an elevated plasma homocysteine concentration was an inclusion criterion, and an elevated serum creatinine concentration was an exclusion criterion. In case of laboratory results outside the reference range set for homocysteine (>50 µmol/L) or creatinine (>150 µmol/L) participants were referred to their general practitioner.

Measurements

Eligible participants were invited for baseline measurements, which were performed during a 1.5-2 hour session at one of the study centres or at the participant's home. The 2-year intervention period started after these baseline measurements. Adherence was assessed by recordings on the research calendar, counts of bi-annually returned tablets, and periodical phone calls with the participants. After two years of intervention, participants are invited for follow-up measurements, in which the baseline measurements are repeated.

Primary outcome

The primary outcome of the trial is time to first osteoporotic fracture. Participants recorded fractures on the research calendar, which was returned every 3 months. Incomplete or unclear data were further inquired by telephone. Furthermore, the research team verified reported fractures with the participants' general practitioner,

hospital physician and/or by radiographs. All fractures are considered osteoporotic, except for head/hand/finger/foot/toe fractures and fractures caused by traffic accidents [38]. The time to fracture is the difference between starting date and date of fracture reported on the calendar or by the general practitioner.

Secondary outcomes

Falls

Falls were recorded weekly on the research calendar. A fall was defined as an unintentional change in position resulting in coming to rest at a lower level or on the ground [39]. Recurrent falling was defined as at least two falls of a participant within six months during the two years of follow-up [40].

Dual Energy X-ray Assessment (DXA)

In two out of three study centres Dual Energy X-ray Assessment (DXA) was performed to measure bone mineral density (BMD) and lean body mass and to assess vertebral fractures, using the Hologic QDR 4500 Delphi device (VUmc, Hologic Inc., USA, CV=0.45%) or the GE Lunar Prodigy device (EMC, GE Healthcare, USA, CV=0.08%). The two devices were cross-calibrated. DXA was performed under standard protocols within four weeks after the participant's start of the intervention.

Total hip, femoral neck and lumbar spine BMD (g/cm^2) were measured. Total hip BMD was measured at the left femur, while in case of a hip prosthesis at the left side, the right side was measured. Instant vertebral assessment (IVA) was performed to detect clinical and non-clinical vertebral fractures. Results were independently evaluated by two researchers, and inconsistencies were discussed.

Furthermore, total body composition was measured. The amount of fat-free soft tissue (i.e. lean mass minus bone mineral content) of the extremities can be used as an indicator of skeletal muscle mass and has been validated in older persons [41].

Quantitative Ultrasound (QUS)

Quantitative ultrasound (QUS) measurements of the calcaneus were performed using a Hologic Sahara bone densitometer (Hologic Inc., USA). Broadband ultrasound attenuation (BUA, dB/MHz, CV=3.7%) and speed of sound (SOS, m/s, CV=0.22%) were measured in duplicate in both the right and the left calcaneus. From these

parameters, the quantitative ultrasound index (QUI, CV=2.6%) and estimated BMD (eBMD) were calculated.

Bone turnover markers

After completion of the study, bone turnover markers will be determined in a subsample in order to obtain better insight in the mechanism underlying the effect of B-vitamin supplementation on bone health. Standard assays will be performed in baseline and follow-up blood samples to measure markers of bone formation and bone resorption, such as procollagen type 1 N-extension peptide (P1NP) and cross-linked carboxyterminal telopeptide of type 1 collagen (CTx).

Physical performance and handgrip strength

Physical performance was measured using three tests; a walking test, a chair stands test, and a balance test. These performance tests are commonly used in elderly people [42-44]. During the timed walking test, participants were asked to walk 3 meters, turn around, and walk back as quickly as possible. During the timed chair stands test the participants rose from and sat down in a chair as quickly as possible for five consecutive times without the use of their arms. Standing balance was assessed with the modified Romberg test in which the participants were asked to maintain balance for 10 seconds in four different positions with increasing difficulty. Each position was performed with eyes open and eyes closed.

Hand grip strength (kg) was measured using a strain-gauged dynamometer (Takei, TKK 5401, Takei Scientific Instruments Co. Ltd., Japan, inter observer CV=5%). Participants were asked to perform two maximum hand grip trials with each hand in standing position with their arms along their body. Maximal hand grip strength was defined as the average of the highest score of the left and right hand.

Vascular parameters

Blood pressure measurements were performed using an Omron M1 plus blood pressure device (Omron Healthcare Europe). In two of the centres vascular structure and function was assessed non-invasively in a subsample by measuring blood pressure, intima-media-thickness (IMT) of the carotid artery, carotid distensibility (DC), aortic pulse wave velocity (PWV) and augmentation index (AIx).

Carotid B-mode ultrasonography is performed using the L105 40 mm 7.5 MHz array transducer (Picus, Pie Medical Equipment, Maastricht, the Netherlands) on the right carotid artery. IMT is evaluated as the distance luminal-intimal interference and the media-adventitial interface (Art.Lab, Esoate Europe, Maastricht, the Netherlands). The vessel wall movement-detector system has been described in detail previously [45]. The system consists of a wall track system and data-acquisition system (Art.Lab, Esoate Europe, Maastricht, the Netherlands). AIx is calculated using arterial tonometry obtained from the right radial, carotid and femoral artery using the Sphygmocor device (Sphygmocor version 7.1, AtCor Medical, Sydney, Australia). PWV is measured with simultaneously three channel ECG recording and recording of the right carotid and femoral artery pulse waveforms. Twenty-four hour ambulatory blood pressure recording was performed using Oscar 2 ambulatory 24 hour blood pressure monitor (SunTech Medical, North Carolina, USA).

Biomarkers of cardiovascular disease and cardiovascular events

Cardiovascular events were defined as cardiovascular mortality, myocardial infarction and stroke. Participants were requested to fill out a questionnaire regarding their cardiovascular history. After completion of the study cardiovascular and inflammatory biomarkers, such as amino-terminal B-type natriuretic peptide (NT-proBNP) and high-sensitivity hsC-reactive protein (hs-CRP) will be measured in baseline and follow-up blood samples.

Cognitive function

We used the Mini-Mental State Examination (MMSE) for a description of global cognitive performance in our study population [46]. In a subsample, i.e. all participants of WU, domain specific cognitive function was assessed using six standardized tests; the Symbol Digit Modalities Test, the Letter Fluency test, the Trail Making Test, the Digit Span Test, the Word Learning Test and the Stroop Colour Word Test. These tests were used to construct the following cognitive domains: attention, working memory, executive function, information processing speed and episodic memory [47].

Depression and Quality of Life

The Geriatric Depression Scale (GDS) was used to measure depressive symptoms [48]. To determine quality of life the EuroQoL EQ-5D [49] and Short Form Health Survey (SF-12) [50] questionnaires were used.

Measurement of covariates

General self-reported health and medication usage

Self-reported medical history, ethnicity, use of medication and of nutritional supplements, current alcohol intake and smoking habits and history of falls and fractures were determined using a questionnaire.

Medication use during the study period was also retrieved from pharmacies. Data included the prescription period, the total amount of drug units per prescription, the prescribed daily number of units, product name, and the Anatomical Therapeutic Chemical (ATC) code.

Physical Activity

Physical activity was measured using the LASA Physical Activity Questionnaire (LAPAQ), which is a validated questionnaire to measure physical activity in elderly people [51]. The activities included walking, cycling, gardening, participation in sports and light and heavy household activities. Frequency and duration of each activity during the last two weeks were assessed. Physical activity was calculated in minutes/day and kcal/day.

Nutritional status and food intake

The Mini Nutritional Assessment (MNA) [52] and the Simplified Nutritional Appetite Questionnaire (SNAQ) [53] were used to screen for malnutrition and appetite loss. Standing height was measured in duplicate to the nearest 0.1 cm with the person standing erect and wearing no shoes. Weight was measured to the nearest 0.5 kg with the person wearing light garments without shoes and empty pockets. In a subsample, i.e. all participants of WU, we estimated dietary intake by a Food Frequency Questionnaire (FFQ) with its main focus on macronutrients, vitamin B_{12}, folate, vitamin D, and calcium. The FFQ was developed by the dietetics group at the department of Human Nutrition, Wageningen University and was derived

from an FFQ which was validated for energy, fat, cholesterol, folate and vitamin B_{12} intake [54, 55].

Genotyping

From the blood samples drawn at baseline, DNA was isolated for genotyping. Subsequently, all samples were genotyped for approximately 700.000 single nucleotide polymorphisms (SNPs) using the Illumina Omni-express array, which has >90% coverage of all common variation in the genome. If known functional SNPs were not tagged well by the array, they were genotyped separately using TaqMan allelic discrimination assays on the ABI Prism 9700 HT sequence detection system. The data will be used in a hypothesis-free genome-wide association study (GWAS) as well as in analyses of genetic variation in known candidate genes.

Data analysis

The data analyses will be performed by following the intention-to-treat procedure (effectiveness study) and the per-protocol-procedure (efficacy study). If necessary, data will be transformed and analyses will be adjusted for the presence of covariates. Time to first fracture will be analysed using Cox Proportional Hazard Models.

Differences in mean change between groups will be analysed with independent sample Student's t-test, ANOVA or other similar tests. Two-sided P values will be calculated and a significance level of 0.05 will be applied.

We did not perform an interim analysis because we did not expect and observe negative side effects of the supplementation and because of the relatively long recruitment period, with most of the participants included in the last year of recruitment. We keep track of any serious adverse events (SAEs) occurring during the duration of the study.

Inclusion and baseline characteristics of the participants

Baseline characteristics of participants in the B-PROOF study are shown in Table 2. During the recruitment, we addressed approximately 69.000 people (Figure 1). This resulted in the screening of 6242 interested persons, of which 3027 were eligible to participate. One hundred and eight participants withdrew consent before start of the intervention resulting in 2919 participants who completed baseline measurements. The mean age of participants at the start of the intervention was 74.1 years

Table 2. Baseline characteristics of the B-PROOF study participants.

	Total (n=2919)	Male (n=1456)	Female (n=1463)
Study location (n)			
-WU	856	499	357
-VUmc	778	301	477
-Erasmus MC	1285	656	629
Age (years)*	74.1 (6.5)	73.4 (6.1)	74.9 (6.8)
Plasma homocysteine (µmol/L)#	14.4	14.6	14.1
	[13.0-16.6]	[13.1-16.8]	[12.9-16.3]
Serum creatinine (µmol/L)#	82.0	90.0	73.0
	[71-94]	[81.0-101.0]	[65.0-84.0]
Weight (kg)#	77.9 (13.3)	83.1 (11.9)	72.7 (12.5)
Height (cm) #	169.3 (9.3)	175.9 (6.6)	162.7 (6.6)
Physical activity (min/day)#	130.0	116.3	142.9
	[84.0-192.9]	[72.5-177.0]	[96.0-205.7]
Years of education*	10.1 (4.0)	10.9 (4.1)	9.2 (3.6)
Smoking (%)			
- Current	9.6	10.8	8.5
- Former	56.5	69.1	44.0
- Never	33.9	20.1	47.6

**Results are presented in mean (standard deviation); #Results are presented in median [interquartile range].*

(SD: 6.5) and 50% was female. Median plasma homocysteine concentration was 14.4 µmol/L (IQR: 13.0-16.6).

DISCUSSION

To our best knowledge, the B-PROOF study is the first intervention study in which the effect of vitamin B_{12} and folic acid supplementation on osteoporotic fractures is studied in a general elderly population. Currently, folic acid fortification is not mandatory in the Netherlands, and it is only applied on small scale in bread substitutes. This intervention is therefore an excellent opportunity to investigate the effect of folic acid and vitamin B_{12} supplementation in a non-fortified population. Positive evidence emerging from this intervention might enable elderly to live into an advanced age with lower fracture risk. Implementation of vitamin B_{12} and folic acid supplementation might therefore reduce the costs of national health services for osteoporosis in the elderly.

Elevated homocysteine concentrations are associated with various health outcomes, but until now there are no large interventions investigating the effect of homocysteine lowering treatment on, for example, physical performance. Therefore, the wide range of secondary outcomes studied in the B-PROOF study is unique. The possibility to perform a GWAS in such a large general elderly population will provide us with relevant data on the underlying mechanisms and genes involved in age-related diseases as osteoporosis and cognitive decline. In addition, DNA analysis gives us the opportunity to focus on the effect of B-vitamins on epigenetic changes.

We have some remarks on the expected outcomes of this study. We expect the effect of folic acid and vitamin B_{12} supplementation to be most beneficial in people with an elevated homocysteine concentration. We therefore only included elderly people with elevated homocysteine concentrations (≥12 µmol/L), but as a consequence, we cannot extrapolate the results to elderly with low to normal homocysteine concentrations (<12 µmol/L). However, 49% of the elderly screened in our study had an elevated homocysteine concentration. This percentage might be higher in the general Dutch elderly population, since people interested in nutrition and health, with a subsequent healthier lifestyle are probably more willing to participate in a long term intervention study. Therefore, the B-PROOF study covers a large segment of the general Dutch elderly population.

Because we supply both folic acid and vitamin B_{12}, it will not be possible to indicate whether the effects of the intervention will be the consequence of folic acid or vitamin B_{12} supplementation or lowering homocysteine concentrations in general. However, since both vitamins play a significant role in homocysteine metabolism, and folic acid supplementation alone might mask a possible vitamin B_{12} deficiency [56], it is the most efficient and safest to supplement both vitamins.

The first longitudinal results of the B-PROOF study will become available in the second semester of 2013.

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Chapter 2

Observational studies on homocysteine and its metabolites

Chapter 2.1

The association between plasma homocysteine levels and bone quality and bone mineral density parameters in older persons

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ABSTRACT

Introduction

High plasma homocysteine levels have been associated with incident osteoporotic fractures, but the mechanisms underlying this association are still unknown. It has been hypothesized that homocysteine might interfere with collagen cross-linking in bone, thereby weakening bone structure. Therefore, we wanted to investigate whether plasma homocysteine levels are associated with bone quality parameters, rather than with bone mineral density.

Methods

Cross-sectional data of the B-PROOF study (n=1227) and of two cohorts of the Rotterdam Study (RS-I (n=2850) and RS-II (n=2023)) were used. Data on bone mineral density of the femoral neck and lumbar spine were obtained in these participants using dual-energy X-ray assessment (DXA). In addition, participants of B-PROOF and RS-I underwent quantitative ultrasound measurement of the calcaneus, as a marker for bone quality. Multiple linear regression analysis was used to investigate the associations between natural-log transformed plasma levels of homocysteine and bone mineral density or ultrasound parameters.

Results

Natural-log transformed homocysteine levels were inversely associated with femoral neck bone mineral density in the two cohorts of the Rotterdam Study (B=– 0.025, p=0.004 and B=– 0.024, p=0.024). In B-PROOF, no association was found. Pooled data analysis showed significant associations between homocysteine and bone mineral density at both femoral neck (B=– 0.032, p=0.010) and lumbar spine (B=– 0.098, p=0.021). Higher natural-log transformed homocysteine levels associated significantly with lower bone ultrasound attenuation in B-PROOF (B=– 3.7, p=0.009) and speed of sound in both B-PROOF (B=– 8.9, p=0.001) and RS-I (B=– 14.5, p=0.003), indicating lower bone quality. Pooled analysis confirmed the association between homocysteine and SOS (B=– 13.1, p=0.016). Results from ANCOVA-analysis indicate that differences in SOS and BUA between participants having a plasma homocysteine level above or below median correspond to 0.14 and 0.09 SD, respectively.

Discussion

In this study, plasma levels of homocysteine were significantly inversely associated with both bone ultrasound parameters and with bone mineral density. However, the size of the associations seems to be of limited clinical relevance and may therefore not explain the previously observed association between plasma homocysteine and osteoporotic fracture incidence.

INTRODUCTION

Osteoporosis is characterized by low bone mass and micro-architectural deterioration of bone tissue, leading to bone fragility and increased fracture risk [1]. Osteoporotic fractures are a major health care problem, since they lead to a significant increase in morbidity and mortality [2]. For example, excess mortality rates in the first year after a hip fracture vary from 12% to 35% [3]. Due to a continuing rise in life expectancy and aging of the population, the economic burden of osteoporotic fractures in Europe is expected to increase substantially in the coming decades; from €36.3 billion in 2000 to €76.8 billion in 2050 [4].

Moderately elevated plasma homocysteine (Hcy) levels have been associated with osteoporotic fracture incidence [5-7]. However, the mechanisms underlying the association between Hcy and osteoporotic fractures have not yet been unraveled. In literature, conflicting results concerning the association between Hcy and bone mineral density (BMD) exist; inverse [8, 9], mixed [10] and no associations [7, 11, 12] have been reported. A recent meta-analysis in women showed no significant association between Hcy and BMD [13]. A meta-analysis in men was not possible. It therefore remains not fully certain whether the major pathway underlying the association between Hcy and osteoporotic fractures includes BMD. It has also been hypothesized that Hcy may interfere with the collagen cross-linking in the bone, thereby weakening bone structure [14]. Since the bone structure and micro-architecture are not completely captured by BMD, which measures the amount of mineralized bone in an area, it has been suggested that quantitative ultrasound (QUS) measurement might be more suitable for determining bone quality [15]. Bone micro-architecture has been shown to be a determinant of QUS-parameters, independent of BMD [16]. In addition, QUS has been proven to predict fracture risk to a similar degree as does BMD measured using dual-energy X-ray assessment

(DXA) [17, 18]. More importantly, both QUS and DXA predict fracture incidence partly independently of each other [19], as was recently confirmed in an updated meta-analysis [20]. Two studies investigating the association between Hcy and QUS parameters have been published [8, 21], showing an inverse association in women only.

Thus, data concerning the association between Hcy and bone quality are relatively scarce, and the association between Hcy and BMD remains inconsistent. We therefore analyzed cross-sectional data from three large Dutch studies to investigate the association between Hcy and BMD. In one of these studies, associations between Hcy and QUS parameters were studied as well.

METHODS

Design and study population

In this study, data of three studies (B-PROOF, Rotterdam Study-I and Rotterdam Study-II) were analyzed, both per cohort and pooled where applicable.

B-vitamins in the PRevention Of Osteoporotic Fractures (B-PROOF)

In the current study, baseline data of a subsample of the B-PROOF-study with data on bone parameters available were used. The B-PROOF-study is a multicenter, double-blind, randomized, placebo-controlled trial investigating the effect of a 2-year daily oral supplementation with 500 µg of vitamin B_{12} and 400 µg of folic acid on fracture incidence. The study population consists of 2919 Dutch men and women aged 65 years and over who have elevated plasma levels of Hcy (12–50 µmol/l) and normal serum creatinine levels (≤150 µmol/l). Details on the B-PROOF study design and population have been described elsewhere [22]. QUS-measurements were performed in a random subsample of persons who were screened for participation, and of whom levels of Hcy were not available yet (n=2185). DXA-measurements were done in a subsample of included participants (Hcy ≥12 µmol/l) who were able to visit one of the study centers. In total, of the participants having Hcy ≥12 µmol/l 627 participants underwent both DXA and QUS-measurements, while 600 underwent DXA only and 618 underwent QUS-measurement only (Figure 1). In addition, QUS-measurements were performed in 940 participants who turned out to be excluded from further participation in the B-PROOF-trial based on the exclusion

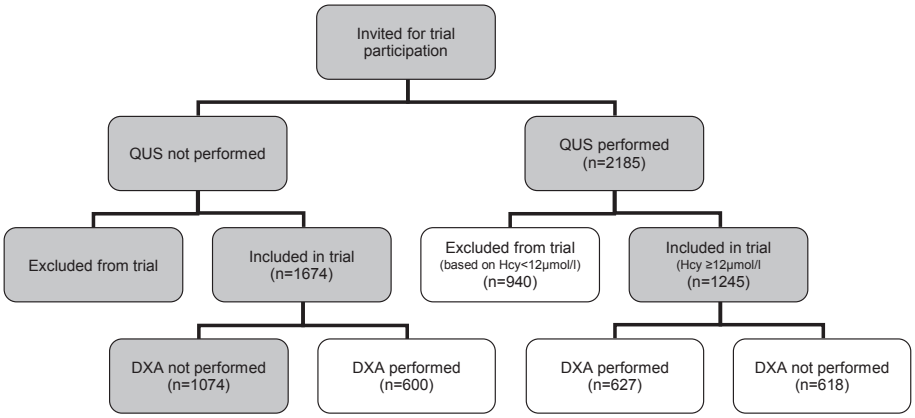

Figure 1. Flow-chart describing number of B-PROOF-participants with data on DXA and/or QUS. (White blocks represent participants with QUS and/or DXA measured at baseline included in the current, cross-sectional analyses.)

criterion of a plasma Hcy-level <12 µmol/l. The Wageningen University Medical Ethics Committee approved the study protocol, and the Medical Ethics committees of Erasmus MC and VUmc gave approval for local feasibility. All participants, including those who were not eligible for the trial, gave written informed consent.

Rotterdam Study (RS-I and RS-II)

The Rotterdam Study is an ongoing, population-based cohort study among people aged 55 years or over, who reside in the Ommoord district of the city of Rotterdam in the Netherlands. The Rotterdam Study was designed to investigate chronic, disabling diseases. Its rationale and design have been described previously [23]. The participants in the current study are part of either the Rotterdam Study-I (RS-I) or the RS-II cohort. Baseline measurements in the RS-I cohort were performed between 1990 and 1993 (RS-I-1). This cohorts' second follow-up visit after baseline took place between 1997 and 1999 (RS-I-3). Measurements and blood drawing performed at this second visit are used for cross-sectional analysis in the current study. Enrollment to the RS-II cohort started in 2000 and baseline data (RS-II-1) collected at that visit are used in the current study. From the RS-I and RS-II cohorts, 2850 and 2023 participants who underwent DXA were included in the analyses, respectively. In addition, QUS-parameters were available in 744 persons from the RS-I cohort. The Rotterdam Study was conducted according to the Declaration of Helsinki and

approved by the medical ethics committee of Erasmus MC. All participants gave written informed consent.

Measurements

Bone mineral density (B-PROOF and Rotterdam Study)

In the B-PROOF-study, BMD-measurements were performed at two study centers (VUmc or Erasmus MC). DXA was used to measure femoral neck (FN) and lumbar spine (LS) BMD (g/cm^2) under standard protocols and within four weeks of the individual's start in the intervention. For all measurements, the Hologic QDR 4500 Delphi device (VUmc (Hologic, USA, CV=0.45%)) or the GE Lunar Prodigy device (Erasmus MC (GE Healthcare, USA, CV=0.08%)) were used. The two devices were cross-calibrated by measuring a European spine phantom (ESP) five times on both machines and all results were adjusted accordingly.

In RS-I and RS-II, BMD at the femoral neck (FN-BMD (g/cm^2)) was assessed by DXA using a Lunar DPX-densitometer (DPX-L, Lunar Corp. Madison, WI, USA) under standard protocols. In addition, in the RS-II cohort, lumbar spine BMD was measured as well.

Quantitative ultrasound measurement (B-PROOF and RS-I)

Quantitative ultrasound (QUS) measurements were performed in the B-PROOF-study and RS-I. Concerning B-PROOF, QUS measurements of the calcaneus were performed using a Hologic Sahara bone densitometer (Hologic, USA) (Erasmus MC, VUmc, WUR) or a CUBA Clinical system (VUmc). Participants were excluded from QUS measurements if edema in the foot/ankle was visibly present, since this is known to affect the measurement [24]. Broadband ultrasound attenuation (BUA, dB/MHz, CV=3.7%) and speed of sound (SOS, m/s, CV=0.22%) were measured in duplo in both the right and the left calcaneus. For each individual, an average was calculated. Measurements were excluded when linearity of the frequency-attenuation relation was violated, since this indicates invalid results.

In RS-I, QUS-measurements were performed as well. Measurements were performed at the baseline visit (RS-I-1), approximately six years prior to blood drawing. BUA and SOS were measured at the right foot using a Lunar Achilles Ultrasound Bone Densitometer (Lunar, USA), which is a system using a water bath.

Blood chemistry (B-PROOF and Rotterdam Study)

In the B-PROOF-study, blood was drawn when participants were in fasting state or had only consumed a light, restricted breakfast. EDTA-blood was placed on ice water immediately and blood was centrifuged within 4 h of venapuncture to prevent a time- and temperature-dependent increase in plasma Hcy. Total plasma Hcy levels were measured using the Architect i2000 RS analyzer (VUmc), HPLC-method (WUR) and LC–MS/MS (EMC). Cross-calibration did not reveal any differences in outcomes between the centers. Serum creatinine was measured with the enzymatic colorimetric Roche CREA plus assay (CV=2%).

In the Rotterdam Study, fasting venous blood samples were drawn from participants to determine levels of Hcy. After withdrawal, EDTA-blood was kept on ice and centrifuged at 4 °C within 2 h. Plasma aliquots were stored at – 80 °C till analysis. Hcy was determined in EDTA-plasma using isotope-dilution liquid chromatography tandem mass spectrometry (LC–MS/MS; Waters Acquity UPLC Quattro Premier XE) by a method adapted from Ducros et al. [25].

Covariates (B-PROOF and Rotterdam Study)

In the B-PROOF-study, weight was measured to the nearest 0.5 kg in all included participants using a calibrated weighing device (SECA 761), while participants were wearing light-weight clothes and no shoes. In addition, duplicate measurements of height to the nearest millimeter were performed using a stadiometer; the average of these measurements was used for analysis. Subsequently, the participant's body mass index (BMI (kg/m^2)) was calculated. In the participants who were excluded from participation in the B-PROOF-trial based on their levels of Hcy and/or creatinine, but in whom ultrasound measurements were performed, height and weight were assessed by self-reporting, using a questionnaire. In all participants, the use of alcohol and tobacco and history of falling were assessed by a questionnaire. Additionally, in all included participants, this questionnaire was checked together with the participant and physical activity was assessed using a validated questionnaire (LASA Physical Activity Questionnaire (LAPAQ)) [26].

In the Rotterdam Study, height and weight were measured at baseline while participants were wearing lightweight clothing and no shoes. Results were used to calculate BMI. Data on smoking behavior, alcohol intake, physical activity and

history of falling at baseline were obtained from all participants during a structured home interview.

Statistical analysis

Distributions of all variables were visually checked for normality and Hcy was natural-log transformed due to presence of skewness. Differences in baseline characteristics between the three studies were assessed using ANOVA for continuous traits and Chi-square for categorical traits. Pearson correlation coefficients between the four (FN-BMD, LS-BMD, BUA and SOS) bone parameters were calculated in RS-I and the B-PROOF-study. Data on the three different studies (B-PROOF, RS-I, RS-II) were analyzed both separately and pooled. For the latter, we adjusted for interaction between cohort and the continuous level of Hcy, since distributions of levels of Hcy were different in the three studies. To investigate the association between Hcy and bone parameters, multiple linear regression analyses were performed. The assumption of linearity between dependent and independent variables was checked and confirmed using partial regression plots. Age, sex and BMI were added as covariates in the regression model. Serum creatinine, alcohol, smoking, fall history, and physical activity were regarded as potential confounders. If their introduction to the model led to a change of 10% or more in beta for Hcy, the covariate was regarded as a confounder and was kept in the model. Additionally, stratification for sex was done to investigate whether associations are sex-specific. Finally, we stratified the participants according to their plasma Hcy levels. ANCOVA was performed to show differences in adjusted bone parameters between participants with a plasma Hcy level below and above median to facilitate interpretation of effect sizes. All analyses were done using IBM SPSS Statistics version 20. Statistical significance was set at $\alpha=0.05$.

RESULTS

Baseline characteristics of the three study populations are shown in Table 1. The RS-II cohort was significantly younger, had lower levels of Hcy and higher FN-BMD compared with the RS-I and B-PROOF studies.

Both in crude analysis and after adjustment for confounders, a significant inverse association was observed between natural log-transformed levels of Hcy and

Table 1. Baseline characteristics of the B-PROOF, RS-I and RS-II study.

	B-PROOF		RS-I		RS-II	p (DXA-samples)
	DXA-sample (n=1227)	QUS-sample (n=2185)	DXA-sample (n=2850)	QUS-sample (n=744)	(n=2023)	
Age (y)	72.9 ±5.7[a]	73.2 ±6.3	72.1 ±6.7[b]	70.3 ±6.1	63.4 ±7.2[c]	<0.001
Gender (% female)	48.4	67.5	56.9	53.6	53.7	<0.001
BMI (kg/m^2)	27.0 ±3.8[a]	25.3 ±3.3	26.9 ±3.9[b]	26.7 ±3.8	27.2 ±4.2[a]	0.017
Hcy (µmol/l) (median, IQR)	14.3 (13.0-16.3)[b]	12.6 (10.6-14.8)	14.3 (11.9-17.4)[a]	13.6 (11.5-16.3)	12.7 (10.9-15.5)[c]	<0.001
Creatinine (µmol/l)	84 ±17[a]	78 ±19	81 ±21[b]	80 ±20	78 ±18[c]	<0.001
FN-BMD (g/cm^2)	0.884 ±0.143[b]	n.a.	0.841 ±0.142[c]	n.a.	0.927 ±0.144[a]	<0.001
L1-L4 BMD (g/cm^2)	1.174 ±0.226	n.a.	n.a.	n.a.	1.155 ±0.192	0.011
BUA (dB/MHz)	69.5 ±16.4	68.2 ±16.7	n.a.	113.1 ±12.4	n.a.	n.a.
SOS (m/s)	1538.9 ±33.0	1537.8 ±34.0	n.a.	1529.9 ±36.0	n.a.	n.a.
Current+former smokers (n (%))	818 (66.6%)	1168 (53.5%)	448 (20.1%)	98 (17.5%)	597 (33.9%)	<0.001
Alcohol consumers (n (%))	1078 (87.9%)	1607 (84.3%)	2323 (81.5%)	637 (86.1)	1721 (85.1%)	0.001
Fall history past 12 months (≥1 fall (n (%)))	374 (32.6%)	582 (33.2%)	678 (23.9%)	190 (25.7%)	429 (21.2%)	<0.001

Values are expressed as mean±SD, unless indicated otherwise. N.a.=not assessed. Cohort differences are indicated by [a,b,c].

Table 2. Multiple linear regression of lnHcy-levels on bone parameters.

		Model 1			Model 2		
	n	B	p	95% CI	B	p	95% CI
B-PROOF							
FN-BMD (g/cm^2)	1190	0.019	0.344	(-0.020;0.058)	-0.003	0.896	(-0.042;0.037)
LS-BMD (g/cm^2)	1223	0.010	0.754	(-0.054;0.074)	-0.025	0.455	(-0.090;0.040)
SOS (m/s)	2183	-13.806	<0.001	(-19.662;-7.950)	-8.924	0.001	(-13.965;-3.882)
BUA (dB/mHz)	2185	-2.069	0.126	(-4.721;0.582)	-3.743	0.009	(-6.541;-0.946)
RS-I							
FN-BMD (g/cm^2)	2850	-0.017	0.034	(-0.033;-0.001)	-0.025	0.004	(-0.041;-0.008)
SOS (m/s)	744	-9.350	0.032	(-17.914;-0.786)	-14.494	0.003	(-23.904;-5.083)
BUA (dB/mHz)	744	-0.107	0.942	(-2.986;2.771)	-1.479	0.360	(-4.648;1.689)
RS-II							
FN-BMD (g/cm^2)	2000	-0.024	0.024	(-0.044;-0.003)	-0.032	0.003	(-0.054;-0.011)
LS-BMD (g/cm^2)	2014	-0.020	0.180	(-0.050;0.009)	-0.026	0.103	(-0.056;0.005)
Pooled analysis							
FN-BMD (g/cm^2)					-0.032	0.010	(-0.056;-0.008)
LS-BMD (g/cm^2)			n.a.		-0.098	0.021	(-0.181;-0.015)
SOS (m/s)					-13.123	0.016	(-23.823;-2.424)
BUA (dB/mHz)					-2.755	0.233	(-7.285;1.776)

Model 1: adjusted for age, sex and BMI.
Model 2 (B-PROOF): additionally adjusted for study region, serum creatinine and type of QUS-device (BUA and SOS only), use of alcohol (FN and LS-BMD only) and smoking (LS-BMD only).
Model 2 (RS-I and RS-II): additionally adjusted for serum creatinine.
*Pooled analysis: adjusted for age, sex, BMI, serum creatinine, cohort, lnHcy*cohort.*

FN-BMD in both the RS-I (B=– 0.025, p=0.004 (adjusted model)) and the RS-II (B=– 0.024, p=0.024 (adjusted model)) cohort (Table 2). This association was not present in the B-PROOF study. Pooled analysis of the data of the three studies, adjusted for age, sex, BMI, serum creatinine, cohort and interaction between level of Hcy and cohort, showed significant inverse associations between natural log-transformed Hcy and FN-BMD (B=– 0.032, p=0.010) and LS-BMD (B=– 0.098, p=0.021). Stratifying the pooled analysis for sex did not show different results for men and women.

Concerning ultrasound parameters in the B-PROOF-study, an inverse association was observed between natural log-transformed Hcy and both SOS (B=– 8.9, p=0.001) and BUA (B=– 3.7, p=0.009) after adjustment for age, sex, BMI, serum creatinine, study center and type of ultrasound device (Table 2). Stratification for sex showed that this association was most pronounced in women (Table 3). In RS-I, natural log-transformed Hcy and SOS were significantly associated (B=– 14.5,

Table 3. Multiple linear regression analysis for the association between lnHcy and QUS-parameters, stratified for sex.

	Males			Females		
	B	p	95% CI	B	p	95% CI
B-PROOF (n=2185)						
SOS (m/s)	-5.039	0.316	(-14.908;4.830)	-11.152	<0.001	(-16.997;-5.307)
BUA (dB/mHz)	-1.277	0.633	(-6.521;3.966)	-5.407	0.001	(-8.718;-2.095)
RS-I (n=744)						
SOS (m/s)	-15.969	0.041	(-31.270;-0.668)	-13.262	0.026	(-24.941;-1.582)
BUA (dB/mHz)	-0.489	0.837	(-5.148;4.170)	-2.543	0.255	(-6.934;1.847)

Model is adjusted for BMI, age, serum creatinine. The B-PROOF data are additionally adjusted for study center and QUS-device.

Table 4. Adjusted means for bone parameters according to plasma Hcy below and above median (studies taken together, ANCOVA).

		Median Hcy	Hcy<median		Hcy>median		Difference	p
	n	(µmol/l)	Mean	SE	Mean	SE	between means	
FN-BMD (g/cm^2)	6039	13.8	0.884	0.003	0.872	0.003	0.012	0.009
LS-BMD (g/cm^2)	3236	13.5	1.172	0.006	1.153	0.006	0.019	0.050
SOS (m/s)	2644	12.8	1538.4	1.183	1533.3	1,030	5.1	0.006
BUA (dB/mHz)	2644	12.8	81.7	0.501	80.6	0.436	1.1	0.132

*FN-BMD and LS-BMD: adjusted for age, sex, BMI, cohort, creatinine, lnHcy*cohort.*
*SOS and BUA: adjusted for age, sex, BMI, creatinine, cohort, lnHcy*cohort.*

p=0.003) after adjustment for confounders. For BUA, the association was not significant (Table 2). Stratification did not reveal clear differences between men and women. A pooled analysis, combining the B-PROOF and RS-I data, confirmed the association between Hcy and SOS (B=– 13.1, p=0.016), but not for BUA (Table 2).

Table 4 shows the mean level of the bone parameters according to the Hcy levels of the participants. Participants having a plasma level of Hcy above median have 0.012 g/cm^2 lower FN-BMD and 0.019 g/cm^2 lower LS-BMD. For SOS and BUA, these differences are respectively 5.1 m/s and 1.1 dB/mHz, corresponding to 0.14 and 0.09 SD, respectively. For FN-BMD, LS-BMD and SOS, differences are statistically significant.

DISCUSSION

Overall, this study shows that plasma Hcy was inversely and modestly associated with both bone ultrasound parameters. However, small associations between Hcy and FN and LS-BMD were also observed.

Concerning BMD, a small but significant association was observed between Hcy and BMD in both the RS-I and the RS-II cohorts, while no such association was observed in the B-PROOF study. A possible explanation for the absence of the association in B-PROOF might be that in this study only participants with levels of Hcy >12 µmol/l were included, creating a narrower range of Hcy. In our opinion, clinical relevance of the strength of the observed associations in the individual cohorts is questionable. Differences in BMD between participants with a plasma level of Hcy below and above median had the size of approximately 0.09 SD. We therefore speculate that the fairly strong relationship between Hcy and osteoporotic fracture risk is not largely explained by effects of Hcy on BMD. This is supported by the recent meta-analysis observing no association between levels of Hcy and BMD in women only [13].

A previous study showed lower BUA in 1267 elderly women with low levels of vitamin B_{12} and high levels of Hcy compared with women with low or normal levels of vitamin B_{12} and normal levels of Hcy [21]. This relationship was not seen in men. In addition, Gerdhem et al. studied 996 women, all aged 75 years and observed up to 2% lower ultrasound results in women with Hcy in the 4th quartile compared with women in the first three quartiles [8]. Our results are in line with these previous findings, as we also observed negative associations which seemed to be more pronounced in women than in men. The results may support the hypothesis of Hcy affecting the process of collagen cross-linking, possibly by blocking the aldehyde groups in collagen [27] which are responsible for cross-linking. However, it should be noted that the differences in our study in BUA and SOS between participants with high and low levels of Hcy were limited to 0.09 and 0.14 SD, respectively. It has been shown that a decrease in BUA of 20.6 dB/mHz, which is more than 1 SD, is associated with a 2.3-fold relative risk of hip fracture [18]. It is therefore expected that the effect we observed is of limited clinical relevance and is possibly not large enough to explain the relationship between Hcy and osteoporotic fracture incidence. In addition, it should be noted that the pooled analysis did not show a

significant association between Hcy and BUA, although an association was present in B-PROOF. The reason for the absence of an association in RS-I and in the pooled analysis, while an association with SOS was observed, is unknown.

A major strength of this study is its size; we investigated Hcy and FN-BMD of 6040 elderly participants in the pooled analysis. A limitation is the cross-sectional design of this study, so reverse causality and residual confounding cannot be ruled out. In addition, it should be noted that the ultrasound measurements in RS-I were done approximately six years before blood drawing and measurement of Hcy took place. Clearly, during this time Hcy may have altered. This may have led to an underestimation of the association between Hcy and BUA/SOS in the RS-I study.

Based on this study, we conclude that there are modest inverse associations between plasma levels of Hcy and bone ultrasound parameters and BMD. However, clinical relevance of these associations is expected to be limited. Moreover, the strength of each of these associations by itself may not be large enough to explain the previously observed strong association between Hcy and osteoporotic fracture incidence. The upcoming results of the B-PROOF-study, a trial with Hcy-lowering B-vitamins on osteoporotic fracture incidence, will be important in further elucidating what explains the association between plasma Hcy levels and fracture risk and in determining causality of relationships.

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Chapter 2.2

The association between plasma homocysteine levels, methylation capacity and incident osteoporotic fractures

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ABSTRACT

Background

An elevated level of plasma homocysteine (Hcy) is a known risk factor for osteoporotic fractures. In addition, Hcy is related to DNA-methylation metabolism. To determine whether the association between Hcy and fractures is explained by an altered methylation capacity, we investigated the associations between levels of s-adenosylmethionine (SAM) and S-adenosylhomocysteine (SAH) and fracture risk.

Methods

We studied 503 females aged 55 years and over from the Rotterdam Study (RS) in whom plasma Hcy, SAM and SAH levels were measured. Bone mineral density (BMD) at the hip was assessed using DXA. Incident fractures were recorded over a mean period of 7.0 years. Cox proportional hazards analysis and linear regression were used to assess relationships between plasma metabolite levels, incident osteoporotic fractures and BMD.

Results

Over a total of 3502 person-years of follow-up, 103 subjects sustained at least one osteoporotic fracture. Whereas incidence of osteoporotic fractures was associated with quartiles of Hcy (p=0.047), it was not associated with quartiles of SAM, SAH or SAM/SAH-ratio (all p for trend >0.6). Stepwise linear regression showed that SAM/SAH-ratio, but not Hcy, was independently associated with hip BMD (β=0.073, p=0.025).

Conclusion

Since SAM, SAH and SAM/SAH-ratio were not associated with osteoporotic fractures, alterations in methylation capacity most likely do not appear to be an important factor in the association between Hcy and fractures.

INTRODUCTION

Osteoporosis is characterized by low bone mass and microarchitectural deterioration of bone tissue, leading to bone fragility and increased fracture risk [1]. Osteoporotic fractures are a major health care problem, since they are associated with significant morbidity and mortality [2]. Excess mortality rates in the first year after a hip fracture vary from 12% to 35% [3]. Due to a rise in life expectancy, the economical burden of osteoporotic fractures in Europe is expected to increase substantially in the coming decades; from €36.3 billion in 2000 to €76.8 billion in 2050 [4].

Mildly elevated plasma homocysteine (Hcy) levels are associated with osteoporotic fractures [5-7]. The mechanism underlying this association has not yet been clarified and literature concerning the presence of an association between Hcy and bone mineral density (BMD) is inconsistent [7, 8]. Hcy is hypothesized to interfere with collagen cross-linking in bone, thereby weakening bone structure [9]. Alternatively, by influencing methylation capacity, Hcy could possibly disturb DNA-methylation [10, 11] and gene-expression, which may lead to changes in bone structure. Methylation capacity is reflected by S-adenosylhomocysteine (SAH) and its precursor S-adenosylmethionine (SAM), two important intermediates in the conversion of methionine to Hcy. SAM donates its methyl group to DNA or other molecules, resulting in the production of SAH. It is known that when Hcy accumulates, the reaction of SAH to Hcy, which is catalyzed by SAH-hydrolase, reverses [12], thereby increasing SAH levels [11]. This results in a lower methylation capacity, which is reflected by a lower SAM/SAH-ratio. The hypothesis that altered methylation capacity might play a role in bone metabolism is supported by the finding that a lower SAM/SAH-ratio in bone correlated with reduced bone strength in hyperhomocysteinemic rats [13].

To determine whether this hypothesis also holds true in humans, we investigated associations between levels of SAM and SAH and incident osteoporotic fractures and femoral neck BMD in older females in the Rotterdam Study. In order to also address potential other mechanisms underlying the association between Hcy and osteoporotic fractures, we investigated the mutual associations between plasma Hcy, SAM, SAH, serum levels of vitamin B_{12} and folate, and methylenetetrahydrofolate reductase-genotype (MTHFR) and the potential associations of B-vitamin levels and MTHFR-genotype with osteoporotic fracture incidence.

METHODS

Setting

Subjects were participants of the Rotterdam Study, which is an ongoing, population-based cohort study among people aged 55 years or over, who reside in the Ommoord district of the city of Rotterdam in The Netherlands. The Rotterdam Study was designed to investigate chronic, disabling diseases. Its rationale and design have been described previously [14]. The Rotterdam Study was conducted according to the Declaration of Helsinki and approved by the medical ethics committee of the Erasmus Medical Center. All subjects gave written informed consent. The subjects in the current study are part of the Rotterdam Study-I (RS-I) cohort. Baseline measurements in the RS-I cohort were performed between 1990 and 1993 (RS-I-1). The second follow-up visit to the research center took place between 1997 and 1999 (RS-I-3) and measurements and blood drawing at that moment serve as baseline for the current study.

Study population

Inclusion criteria for the current study were female sex, age of 55 years or over at enrollment in RS-I, and a negative history for hip fracture, cancer, cardiovascular disease, dementia and stroke between RS-I-1 and RS-1-3. Maximal duration of follow-up for fractures was 9.6 years.

Clinical measurements

At time of blood drawing at RS-I-3, bone mineral density at the femoral neck (FN-BMD, g/cm^2) was assessed by dual-energy X-ray assessment (DXA) using a Lunar DPX-densitometer (DPX-L, Lunar Corp. Madison, WI, USA) under standard protocols. Height and weight were measured at baseline while subjects were wearing lightweight clothing and no shoes. Body mass index (BMI, kg/m^2) was calculated by dividing the subject's weight (kg) by the squared height (m).

Assessment of osteoporotic fractures

General practitioners continuously monitored participants for incident fractures, which were reported by means of a computerized system. Events were classified independently by two research physicians according to the International Statistical

Classification of Diseases and Related Health Problems, 10th Revision (ICD-10-CM). An expert in osteoporosis reviewed all coded events for final classification. Follow-up started at RS-I-3 and ended on December 31st, 2006. Incident osteoporotic fractures were defined as all incident fractures, except for fractures of hand, foot, face or skull, and for high-trauma and cancer-related fractures.

Blood chemistry

Fasting venous blood samples were drawn from participants to determine levels of Hcy, SAM and SAH in EDTA-plasma and vitamin B_{12} and folate in serum. After withdrawal, EDTA-blood was kept on ice and centrifuged at 4 °C within 2 h. Plasma aliquots were stored at – 80 °C till analysis. In addition, MTHFR 677 C → T polymorphism (rs1801133) was determined from isolated DNA as described previously [15].

Hcy was determined in EDTA-plasma using isotope-dilution liquid chromatography tandem mass spectrometry (LC–MS/MS; Waters Acquity UPLC Quattro Premier XE) by a method adapted from Ducros et al. [16]. For chromatographic separation, we used a Waters Symmetry C_8 column (2.1 × 100 mm, reference WAT 058961, Waters, Etten-Leur) with a precolumn (Waters, reference 205000343). The column was eluted at 0.25 ml/min and no splitter was used. Calibration was performed with aqueous standards because they gave similar results as plasma-based standards.

SAM and SAH were also determined using LC–MS/MS by a method adapted from Gellekink et al. [17]. In short, non-acidified EDTA-plasma was stored at – 80 °C and 200 µl of plasma was used for sample clean-up. Samples (10 µl) were injected on a 50 × 2.1 mm Atlantis C_{18} column (Waters) and eluted in a gradient of methanol in aqueous acetic acid (0.1%). The retention times were 0.6 min (SAM) and 1.4 min (SAH). Standards were dissolved in 1 mmol/l HCl; pool sera were SAM and SAH depleted by solid phase extraction and spiked with the calibrator. Calibration curves for SAM and SAH were linear till 500 nmol/l.

In serum, vitamin B_{12} and folate were measured using electrochemiluminescence immunoassay (Modular E170, Roche, Almere, The Netherlands).

Genomic DNA was isolated using the salting out method. MTHFR 677 C → T polymorphism was determined using a Taqman assay as described previously [15]. Primers and probes are available on request.

Statistical analysis

Distributions of all variables were visually checked for normality and natural logarithms of the variables Hcy, SAM, SAH, SAM/SAH-ratio, vitamin B_{12} and folate were taken. Pearson correlation coefficients between Hcy and the related metabolites were calculated. To estimate the hazard ratios for osteoporotic fractures within quartiles of Hcy, SAM, SAH, SAM/SAH-ratio, serum levels of folate and B_{12} and MTHFR 677 C $\rightarrow$ T mutation, we conducted Cox proportional hazards analyses, adjusted for age and BMI. We adjusted for these variables since they are known confounders in the relationship between Hcy and osteoporotic fractures. In a second model, additional adjustment for FN-BMD was performed to investigate whether potential associations between metabolites and fractures were explained by differences in FN-BMD at time of measurement of metabolites.

In addition, to identify determinants of FN-BMD, multiple linear regressions were performed. For all statistical analyses, SPSS version 17 was used. The level of statistical significance was set at 0.05.

RESULTS

Baseline characteristics

We included 503 women in this study; mean follow-up time for fracture was 7.0 (SD 2.3) years. Table 1 shows the baseline characteristics of the participants. In total, there were 3502 person-years of follow-up, during which 103 subjects sustained at least one osteoporotic fracture. Median levels of Hcy, vitamin B_{12} and folate were

Table 1. Baseline characteristics of study subjects (n=503).

	Median	Range
Age (y)	68.5	61.3-74.9
BMI (kg/m^2)	27.0	16.8-45.1
FN-BMD (g/cm^2)	0.83	0.38-1.38
Hcy (µmol/l)	9.3	3.5-29.7
Folate (nmol/l)	17.4	6.1-45.4
B_{12} (pmol/l)	328	83-1476
SAM (nmol/l, n=489)	85.6	53.1-198.3
SAH (nmol/l, n=489)	17.2	9.6-43.5
SAM/SAH	5.1	3.0-8.7

Female reference values [18]: Hcy 6-19 µmol/l; folate 8-28 nmol/l; B_{12} 145-637 pmol/l, SAM 70-128 nmol/l; SAH 9-20 nmol/l; SAM/SAH 4.7-9.0.

within the normal ranges. In addition, levels of SAM and SAH were also within normal ranges, indicating reliable measurement of these instable metabolites.

Distribution of MTHFR 677 C → T genotype frequencies did not deviate from Hardy–Weinberg equilibrium (p=0.05). Of the 486 MTHFR 677 C → T genotyped subjects, 41.8% were homozygous for the C-allele, 48.8% were heterozygous, and 9.5% were homozygous for the T-allele. T-allele frequency was 33.8%, which is similar to other reports in Caucasians [19, 20]. We checked correctness of the genotyping assay by including 5% duplicates and found no discrepancies. Comparing levels of Hcy, folate, B_{12}, SAM, SAH and SAM/SAH-ratio using one-way ANOVA showed that Hcy and folate differed significantly across genotype groups (Table 2). Confirming previous observations, Hcy was 0.8 µmol/l higher and folate was 3.8 nmol/l lower in TT-individuals compared with CC-individuals. SAM and SAH levels did not significantly differ between MTHFR 677 C → T genotypes.

Table 2. Levels of homocysteine and related metabolites in individuals with different MTHFR 677 C→T genotypes.

	MTHFR 677 C→T genotype			
	CC	**CT**	**TT**	**p[a]**
Hcy (µmol/l)	9.3	9.4	10.1	0.047
Folate (nmol/l)	17.9	17.5	14.1	0.013
B_{12} (pmol/l)	330	321	353	0.158
SAM (nmol/l)	85.6	86.4	87.3	0.916
SAH (nmol/l)	17.6	17.0	15.9	0.644
SAM/SAH	5.0	5.1	5.4	0.354

[a]P-values are based on one-way ANOVA using ln-transformed variables.

Hcy and related metabolites

Figure 1 shows statistically significant correlations between Hcy, SAM, SAH, SAM/SAH-ratio, vitamin B_{12} and folate. Plasma Hcy levels correlated negatively with vitamin B_{12} and folate, while the B-vitamins correlated positively, but weakly, with each other. With increasing levels of Hcy, SAM and SAH both increased significantly, while the SAM/SAH-ratio declined. Folate levels were positively but weakly correlated with SAM/SAH-ratio.

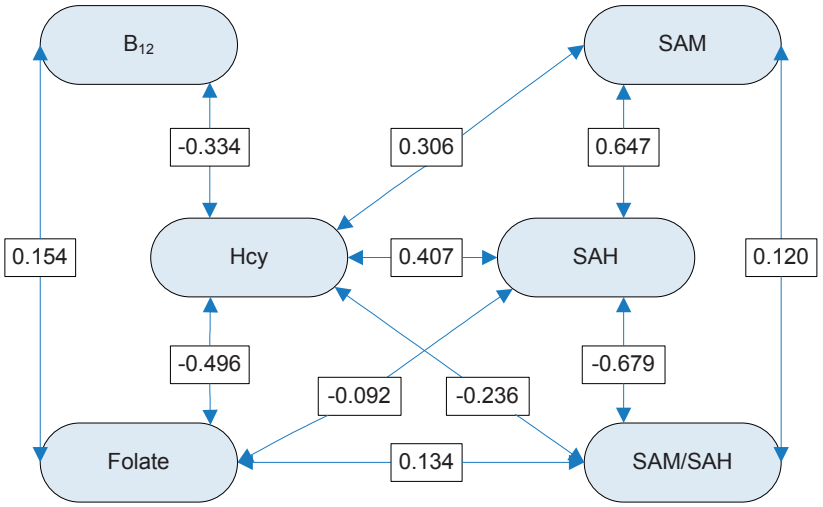

Figure 1. Statistically significant Pearson's correlations between metabolites in the Hcy-pathway.

Hcy, related metabolites and incident osteoporotic fractures

In Table 3, results from Cox-regression on osteoporotic fractures are shown. Quartiles of Hcy significantly predicted incident osteoporotic fractures, however, after adjusting for FN-BMD this effect attenuated slightly. Quartiles of SAM/SAH-ratio, SAH or SAM did not predict incident osteoporotic fractures after adjustment for age and BMI (model 1), or age, BMI and FN-BMD (model 2). When blood parameters were entered in the model as continuous variables, no significant associations with incident fractures were observed.

Using Cox regression models 1 and 2, no associations between osteoporotic fracture incidence and MTHFR 677 C → T genotype (p for trend >0.37) or serum folate (p for trend >0.15) or B_{12} levels (p for trend >0.14) were observed (data not shown). In addition, no effect of interaction between MTHFR 677 C → T genotype and folate levels was seen.

When using a multivariate model including age, BMI, MTHFR 677 C → T genotype and the continuous blood parameters Hcy, SAM/SAH-ratio, folate and B_{12} all together in one model, none of these individual parameters contributed significantly to the prediction of osteoporotic fractures (data not shown).

Table 3. Hazard ratios for osteoporotic fractures (OF) in quartiles of Hcy, SAM, SAH and SAM/SAH.

		Number of subjects with OF/ total number of subjects	Percentage of subjects with OF	Model 1[a]		Model 2[b]	
				HR for OF	95% CI	HR for OF	95% CI
Hcy (µmol/l)	<7.9	18/128	14.1	1.00		1.00	
	7.9-9.4	23/125	18.4	1.27	0.68-2.35	1.28	0.69-2.38
	9.4-11.6	36/134	26.9	1.96	1.10-3.46	1.82	1.02-3.22
	>11.6	26/116	22.4	1.64	0.89-3.02	1.50	0.81-2.77
	p (trend)			0.047		0.107	
SAM (nmol/l)	<78.3	25/122	20.5	1.27	0.69-2.37	1.27	0.68-2.37
	78.3-86.2	24/123	19.5	1.19	0.64-2.19	1.32	0.71-2.45
	86.2-97.9	28/122	23.0	1.46	0.83-2.57	1.68	0.95-2.98
	>97.8	21/122	17.2	1.00		1.00	
	p (trend)			0.603		0.635	
SAH (nmol/l)	<15.0	24/127	18.9	1.00		1.00	
	15.0-17.2	28/119	23.5	1.25	0.72-2.17	1.21	0.70-2.10
	17.2-20.2	22/122	18.0	0.96	0.54-1.72	0.94	0.52-1.68
	>20.2	24/121	19.8	0.99	0.55-1.79	0.88	0.49-1.60
	p (trend)			0.734		0.938	
SAM/SAH	<4.5	25/123	20.3	1.03	0.58-1.82	0.92	0.52-1.64
	4.5-5.1	24/122	19.7	1.06	0.60-1.89	1.04	0.58-1.85
	5.1-5.7	26/123	21.1	1.10	0.60-1.89	1.11	0.63-1.95
	>5.7	23/121	19.0	1.00		1.00	
	p (trend)			0.971		0.712	

[a] Model 1: Adjusted for age and BMI, [b] Model 2: Adjusted for age, BMI and FN-BMD.

Hcy, related metabolites and FN-BMD

We performed regression analyses of FN-BMD on all blood parameters and MTHFR 677 C → T genotype, adjusted for age and BMI (Table 4, model 1). The results show that Hcy, folate, SAM/SAH-ratio and SAH were all significantly associated with FN-BMD in the expected directions. We next tested all the parameters in a multivariate forward stepwise regression analysis to assess independent determinants of FN-BMD (Table 4, model 2). The analysis shows that only age, BMI, SAM/SAH-ratio and folate levels contributed significantly to the determination of FN-BMD, while levels of B_{12} and Hcy and MTHFR 677 C → T genotype did not. Inclusion of current alcohol use, smoking status and level of education did not change these results (data not shown).

Table 4. Multiple linear regression on FN-BMD (g/cm^2).

	Model 1		Model 2	
	B	**p**	**B**	**p**
Age (y)	n.a.	n.a.	-0.005	<0.001
BMI (kg/m^2)	n.a.	n.a.	0.008	<0.001
lnHcy (µmol/l)	-0.046	0.022		
lnSAM/SAH	0.085	0.007	0.073	0.025
lnSAM (nmol/l)	0.002	0.952		
lnSAH (nmol/l)	-0.053	0.036		
lnFolate (nmol/l)	0.038	0.008	0.034	0.021
lnB_{12} (pmol/l)	-0.008	0.542		
MTHFR-genotype				
- CT vs. CC	0.008	0.509		
- TT vs. CC	-0.013	0.501		

Model 1: Linear regression adjusted for age and BMI.
Model 2: Stepwise linear regression (age, BMI, MTHFR 677 C→T, folate, B_{12}, SAM/SAH and Hcy).

DISCUSSION

This study showed that indicators of methylation capacity (plasma SAM, SAH and their ratio) were not associated with incident osteoporotic fractures in healthy, elderly women, while levels of Hcy were. This suggests that methylation capacity is not an important factor in the association between plasma Hcy and osteoporotic fractures. We further showed that SAM/SAH-ratio significantly associated with FN-BMD independently of age, BMI and serum folate levels.

To the best of our knowledge, circulating levels of SAM/SAH-ratio have not been investigated previously in relation to osteoporotic fracture risk in humans. However, some studies have been conducted on related outcome measures. Holstein et al. investigated associations of SAM, SAH and their ratio with bone morphology, all measured in the hip bone of patients undergoing hip replacement due to osteoarthritis (n=82) [21]. Higher levels of SAH and Hcy, but unexpectedly also of SAM, turned out to be associated with impaired cancellous bone structure. However, no significant association with SAM/SAH-ratio was found. In addition, Herrmann et al. investigated effects of a high homocystine diet in rats [13]. This diet decreased SAM/SAH-ratio in bone as well as plasma. In addition, bone SAM/SAH-ratio was positively correlated with bone strength in these rats. We did not observe a signifi-

cant association between SAM/SAH-ratio and osteoporotic fractures in the current study. This may be due to the fact that, in general, our study population had normal SAM/SAH-ratios, similar to previously observed values in healthy adults [17]. In addition, levels of Hcy were low to normal, indicating sufficient methylation capacity. Alternatively, the effects of SAM/SAH-ratio on fracture incidence might have been too small to detect in our study.

Interestingly, although it was not predicting fractures, high SAM/SAH-ratio did associate independently and significantly with high FN-BMD, a finding which has not been observed previously. This observation might be explained by results from an in vitro study which showed that decreased SAM/SAH-ratio, caused by inhibition of SAH-hydrolase, inhibited osteoblast differentiation and extracellular matrix calcification [22]. We may speculate that the effect of SAM/SAH-ratio on BMD was not large enough to affect fracture incidence, which is known to be multifactorial. In addition, possible residual confounding effects of lifestyle factors, co-morbidities or drug use cannot be fully ruled out. Next to SAM/SAH-ratio, also folate levels were independently associated with FN-BMD, while vitamin B_{12} levels were not. Previous studies on effects of these vitamins on BMD have shown inconsistent results, as was reviewed by Herrmann et al. [23]. Our results may suggest folate to have a direct effect on BMD, bypassing the Hcy-pathway.

The association between Hcy and osteoporotic fracture risk observed in earlier studies by us [7] and others [5, 6] was confirmed by our results. Hcy did not independently associate with FN-BMD, a phenomenon which also has been observed previously [7, 24, 25], though not consistently [19]. Since BMD is not the only aspect of bone strength, effects of Hcy or SAM/SAH-ratio on other parameters reflecting bone quality, such as bone ultrasound attenuation, remain to be investigated.

We did not observe any associations between MTHFR 677 C $\rightarrow$ T genotype and BMD or fracture incidence. A meta-analysis on the association between MTHFR 677 C $\rightarrow$ T genotype and BMD showed that, overall, the TT-genotype was associated with a slightly lower BMD than the CT/CC-genotypes, an effect which was more pronounced in women than in men [26]. However, results from individual studies were inconsistent. Concerning fractures, again inconsistent results have been observed. While some studies observed a deleterious effect in individuals with the MTHFR 677 TT genotype on fractures [20, 27], others observed no [28, 29] or even

a protective effect [30]. In our study, lack of power might have been a cause for absence of associations.

A limitation of this study is the fact that the study population was relatively healthy and considerably homogenous and that subjects had somewhat low and narrowly distributed Hcy levels. This might have reduced the power to find any associations, and may explain why the association between Hcy and osteoporotic fracture incidence was somewhat weaker than observed previously [7]. In addition, levels of SAM/SAH are not informative concerning gene-specific methylation. However, the fact that the ratio of SAM/SAH in plasma has been correlated with other outcomes previously [31, 32] shows that it is a relevant pathway to study further and justifies investigating its association with bone phenotypes as well. However, additional gene-specific effects of methylation cannot be ruled out based on this study. In addition, because levels of SAM and SAH are known to be tissue-specific, the circulating levels of SAM and SAH that were examined in the current study might not fully reflect the local levels in the bone. However, the fact that we did find an association between plasma SAM/SAH-ratio and FN-BMD might indicate that the problem of tissue-specificity was small.

Within our study, plasma samples were prepared within half an hour of sampling and were immediately frozen in liquid nitrogen. Storage thereafter was at – 80 °C. Gellekink et al. [17] discussed the importance of acidifying plasma in order to prevent SAM from converting to SAH preanalytically. However, we showed earlier that this is not necessary when EDTA whole blood is rapidly separated and stored at – 80 °C and appropriate measures are taken when thawing and analyzing the stored plasma samples [33]. The fact that the ratios of SAM to SAH we measured in our study population are within the normal range supports the assumption that absence of acidification did not lead to any problems.

In conclusion, this study shows that a higher plasma SAM/SAH-ratio independently predicts a higher FN-BMD, but is not associated with a decrease in osteoporotic fracture risk. To gain more insight into the association between SAM/SAH-ratio and FN-BMD, it would be interesting to investigate DNA-methylation patterns of genes known to influence BMD in relation to Hcy and SAM/SAH-ratio. The B-PROOF-study, a currently running multi-center trial in which the effect of supplementation with folic acid and vitamin B_{12} is investigated, might provide more insight into the

potentially causal role of Hcy in the occurrence of osteoporotic fractures and its effects on bone quality and DNA-methylation [34].

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Chapter 3

Results of a homocysteine-lowering intervention: B-PROOF

Chapter 3.1

Effect of daily vitamin B_{12} and folic acid supplementation on fracture incidence in elderly with an elevated plasma homocysteine level: B-PROOF, a randomized controlled trial

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ABSTRACT

Background

Elevated plasma homocysteine levels are a risk factor for osteoporotic fractures. Lowering homocysteine with vitamin B_{12} and folic acid supplementation may reduce fracture risk.

Objective

This study (B-PROOF) aimed to determine whether vitamin B_{12}/folic acid supplementation reduces osteoporotic fracture incidence in hyperhomocysteinemic elderly.

Design

It is a double-blind, randomized, placebo-controlled trial including 2,919 participants aged ≥65 years with elevated homocysteine levels (12-50 µmol/L). Participants were assigned to daily 500 µg vitamin B_{12} and 400 µg folic acid or placebo supplementation for two years. Both tablets also contained 600 IU vitamin D_3. Primary endpoint was time-to-first osteoporotic fracture. Exploratory, pre-specified subgroup analyses were performed among men and women, and persons below and above age 80y. Data were analyzed according to intention-to-treat and per-protocol principles.

Results

Osteoporotic fractures occurred in 61 persons (4.2%) in the intervention group compared with 75 (5.1%) in the placebo group. Osteoporotic fracture risk was not significantly different between groups in the intention-to-treat analyses (Hazard Ratio (HR)=0.84, 95%CI 0.58-1.22) or per-protocol analyses (HR=0.82, 95%CI 0.55-1.22). For persons >80 years, in per-protocol analyses, osteoporotic fracture risk was lower in the intervention group compared with placebo (HR=0.28, 95%CI 0.10-0.74). The total number of adverse events (including mortality) did not differ between groups. However, 63 vs. 42 participants in the intervention and placebo group, respectively, reported incident cancer (HR=1.55, 95%CI 1.04-2.30).

Conclusion

These data show that combined vitamin B_{12}/folic acid supplementation had no effect on osteoporotic fracture incidence in this elderly population. Exploratory subgroup analyses suggest a beneficial effect on osteoporotic fracture prevention in compliant persons >80 years. However, treatment was also associated with increased incidence of cancer, although this possible adverse effect should be interpreted with caution. Therefore, vitamin B_{12}/folic acid supplementation cannot be recommended at present for fracture prevention in elderly people.

INTRODUCTION

Osteoporosis is a chronic, multifactorial disorder, characterized by low bone mass and micro-architectural deterioration of bone tissue with fractures as a major consequence [1]. Fractures lead to pain, impairment in physical and social functioning, loss of quality of life and an increased risk of mortality [2]. Because of further ageing of the population, the number of fractures and their socio-economic burden is expected to rise substantially in the coming decades [3].

An elevated circulating plasma homocysteine (Hcy) concentration has been identified as an independent risk factor for osteoporotic fractures in observational studies [4-10], a finding that is consistent with meta-analyses [11, 12] and mechanistic studies [13, 14]. Elevated Hcy concentrations (≥15 µmol/L) are prevalent in 30-50% in persons >65y [15, 16]. Treatment with vitamin B_{12} and folic acid, both playing a central role in the Hcy metabolism [17], is effective in normalizing Hcy concentrations [18, 19]. Three randomized controlled trials investigated the effect of B-vitamin supplementation on fracture risk [20-22]. Among stroke survivors, a large protective effect of 2y supplementation of 1.5 mg vitamin B_{12} and 5 mg folic acid was observed on hip fracture risk in the trial of Sato and colleagues [21]. However, in the HOPE-2 trial no effect of 5y supplementation of 1 mg vitamin B_{12}, 2.5 mg folic acid and 50 mg vitamin B_6 was observed on fracture incidence among persons with high cardiovascular risk [22]. In the VITATOPS-study, also no effect of treatment with 2 mg folic acid, 25 mg vitamin B_6 and 500 µg vitamin B_{12} during a mean of 2.8y on osteoporotic fracture incidence was observed in patients with cerebrovascular disease [20]. Given the conflicting results and low generalizability to the general older population, further investigation is needed.

We conducted the B-vitamins for the PRevention Of Osteoporotic Fractures (B-PROOF) study to assess the efficacy (through intention-to-treat analysis) and effectiveness (through per-protocol analysis) of two-year oral supplementation with 500 µg vitamin B_{12} and 400 µg folic acid in the prevention of osteoporotic fractures in Dutch elderly people with elevated plasma Hcy concentrations.

SUBJECTS AND METHODS

Study design

B-PROOF is a randomized, placebo-controlled, double-blind multi-center trial, of which the design and methods have been described in detail previously [23]. The B-PROOF study is registered with the Netherlands Trial Register (NTR1333) and with ClinicalTrials.gov (NCT00696414).

Setting and Participants

The included participants had to be independently or assistedly living but not residing in a nursing home, aged 65y or older with elevated Hcy concentrations (12-50 µmol/L). Exclusion criteria were a serum creatinine concentration >150 µmol/l, cancer diagnosis in the past 5 years and severe immobility (being bedridden or using a wheelchair permanently). In total, 2,919 participants were included.

Randomization and Intervention

Participants were randomized in a 1:1 ratio to receive daily either an oral tablet containing 500 µg B_{12} and 400 µg folic acid or a placebo tablet. Tablets in both treatment arms contained 600 IU vitamin D_3 to ensure a normal vitamin D status [24]. The intervention and placebo tablets were indistinguishable in taste, smell and appearance. Randomization was stratified for study center, sex, age (65-80y, >80y) and concentration of Hcy (12-18 µmol/L, >18 µmol/L). The intervention period comprised two years. As planned at the start of the B-PROOF study [23], to increase power, participants who finished their intervention more than one year before the end of the study (n=678) were invited to extend their participation with one year. In total, 393 participants agreed and extended their participation. Participants with extended follow-up (n=393) were significantly older (75.3 vs. 73.9 yrs, $p<0.001$) and had higher serum methylmalonic acid (MMA) (0.23 vs. 0.22 µmol/L, $p=0.021$) as

compared with participants without extended follow-up (n=2526). There were no differences with regard to sex, Hcy, vitamin B_{12}, folate and holotranscobalamin (holoTC) concentrations. The Medical Ethics Committee of Wageningen UR approved the study protocol and the Medical Ethics Committees of Erasmus MC and VU University Medical Center gave approval for local feasibility. All participants gave written informed consent.

Outcomes and Follow-up

At baseline and 2y follow-up, a broad set of measurements was performed. In the current paper, the primary outcome of the study is reported, i.e. time-to-first osteoporotic fracture, as well as the secondary outcome time-to-first of any fracture, and adverse events.

Fracture assessment

Fractures were reported by the participants on a study calendar which was returned every three months during the intervention period. Additionally, participants were asked for the occurrence of fractures at the follow-up measurement using a structured questionnaire. All reported fractures were verified with the participants' general practitioner or hospital. Fractures were classified as osteoporotic or non-osteoporotic. Osteoporotic fractures were defined as all fractures except for head, hand, finger, foot or toe fractures, fractures caused by traffic accidents and fractures caused by cancer [25].

Subjects who dropped out of the study, or who were unable to complete the follow-up measurements were contacted around the end of the follow-up period to obtain information on incident fractures. In case this was not successful, a participant was regarded as lost to follow-up, and date of last contact was recorded. Date, type and cause of fracture were recorded and verified.

Compliance

Every six months, new tablets were sent to the participants and they were requested to return any remaining tablets. Participants were defined as compliant when at least 80% of the tablets had been taken during the intervention period, as indicated by the returned tablets.

Adverse events

Adverse events, that is, ill-health related conditions, were recorded by participants on the study calendars. In addition, all events reported to the study team by phone or otherwise were recorded. In case participants during the study reported a cancer diagnosis of any type, except for non-melanoma skin cancer, they were excluded from further tablet use. Also at the end of the intervention period, participants were asked whether they had been diagnosed with cancer during the trial. Reported cases of cancer, except for non-melanoma skin cancer, were verified with the participants' general practitioner or hospital. In case a participant deceased during the study period, this was reported by relatives.

Baseline characteristics

Height and weight were measured and information on demographic factors, lifestyle characteristics, medication use and medical history were obtained using a questionnaire. Anti-osteoporotic medication use included use of bisphosphonates, strontium-ranelate, selective estrogen-receptor modulators, estrogens, androgens, denosumab or teriparatide at baseline. Plasma Hcy and serum creatinine were determined [23], and serum 25(OH) vitamin D were measured [26]. For Hcy, follow-up concentrations were measured as well. In addition, baseline serum vitamin B_{12} and folate were determined using immunoelectrochemiluminescence assay (Elecsys 2010, Roche, Almere, the Netherlands). Serum holoTC was determined by the AxSYM analyser (Abbott Diagnostics, Hoofddorp, the Netherlands) and serum MMA was measured by LC-MS/MS.

Statistical analyses

Fracture rate was estimated to be 5-6% in untreated elderly people (either independently or institutionalized). With an expected fracture rate reduction of 34% in the intervention group, a power of 80%, and a significance level of 0.05 (one-sided), 1,500 participants per treatment group were required [23].

Statistical analyses were performed before the treatment code was revealed. Baseline characteristics of the treatment groups were compared with Chi-square tests for categorical data and unpaired Student's t-tests for continuous data. Non-parametric tests were applied if the distribution was skewed. Difference between

the treatment groups in change of Hcy after two years was tested with an unpaired Student's t-test.

To assess the efficacy of the supplementation the primary analysis was based on the intention-to-treat (ITT) principle, including all subjects who agreed to start the treatment and completed the baseline measurements. To assess the effectiveness of the supplementation pre-specified per-protocol (PP)-analyses were performed that included only data from subjects who were compliant to the study protocol. For drop-outs, the time until drop-out was used in these analyses.

Time-to-event data were analyzed using the Kaplan-Meier approach and the log-rank test. Hazard ratios (HR) and 95% confidence intervals (95%CI) were calculated with the use of crude and adjusted (for age, sex, study center, baseline Hcy and HoloTC) Cox proportional-hazards models. Individual time of follow-up was calculated as the time until the first fracture (primary outcome: osteoporotic or secondary outcome: any type), end of intervention period, date of lost-to-follow-up, or death, whichever came first. Log-minus-log plots were used to check the proportional hazard models assumption, which was not violated.

The difference in number of persons who reported at least one adverse event between treatment groups was tested using Chi-square. For time-to-cancer, post-hoc analyses were performed following the same approach as the fracture analyses. Additionally, sensitivity-analyses were done including cancer cases that could not be fully verified, and this was repeated after excluding relapse cancer cases.

For all outcomes, interaction with treatment was tested for the pre-specified covariates sex, baseline age below and above 80y, and plasma Hcy below and above 18 µmol/l. In case of significant interaction ($p<0.1$), subgroup analyses were performed.

Statistical significance was set at $\alpha=0.05$. Data were analyzed using IBM SPSS Statistics 20 (SPSS Inc., Chicago, Illinois).

RESULTS

Characteristics of the participants

Analyses included 2,919 participants (Figure 1) (49.9% men, mean age=74.1y, median plasma Hcy concentration=14.4 µmol/L). At baseline, no significant differences between the intervention (n=1,461) and placebo (n=1,458) group were observed, except for a 3% higher holoTC concentration in the intervention group (p=0.028) (Table 1). Mean change in Hcy concentrations was -4.4 µmol/L in the intervention vs. -0.2 µmol/L in the placebo group (p<0.001) (Table 2).

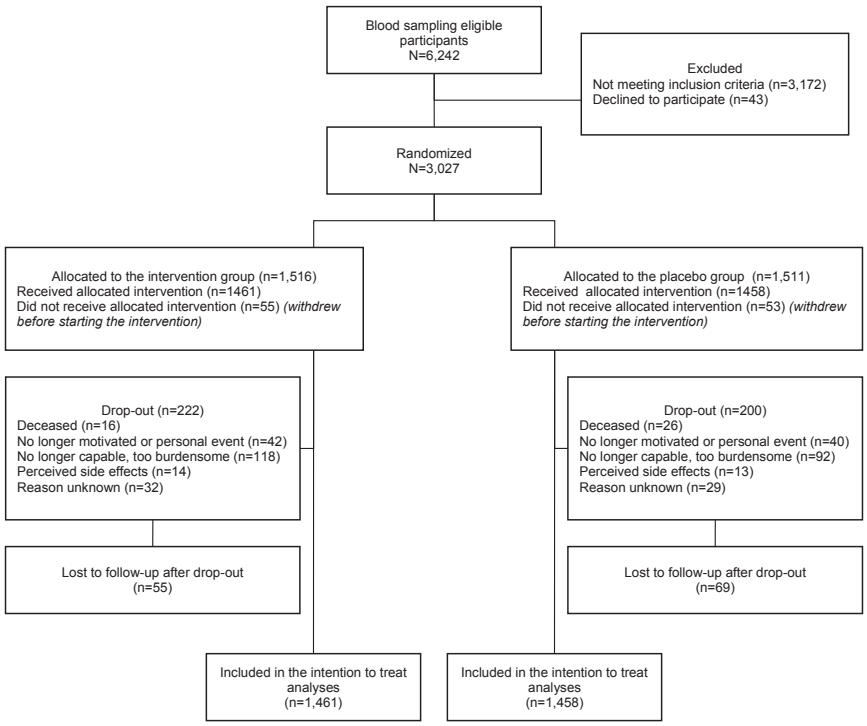


Figure 1. Screening, randomization and follow-up in the B-PROOF study.
Note: the number of participants that dropped out because they deceased does not equal the total number of deceased participants; some participants (n=37) dropped out for other reasons and deceased after drop-out (Intention-to-treat).

Table 1. Baseline characteristics of the B-PROOF study population (n=2,919).

	Placebo group (N=1,458)	**Intervention group (N=1,461)**
Age (y) [1]	74.2 (6.4)	74.0 (6.6)
Sex (% women) [2]	49.7	50.4
Study center [2]		
- WUR (%)	29.6	29.2
- VUmc (%)	26.8	26.4
- EMC (%)	43.6	44.4
History of fracture (% yes) [2,3]	42.9	41.3
Height (cm) [1]	169.2 (9.3)	169.4 (9.4)
Weight (kg) [1]	77.8 (13.3)	77.9 (13.3)
Current smoker (%) [2]	9.7	9.5
Alcohol use [3]		
- Light (%)	66.8	68.0
- Moderate (%)	29.0	28.5
- Excessive (%)	4.2	3.5
Physical activity (min/day) [2,4]	131 [86-193]	126 [81-190]
Education [2]		
- Low (%)	53.6	52.4
- Intermediate (%)	21.1	21.1
- High (%)	25.4	26.5
B_{12} and/or folic acid supplement use (% yes) [2,3]	15.8	15.3
Vitamin D supplement use (% yes) [2,3]	19.7	18.3
Osteoporotic medication use (% yes) [2,3]	7.1%	7.8%
Biochemical analyses:		
Homocysteine (µmol/L) [4]	14.5 [13.0-16.7]	14.3 [13.0-16.5]
Vitamin B_{12} (pmol/L) [4]	266 [204-343]	267 [213-341]
Folate (nmol/L) [4]	18.9 [14.8-24.5]	18.8 [14.9-24.7]
Methylmalonic acid (µmol/L) [4]	0.23 [0.18-0.31]	0.22 [0.18-0.30]
Holotranscobalamin (pmol/L) [4]	63.0 [45.0-84.0]	65.0 [48.0-86.0][5]
25(OH) vitamin D [1]	55.8 (23.9)	55.5 (25.8)
Creatinine (µmol/L) [1]	84.1 (18.0)	83.9 (18.6)

[1]Presented as mean (SD), difference tested using t-test. [2]Presented as percentages, differences tested using Chi-squared test. [3]Data based on self-report. [4]Presented as median [interquartile range], differences tested using Mann-Whitney U test. [5]P <0.05. WUR=Wageningen UR, VUmc=VU University Medical Center, EMC=Erasmus MC.

Table 2. Levels of plasma homocysteine concentrations (µmol/L) at baseline and after follow-up according to treatment group, both for the complete study sample and per age category.

		Placebo group				Intervention group				
		Baseline homocysteine (µmol/L) [1]	Follow-up homocysteine (µmol/L) [1]	2-yr change (µmol/L) [2]	N	Baseline homocysteine (µmol/L) [1]	Follow-up homocysteine (µmol/L) [1]	2-yr change (µmol/L) [2]	N	p-value
Intention-to-treat										
	Total sample	14.4 [13.0-16.5]	14.3 [12.4-17.0]	-0.2 (4.1)	1,299	14.2 [13.0-16.4]	10.3 [8.9-12.0]	-4.4 (3.3)	1,296	<0.001
	Age ≤80y	14.2 [12.9-16.1]	14.0 [12.2-16.5]	-0.3 (3.8)	1,107	14.1 [12.9-16.0]	10.2 [8.8-11.8]	-4.3 (3.1)	1,114	<0.001
	Age >80y	15.6 [13.4-18.4]	16.4 [14.1-20.0]	0.7 (5.2)	192	15.4 [13.7-18.5]	11.2 [9.5-13.5]	-4.5 (4.5)	182	<0.001
Per-protocol										
	Total sample	14.4 [13.0-16.4]	14.3 [12.3-16.9]	-0.2 (4.0)	1,231	14.2 [13.0-16.3]	10.2 [8.9-11.8]	-4.5 (3.1)	1,240	<0.001
	Age ≤80y	14.2 [12.9-16.1]	14.0 [12.1-16.5]	-0.3 (3.7)	1,052	14.1 [12.9-16.0]	10.2 [8.8-11.7]	-4.4 (3.0)	1,076	<0.001
	Age >80y	15.6 [13.4-18.4]	16.2 [14.0-19.9]	0.6 (5.2)	179	15.3 [13.6-18.2]	10.9 [9.4-13.2]	-5.0 (3.6)	164	<0.001

[1]Presented as median [interquartile range]. [2]Presented as mean (SD), difference tested using t-test.

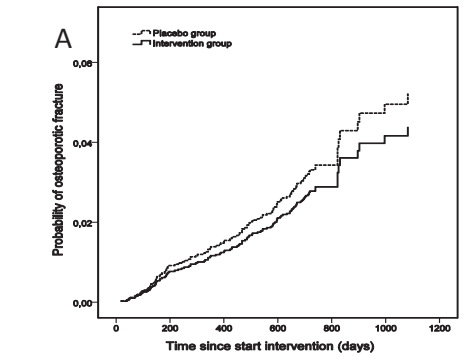
Primary endpoint: Osteoporotic fractures

In the ITT-analyses, 52 persons sustained a total of 61 osteoporotic fractures (fracture rate=2.0/100 person-y) in the intervention group vs. 61 persons with 75 osteoporotic fractures (fracture rate=2.5/100 person-y) in the placebo group (incidence rate ratio=0.80, 95%CI=0.55 to 1.16). Two fractures could not be verified, and were considered as non-case. Time-to-first osteoporotic fracture was not significantly different between the intervention and placebo group (log-rank p=0.396). Cox proportional-hazard models adjusted for age, sex, study center, baseline plasma

Table 3. Fracture and cancer rates and Hazard Ratios as measures of the association of treatment with time-to-first osteoporotic fracture, time-to-first fracture of any type, and time-to-cancer diagnosis in the total group and subgroups, derived from Cox proportional-hazards analyses; intention-to-treat and per-protocol analyses.

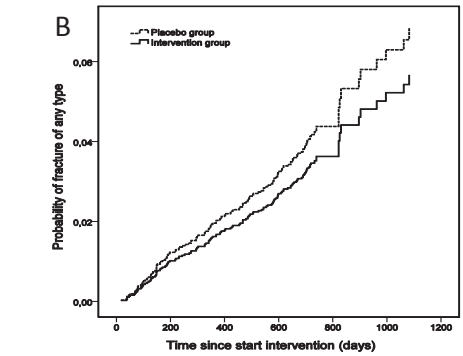
Outcome	Placebo group		Intervention group		Crude model		Adjusted model	
Intention-to-treat analysis	**N**	**Rate/100 person-y**	**N**	**Rate/100 person-y**	**Hazard Ratio (95%CI)**	**p-value**	**Hazard Ratio (95%CI)**	**p-value**
Osteoporotic fracture (n=2,919)	75	2.5	61	2.0	0.85 (0.59-1.23)	0.385	0.84 (0.58-1.22) [1]	0.362
Any type of fracture (n=2,919)	94	3.1	79	2.6	0.84 (0.60-1.17)	0.298	0.83 (0.59-1·16) [1]	0.279
Cancer (n=2,906)	42	1.4	63	2.1	1.56 (1.05-2.31)	0.029	1.55 (1.04-2.30) [1]	0.032
Age ≤80y (n=2,416)	36	1.4	49	1.9	1.33 (0.86-2.04)	0.201	1.30 (0.85-2.01) [2]	0.231
Age >80y (n=490)	6	1.2	14	2.9	3.66 (1.21-11.11)	0.022	3.68 (1.21-11.24) [2]	0.022
Men (n=1,450)	29	1.9	32	2.1	1.17 (0.70-1.96)	0.551	1.15 (0.69-1.94) [3]	0.585
Women (n=1,456)	13	0.9	31	2.0	2.35 (1.23-4.50)	0.010	2.34 (1.22-4.46) [3]	0.010
Per-protocol analysis	**N**	**Rate/100 person-y**	**N**	**Rate/100 person-y**	**Hazard Ratio (95%CI)**	**p-value**	**Hazard Ratio (95%CI)**	**p-value**
Osteoporotic fracture (n=2,661)	62	2.3	48	1.8	0.82 (0.55-1.22)	0.326	0.82 (0.55-1.22) [1]	0.327
Age ≤80y (n=2,263)	36	1.6	41	1.8	1.07 (0.68-1.68)	0.769	1.08 (0.69-1.71) [2]	0.728
Age >80y (n=398)	26	6.0	7	1.9	0.30 (0.11-0.82)	0.018	0.28 (0.10-0.74) [2]	0.011
Any type of fracture (n=2,661)	84	3.0	68	2.4	0.81 (0.57-1.16)	0.255	0.81 (0.57-1·16) [1]	0.250
Age ≤80y (n=2,263)	55	2.3	60	2.5	1.01 (0.68-1.51)	0.957	1.02 (0.68-1.52) [2]	0.930
Age >80y (n=398)	29	6.4	8	2.0	0.29 (0.11-0.77)	0.013	0.26 (0.10-0.71) [2]	0.008
Cancer (n=2,651)	27	1.0	43	1.6	1.66 (1.02-2.70)	0.042	1.67 (1.02-2.71) [1]	0.040
Age ≤80y	23	1.0	35	1.5	1.50 (0.89-2.54)	0.131	1.50 (0.88-2.53) [2]	0.135
Age >80y	4	0.9	8	2.1	2.89 (0.77-10.87)	0.118	2.75 (0.73-10.39) [2]	0.137
Men	18	1.3	24	1.7	1.43 (0.77-2.67)	0.257	1.49 (0.80-2.77) [3]	0.213
Women	9	0.7	19	1.4	2.09 (0.94-4.61)	0.069	2.03 (0.92-4.49) [3]	0.080

[1]Adjusted for age, sex, study center, and baseline levels of homocysteine and holotranscobalamin, [2]Adjusted for sex, study center, and baseline levels of homocysteine and holotranscobalamin, [3]Adjusted for age, study center, and baseline levels of homocysteine and holotranscobalamin, 95%CI=95% Confidence Interval. Stratified analyses were performed if the interaction of sex, age, homocysteine, or study center with treatment was significant (p<0.10).



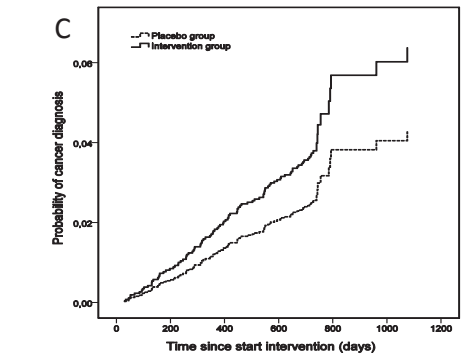
No. at risk

Intervention	1461	1431	1431	1380	178	171	0
Placebo	1458	1421	1396	1364	200	189	0



No. at risk

Intervention	1461	1425	1403	1369	178	171	0
Placebo	1458	1419	1389	1357	200	188	0



No. at risk

Intervention	1454	1424	1401	1374	188	182	0
Placebo	1452	1426	1400	1378	209	203	0

Figure 2. Time-to- A) first osteoporotic fracture, B) first fracture of any type, and C) cancer diagnosis, adjusted for age, sex, study center, plasma homocysteine and serum holotranscobalamin, derived from Cox proportional-hazards analysis (Intention-to-treat).

Hcy, and serum holoTC showed that persons in the intervention group did not have a significantly lower probability to sustain an osteoporotic fracture than persons in the placebo group (HR=0.84, 95%CI 0.58 to 1.22) (Figure 2A, Table 3). PP-analysis was performed among 2,661 compliant participants, including 91.4% of participants in the intervention group vs. 90.9% of the placebo group. Fracture rates are presented in Table 3. Multivariable Cox proportional-hazard models did not show a significantly different osteoporotic fracture risk between the intervention group and placebo group (HR=0.82, 95%CI 0.55 to 1.22) (Table 3).

Secondary endpoint: Any type of fractures

The rate of fractures of any type in the intervention group vs. the placebo group was 2.6/100 person-y vs. 3.1/100 person-y, respectively. No significant effects of the intervention in both the ITT- and the PP-analyses were observed (HR=0.83, 95%CI 0.59 to 1.16 and HR=0.81, 95%CI 0.57 to 1.16, respectively) (Figure 2B, Table 3). Specific types of fractures are shown in Table 4.

Table 4. Total number of fractures during the intervention period per fracture type according to treatment group and age category.

	Placebo group			Intervention group		
	Total sample (N=1,458)	**Age ≤80y (N=1,205)**	**Age >80y (N=253)**	**Total sample (N=1,461)**	**Age ≤80y (N=1,220)**	**Age >80y (N=241)**
Head	5	5	0	2	2	0
Arm	11	8	3	13	10	3
Elbow	1	1	0	0	0	0
Wrist	12	8	4	16	14	2
Hand	4	4	0	2	2	0
Fingers	1	1	0	4	3	1
Rib	11	7	4	13	13	0
Vertebra	15	8	7	7	3	4
Pelvis	6	0	6	1	0	1
Hip	13	8	5	8	5	3
Leg	5	2	3	2	0	2
Ankle	7	7	0	6	5	1
Foot	3	3	0	3	3	0
Toe	0	0	0	2	2	0
Total	94	62	32	79	62	17

Exploratory analyses

Interactions of treatment group with age, sex and Hcy concentration were not significant in the ITT-analyses for both osteoporotic fractures and any type of fractures. In the PP-analyses, a significant interaction effect with age was observed (p=0.020 and p=0.018 for respectively osteoporotic fractures and any type of fractures). Persons >80y in the intervention group had a lower probability of sustaining an osteoporotic fracture (HR=0.28, 95%CI 0.10 to 0.74) compared with the placebo group (Table 3). The number needed to treat was 25 (for two years). Results were similar when any type of fractures was considered. No effect was observed in persons <80y and no statistically significant interaction was observed with sex and Hcy concentrations.

Adverse events

Mortality did not differ between the intervention and placebo group (n=37 vs. n=42 respectively, p=0.571, ITT). In the total number of adverse events, no difference was observed between treatment groups (p=0.862). However, 63 participants in the intervention group and 42 participants in the placebo group reported a new, subsequently verified diagnosis of cancer during the intervention period (Chi-square p=0.038). The HR was 1.55 (95%CI 1.04 to 2.30) (ITT, Table 3, Figure 2C). Verification of cancer diagnosis was not possible in 13 cases. PP-analyses (Table 3) and sensitivity analyses (data not shown) provided similar results.

Interaction effects with age (p=0.085) and sex (p=0.090) were observed (ITT). Corresponding subgroup analyses revealed that the effect was more pronounced in participants aged >80y (HR=3.68, 95%CI 1.21 to 11.24), and in women (HR=2.34, 95%CI 1.22 to 4.46). Differences mainly appeared for colorectal cancer (14 in intervention group vs. 5 in placebo) and other gastro-intestinal cancers (7 vs. 1, respectively) (data not shown).

DISCUSSION

Daily supplementation of 500 µg vitamin B_{12} and 400 µg folic acid – in addition to 600 IU vitamin D_3 - for 2 years did not significantly reduce osteoporotic fracture risk in elderly aged ≥65y with an elevated plasma Hcy concentration. Pre-specified subgroup analysis suggested a reduction of fractures among those aged >80y

who were compliant in taking the supplement. Mortality did not differ between treatment groups over the two-year intervention period. However, supplementation was associated with a higher cancer incidence, especially colorectal and other gastro-intestinal cancers. The subgroup analysis in persons aged >80y who were compliant and the analyses on mortality and cancer should be considered as exploratory analyses as the study was only powered to detect differences in fracture risk.

Compared to the Sato trial [21] (a strong treatment effect on fractures) and the HOPE-2 [22] and VITATOPS trials [20] (no treatment effect), differences in study design and population with B-PROOF should be noted. Regarding baseline health status, the Sato trial included a very specific, high fracture risk population consisting of post-ischemic stroke, hemiplegic patients. HOPE-2 and VITATOPS included participants with a history of vascular or cerebrovascular disease, while B-PROOF included participants primarily based on elevated Hcy concentrations. Also, median Hcy concentrations differed substantially between the studies: 19.9 µmol/L in the Sato trial, 11.5 µmol/L in the HOPE-2 trial, 14.3 µmol/L in VITATOPS and 14.4 µmol/L in B-PROOF. In addition, dietary patterns, presence of fortified food and/or supplement use might contribute to differences between the populations. Mean age did not differ substantially between the studies. Whereas sex distribution was similar for the Sato trial and B-PROOF (53% vs. 50% women), fewer women participated in HOPE-2 (28%) and VITATOPS (36%). However, we did not find evidence for an interaction between sex and treatment in our study. Comparison of dose and duration across the three trials indicates that higher supplementation dose and longer study duration did not result in more favorable outcomes, therefore not explaining the differences in results. Concluding, in the Sato trial, Hcy concentrations were higher and the general health status of the participants was worse, resulting in a higher *a priori* fracture risk than for participants in HOPE-2, VITATOPS, and B-PROOF.

In the current study, only a significant effect of B-vitamins on fracture risk was observed in compliant persons aged >80y. It is known that Hcy concentrations increase with age, and therefore baseline Hcy concentrations or change in Hcy concentrations might provide a possible explanation for the results. On the one hand, we did not observe significant interaction of baseline Hcy concentration (below and above 18 µmol/L) with treatment. Interestingly, on the other hand, Hcy concentrations appeared to decrease more in compliant persons >80y compared

with persons <80y (Table 2, post-hoc analysis), especially when taking into account the changes over time as shown in the placebo group. As these analyses should be considered exploratory, future studies are needed to examine this further.

The observation of a significantly higher cancer incidence in the intervention group than in the placebo group was unexpected. It is important to note that B-PROOF was not designed to study cancer as primary outcome. The limited follow-up time of 2 years, for instance, does not allow firm conclusions about cancer development and long-term cancer risk. The results of the present trial differ from the B-Vitamin Treatment Trialists' collaboration meta-analysis of 13 trials, involving 49,621 individuals, which reported that allocation to folic acid had no significant effects on overall cancer incidence (1904 in the folic acid group vs 1809 in the control group, rate ratio (RR) 1.06, 95%CI 0.99 to 1.13), or on cancer incidence at any site [27]. This meta-analysis, primarily involving participants at high risk of cardiovascular disease, tested the effect of a mean daily dose of folic acid of 2 mg (range 0.5-5 mg) for an average duration of 5.2 years [27]. These findings were consistent with two previous meta-analyses (RR 1.05, 95% CI 0.99 to 1.11 and RR 1.07, 95% CI 1.00 to 1.14) [28, 29]. The higher cancer risks observed in B-PROOF may reflect the effects of chance as they were based on only 105 incident cancer events compared with 3713 cancer events in the B-Vitamin Treatment Trialists' collaboration meta-analysis [27]. However, cancer risk after B-vitamin treatment should be a point of attention in future studies.

The dose of folic acid provided in B-PROOF (400 µg) was relatively low and well below the tolerable upper intake level for folic acid of 1 mg per day in Europe [30]. In addition, no national mandatory folic acid food fortification exists in the Netherlands. Subgroup analysis in two of the three meta-analyses on this potential side-effect showed that increased risk of cancer was mainly seen in low-dose (≤1mg/day) supplementation rather than in high-dose supplementation, while a dose-response effect was absent [28, 29]. However, it should be noted that the low-dose trials all had doses above ours (ranging from 0.5 to 1.0 mg) and in addition, dose-related effects were absent in the third meta-analysis [27].

Regarding vitamin B_{12} and vitamin D_3, to date, little is known about the possible relation between vitamin B_{12} and cancer risk or the interaction between folic acid, B_{12} and/or vitamin D_3 and cancer risk.

Folate is required for DNA-synthesis and DNA-methylation, processes that are also important in cancer initiation and progression. It has been hypothesized that folic acid prevents against the initiation of cancer, while it enhances growth and progression of established neoplastic cells [31]. As shown in Figure 2C, the curves for cancer incidence diverge shortly after the start of the intervention. This fits with the hypothesis of an effect on cancer progression, rather than cancer induction. This idea is supported by the observation that the effect on cancer incidence in our study was most pronounced in persons aged >80y, among whom the presence of latent cancer is speculated to be more likely. The fact that our study population was older (mean age 74y) than the populations in the three meta-analyses (mean population ages ranging from 26y to 69y [29]) may therefore also explain the higher overall HR observed in our study. Further research into the effects of folic acid on cancer progression is warranted, especially in the oldest old.

The major strengths of B-PROOF are its double-blind randomized placebo-controlled design and the use of clinical endpoints. It is the first trial primarily designed to study the effect of B-vitamin supplementation on fracture prevention in an elderly population with mildly elevated Hcy concentrations. Another strength is the high compliance with the allocated treatment. A limitation of the study is that we included 2,919 instead of 3,000 participants as indicated by our sample size calculation. As described before, there was a pre-planned prolonged follow-up, which included in total 393 persons, to increase power. These persons were slightly different (in terms of age and MMA status) compared with the persons without extended follow-up, with potentially a slightly higher *a priori* risk of fractures. However, the subgroup was too small for further sub-analyses. Both the occurrence of a fracture and the diagnosis of cancer were based on self-report, which could be regarded as a limitation. However, structured questionnaires were used and the diagnoses were verified with the participant's general practitioner or hospital. Potential underreporting is expected to be non-differential for treatment groups. In addition, it should be noted that multiple statistical tests have been performed. Although they were pre-specified, the occurrence of false-positive findings cannot be ruled out.

In conclusion, an overall effect of supplementation of vitamin B_{12} and folic acid in reducing fracture risk in elderly with elevated Hcy concentrations was not observed in B-PROOF. Exploratory analyses suggested a reduced fracture risk in elderly

aged >80y who were compliant in taking the supplement. On the other hand, supplementation of vitamin B_{12} and folic acid was also associated with higher cancer risk, although these results should be treated with caution as they have not been observed in meta-analyses of previously available trials with folic acid. Hence, vitamin B_{12} and folic acid supplementation cannot be recommended for fracture prevention.

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Chapter 3.2

Effect of vitamin B_{12} and folic acid supplementation on bone mineral density and quantitative ultrasound parameters in older people with an elevated plasma homocysteine level: B-PROOF, a randomized controlled trial

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Submitted

ABSTRACT

High plasma homocysteine levels are associated with increased osteoporotic fracture incidence. However, the mechanism remains unclear. We investigated the effect of homocysteine-lowering vitamin B_{12} and folic acid treatment on bone mineral density (BMD) and calcaneal quantitative ultrasound (QUS) parameters within the B-PROOF study. This randomized, double-blind, placebo-controlled multi-center trial included participants aged ≥65 years with plasma homocysteine levels between 12-50 µmol/l. The intervention comprised 2-year supplementation with either a combination of 500 µg B_{12}, 400 µg folic acid and 600 IU vitamin D_3 or placebo with 600 IU vitamin D_3 only. In total, 1111 participants underwent repeated dual-energy X-ray assessment and 1165 participants QUS. Femoral neck (FN) BMD, lumbar spine (LS) BMD, calcaneal broadband ultrasound attenuation (BUA) and calcaneal speed of sound (SOS) were assessed. After two years of intervention, FN-BMD and BUA had significantly decreased, while LS-BMD significantly increased (all $p<0.01$) and SOS did not change in either treatment arm. ANCOVA-analyses showed that no statistically significant differences between the intervention and placebo group were present for FN-BMD ($p=0.24$), LS-BMD ($p=0.16$), SOS ($p=0.67$) and BUA ($p=0.96$). However, for BUA an interaction effect with age was observed among compliant participants. Subgroup analyses among compliant persons >80 years revealed a small positive effect of the intervention on BUA at follow-up (estimated marginal mean 64.4 dB/MHz for the intervention group and 61.0 dB/MHz for the placebo group, $p=0.04$ for difference). In conclusion, this study showed no overall effect of treatment with vitamin B_{12} and folic acid on BMD or QUS parameters in elderly, mildly hyperhomocysteinemic persons, but suggests a small beneficial effect on BUA in persons >80 years who were compliant in taking the supplement.

INTRODUCTION

Approximately a decade ago, plasma levels of homocysteine (Hcy) were discovered to be positively associated with incident osteoporotic fractures [1, 2]. Vitamin B_{12} and/or folate are important co-factors in the remethylation of Hcy to methionine and high plasma Hcy levels are often caused by vitamin B_{12} and/or folate deficiency [3]. Subsequent supplementation with these vitamins has been shown to be effec-

tive in reducing levels of Hcy [4]. Supplementation was therefore hypothesized to be associated with a lower fracture incidence as well. However, intervention studies with B-vitamin supplementation observed inconsistent effects on fracture prevention [5-8]).

The potential mechanism underlying the association between Hcy and fractures remains to be determined. One of the hypotheses concerns the role of bone mineral density (BMD) in this association. Previously, cross-sectional studies on the relation between Hcy and BMD showed conflicting results (e.g. [9-11]). Moreover, two trials investigated the effect of B-vitamin supplementation on BMD, and both observed no effects [6, 12]. However, these trials were limited either in size (n=47) [12] or in generalizability (hemiplegic post-stroke patients) [6] and both used fairly high doses of B-vitamins.

Alternatively, Hcy is thought to interfere with collagen cross-linking in bone, thereby reducing bone quality. This suggestion is supported by clinical observations in patients with homocystinuria, among whom bone collagen profiles are disturbed [13]. Previous cross-sectional data indeed showed inverse associations between Hcy and bone quality, as reflected by quantitative ultrasound (QUS) parameters [14-16]. However, intervention studies on the effect of B-vitamin supplementation on those QUS parameters are lacking.

The current study investigated the effects of vitamin B_{12} and folic acid supplementation on BMD and QUS parameters, that is broadband ultrasound attenuation (BUA) and speed of sound (SOS), in a large, mildly hyperhomocysteinemic, but otherwise general elderly population.

MATERIALS AND METHODS

Study design

The B-PROOF study is a double-blind, randomized, placebo-controlled multicenter trial. It was primarily designed to investigate the effect of 2-year oral supplementation with 400 µg folic acid and 500 µg vitamin B_{12} on osteoporotic fracture incidence in hyperhomocysteinemic persons aged 65 years and over [17]. Participants in both treatment arms additionally received 600 IU of vitamin D_3 daily. Participants (n=2919) were randomly assigned to the treatment groups in a 1:1 ratio while stratifying for study centre, sex, age (65-80 years, >80 years), and Hcy level (12-18 µmol/L,

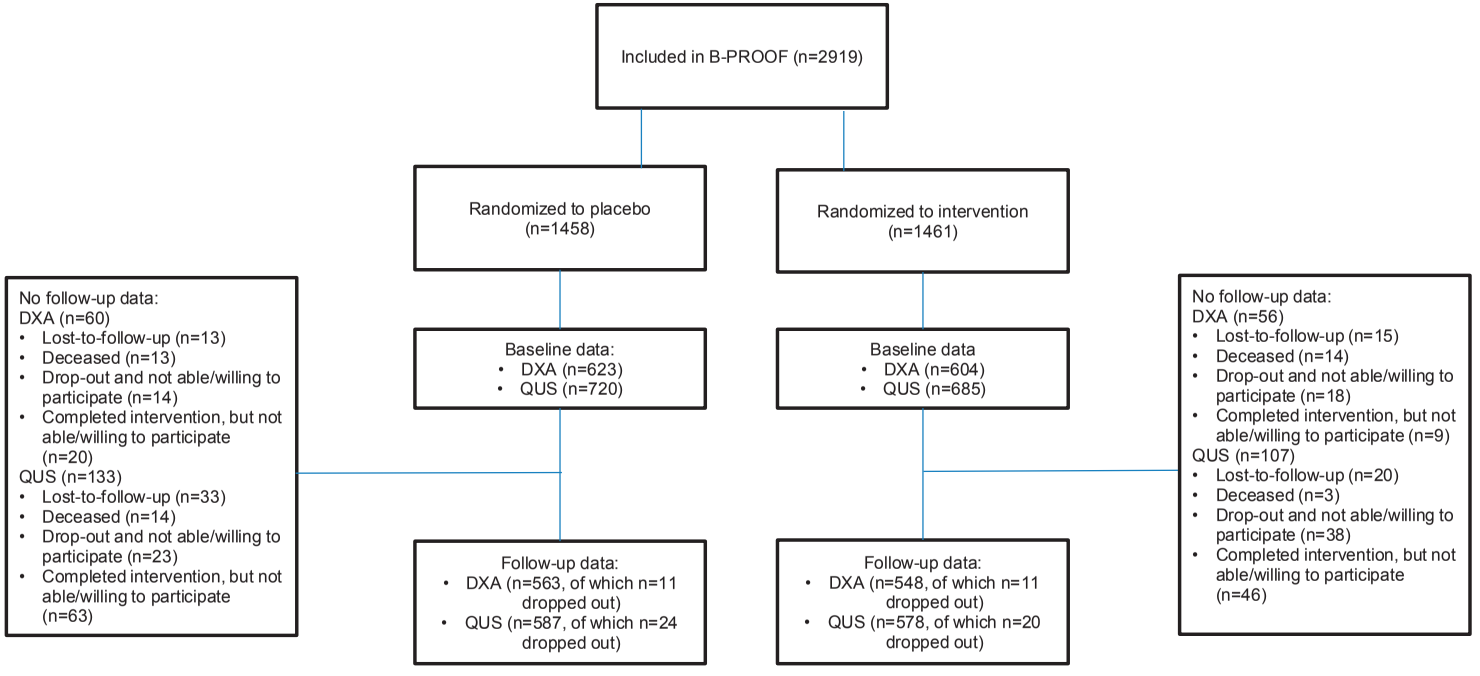

Figure 1. Flow-chart regarding DXA and QUS-measurements in the B-PROOF study.

≥18 µmol/L). The random allocation sequence and randomization were generated and performed using SAS 9.2 by an independent research dietician. Intervention and placebo tablets were indistinguishable in taste, smell and appearance. Both the participants and all researchers and research assistants were blinded to the study treatment. Treatment effects on BMD and QUS were predefined secondary outcomes [17]. Recruitment of participants took place between September 2008 and March 2011. Details of the B-PROOF study were described previously [17]. The B-PROOF study has been registered with the Netherlands Trial Register http://www.trialregister.nl under identifier NTR 1333 since June 1, 2008 and with ClinicalTrials.gov under identifier NCT00696514 since June 9, 2008. The Medical Ethics Committee of Wageningen University (WU) approved the study and local feasibility was given by the Medical Ethics Committees of VU University Medical Center (VUmc) and Erasmus MC. The study was performed in accordance with the Declaration of Helsinki and all participants gave written informed consent.

Study population

Inclusion criteria were an age of 65 years or over at baseline and a plasma Hcy level between 12.0 and 50.0 µmol/l. Exclusion criteria were a level of serum creatinine of >150 µmol/l, the presence of cancer in the past five years (excluding non-melanoma skin cancer), use of high doses of B-vitamins (intramuscular injections of vitamin B_{12} and/or folic acid intake >300 µg/day) or permanent use of a wheel chair. For BMD measurements, participants had to be able to visit one of the study centers. Figure 1 shows the flow-chart of the study sample.

Basic characteristics

At baseline, height was measured without shoes to the nearest millimeter using a stadiometer. Weight was measured while the participant wore light clothes and no shoes. Body mass index was calculated as weight/height^2. Structured questionnaires were used to assess fracture history, current use of medication and supplements, level of education, use of alcohol and current smoking behavior[17]. Anti-osteoporotic medication use defined as the use of bisphosphonates, strontium-ranelate, selective estrogen-receptor modulators, estrogens, androgens, denosumab or teriparatide. Blood drawing was done when the participant was in a fasted state or had consumed a light, restricted breakfast. EDTA-blood was placed

on ice immediately after drawing. Plasma Hcy, serum creatinine, folate, vitamin B_{12}, holotranscobalamin and methylmalonic acid and methylenetetrahydrofolate reductase (MTHFR)-genotype were determined; details of the methods used have been described previously [8, 17].

Dual-energy x-ray (DXA) assessment

In a subsample of 1227 participants, DXA was performed at baseline. Of these participants, 1111 persons also underwent a DXA after the 2 years of intervention (Figure 1). DXA was performed in two of the three study centers. In VUmc, a Hologic QDR 4500 Delphi device (Hologic Inc., USA, CV=0.45%) was used. In Erasmus MC, a GE Lunar Prodigy device (GE Healthcare, USA, CV=0.08%) was used.

In Erasmus MC, during the intervention period, a new scanner of the same type was installed. Follow-up measurements for participants who were measured using the new device at follow-up were adjusted for results of a cross-calibration with the old system. A participant's baseline and follow-up measurement always took place in the same study center.

A scan of the femur was made to determine the BMD at the femoral neck. The left hip was scanned, but in case a prosthesis was present, the right hip was scanned. A scan of the lumbar spine was made to assess BMD in the vertebrae L1 to L4. Measurements were performed according to manufacturer's protocols.

QUS parameters

QUS parameters of the calcaneus were measured using the portable Hologic Sahara bone densitometer (Hologic, USA) (Erasmus MC, VUmc, WU) or the portable CUBA Clinical system (McCue Ultrasonics, UK) (VUmc). At baseline, QUS measurements were performed in 1405 participants. Repeated QUS was available in 1165 participants (Figure 1). Measurements of both the left and right calcaneus were performed in duplo. Mean broadband ultrasound attenuation (BUA, CV=3.7%) and speed of sound (SOS, CV=0.22%) were calculated as the average of these four measurements. Measurements were excluded if the expected linear frequency-attenuation relation was violated, because this indicates invalid results.

Compliance

Participants were asked to return remaining study tablets every 6 months during their 2-year intervention period. Participants were regarded as compliant to the study treatment when at least 80% of the tablets had been taken during the intervention period, as indicated by the number of returned tablets. Compliance of participants who dropped out of the study was calculated over the planned full study period of 2 years.

Adverse events

Adverse events were reported by the participants on their study calendar or via telephone, as has been described previously [8].

Sample size calculation and statistical analyses

Based on an expected increase in BMD of 0.027 g/cm^2 (extrapolated from [18]), who observed a one-year-change in spinal BMD of 0.0135 when folate levels increased with 15 nmol/l) between the two treatment groups, an SD of 0.18 g/cm^2 and a power of 80% to detect this difference, we estimated that 541 participants had to be included in both treatment arms. Similarly, a decline in BUA of 2.1 dB/MHz is expected in 2 years in the placebo group, and we expect this decline to be prevented in the intervention group (extrapolated from [19]). With a difference of 2.1 dB/MHz and an SD of 9.4, 316 participants per group would be needed.

All statistical analyses were performed according to a predefined analysis plan. Differences in baseline characteristics between the two treatment groups were tested using a t-test for continuous traits and a Chi-squared test for categorical traits. If a variable was non-normally distributed, a Mann-Whitney U test was used.

In the primary intention-to-treat analyses, all participants of whom both baseline and follow-up data were available were included. In the secondary per-protocol analyses, only compliant participants were included.

Paired t-tests were done to assess the difference within treatment groups between baseline and follow-up for all outcomes. To test the difference in outcomes after two years of treatment between the intervention group and the placebo group, analysis of covariance (ANCOVA) was performed. In addition to the baseline value of the outcome of interest (FN-BMD, LS-BMD, BUA, or SOS), sex and age were entered as covariate in the basic model. This was defined as the primary analysis.

Next, other potential confounders, defined by a p-value of the difference between the treatment arms <0.2, were entered in the model. They were retained in the fully adjusted model if they changed F of the treatment in the basic model with at least 10%. This was done for each outcome separately. For BMD, analyses were repeated after stratification for study center, since both centers used different DXA-devices, which are known to produce systematically different results.

Interactions between treatment and baseline age, sex, and Hcy were investigated. Stratified analyses were performed if the interaction term was statistically significant. All statistical analyses were performed using IBM SPSS Statistics 20. Level of significance was set at α=0.05.

RESULTS

Table 1 shows the general characteristics at baseline of 1111 participants with repeated DXA and of 1165 participants with repeated QUS. At baseline, LS-BMD was higher in the intervention group compared with the placebo group (1.14 vs. 1.11 g/cm^2, respectively, p=0.03). In the BMD-sample, levels of serum holotranscobalamin were slightly higher in the intervention group (70 vs. 65 µmol/l, p=0.03). In the QUS-sample, participants in the placebo group more often had a positive fracture history (45% vs. 35%, p<0.01).

A total of 611 participants had both FN-BMD as well as QUS available at baseline and at follow-up. At baseline, FN-BMD correlated significantly with both BUA (r=0.48, p<0.01) and SOS (r=0.42, p<0.01).

For the BMD-sample, median levels of Hcy changed from 14.3 to 10.5 µmol/l in the intervention group and from 14.3 to 14.4 µmol/l in placebo during the intervention period (p<0.01 for difference in change between groups). For the QUS-sample, median levels went from 14.2 to 10.2 µmol/l, and remained 14.3 µmol/l, respectively (p=<0.01 for difference between groups).

BMD effects

Baseline and follow-up BMD per treatment group are shown in Table 2. FN-BMD significantly decreased in both treatment groups. On the contrary, LS-BMD increased significantly in both treatment groups. Estimated mean BMD at follow-up in both the femoral neck (0.84 g/cm^2 (95% CI 0.834;0.839) in the intervention group

Table 1. Baseline characteristics for B-PROOF participants with BMD at baseline and follow-up (N=1111) and for participants with QUS at baseline and follow-up (n=1165).

	BMD		QUS	
	Placebo	**Intervention**	**Placebo**	**Intervention**
	N=563	**N=548**	**N=587**	**N=578**
Age (y)[a]	72.8 (5.4)	72.4 (5.6)	73.3 (73.3)	73.4 (73.4)
Sex (% female)	48.3	48.2	57.4	53.8
Hcy (µmol/l)[b]	14.3 [12.9-16.3]	14.3 [12.9-16.0]	14.3 [12.9-16.4]	14.2 [13.0-16.1]
Creatinine (µmol/l)[b]	80 [71-93]	82 [71-93]	79 [70-92]	82 [70-93]
Folate (nmol/l)[b]	19.1 [14.8-25.4]	19.8 [15.4-24.8]	19.1 [14.8-24.5]	18.9 [15.6-24.6]
B_{12} (pmol/l)[b]	269 [204-343]	286 [218-348]	268 [204-352]	270 [216-346.3]
Methylmalonic acid (µmol/l)[b]	0.21 [0.17-0.29]	0.21 [0.17-0.28]	0.22 [0.18-0.30]	0.23 [0.18-0.30]
Holotranscobalamin (pmol/l)[b]	65 [47-88][c]	70 [50-91][c]	65 [45-85]	66 [49-88]
MTHFR-genotype (%)				
CC	43.1	47.9	43.2	47.4
CT	41.9	40.1	46.3	39.2
TT	15.0	12.0	10.5	13.4
Height (cm)[a]	169.9 (8.9)	170.4 (9.0)	168.5 (8.8)	168.9 (9.2)
Weight (kg)[a]	77.7 (12.9)	78.5 (13.0)	76.7 (12.2)	76.6 (12.5)
BMI (kg/m^2)[a]	26.9 (3.9)	27 (3.8)	27.0 (3.9)	26.8 (3.8)
Smoking status (%)				
Current	8.7	8.4	7.5	10.0
Former	58.6	56.9	55.2	56.2
Never	32.7	34.7	37.3	33.7
Alcohol consumption (%)				
No/light	62.9	64.4	64.9	67.3
Moderate	31.8	31.2	30.7	28.4
Excessive	4.8	3.6	3.9	3.5
Very excessive	0.5	0.7	0.5	0.9
Level of education (%)				
Low	54.8	52.2	53.6	52.6
Middle	19.9	18.8	22.2	20.4
High	25.3	29.0	24.2	27.0
Study center (%)				
VUmc	35.7	32.5	35.4	36.2
Wageningen UR	-	-	20.4	21.1
Erasmus MC	64.3	67.5	44.1	42.7
Users of folic acid and/or vit. B_{12} (%)	17.1	14.6	17.4	14.4
Osteoporotic medication use (%)	6.4	7.5	8.9	10.4
Positive fracture history (%)	41.4	39.1	45.0[c]	35.3[c]
FN-BMD (g/cm^2)[a]	0.84 (0.15)	0.85 (0.17)	-	-

Table 1. *Continued*

	BMD		QUS	
	Placebo	Intervention	Placebo	Intervention
	N=563	N=548	N=587	N=578
T-score FN-BMD[a]	-1.23 (0.93)	-1.15 (1.04)	-	-
LS-BMD (g/cm^2)[a]	1.11 (0.22) [c]	1.14 (0.25) [c]	-	-
T-score LS-BMD[a]	-0.3 (1.7)	-0.1 (1.9)	-	-
BUA (dB/MHz)[a]	-	-	70.9 (16.8)	71.8 (17.6)
SOS (m/s)[a]	-	-	1537 (31)	1539 (33)

[a]Presented as mean (standard deviation). [b]Presented as median [interquartile range]. [c]P-value<0.05.
BMD=bone mineral density, QUS=quantitative ultrasound, BMI=body mass index, FN=femoral neck, LS=lumbar spine, MTH FR=methylenetretrahydrofolate reductase

Table 2. Bone mineral density (n=1111) and quantitative ultrasound parameters (n=1165) at baseline and follow-up.

	Placebo			Intervention		
	Baseline	Follow-up	p-value	Baseline	Follow-up	p-value
FN-BMD (g/cm^2)	0.84 (0.15)	0.83 (0.15)	<0.01	0.85 (0.17)	0.85 (0.17)	<0.01
LS-BMD (g/cm^2)	1.11 (0.22)	1.12 (0.22)	<0.01	1.14 (0.29)	1.15 (0.25)	<0.01
BUA (dB/MHz)	70.9 (16.8)	68.5 (17.4)	<0.01	71.8 (17.6)	69.4 (17.9)	<0.01
SOS (m/s)	1537 (31)	1537 (33)	0.25	1540 (34)	1539 (35)	0.46

FN=femoral neck, LS=lumbar spine, BMD=bone mineral density, BUA=broadband ultrasound attenuation, SOS=speed of sound. Presented as mean (standard deviation).

vs 0.83 g/cm^2 (95% CI 0.831;0.837) in placebo (p=0.24)), and lumbar spine (1.14 g/cm^2 (95% CI 1.134;1.142) vs 1.13 g/cm^2 (95% CI 1.130;1.138), respectively, p=0.16) were not significantly different between treatment groups (Figure 2). This did not change after adjusting for other potential confounders (holotranscobalamin and vitamin B_{12}). No statistically significant interaction was observed. When the analyses were stratified for study center, as pre-specified, similar results were obtained. For FN-BMD, in VUmc, estimated means after 2 years were 0.717 (95% CI 0.712;0.722) and 0.719 (95% CI 0.714;0.724) g/cm^2 in the placebo and intervention groups, respectively. In Erasmus MC, these values were 0.896 (95% CI 0.892;0.899) and 0.898 (95% CI 0.895;0.902) g/cm^2, respectively. For LS-BMD, in VUmc, estimated means after 2 years were 1.018 (95% CI 1.011;1.024) and 1.017 (95% CI 1.010;1.024) g/cm^2 in the placebo and intervention groups, respectively. In Erasmus MC, correspond-

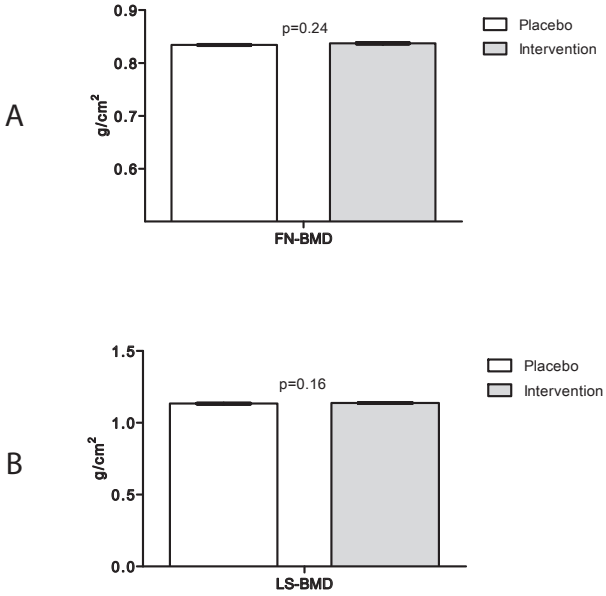

Figure 2. Estimated mean FN-BMD (A) and LS-BMD (B) after 2 years of intervention, adjusted for baseline FN-BMD/LS-BMD, age and sex.

ing values were 1.202 (95%CI 1.197;1.207) and 1.208 (95% CI 1.203;1.212) g/cm^2. All differences were non-significant.

In the per-protocol analyses, 1069 participants were included, and results were similar to the intention-to-treat analyses (data not shown).

QUS effects

A significant two-year decline in BUA was observed in both the intervention group and the placebo group (both p<0.01), whereas SOS levels did not change significantly in any of the groups (Table 2). Changes in BUA and SOS were not significantly different between treatment groups after adjustments for age, sex, and baseline values of BUA/SOS (Figures 3A and 3B). The estimated marginal means for BUA were 69.0 dB/MHz (95% CI 68.4; 69.6) in both the intervention group and in the placebo group (p=0.96), and the estimated marginal means for SOS were 1538.1 m/s (95% CI 1536.6; 1539.6) in the intervention group vs. 1537.6 m/s (95% CI 1536.2;

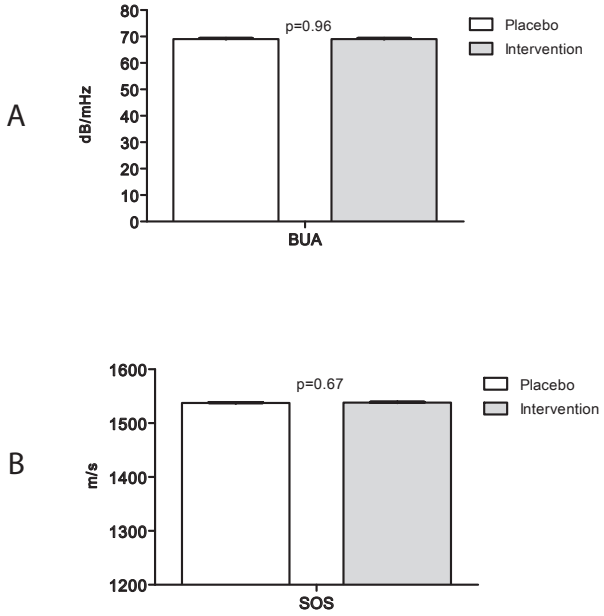

Figure 3. Estimated mean BUA (A) and SOS (B) after 2 years of intervention, adjusted for baseline BUA/SOS, age and sex.

1539.1) in the placebo group (p=0.67). Additional adjustments for fracture history, holotranscobalamin, smoking, vitamin B supplement use and MTHFR genotype (BUA), or fracture history, smoking and MTHFR-genotype (SOS) did not change the findings (data not shown). No interactions with age, sex, and baseline Hcy concentration were observed. Results of the per-protocol analyses, including 1097 participants, did not substantially differ from the intention-to-treat analyses (data not shown). Yet, in the analyses with BUA as outcome, the interaction with age was significant (p=0.02). Stratified analyses showed no effect among persons ≤80 years, but among persons >80 years, a significant beneficial effect of the treatment was observed (p=0.04, Figure 4). The estimated marginal means were 64.4 dB/MHz (95% CI 62.1; 66.6) in the intervention group vs 61.0 dB/MHz (95% CI 58.8; 63.3) in the placebo group.

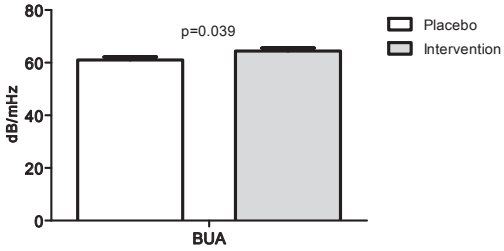

Figure 4. Estimated mean BUA among compliant persons >80y after 2 years of intervention, adjusted for baseline BUA, age and sex.

DISCUSSION

This randomized controlled trial did not show an overall effect of 2-year oral folic acid and vitamin B_{12} supplementation on BMD and QUS parameters compared with the placebo. In a subgroup of persons >80 years who were compliant with the study protocol, a small but statistically significant positive effect of the B-vitamin intervention was observed on BUA.

This study is the first trial investigating the effects of vitamin B_{12} and folic acid on QUS. Moreover, effects on BMD have not been studied before in a large, mildly hyperhomocysteinemic, but otherwise general older population. Two previous trials have been conducted, showing results that are in concordance with our findings. A Japanese trial investigated the effect of 1.5 mg vitamin B_{12} and 5 mg folic acid on hip fracture incidence and metacarpal BMD in hemiplegic post-stroke patients. In that study, no effect of a 2-year treatment on BMD was observed, while fracture incidence was strongly and significantly reduced in this specific population [6]. In addition, a small trial (n=47) has been performed which investigated the effect of a 1-year treatment with vitamin B_{12}, B_6 and folic acid on BMD among osteoporotic patients [12]. Overall, no effects were observed in that study. However, in participants with Hcy >15 µmol/l (n=8 in the intervention group), a significant increase in T-score was seen. In our study, no interaction effect of the treatment with baseline Hcy levels was observed. It should be noted that in comparison to our study, Hermann et al. used higher doses (2.5 mg folic acid, 25 mg B_6 and 500 µg B_{12}) [12].

QUS parameters are largely determined by BMD, but bone microarchitecture is an important determinant as well, independent of BMD [20]. QUS has been shown

to be an independent predictor for fracture risk [21]; a decrease of 1 SD in BUA has been associated with a 1.4 fold increased risk of any clinical fracture [21]. We observed a mean difference in BUA of 3.4 dB/MHz (5.2% of mean baseline BUA) between the intervention and placebo group among compliant persons >80 years. Because the spreading of BUA is relatively large (SD=17.1), the observed effect will be of minor importance on population level. However, when applying a longer duration of intervention, it might become clinically relevant.

Recently, we have shown within the B-PROOF study that fracture incidence was lower in the intervention group compared with placebo only when specifically addressing compliant participants aged 80 years or over [8]. The currently reported change in BUA might partly explain this age-specific treatment effect. Unfortunately, we were not able to test this hypothesis due to a too low absolute number of fractures among participants in this age category of whom BUA data were available (n=23). Alternatively, the lack of an effect on BMD does not completely rule out the possibility of BMD as a mediator. Participants of the DXA-subsample had to be able to visit one of the study centers and may therefore not be fully representative of the complete study population: as compared to the total sample, the DXA-subsample was significantly younger (mean age 72.6 vs 74.1, $p<0.01$), with a lower percentage of persons aged >80 years (9.0% vs 16.9%, $p<0.01$). In line with this, the subgroup of persons aged >80 years with DXA was also significantly younger than the subgroup of the complete study population (mean age 83.9 vs 85.1, $p<0.01$). The somewhat selective sample hampers definite conclusions about the absence of an effect of B-vitamins on BMD in persons >80 years.

It should be noted that LS-BMD increased in both treatment groups during 2 years of intervention, while FN-BMD decreased. In older persons, an increase in LS-BMD can be expected due to, for instance, degenerative changes of the spine [22, 23]. Our observation therefore supports the presumption that LS-BMD may not be a valid indicator of osteoporosis at high age [24].It could be regarded as a limitation that baseline levels of BMD in this randomized controlled trial differed significantly between the intervention and placebo group. However, we adjusted for baseline BMD, and therefore we assume that this did not influence the results of the analyses. Another limitation of the study is the fact that all participants received 600 IU vitamin D_3 daily, which is in line with the guidelines of the Dutch Health Council [25]. In the past, vitamin D supplementation with 400 IU daily has been

shown to influence BMD up to 2.6% [26, 27]. Effects of vitamin D may therefore have masked the possibly small effects of vitamin B_{12} and folic acid on BMD.

From the current study we conclude that there is no overall effect of 2-year treatment with vitamin B_{12} and folic acid on BMD or QUS in hyperhomocysteinemic elderly people. Among elderly >80 years who were compliant in taking the supplement, a positive effect of the treatment on BUA was observed. This might partly explain the previously reported reduction in fracture risk in the same subgroup [8]. It is important to note that an adverse effect of our treatment with vitamin B_{12} and folic acid on cancer incidence was observed, as has been published previously [8], implying caution in designing further research. Nonetheless, research on effects of B-vitamin treatment on other mechanisms, for instance on bone markers, computed tomography, or potentially the relatively new assessment of trabecular bone score, is warranted to reveal the additional pathways by which vitamin B_{12} and folic acid exert a potential anti-fracture effect in hyperhomocysteinemic elderly.

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Chapter 3.3

Associations of osteocalcin and osteopontin with homocysteine, bone mineral density and fractures, and the effect of a homocysteine-lowering treatment on their levels

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Chapter 4

A Mendelian randomization approach

Chapter 4.1

The association of plasma homocysteine levels with fractures and bone mineral density: a Mendelian randomization approach

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Chapter 5

General discussion

The objective of this thesis was to gain more insight into the potential role of homocysteine on incidence of fractures and other measures of bone health. To this end, we made use of both cross-sectional and longitudinal observational data. Moreover, we set up a large clinical trial to investigate the effects of a homocysteine-lowering treatment on fracture incidence and bone parameters.

MAIN FINDINGS

B-PROOF: A homocysteine-lowering intervention

In the past decades, high plasma homocysteine levels have been associated with increased risk of, e.g., cardiovascular disease [1] and cognitive impairment [2]. However, trials with folic acid and/or vitamin B_{12} that were designed to reduce homocysteine levels to prevent cardiovascular disease showed disappointing results [3, 4]. In 2004, the first evidence was published that plasma levels of homocysteine were also positively associated with incident fractures [5, 6]. In the years thereafter, this observation was replicated in other studies, and recently a meta-analysis reconfirmed these findings; an increase of 1 µmol/l of plasma homocysteine was associated with 4% increased fracture risk [7]. Up till now, four clinical trials [8-11], including B-PROOF, have been published that investigated the effect of supplementation with folic acid/vitamin B_{12} on fracture incidence. An overview of these trials is given in Table 1.

As can be seen, only in the first trial a significant and very impressive reduction of (hip) fractures was observed. This finding was not replicated in the other three trials. The positive trial included persons with both much higher baseline homocysteine levels and a higher *a priori* fracture risk than the other trials, and the results are based on a relatively small number of fractures. This might explain the differences in results. It should also be noted that the four trials use different interventions and doses, however, the fact that the reduction in homocysteine in B-PROOF is similar to HOPE-2 and VITATOPS suggests that low dose supplementation is sufficiently effective in reducing homocysteine. The larger reduction in homocysteine that was observed in the Sato study is probably mainly caused by the high levels of homocysteine at baseline. Next, in HOPE-2 and VITATOPS some included participants live in countries with mandatory folic acid fortification, which could have attenuated effects of the homocysteine-lowering intervention. This is not the case for the Japa-

nese and Dutch study populations. Finally, it should be noted that only B-PROOF was primarily designed to investigate the effect on fractures.

It would be of special interest to perform a meta-analysis of the available trials in the future, especially since power to detect effects on fractures per study is relatively low. For example, B-PROOF was powered to detect a reduction in osteoporotic fracture incidence of 34% [12], which might be regarded as over-optimistic given the current insights. More importantly, besides the exceptionally strong findings of Sato et al., a trend towards beneficial treatment effects was also observed in both VITATOPS and B-PROOF, although this was not statistically significant in either study. Furthermore, it is noteworthy that within B-PROOF a significant and beneficial effect (HR=0.30, p=0.018) of the intervention on fracture incidence was observed in a subgroup of participants who were at least 80 years old and who were compliant to the study treatment. This age group might thus be more susceptible to effects of a homocysteine-lowering intervention, a hypothesis which seems to be in line with observations in cardiovascular disease; homocysteine becomes of increased importance as a risk indicator for cardiovascular disease in the oldest old (>85 years) [13]. In support of this, we also observed a relatively strong reduction of the homocysteine levels in this subgroup, which also had a slightly higher baseline level of homocysteine. Therefore, effects of homocysteine and its reduction especially merit further investigation in persons older than approximately 80 years.

Within our study, it was unexpectedly observed that intervention with folic acid and vitamin B_{12} was associated with a higher cancer incidence. Previously, a large meta-analysis (n=49,621 participants) reported no significant effect of folic acid treatment on cancer incidence, although the hazard ratio (HR=1.06) was only borderline non-significant (95% CI 0.99-1.13, p=0.10) [14]. In our study, the increase in cancer appeared most strongly in persons older than 80 years (HR=3.66, p=0.022). Unfortunately, in the meta-analysis, cohorts were included with participants of lower age. Thus, it cannot be excluded that effects of folic acid in the oldest old are different and/or stronger than in younger persons. A new, stratified meta-analysis, investigating potential age-specific effects on cancer incidence, is therefore warranted.

Within B-PROOF, not only folic acid was supplemented, but also vitamin B_{12}. Unfavorable effects of vitamin B_{12} supplementation on cancer incidence therefore cannot be ruled out. Such an association has not been studied as extensively as

for folic acid. Vitamin B_{12} levels have been shown to be positively associated with prostate cancer in a meta-analysis [15]. However, a meta-analysis for breast cancer reported no significant associations [16], while for cervical neoplasms, a protective effect of high vitamin B_{12} levels was observed in a small meta-analysis [17]. Thus, we cannot draw conclusions concerning the role of vitamin B_{12} in the unfavorable cancer results in B-PROOF.

The results of B-PROOF imply that caution is needed when further research on potential beneficial effects of homocysteine-lowering treatment is designed. For instance, performing interim analyses or using supplements not containing folic acid may be considered. In addition, it is recommended to further investigate the long-term effects of the intervention within B-PROOF on cancer incidence, especially since it was hypothesized that folic acid may influence cancer progression, rather than initiation. This hypothesis is not only supported by the fact that we saw an effect on cancer within B-PROOF immediately after the start of the intervention, but also by observations that after starting mandatory folic acid food fortification in the United States and Canada, a temporary increase in colorectal cancer incidence was seen [18], while on the longer term a decrease in colorectal cancer was recently reported [19]. It is conceivable that high levels of folate inhibit the initiation of cancer by ensuring proper DNA-synthesis, while they stimulate latent malignancies by meeting their high folate demands needed to facilitate a higher proliferation rate [18]. If this hypothesis is indeed true, one would expect the unfavorable effect on cancer within B-PROOF to diminish over time, possibly even followed by a favorable treatment effect on the longer term if cancer initiation has been inhibited.

Potential mechanisms

As discussed above, intervention studies aiming to reduce fracture incidence by lowering levels of homocysteine show inconclusive results. Although this may be due to differences in study design, it could reflect the absence of a causal relation between homocysteine and fractures. Therefore, it is of importance to also investigate potential underlying mechanisms to gain insights into whether or not homocysteine could be causally related to fractures. In this thesis, several potential mechanisms of how homocysteine may influence bone metabolism were examined; here, the roles of quantitative ultrasound parameters, bone mineral density (BMD), bone turnover, and DNA-methylation will successively be addressed.

Table 1. Overview of randomized controlled trials investigating preventive effect on fractures of intervention with B-vitamins.

Study, year of publication, [ref]	n	Primary study outcome	Main fracture outcome	n of fractures	Main inclusion criteria	Age (y, mean (SD))	Sex (% female)	Country of study	Ethnicity
Sato, 2005, [9]	628	Unknown	Hip fracture	33 (fractures)	≥65y, residual hemiplegia after stroke	71.4 (5)	53.8	Japan	No information
HOPE 2, 2007, [10]	5522	Composite of CVD-death, nonfatal MI or stroke	Any type of fracture	350 (fractures)	≥55y, pre-existing CVD or DM+CVD risk factor	68.9 (7)	28.2	Canada, USA, Brazil, Western Europe, Slovakia	No information
VITATOPS, 2013, [8]	8164	Composite of stroke, MI or death due to vascular causes	Osteoporotic fracture	145 (persons)	Recent stroke or transient ischemic attack	62.2 (12)	36	Multiple (n=20)	Western European, Oriental, and Asian
B-PROOF, 2014, [11]	2919	Osteoporotic fractures	Osteoporotic fracture	103 (persons)	≥65y, plasma Hcy ≥12 µmol/l, serum creatinine <150 µmol/l	74.1 (7)	50.1	Netherlands	Mainly Caucasian

CVD=cardiovascular disease, DM=diabetes mellitus, MI=myocardial infarction.

Firstly, it has been hypothesized that homocysteine influences collagen cross-linking [20], thereby weakening the structure of the bone. As a proxy of this, we used quantitative ultrasound measurements, of which the parameters have been associated with bone micro-architecture [21]. While levels of homocysteine cross-sectionally associated negatively with these parameters **(Chapter 2.1)**, we did not observe an effect of the treatment with vitamin B_{12} and folic acid **(Chapter 3.2).** However, a small effect was observed on BUA in persons >80 years of age who were compliant to the study treatment. This might support the findings concerning a protective effect of the homocysteine-lowering intervention on fractures in this same subsample, although the clinical relevance of the effect size on BUA may be limited. To our knowledge, this is the first trial investigating such effects. It should be noted that although quantitative ultrasound parameters are known to be related to micro-architectural structure of the bone [21], and predict fractures partly independently of BMD [22], it remains difficult to grasp what they actually represent.

Mean baseline Hcy (µmol/l)	Intervention				Placebo	Follow-up duration (y)	Change in Hcy (%)		Result
	Folic acid (mg)	B_{12} (mg)	B_6 (mg)	D_3 (IU)			Intervention	Placebo	
19.9	5	1.5	-	-	Double placebo	2	-38.1%	+31.2%	RR: 0.22 (0.09-0.53)
11.5	2.5	1	50	-	Matching placebo	5	-19%	+7%	HR: 1.01 (0.82-1.24)
14.2	2	0.5	25	-	Matching placebo	2.8	-23%	+7%	RR: 0.86 (0.62-1.18)
14.4	0.4	0.5	-	600	600 IU vitamin D_3	2	-29%	-1%	HR: 0.84 (0.58-1.22)

It would be interesting to take bone biopsies to assess the level of homocysteine in bone [23] and perform micro-CT (computed tomography) to visually inspect the changes at the microarchitectural level after an intervention like B-PROOF's. In addition, it may be informative to perform high resolution peripheral quantitative CT in vivo. However, drawbacks of these methods are the invasiveness and the use of higher-dose ionizing radiation.

A second hypothesis proposes direct effects of homocysteine levels on BMD. Whether homocysteine influences fracture incidence via BMD has been under debate in the last few years; results were conflicting, but recently a meta-analysis in females showed no significant association [7]. We investigated this second potential mechanism in **Chapters 2.1 and 3.2.** Cross-sectionally, a negative association between plasma homocysteine levels and BMD at both the femoral neck and lumbar spine was observed. However, the effect size was very small. Moreover, no effect of the B-PROOF intervention on BMD in either femoral neck or lumbar

spine was observed. In persons older than 80 years, an effect was also absent. The results of this intervention are in line with findings from previous trials [24, 25]. Therefore, BMD does not appear to be part of the mechanism that could underlie the potentially causal relation between homocysteine and fractures.

Thirdly, we assessed the associations between homocysteine and bone turnover markers (osteocalcin and osteopontin) and the effect of the homocysteine-lowering treatment on these markers. Supplementation with vitamin B_{12} and folic acid did not influence levels of these markers **(Chapter 3.3).** While osteocalcin is an established marker of bone formation [26], which was also associated negatively with femoral neck BMD and borderline significantly positively with fractures in our study, the effects of osteopontin are not that comprehensible. In vitro, it was seen that homocysteine stimulates pre-osteoblastic production and expression of osteopontin [27], but osteopontin is known to be rather pleiotropic [28]. Possibly, assessing a marker of bone resorption, such as the C-terminal telopeptide, would have been more appropriate to exclude a dissociation between bone formation and resorption, but this marker was not available, and such an effect is not very likely.

Lastly, it has been hypothesized that homocysteine could influence DNA-methylation, thereby exerting its influence on fractures. We investigated this hypothesis in **Chapter 2.2** using data of the Rotterdam Study. The ratio of plasma SAM/SAH, which is regarded as a measure for overall methylation capacity, was associated with femoral neck BMD, but not with incident fractures. It should be noted that although SAM/SAH-ratio may reflect methylation capacity, it is known to be tissue-specific and it does not necessarily reflect methylation of specific genes. To our knowledge, this has been the only study in humans so far investigating this phenomenon. Thus, the role of DNA-methylation in the association between homocysteine and fractures merits more investigation; especially the response in gene-specific methylation to the intervention with vitamin B_{12} and folic acid would be interesting to investigate further, as well as specific effects in the oldest old.

Mendelian randomization approach

A more fundamental way to assess causality in relationships is to use the approach of Mendelian randomization. We used this approach within B-PROOF and the GEFOS-consortium (a large-scale global collaboration investigating genetic factors

for osteoporosis) to investigate whether gene variants known to predict plasma homocysteine levels are also related to fracture incidence. If such an association is present, it would support the hypothesis of homocysteine being causally related to incident fractures, since presence of reverse causation and confounding is unlikely when dealing with genetic markers. However, our analysis showed that there was no significant association between a risk score combining the effect of 18 single nucleotide polymorphisms (predicting levels of homocysteine) and risk of fractures or FN-BMD, therefore not supporting causality. However, previous studies did observe associations between the MTHFR C677T genotype and fractures. Inclusion of cohorts originating from countries in which folic acid fortification of staple foods is mandatory may have distorted our findings, since a gene-environment interaction between the MTFHR C677T genotype and plasma folate levels on levels of homocysteine is well-known [29]. This merits further investigation. In addition, the low amount of variance in homocysteine that is explained by the genetic risk score decreases power to observe associations.

So…

Is homocysteine truly a risk factor for osteoporotic fractures, or is it merely a risk indicator, or by others nicely typed as 'innocent bystander' [30] or just 'an expensive creatinine' [31]? The ambiguity of the results presented in this thesis prevent us from drawing firm conclusions about this. In summary, we investigated several pathways that could potentially be underlying the observed association between homocysteine and incident osteoporotic fractures. Based on these results, none of these pathways clearly arose to be of importance in this association. However, although the overall effects of the B-PROOF intervention on fractures were not statistically significant, there does appear to be a trend towards a preventive effect of B-vitamins, especially in the oldest old and such an age-specific trend was also seen for bone ultrasound attenuation.

METHODOLOGICAL CONSIDERATIONS: B-PROOF, WHAT IF…

Talking in retrospect is always easy. So let's start with that. What if B-PROOF had had an intervention period of 10 years? What if... 5000 participants were included in the trial? What if the cut-off level for homocysteine had been 15 µmol/l? Or if

it was even raised to 20 µmol/l? What if the cut-off level for age had not been 65, but 70 years? It all comes down to power. As in every trial, in designing B-PROOF several compromises had to be made to keep the study feasible, both practically and financially. Unfortunately, the results concerning the primary outcome can be regarded to be inconclusive to some extent, and with the current knowledge, some of these decisions possibly would have been made differently.

The intervention within B-PROOF consisted of vitamin B_{12} and folic acid. However, also riboflavin (vitamin B_2) and pyridoxin (vitamin B_6) are co-factors in the metabolism of homocysteine. One could argue that the trial design should have also included these vitamins. However, it is known that especially folate/folic acid supplementation, and to a lesser extent also vitamin B_{12}, are the strongest influencers of plasma homocysteine levels [32]. Addition of B_6 to these vitamins did not have an additional effect on homocysteine levels [32]. Vitamin B_2 supplementation has shown to be ineffective in lowering homocysteine levels [33, 34]. Moreover, especially for vitamin B_{12}, intake and status are of important public health concern in the Netherlands, specifically in the elderly [35]. Reassuringly, the intervention has proven its effect by lowering plasma homocysteine by 4.4 µmol/l on average, while levels in the placebo group remained largely unchanged (-0.2 µmol/l), showing that the intended lowering of homocysteine was indeed accomplished. A recent meta-analysis showed a reduction of 4% in fractures for every 1 µmol/l decrease in homocysteine [7], which implies that within B-PROOF a fracture reduction of 4.2*4%=16.8% could be obtained. The observed HR of 0.84 matches perfectly with these expectations.

Finally, 600 IU of vitamin D_3 were supplemented to both the intervention and placebo group. This was done for ethical purposes; the Dutch Health Council recommends vitamin D supplementation for women above 50 years of age (400 IU) and for men and women above 70 years of age (400 IU) [36], the latter was even raised to 800 IU in 2012 [37]. However, although we do not expect interactions between the B-vitamin intervention and vitamin D_3 to have occurred for any of the study outcomes, this cannot be fully excluded either. In addition, the expected separate effect of vitamin D_3 supplementation may have attenuated any effects of the lowering of homocysteine.

CLINICAL IMPLICATIONS

Fractures have large implications, both for the individual and for society. The individual patient experiences pain, loss of quality of life, immobilization, and has a risk of dying sooner [38]. In addition, the costs of a fracture are high; in the Netherlands, a hip fracture is estimated to cost €14,000 [39]. Currently, several treatments are available to combat osteoporosis and recurrent fractures. For example, bisphosphonates are first-line therapy in case of osteoporosis, and they are able to prevent fractures (RR=0.30-0.80) by inhibiting bone resorption [40]. However, current problems in osteoporosis treatment are, besides the suboptimal screening, also the very low adherence to medication use, partly because of its side effects [40], such as gastro-intestinal problems. Therefore, next to medication, nutritional advices and other preventive strategies are all the more important. Concerning vitamin D, the Dutch Health Council already recommends vitamin D supplementation, since it is known that 41-78% of women above 50 years are vitamin D deficient (<50 nmol/l) [37]. In analogy, the screening of participants for the B-PROOF study showed that approximately 48% of screened participants had mildly elevated plasma homocysteine levels. Prevention of fractures via lowering homocysteine would therefore be an interesting strategy in clinical practice, especially since it is a preventive measure that is not merely a general lifestyle guideline, but is personalized for the patient based on his plasma homocysteine level. The results of the B-PROOF trial show that this might be a relevant strategy in the oldest old. However, since we also observed an unfavorable effect of the intervention on malignancies, this strategy clearly cannot be advised yet based on the current evidence.

FUTURE DIRECTIONS

Taken together, the results reported in this thesis temper the expectations of homocysteine being a modifiable causal factor for fractures. Firstly, no clear mechanisms were found which could support causality in the association between homocysteine and fractures. It should be noted that especially the roles of (tissue-specific) DNA-methylation and bone microarchitecture need further investigation. Secondly, the intervention with vitamin B_{12} and folic acid did not have significant effects on the overall population. However, the effect sizes were of a clinically rel-

evant size (HR=0.84), and in a subgroup of compliant participants above 80 years of age, the effect was substantially larger and statistically significant. Possibly, due to issues discussed above, we had limited power to detect beneficial effects of the treatment. Therefore, a meta-analysis on the currently reported four RCT's could provide more insights, and future research on this topic may need to focus on the oldest old as results were most promising in this subgroup. Clearly, the fact that we observed a higher cancer incidence in B-PROOF's intervention group as compared with the placebo group is of concern. To follow-up both fractures and cancer, it is recommended that both these outcomes are going to be monitored within B-PROOF for a longer duration. Plans for this expansion of the study are currently being made.

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Chapter 6

Summary/samenvatting

6.1 SUMMARY

Osteoporosis is a multi-factorial disease, characterized by low bone mass and deterioration of bone micro-architecture. Due to aging of the world's population, the problem of osteoporosis is expected to increase in the coming decades. In 2004, first evidence was published concerning a potential role of the amino acid homocysteine in fracture incidence; high plasma homocysteine levels were associated with higher risk of osteoporotic fractures. However, whether this relationship was causal remained to be determined. Since it is also known that homocysteine levels can be reduced by supplementation with folic acid and vitamin B_{12}, two vitamins that are essential in the conversion of homocysteine to methionine, homocysteine is a potential modifiable risk factor for fractures and/or osteoporosis. In this thesis, the role of homocysteine in bone and potential protective effects of a homocysteine-lowering treatment are addressed.

Chapter 1.1 provides a general introduction to this thesis, complemented by **Chapter 1.2**, in which the design of the B-PROOF study is discussed in detail. In short, the B-PROOF study is a large (n=2919) randomized controlled trial, for which hyperhomocysteinemic (12-50 µmol/l) men and women aged 65 years and over were randomized to either receive 400 µg of folic acid and 500 µg of vitamin B_{12} daily, or placebo. All participants received 600 IU of vitamin D3 daily. Incident fractures, but also (amongst others) bone mineral density, quantitative ultrasound measurements and blood parameters at baseline and after 2 years of follow-up were assessed.

Chapter 2 describes cross-sectional associations within B-PROOF and within the Rotterdam Study. The Rotterdam Study is a large prospective cohort study in Ommoord, Rotterdam, among middle-aged and elderly men and women. In **Chapter 2.1**, we address the cross-sectional associations of plasma homocysteine levels with bone mineral density and ultrasound parameters. Inverse associations were observed, however based on the effect size they appeared to be of limited clinical relevance. **Chapter 2.2** focuses on the association of the ratio of s-adenosylmethionine to s-adenosylhomocysteine with bone mineral density and incident fractures

within a female sample of the Rotterdam Study. Although an association with bone mineral density was seen, no association of this ratio with fractures was observed.

In **Chapter 3**, effects of the B-PROOF intervention are reported. In **Chapter 3.1**, the effects of the intervention with vitamin B_{12} and folic acid on fracture incidence are described. In the overall study population, no statistically significant beneficial effects of the treatment on fracture incidence were seen. However, exploratory analyses in compliant participants older than 80 years did show a protective effect of the treatment on fractures. Notably, we also observed an unfavorable association of the intervention with cancer incidence; participants who received vitamin B_{12} and folic acid reported significantly more often cancer than participants in the placebo group. **Chapter 3.2** presents the effect of the intervention on bone mineral density and quantitative ultrasound parameters. In general, no effects of the treatment on any of the parameters were observed. In a subgroup of compliant participants aged 80 years and over, a small beneficial effect on bone ultrasound attenuation was seen. **Chapter 3.3** focuses on osteocalcin and osteopontin, two markers reflecting bone turnover. Both were inversely associated with bone mineral density, and osteocalcin was borderline significantly associated with incident fractures. The intervention with folic acid and vitamin B_{12}, which showed to be effective in reducing homocysteine levels, had no effect on levels of osteocalcin and osteopontin, indicating that bone turnover does not appear to play an important role in the previously observed association between homocysteine and incident fractures.

Chapter 4 discusses the results of a Mendelian randomization approach. Using data of B-PROOF and of the GEFOS-consortium, we investigated whether a combination of genes that are known to predict plasma homocysteine levels is able to predict fractures. Such an association is not expected to be influenced by confounding. We did not observe an association of these genes with fractures. These data therefore do not support a truly causal role of homocysteine in the occurrence of fractures.

In the general discussion (**Chapter 5**), the main findings of this thesis are discussed and suggestions for further research are given.

6.2 SAMENVATTING

Osteoporose (botontkalking) is een aandoening met diverse oorzaken die wordt gekenmerkt door een lage botmassa en een verslechtering van de micro-architectuur van het bot. Ten gevolge van de vergrijzing van de wereldbevolking bestaat de verwachting dat het probleem van osteoporose in de komende decennia toe zal nemen. In 2004 werd het eerste bewijs gepubliceerd voor een mogelijke effect van het aminozuur homocysteine op het vóórkomen van osteoporotische fracturen; een hoog plasma homocysteinegehalte werd in verband gebracht met een verhoogd risico op osteoporotische breuken. Echter, het is onzeker of dit verband ook daadwerkelijk oorzakelijk is. Het is bekend dat het homocysteinegehalte omlaag kan worden gebracht door middel van suppletie met foliumzuur en vitamine B_{12}, twee vitamines die essentieel zijn bij de omzetting van homocysteine in methionine. Homocysteine vormt mogelijk dan ook een risicofactor voor osteoporotische fracturen die beïnvloedbaar is. In dit proefschrift wordt de rol van homocysteine in het bot en de mogelijk beschermende rol van homocysteine-verlagende vitaminesuppletie besproken.

Hoofdstuk 1.1 betreft een algemene introductie in het onderwerp, aangevuld met **Hoofdstuk 1.2**, waarin de opzet van de B-PROOF-studie wordt besproken. In het kort betreft B-PROOF een grote (n=2919) gerandomiseerde, gecontroleerde interventiestudie. Mannen en vrouwen van 65 jaar en ouder met een verhoogd homocysteinegehalte (12-50 µmol/l) werden gerandomiseerd over de twee interventie-armen: één groep ontving gedurende twee jaar dagelijks 400 µg foliumzuur en 500 µg vitamine B_{12}, terwijl de andere groep een placebo-tablet innam. Alle deelnemers ontvingen daarnaast 600 IE vitamine D_3. Er werd informatie verzameld over de fracturen die tijdens de studie ontstonden, daarnaast werden aan het begin en aan het einde van de studie botdichtheidsmetingen en een echo van de hiel uitgevoerd en werden diverse bepalingen in het bloed gedaan.

Hoofdstuk 2 beschrijft cross-sectionele associaties binnen B-PROOF en de Rotterdam Studie. De Rotterdam Studie is een grote prospectieve cohort-studie bij mannen en vrouwen van middelbare en oudere leeftijd in Ommoord, Rotterdam. In **Hoofdstuk 2.1** wordt de cross-sectionele associatie van plasma homocystei-

negehaltes met botdichtheid en metingen met ultrasoon geluid behandeld. Er werden omgekeerde verbanden gezien, echter, deze verbanden waren dusdanig klein, dat de klinische relevantie ervan beperkt lijkt. **Hoofdstuk 2.2** richt zich op het verband van de ratio van s-adenosylmethionine en s-adenosylhomocysteine met botdichtheid en fracturen binnen een groep vrouwen uit de Rotterdam Studie. Hoewel we een associatie van deze ratio met botdichtheid zagen, bleek er met fracturen geen verband te zijn.

In **Hoofdstuk 3** worden de effecten van de B-PROOF-interventie gerapporteerd. De effecten van vitamine B_{12}/foliumzuursuppletie op het vóórkomen van fracturen zijn te lezen in **Hoofdstuk 3.1**. In de gehele onderzoekspopulatie zagen we geen statistisch significante effecten van suppletie op de fractuurincidentie. Echter, analyses in therapietrouwe deelnemers die ouder dan 80 jaar waren lieten wel een beschermend effect van suppletie op botbreuken zien. Er dient echter ook te worden opgemerkt dat we een ongunstige associatie van de suppletie met het vóórkomen van kanker zagen; deelnemers die vitamine B_{12}/foliumzuursuppletie ontvingen, meldden significant vaker kanker dan de deelnemers in de placebo-groep. **Hoofdstuk 3.2** beschrijft de effecten van de interventie op botdichtheid en metingen met ultrasoon geluid die de botkwaliteit dienen te weerspiegelen. Er werden geen effecten van suppletie op deze parameters gevonden, echter, in een subgroep van therapietrouwe deelnemers die ouder dan 80 jaar waren werd wel een gunstig, maar klein, effect gezien op één van de maten van botkwaliteit. **Hoofdstuk 3.3** richt zich op osteocalcine en osteopontine, twee markers die botombouw weerspiegelen. Beide markers waren omgekeerd geassocieerd met botdichtheid, daarnaast leek osteocalcine ook geassocieerd te zijn met fracturen, echter dit laatste effect was niet statistisch significant. Suppletie met foliumzuur en vitamine B_{12} bleek weliswaar effectief in het verlagen van het homocysteine-gehalte, maar het had geen effect op de osteocalcine- en osteopontinegehaltes. Dit suggereert dat botombouw geen belangrijke rol lijkt te spelen in het eerder waargenomen verband tussen het homocysteinegehalte en fracturen.

In **Hoofdstuk 4** worden de resultaten van een analyse op basis van Mendeliaanse randomisatie beschreven. Met behulp van data van de B-PROOF-studie en van het GEFOS-consortium is onderzocht of een combinatie van genen, waarvan bekend

is dat ze het plasma homocysteinegehalte beïnvloeden, tevens fracturen kan voorspellen. Er werd echter geen verband tussen deze genen en fracturen waargenomen. Dit ondersteunt dan ook niet de hypothese van een causaal verband tussen homocysteine en het vóórkomen van fracturen.

In de algemene discussie (**Hoofdstuk 5**) worden ten slotte de belangrijkste bevindingen uit dit proefschrift besproken en worden suggesties voor verder onderzoek gedaan.

Appendix

LIST OF ABBREVIATIONS

B-PROOF=B-vitamins for the PRevention Of Osteoporotic Fractures
BMD=bone mineral density
BMI=body mass index
BUA=bone ultrasound attenuation
CBS=cystathionine β synthase
CV=coefficient of variation
DNA=desoxyribonucleic acid
DXA=dual-energy X-ray absorptiometry
FN-BMD=femoral neck bone mineral density
GEFOS=GEnetic Factors for OSteoporosis
GRS=genetic risk score
GWAS=genome-wide association study
HR=hazard ratio
HoloTC=holotranscobalamin
Hcy=homocysteine
IF=intrinsic factor
ITT=intention-to-treat
LS-BMD=lumbar spine bone mineral density
MTHFR=methylenetetrahydrofolate-reductase
MMA=methylmalonic acid
PP=per protocol
QUS=quantitative ultrasound
SAH=s-adenosylhomocysteine
SAM=s-adenosylmethionine
SNP=single-nucleotide polymorphism
SOS=speed of sound
RCT=randomized controlled trial
RS=Rotterdam study

DANKWOORD

Een proefschrift schrijf je niet alleen…

Geachte prof. Uitterlinden, beste André. In het begin van mijn promotietraject was onze samenwerking nog van beperkte omvang. Samen met de rest van het team was ik druk bezig met het verzamelen van alle deelnemers en data, maar van een afstandje bleef je natuurlijk op de hoogte. Naar het einde toe werd de samenwerking intensiever en bleek ook dat je er voor B-PROOF was toen het nodig was. Je nooit aflatende enthousiasme voor B-PROOF en voor wetenschap in het algemeen was enerzijds erg aanstekelijk, maar kon me aan de andere kant ook laten twijfelen of dit 'het wel was' voor mij. Het lijkt me geweldig om zo'n passie voor je werk te hebben als jij! Enorm bedankt voor alle adviezen en je positiviteit.

Geachte dr. Van der Velde, beste Nathalie, ook jou wil ik hartelijk danken voor alles wat je in de afgelopen jaren voor mij hebt gedaan. Het belangrijkste voor mij was wel het altijd in mij gestelde vertrouwen, ook op momenten dat dat er bij mijzelf even aan ontbrak. Je hielp me de grote lijnen te blijven zien en de grote brij aan werk stap voor stap aan te pakken. Mede daardoor ligt dit proefschrift nu hier!

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ABOUT THE AUTHOR

Curriculum vitae

Anke Enneman was born on August 30, 1985 in Bathmen, the Netherlands. After completing secondary school at De Waerdenborch in Holten, she started studying Nutrition and Health at Wageningen University. She obtained both the Bachelor's degree (cum laude, 2006) and the Master's degree (2008). As part of her studies, she completed internships at Friesland Foods and at CeSSIAM in Guatemala. After working as a research assistant at Erasmus MC for some months after graduating, she got appointed as PhD-candidate at the section of Geriatrics in Erasmus MC, Rotterdam, the Netherlands. She worked on the B-PROOF study, the trial on which this thesis is largely based. In addition, she supervised five Bsc./Msc. level students. Currently, she is appointed as data manager/research coordinator at the Van Creveldkliniek in Utrecht, the Netherlands. She lives together with Maarten Braem and together they have a son and a second baby on the way.

PHD-PORTFOLIO

General courses	Year	Workload ECTS)
Biomedical English Writing and Communication	2011	1.5
BROK ('Basiscursus Regelgeving Klinisch Onderzoek')	2011	1.0
Research Integrity	2011	0.15
Specific courses		
Genome Wide Association Analysis	2009	1.0
Principles of Genetic Epidemiology	2009	1.0
Genomics in Molecular Medicine	2009	1.0
Stralingshygiëne	2009	0.5
SNP-course	2009	2.0
MS Access	2010	0.25
SNP-course	2011	2.0
Survival Analysis	2011	1.0
Regression Analysis	2011	1.0
Seminars and workshops		
Weekly scientific seminars Genetics Lab	2009-2014	
CPO Autumn Symposium	2009	0.25
Coeur Research Seminar	2011	0.25
PhD-day	2012	0.25
Orientation on Career	2013	1.0
(Inter)national conferences		
International Congress of Nutrition, Bangkok	2009	1.0 (two posters)
Geriatriedagen, Rotterdam	2010	1.0
Wetenschapsdagen, Antwerpen	2010	1.0 (poster)
Geriatriedagen, Den Bosch	2011	1.0 (poster)
Wetenschapsdagen, Antwerpen	2011	1.0 (poster)
International Association of Gerontology and Geriatrics, Bologna	2011	1.0 (oral)
KNAW-conference 'From-DNA-variations to phenotype', Rotterdam	2011	1.0
International Conference on Homocysteine Metabolism, Lisbon	2011	1.0 (oral)

European Union Geriatric Medicine Society	2011	0.25 (poster)
Geriatriedagen, Den Bosch	2012	1.0 (poster)
Wetenschapsdagen, Antwerpen	2012	1.0 (poster)
Netherlands Consortium for Healthy Aging, Amersfoort	2012	1.0 (poster)
Netherlands Consortium for Healthy Aging, Den Haag	2012	1.0
European Calcified Tissue Society, Stockholm	2012	1.0 (poster)
MolMed day	2012	0.25 (poster)
Fragility Fracture Care symposium, Den Haag	2013	0.5 (oral)
PCDI-Retreat, Heeze	2013	1.0
International Association of Gerontology and Geriatrics, Seoul	2013	1.0 (oral)
Nederlandse Vereniging voor Calcium- en Botstofwisseling	2013	0.5 (oral)
Netherlands Consortium for Healthy Aging, Den Haag	2013	0.5 (poster)
Wetenschapsdagen, Antwerpen	2014	1.0 (oral)
Geriatriedagen, Den Bosch	2014	1.0 (oral

Teaching

Supervision 5 BSc./MSc.-students	2009-2012	10.0

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