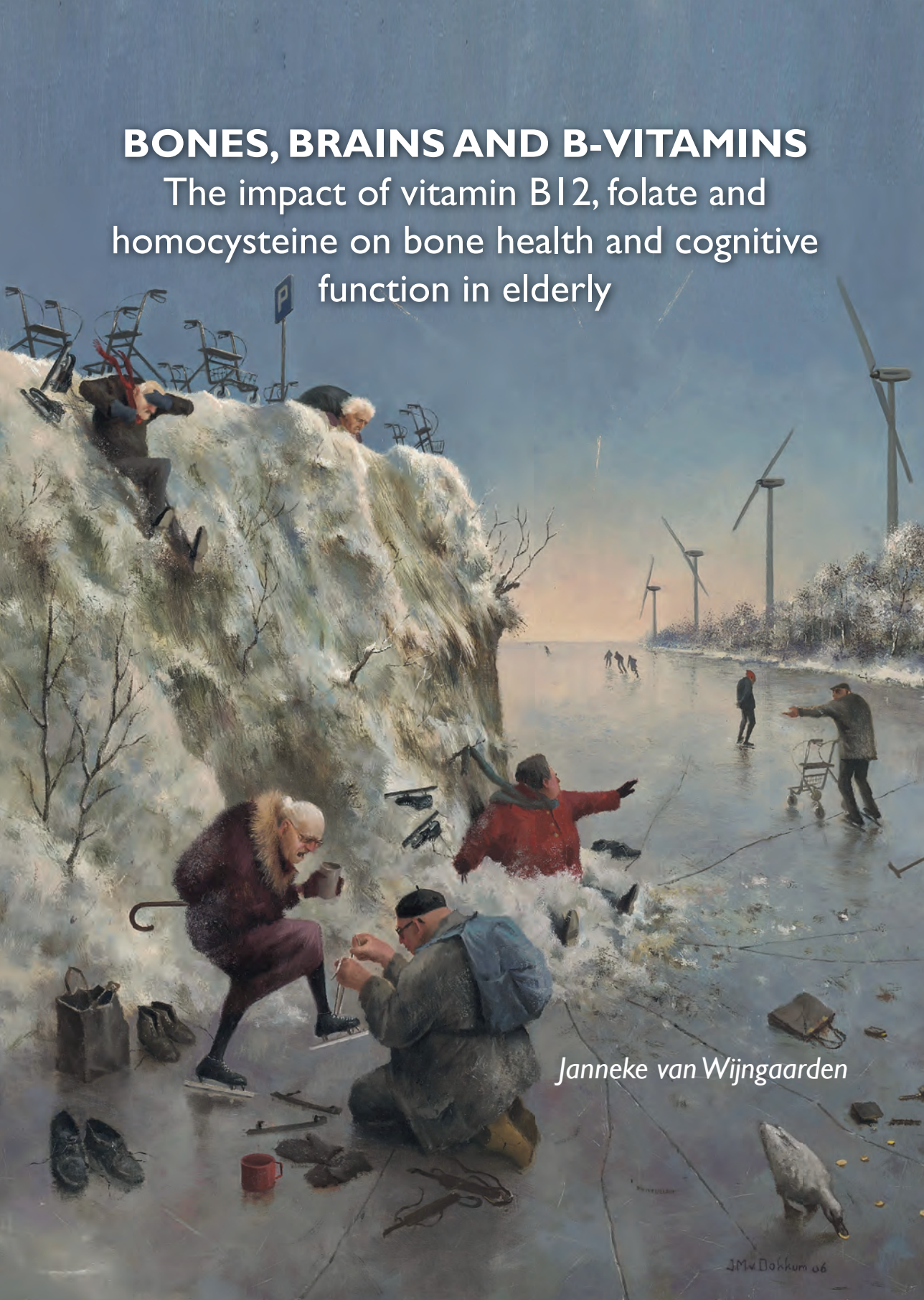
BONES, BRAINS AND B-VITAMINS

The impact of vitamin B12, folate and homocysteine on bone health and cognitive function in elderly



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ABSTRACT

Background

An elevated homocysteine level has been indicated as a risk factor for cardiovascular disease, cognitive decline, and fractures. Supplementation of vitamin B_{12} and folic acid in order to normalize homocysteine levels might be of substantial public health importance as this might reduce the risk for several age-related conditions. This thesis focuses on two health outcomes frequently associated with elevated homocysteine levels and low levels of vitamin B_{12} and folate: osteoporosis and cognitive decline later in life.

Methods

Findings are presented in the context of a model which links dietary intake to biomarkers of nutritional status and subsequently to health outcomes. Two systematic reviews with meta-analyses investigated the current status of knowledge about the association of vitamin B_{12} intake and status with cognitive function, and the association of homocysteine, vitamin B_{12} and folate status with bone health. Baseline data of the B-PROOF study were used to assess 1) the association of vitamin B_{12} intake with status according to four biomarkers (vitamin B_{12}, holotranscobalamin (holoTC), methylmalonic acid (MMA) and homocysteine), 2) the mutual association among these four vitamin B12 biomarkers and 3) the association between homocysteine, vitamin B12 biomarkers, folate and cognitive function. The effect of 2-year daily vitamin B12 (500 μg) and folic acid (400 μg) supplementation on fracture risk was assessed in the B-PROOF study, a large (N=2919) randomized controlled trial in elderly people (aged ≥65 years) with an elevated homocysteine level (≥12.0 μmol/L).

Results

The systematic review of the literature showed no or inconsistent associations of vitamin B_{12} intake with cognitive function. Furthermore, serum vitamin B_{12} was not associated with risk of dementia, global cognition or memory. Studies on MMA and holoTC reported significant associations with risk of dementia, Alzheimer's disease and global cognition. A meta-analysis showed that serum/plasma vitamin B_{12} per 50 pmol/L was borderline significantly associated with a lower fracture risk (RR=0.96, 95% CI = 0.92-1.00) and that homocysteine was significantly associated with a higher fracture risk (RR=1.04, 95% CI = 1.02-1.07). Meta-analyses regarding vitamin B_{12}, folate and homocysteine levels and BMD did not show significant associations.

In the B-PROOF study a doubling of vitamin B_{12} intake was associated with 9% higher levels of vitamin B_{12}, 15% higher holoTC, 9% lower MMA and 2% lower homocysteine, saturation of biomarkers occurs with dietary intakes of >5 μg B_{12}. Levels of MMA and homocysteine were higher when vitamin B_{12} levels were below 330 pmol/L and when holoTC levels were below 100 pmol/L, with a steep elevation when levels of vitamin B_{12} and HoloTC were below 220 and 50 pmol/L respectively. At baseline, levels of vitamin B_{12} and holoTC were not associated with cognitive function in any cognitive domain. Levels of homocysteine (β= -0.009), folate (β= 0.002), MMA (β= -0.163) and the wellness score – a vitamin B_{12} biomarker combination score - (β= 0.048) were significantly associated with the domain of episodic memory. Additionally, homocysteine (β= -0.015) and the wellness score (β= 0.103) were significantly associated with the domain information processing speed.

The B-PROOF intervention did not lower the risk of fracture in the total population (HR=0.84, 95% CI = 0.58-1.22). Per protocol subgroup analysis of elderly aged >80 years, showed a lower risk of fracture in the intervention group (HR=0.28, 95% CI 0.10-0.74). We observed more cancer cases in the intervention group (HR=1.55, 95% CI = 1.04-2.30) compared to the placebo group. We cannot rule out the possibility of accelerated cancer progression as a possible negative side effect.

Conclusion

Our literature reviews and observational data confirm an association of levels of homocysteine, vitamin B_{12} and folate with cognitive function and fracture risk in elderly. Supplementation with vitamin B_{12} and folic acid did not lower the risk of fracture in the total study population. Though positive effects on fracture incidence emerged in elderly aged >80 years, these benefits should be weighed against potential risks.

TABLE OF CONTENTS

I

General introduction

The world population is ageing rapidly. The number of people aged 65 years or older in the Netherlands is expected to grow from 2.6 million in 2010 to 4.5 million in 2050 [1], and worldwide from about 810 million in 2012 to more than 2 billion in 2050, or 22% of the total world population [2]. With ageing, the prevalence of age-related diseases and disabilities increases, together with the subsequent burden to individuals and society. Strategies to prevent or treat age-related disabilities are therefore important for public health. The maintenance of an optimal nutritional status and interventions to improve nutritional status may contribute to the health and well-being of the elderly and could therefore be included in these strategies.

Elevated homocysteine levels are prevalent in 30-50% of Dutch elderly people [3-5], mainly due to low vitamin B_{12} and folate status. An elevated homocysteine level has been indicated as a risk factor for cardiovascular disease, cognitive decline, and fractures [6]. Supplementation of vitamin B_{12} and folic acid in order to normalize homocysteine levels might be of substantial public health importance as this could theoretically reduce the risk of several age-related conditions. In this thesis we focus on two health outcomes frequently associated with elevated homocysteine levels and low levels of vitamin B_{12} and folate with great public health importance in elderly: osteoporosis and cognitive decline.

HOMOCYSTEINE METABOLISM AND RELATED B-VITAMINS

Homocysteine

Homocysteine is a sulphur-containing amino acid which is formed during catabolism of the essential amino acid methionine as a product of numerous transmethylation reactions. Methionine is converted to S-adenosylmethionine (SAM), an important donor of methyl groups, which is subsequently demethylated to S-adenosylhomocysteine (SAH), which is then hydrolyzed to homocysteine (Figure 1). Homocysteine plays a central role in two metabolic pathways: remethylation and transsulfuration. In the remethylation pathway homocysteine is remethylated to methionine, a reaction catalyzed by methionine synthase, which uses vitamin B_{12} (cobalamin) as a co-factor and 5-methyl-tetrahydrofolate (5-MTHF) as a methyl donor. This remethylation takes place in most tissues. In the liver and kidneys, homocysteine is also remethylated by betaine-homocysteine methyl transferase which uses betaine as a methyl donor. In the transsulfuration pathway, limited to the liver and kidneys, homocysteine is irreversibly converted to cystathionine by cystathionine β-synthase, which requires vitamin B6 (pyridoxine) as a co-factor. Cystathionine is further hydrolysed by gamma-cystathionase to cysteine [6-8].

Levels of homocysteine are mainly determined by age, sex and renal function: homocysteine levels increase with age, men have higher levels than women and an impaired renal function raises homocysteine levels [9]. Furthermore, levels of homocysteine largely depend on the B-vitamins required in the homocysteine metabolism. Dietary intake and status of vitamin B_{12} and folate, but also of vitamin B2 and B6 are inversely associated with homocysteine levels. The MTHFR 677C>T polymorphism is associated with reduced MTHFR enzyme activity [10], resulting in about 25% higher homocysteine levels in individuals with the TT genotype in comparison to individuals with the CC genotype [11].

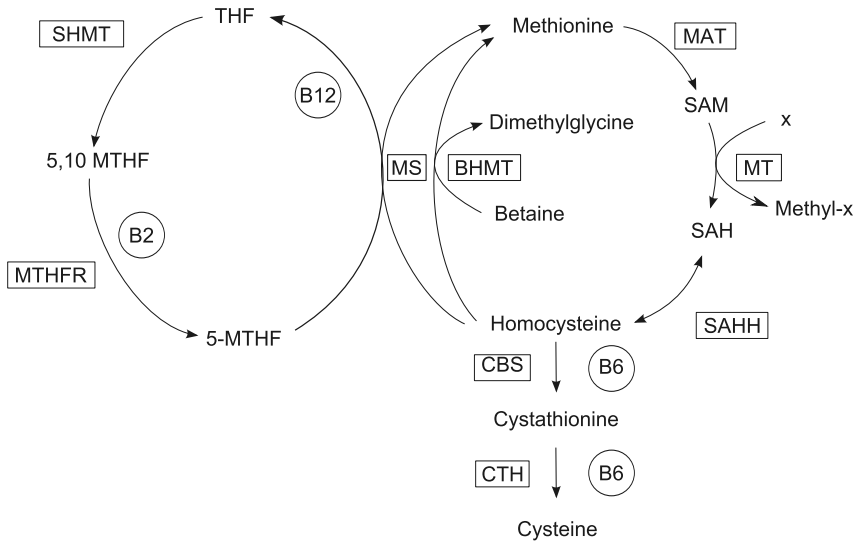

Figure 1. Schematic representation of the folate cycles and homocysteine metabolism. Hcy: homocysteine, 5-MTHF: 5-methyl-tetrahydrofolate, 5,10 MTHF: 5,10-methylene-tetrahydrofolate, SAH: S-adenosylhomocysteine, SAM: S-adenosylmethionine, THF: tetrahydrofolate Enzymes: BHMT: betaine-homocysteine methyltransferase, CBS: cystathionine β-synthase, CTH: cystathionine γ-lyase, MAT: methionine-adenosyltransferase, MS: methionine synthase, MT: methyltransferases, MTHFR: methylenetetrahydrofolate reductase, SAHH: S-adenosylhomocysteine hydrolase, SHMT: serine-hydroxymethyltransferase, B-vitamins as co-factor: B_2: riboflavin (as FAD: flavin adenine dinucleotide), B_6: pyridoxine, B_{12}: cobalamin

Homocysteine and cardiovascular disease

Homocystinuria is a rare disease caused by inborn errors in the homocysteine metabolism, leading to severely elevated homocysteine levels. In the 1960s it was discovered that patients with homocystinuria suffered, among other symptoms, from arterial damage. Concluding from this observation, McCully postulated the 'homocysteine theory': the theory that homocysteine or one of its derivatives is toxic for the vascular wall [12].

In the following decades observational research indicated elevated homocysteine levels as an independent risk factor for cardiovascular diseases [13]. However, evidence from homocysteine-lowering intervention studies with clinical endpoints was lacking for a long time, frustrating the discussion about the causal relation between homocysteine and cardiovascular disease.

When finally, in the course of the first decade of the 21st century, the results of large intervention trials – predominantly secondary prevention trials – were published, they were somewhat disappointing, as none of the interventions showed an overall preventive effect of lowering homocysteine levels with B-vitamins on cardiovascular health outcomes [14-22].

In addition, a meta-analysis of 12 randomized controlled trials, including the above mentioned trials, involving a total of 47,429 participants showed no effect of homocysteine lowering intervention on non-fatal myocardial infarction (pooled RR 1.02 (95% confidence interval 0.95;1.10), stroke (pooled RR 0.91, 95% CI 0.82;1.01) or death by any cause (pooled RR 1.01 (95% CI 0.96-1.07) [23]. Yet, the effect of folic acid on stroke is equivocal as some studies observed a protective effect of folic acid supplementation on stroke [24].

The failure of well-designed intervention studies to confirm homocysteine as a causal risk factor for cardiovascular disease destabilizes the homocysteine theory. To date, there is still no consensus whether elevated levels of homocysteine are the true cause of the observed negative health effects, or low levels of vitamin B_{12} or folate, renal insufficiency or another, yet unknown cause.

Next to cardiovascular diseases, research was extended to other health outcomes. Associations of elevated homocysteine levels were observed with, among others, increased fracture risk, impaired cognitive function and Alzheimer's disease in several observational studies [6, 25-28].

Vitamin B_{12}

Vitamin B_{12} is a water soluble vitamin, present mainly in foods of animal origin, such as meat, fish, eggs, liver, shellfish and dairy products. Vitamin B_{12} is essential for the development and myelination of the central nervous system and maintenance of its normal function. Besides its role as a cofactor for methionine synthase (Figure 1), vitamin B_{12} also acts as a cofactor for methylmalonyl coenzyme A (CoA) mutase, which catalyzes the conversion of methylmalonyl-CoA into succinyl CoA. In vitamin B_{12} deficiency the metabolites homocysteine and methylmalonic acid (MMA) accumulate.

Vitamin B_{12} deficiency is common in elderly, with a prevalence of marginal vitamin B_{12} status around 20% [29, 30]. Symptoms of vitamin B_{12} deficiency include haematological effects like megaloblastic anemia, gastrointestinal effects like glossitis and neurological manifestations such as peripheral neuropathy, myelopathy, subacute combined degeneration, delirium, depression, behavioural disorders and cognitive impairment [31-33]. The diagnosis of clinical vitamin B_{12} deficiency was originally based on the presence of severe megaloblastic anaemia combined with neuropsychological symptoms, but Lindenbaum et al. have showed that neurological symptoms such as cognitive impairment also occurred in the absence of haematological signs [34]. Currently, vitamin B_{12} deficiency is determined by measuring biomarkers of vitamin B_{12} status, although there is no consensus about the best biomarker or biomarkers and accompanying cut-off values [35-37].

Vitamin B_{12} status can be evaluated by serum or plasma total vitamin B_{12} (total B_{12}) levels, holotranscobalamin (holoTC) and the metabolites homocysteine and MMA [38]. Total B_{12} is most commonly used as a marker for vitamin B_{12} status, although MMA is considered a better marker, yet

more expensive and therefore not regularly measured [35, 38, 39]. HoloTC is the fraction of circulating vitamin B_{12} that can be taken up by the cells, and is considered as an early marker for vitamin B_{12} deficiency [40]. HoloTC is a relatively new marker and currently not widely used in clinical practice [40, 41]. Homocysteine levels are, though a sensitive marker, not specific for vitamin B_{12} deficiency, since homocysteine levels are not only affected by vitamin B_{12} status, but also, among others, by folate status [38, 42].

Folate and folic acid

Folate is a vitamin present in many food items, such as green leafy vegetables, fruits, meat and dairy products. Folate is a generic term for a family of compounds including folates naturally occurring in foods, and folic acid, a synthetic form used in food fortification and supplements. Folate accepts and donates one-carbon groups, which makes it important for DNA synthesis, DNA methylation and for the conversion of amino acids, such as in the homocysteine metabolism [7]. Folate from the diet is metabolized to 5-methyl-tetrahydrofolate (5-MTHF) which acts as a methyl donor in the homocysteine metabolism (Figure 1). In the conversion of 5,10-methylene-tetrahydrofolate (5,10-MTHF) to 5-MTHF by the enzyme methylene tetrahydrofolate reductase (MTHFR), another B-vitamin, vitamin B2 (riboflavin), plays a role since flavin adenine dinucleotide (FAD), a metabolite of vitamin B2, serves as a cofactor for MTHFR [8].

High intakes of folic acid around the conception have been shown to reduce the risk of congenital neural tube defects [43, 44]. To ensure a sufficient folate status for women of childbearing age, the fortification of flour with folic acid is nowadays mandatory in over 60 countries worldwide [45]. In the Netherlands, there is up to now no mandatory fortification with folic acid, though several food products, such as breakfast cereals, are fortified. Women who have a wish to become pregnant are advised to take a daily supplement with 400 μg of folic acid periconceptionally [46].

Folate status can be evaluated by measuring folate in serum or in erythrocytes. It is generally established that serum folate reflects short-term folate intake, and erythrocyte folate reflects long-term intake [47], but serum folate levels are nowadays acknowledged as an adequate marker for folate status in epidemiological studies [48].

Supplementation with folic acid is highly effective in lowering homocysteine levels (25% decrease) [49], but the intake of high doses of folic acid alleviates vitamin B_{12}-related anaemia and may therefore delay the diagnosis of vitamin B_{12} deficiency [50]. As vitamin B_{12} deficiency is common in elderly the addition of vitamin B_{12} next to folic acid supplementation could be recommended. Vitamin B_{12} supplementation contributes further to the lowering of homocysteine levels with an additional 7% [49, 51]. The addition of vitamin B6 next to folic acid and vitamin B_{12} supplementation does not contribute further to lowering homocysteine levels [49]. We therefore focus in this thesis on homocysteine, vitamin B_{12} and folate.

AGE-RELATED HEALTH OUTCOMES: OSTEOPOROSIS AND COGNITIVE DECLINE

Osteoporosis

Osteoporosis is a chronic, multifactorial disorder which is characterized by low bone mass and micro- architectural deterioration of bone tissue [52]. A more pragmatic approach to the definition of osteoporosis is the occurrence of an osteoporotic fracture, since the occurrence of a fracture is in most cases the first clinical sign of the presence of osteoporosis. Fractures lead to pain, decreases in physical and social functioning, loss of quality of life and increased mortality in the case of hip fractures [53]. Osteoporotic fractures are a major cause of morbidity and disability in elderly.

Risk factors for osteoporosis

The etiology of osteoporosis is complex and influenced by multiple risk factors, including non-modifiable risk factors as well as lifestyle and dietary factors.

Important non-modifiable factors for an increase in osteoporosis risk include: an increasing age; sex: women have a higher risk than men; a family history of fracture; the occurrence of a previous fracture; ethnicity: osteoporosis is more prevalent in Caucasian and Asian populations; the long-term use of glucocorticoids; and the presence of rheumatoid arthritis [54].

Lifestyle factors influencing osteoporosis risk include: high intakes of alcohol, smoking, low body mass index (BMI), sedentary lifestyle and frequent falls. Well established nutritional factors include low intakes of vitamin D, calcium, and protein [54]. A combination of vitamin D and calcium supplementation have been shown to decrease the incidence of fractures [55] and increased physical activity lowers the risk of osteoporosis [56-58]. Several observational studies showed that elevated homocysteine levels and low levels of vitamin B_{12} and folate are associated with higher fracture risk in elderly people [25, 26, 59-63].

Two randomized controlled trials (RCTs) investigated the effect of B-vitamin supplementation on fracture risk as a secondary outcome [64, 65]. These studies showed conflicting results and had a low generalizability to the older population. Sato et al. observed a large protective effect of 2-year daily supplementation of 1.5 mg vitamin B_{12} and 5 mg folic acid on hip fracture risk [64]. In the HOPE-2 trial no effect of 5-year daily supplementation of vitamin B_{12} (1 mg), folic acid (2.5 mg) and vitamin B6 (50 mg) was observed on fracture incidence [65]. Evidence that B-vitamin supplementation may lower the risk of fracture is therefore still scarce: there is a need for well-designed RCTs to establish the possible role for B-vitamins in fracture prevention.

Mechanisms underlying the association between homocysteine, vitamin B_{12}, folate and osteoporosis

There are several suggested mechanisms for the association between homocysteine, vitamin B_{12} and folate with bone health. Homocysteine may interfere with collagen cross-linking. Cross-links are important for the stability and strength of the collagen network and interference in cross-link formation

could cause an altered bone matrix, resulting in more fragile bones [66]. Vitamin B_{12} has been shown to stimulate osteoblast (bone forming cell) proliferation and alkaline phosphatase activity [67] and vitamin B_{12} deficiency has been associated with impaired functional maturation of osteoblasts [68, 69]. Other research shows evidence of osteoclast (bone resorption cell) stimulation in the presence of high homocysteine and low vitamin B_{12} concentrations [70, 71]. The role of folate in bone metabolism is likely to be indirectly related via elevated homocysteine levels [72].

Cognitive decline and dementia

Cognitive functioning is the process of receiving, processing, storing and using information. Cognitive functioning decreases with ageing. This decrease includes normal, age-related cognitive decline, but also more rapid cognitive decline, leading to cognitive impairment, and dementia or Alzheimer's disease.

Dementia is a syndrome due to disease of the brain, usually chronic, characterized by a progressive, global deterioration in intellect including memory, learning orientation, language, comprehension and judgment [73]. Alzheimer's disease is the most common form of dementia and contributes to about 60–70% of dementia cases. Other major contributors include vascular dementia, dementia with Lewy bodies, and a group of diseases that contribute to frontotemporal dementia. The total number of people with dementia worldwide is estimated at 35.6 million in 2010 and is projected to nearly double every 20 years, to 65.7 million in 2030 and 115.4 million in 2050 [73].

Risk factors for cognitive decline and dementia

Multiple risk factors have been identified for cognitive decline and dementia. Major non-modifiable risk factors include: an increasing age; sex: women have a higher risk than men; carrying the apolipoprotein E4 allele; and the presence of vascular pathologies such as high blood pressure, diabetes and cardiovascular disease [74].

Lifestyle factors that affect cognitive decline and dementia include: education level: a higher education level seems protective against cognitive decline and dementia; marital status and a social network: being married or living together and having a large social network with a lot of activities seems to be protective [74]. Smoking, high alcohol consumption, a low physical activity level, a higher BMI in midlife and an accelerated decline in BMI in later life are risk factors [74]. Nutritional factors associated with a protective effect on the development of cognitive decline and dementia include high intakes of dietary vitamin E, omega-3 fatty acids and fatty fish [75]. Higher intakes of saturated fatty acids seem to increase the risk for cognitive decline and dementia [75]. Evidence however is primarily observational, intervention studies are lacking or show inconclusive results. Elevated levels of homocysteine and low levels of vitamin B_{12} and folate have been associated with cognitive impairment and an increased risk of dementia [75-77]. Here, the level of evidence is also mainly observational; intervention studies with vitamin B_{12} and folic acid supplementation are of miscellaneous quality and show inconclusive results [77].

Mechanisms underlying the association between homocysteine and cognitive decline

Several mechanisms have been postulated by which homocysteine, vitamin B_{12} and folate may affect cognitive function, the most important being: Hypomethylation as a result of low vitamin B_{12} and folate levels and elevated homocysteine levels causes myelin damage and disturbed neurotransmitter metabolism [78]; homocysteine is suggested to be neurotoxic [79]; in addition, high levels of homocysteine could cause structural vascular changes in the brain [80], leading to brain atrophy and white matter hyperintensities [81, 82]; high levels of MMA may also induce neurological damage [83].

RATIONALE AND OUTLINE OF THIS THESIS

This thesis largely builds on the B-PROOF study and its related evidence base. The B-PROOF study is a large, multicenter RCT, initiated to investigate the effect of 2-year vitamin B_{12} and folic acid supplementation on fracture risk in elderly people (aged ≥65 years) with an elevated homocysteine level (≥12 μmol/L). At baseline, blood samples were obtained and participants (N=2919) filled out questionnaires about their health and lifestyle and underwent a broad screening including measurements of anthropometry, cognitive function and physical function. This resulted in an extensive dataset, which was used to describe several cross-sectional associations. Furthermore, we performed systematic literature reviews with meta-analyses to give an overview of the evidence available on the association of B-vitamins with bone health and cognitive function. These reviews were written within the context of the EURRECA network of excellence, which aimed at harmonizing the process of setting micronutrient recommendations across Europe with special focus on vulnerable population groups, including elderly people [84, 85].

Chapter 2 describes the design of the B-PROOF study. In **Chapter 3** we explored the association of vitamin B_{12} intake with 4 biomarkers for vitamin B_{12} (total B_{12}, holoTC, MMA and homocysteine) at baseline of the B-PROOF study. Two systematic reviews with meta-analyses investigate the association of vitamin B_{12} intake and status with cognitive function (**Chapter 4**), and the association of homocysteine, vitamin B_{12} and folate status with fracture incidence and BMD (**Chapter 5**). In **Chapter 6** we analyzed the baseline association of vitamin B_{12} and folate status with cognitive function in the B-PROOF study population. **Chapter 7** describes the effect of 2-year vitamin B_{12} and folic acid supplementation on fracture risk in elderly people: the main outcome of the B-PROOF study. In the final chapter, **Chapter 8**, we summarize the main findings of the research conducted for this thesis and reflect on our methodology and opportunities for future research.

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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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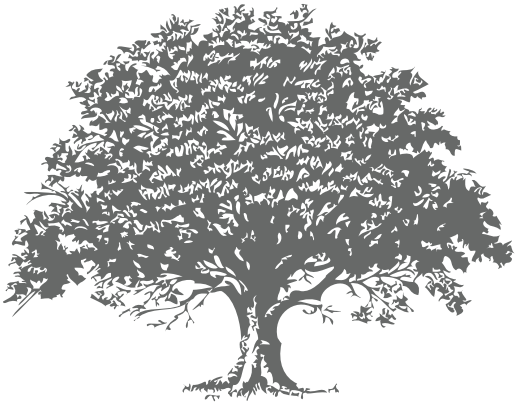

B·PROOF

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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

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 (≥12μmol/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 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 (NTR 1333) 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 independent 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 (≥15μmol/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

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 (≥ 12μmol/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 (www.trialregister.nl) 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 [10], 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.

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, 10] 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, 10].

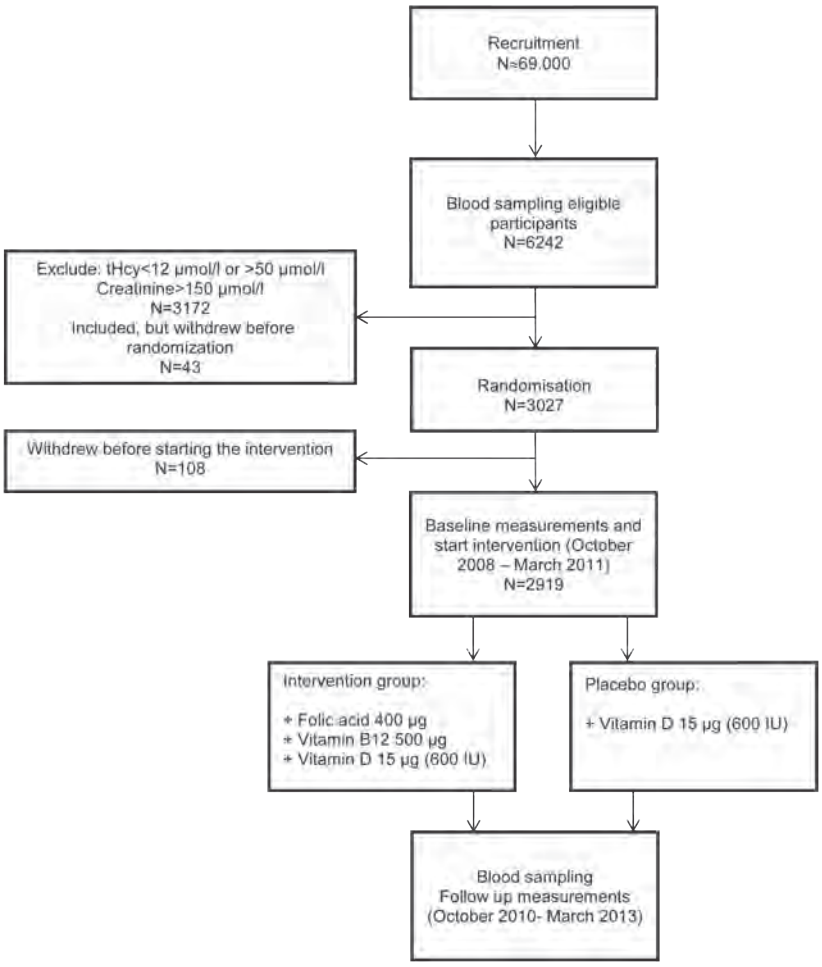

Figure 1. Recruitment and baseline measurements in participants of the B-PROOF study

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

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/cm2) 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 40mm 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 (SD: 6.5) and 50% was female. Median plasma homocysteine concentration was 14.1 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.

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 [13.0-16.6]	14.6 [13.1-16.8]	14.1 [12.9-16.3]
Serum creatinine (μmol/L)#	82.0 [71-94]	90.0 [81.0-101.0]	73.0 [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 [84.0-192.9]	116.3 [72.5-177.0]	142.9 [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].

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.

COMPETING INTERESTS

The B-PROOF study has received funding so far from NZO (Dutch Dairy Association), Zoetermeer, and Orthica, Almere, the Netherlands. The sponsors have no role in the design or implementation of the study, data collection, data management, data analysis, data interpretation, or in the preparation, review, or approval of the manuscript.

AUTHORS' CONTRIBUTIONS

JPVW, EMBB, KMAS, AWE, SCVD implement the practical realisation of the study.

RAMDR, LCPGMDG and PL designed and initiated the trial. LCPGMDG is the principal investigator. LCPGMDG, PL and AGU represent the scientific committee of the B-PROOF study. RAMDR is the overall trial coordinator and NMVS and NVDV are local trial coordinators. RAMDR, NMVS, NVDV, JPVW, AWE, SCVD, KMAS, MCZ, JBJVM and JB planned and coordinated the study, JPVW, AWE, SCVD, KMAS and EMBB are responsible for data collection and management and perform statistical analyses, interpret results, JPVW drafted the manuscript. All authors assisted in interpretation of the results, critically reviewed the manuscript, and approved the final draft.

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3

Associations of vitamin B12 intake and related biomarkers in a Dutch elderly population

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ABSTRACT

Background

Vitamin B_{12} status is measured by four plasma/ serum biomarkers: total vitamin B_{12}, holotranscobalamin (holoTC), methylmalonic acid (MMA) and homocysteine (tHcy). Associations of B_{12} intake with holoTC and tHcy and associations between all four biomarkers have not been extensively studied. This study therefore investigates associations between B_{12} intake and biomarkers and associations between biomarkers.

Methods

Levels of total B_{12}, HoloTC, MMA and tHcy were determined in 2919 elderly people (≥65 years) with elevated tHcy levels (≥12 μmol/L). B_{12} intake was assessed in a subsample. Multivariate regression analysis assessed the association between intake and status. With restricted cubic spline plots we explored the dose-response association between B_{12} intake and biomarkers, and the association of total B_{12} and holoTC with tHcy and MMA.

Results

A doubling of B_{12} intake was associated with 9% higher total B_{12}, 15% higher HoloTC, 9% lower MMA and 2% lower tHcy. Saturation of biomarkers occurs with dietary intakes of >5 μg B_{12}. Spline regression showed that levels of MMA and tHcy started to rise when vitamin B_{12} levels fall below 330 pmol/L and with HoloTC levels below 100 pmol/L, with a sharp increase with levels of B_{12} and HoloTC below 220 and 50 pmol/L respectively.

Conclusion

In this study we observed a significant association between vitamin B_{12} intake and vitamin B_{12} biomarkers and between the biomarkers. The observed inflections for total B_{12} and holoTC with MMA and tHcy could indicate cut-off levels for further testing for B_{12} deficiency and determining subclinical B_{12} deficiency.

INTRODUCTION

Vitamin B_{12} is water-soluble vitamin, essential for neurological functioning and the production of cells, and is present in foods of animal origin. Vitamin B_{12} status is of concern in the elderly population, as it is estimated that about 20% of the elderly have a suboptimal vitamin B_{12} status [1-3].

Vitamin B_{12} status can be evaluated by serum or plasma total vitamin B_{12} (total B_{12}) levels, holotranscobalamin (holoTC) and the metabolites homocysteine (tHcy) and methylmalonic acid (MMA) [4]. HoloTC is the fraction of circulating vitamin B_{12} that can be taken up by the cells [5]; the metabolites homocysteine (tHcy) and MMA accumulate with vitamin B_{12} deficiency, due to a lack of vitamin B_{12} as cofactor for the enzymes methionine synthase and methylmalonyl-CoA mutase, respectively. Methionine synthase remethylates homocysteine to methionine and methylmalonyl-CoA mutase converts methylmalonyl-Coa to succinyl CoA.

Total B_{12} is most commonly used as a marker for vitamin B_{12} status, although MMA is considered a better marker, yet more expensive and therefore not regularly measured [4, 6, 7]. HoloTC is a relatively new marker and currently not widely used in clinical practice [5, 8]. As tHcy level is not only affected by vitamin B_{12} status, but, among others, also by folate status, elevated tHcy levels are, though a sensitive marker, not specific for vitamin B_{12} deficiency, but also for folate deficiency [4, 9].

Levels of biomarkers for vitamin B_{12} status depend on vitamin B_{12} intake from food and supplements and on body stores. Until now, research on the association of daily vitamin B_{12} intake with biomarker status focused mainly on total B_{12} and MMA as a biomarker for vitamin B_{12} status[10-15]. Less is known about the association between vitamin B_{12} intake and levels of HoloTC and tHcy. Furthermore, studies investigating associations between biomarkers of vitamin B_{12} generally focus on only two or three of the biomarkers [16, 17]. In this study, we explored: 1) the association between vitamin B_{12} intake and four biomarkers of vitamin B_{12} status (total B_{12}, HoloTC, MMA and tHcy), and 2) the mutual association among the four biomarkers for vitamin B_{12} status in elderly people participating in the B-PROOF study. We furthermore assessed the prevalence of atrophic gastritis, as this condition is postulated as an important cause for vitamin B_{12} deficiency in elderly [2].

MATERIALS AND METHODS

Subjects

In this cross-sectional study, baseline data of the B-PROOF study were used. The B-PROOF study is a randomized controlled double blind intervention study investigating the effect of daily vitamin B_{12} and folic acid supplementation on fracture risk in a general elderly population (≥65 years) with elevated homocysteine levels (≥12 μmol/l). The study population and the design of the study have been described in detail elsewhere [18].

The study was carried out in three study centers in The Netherlands; Erasmus MC (EMC, Rotterdam), VU University Medical Center (VUmc, Amsterdam) and Wageningen University (WU, Wageningen). A total number of 2919 people were included in the intervention. In this paper we report dietary intake data from the Wageningen subsample and biomarker data for the whole population. All participants gave written informed consent before the start of the study. The Medical Ethics committee of WU approved the study protocol, and the Medical Ethics committees of EMC and VUmc gave approval for local feasibility.

Dietary intake

Habitual dietary intake was estimated by a Food Frequency Questionnaire (FFQ), which was developed to assess the intake of energy, macronutrients, type of fat, vitamin B_{12}, folate, vitamin D, and calcium. This FFQ was based on a questionnaire which was developed and validated for the assessment of energy, total fat, fatty acids and cholesterol [19]. This basic FFQ was updated using the Dutch National Food Consumption Survey of 1998 and extended with questions to estimate folate, vitamin B_{12}, calcium, and vitamin D intake [20]. In addition, new foods on the market relevant for the purpose of the FFQ were included. Finally, the FFQ consisted of 190 food items and which covers at least 90% of energy and nutrient intake [21]. Furthermore, use of vitamin B_{12}, folic acid and vitamin D supplements apart from the study supplements was registered as well. The FFQ was sent to all participants at the WU (n=856).

Biochemical markers: laboratory analysis

Blood samples were obtained from participants in a standardized way in a fasted state or after a light breakfast. Plasma tHcy was measured using the Architect i2000 RS analyser (VUmc, intra assay CV=2%, inter assay CV=4%), HPLC method (WU, intra assay CV=3.1%, inter assay CV=5.9%) and LC-MS/MS (EMC, intra assay CV=5.5% at 14.1 μmol/L, inter assay CV=1.4% at 13.7 μmol/L). According to cross-calibration, outcomes of the three centres did not differ significantly (results not shown). Serum vitamin B_{12} and folate were measured using immunoelectrochemiluminescence assay (Elecsys 2010, Roche, Almere, The Netherlands) (intra assay CV vitamin B_{12} 5.1% at 125 pmol/L and 2.9% at 753 pmol/L; intra assay CV folate: 5.9% at 5.7 nmol/L and 2.8% at 23.4 nmol/L) [22]. Serum HoloTC was determined by the AxSYM analyser (Abbott Diagnostics, Hoofddorp, The Netherlands) (intra assay CV<8%) and serum MMA was measured by LC-MS/MS (intra assay CV=8.1% at 0.18 μmol/L, inter assay CV=1.6% at 0.24 μmol/L) [23]. Serum creatinine was measured with the enzymatic colorimetric Roche CREA plus assay (intra assay CV=2%). DNA was isolated from buffy coats to determine MTHFR C677T genotype using the Illumina Omni-express array.

Serum pepsinogen I (PGI) and II (PGII) concentrations were measured at WU in a subsample (n=762), by ELISA technique (Epitope Diagnostics, San Diego, CA, USA) (intra assay CV<10%).

Other variables

Smoking (never, former, and current smoker) and alcohol consumption (none, light, moderate, and excessive) were based on self-report. Classification of alcohol use was based on the number of days per week alcohol was consumed and the number of drinks per time.

Statistical analysis

Data are presented as mean with SD or median with interquartile range. Associations between vitamin B_{12} intake and status and between status markers were examined using Spearman's correlation coefficient, because biomarker data were not normally distributed. As levels of tHcy are also influenced by levels of folate [24], the correlation between vitamin B_{12} and tHcy was adjusted for serum folate level. Both intake and status markers were transformed to their natural-log as we assumed a dose response association between vitamin B_{12} intake and status with a saturation of biomarkers at higher levels of intakes [15]. With the log-transformed variables we performed multivariate regression analysis. Based on their biological relevance and their contribution to a change in the regression coefficient of the variable of interest of at least 10%, age, sex, smoking status, alcohol intake and supplement use were included as covariates in the multivariate linear regression analysis. Betas are interpreted as follows: when vitamin B_{12} intake was e-times higher, biomarker status was $(e\beta -1)*100\%$ higher, consequently, a doubling of vitamin B_{12} intake was associated with a higher total B_{12} status of approximately $(2\beta-1)*100\%$.

We used restricted cubic spline plots to explore the dose-response association between vitamin B_{12} intake and status markers, and the association among serum vitamin B_{12} and tHcy, MMA and holoTC. With these splines we were able to estimate visually an optimal vitamin B_{12} intake per biomarker status and to estimate a breakpoint in the association between total B_{12} status, HoloTC, MMA and tHcy. Following the analyses of Vogiatzoglou et al. we excluded the lowest and highest 2.5 percentiles of total B_{12} and HoloTC in the status-status spline to smoothen the spline [17]. Cubic spline functions were tested in regression models at three, four and five knots using spline plots and likelihood ratio tests [25].

All analyses were performed using SAS statistical software, version 9.2 (SAS institute Inc, Cary, NC), except the restricted cubic splines, which were performed using R statistical software version 2.12.2 (www.R-project.org). Significance was accepted at $p<0.05$.

RESULTS

The study population comprises 2919 participants, with a mean age of 74 ± 7 years and 50% were men (Table 1).

Table 1. Characteristics of the study population

Characteristic	Value[a]
Demographic measures (n=2919)	
Age (y)	74.1 ± 6.5
Male	1459 (50%)
Education:	
-low	53%
-middle	21%
-high	26%
Current smoker	10%
BMI (kg/m^2)	27.1 ± 4.0
MTHFR genotype	
677 CC	46%
677 CT	41%
677 TT	13%
Biochemical measures (n=2919)	
Plasma tHcy (µmol/L)	14.4 (13.0 - 16.6)
Serum holoTC (pmol/L)	64.0 (46.0 -85.0)
Serum total B12 (pmol/L)	266.4 (208.6-342.5)
Serum MMA (µmol/L)	0.23 (0.18 - 0.30)
Serum folate (nmol/L)	18.7 (14.8 - 24.0)
Serum creatinine (µmol/L)	84.0 ± 18.3
Pepsinogen (n=720):	
pepsinogen I (ng/ml)	205.8 ± 102.5
pepsinogen II (ng/ml)	15.5 ± 10.6
PGI/PGII (ratio)	15.3 ± 7.7
% atrophic gastritis (ratio <1.6)	3%
Dietary intake (n=603)	
Dietary vitamin B12 intake (µg/day)	4.1 ± 2.0
Dietary folate intake (µg/day)	192 ±54
Energy intake (kcal/day)	2006 ± 473

BMI, Body Mass Index; MTHFR, Methylenetetrahydrofolate Reductase; tHcy, total homocysteine; holoTC, holotranscobalamin; MMA, methylmalonic acid

[a] Values are presented as mean ± SD, number (%) or median (IQR)

Vitamin B_{12} intake

Six hundred sixty four (664) participants (78%) returned their FFQ; after excluding data from 61 participants with unrealistically high or low energy intake as defined by Goldberg [26] valid intake data of 603 participants were available. Mean dietary intake of vitamin B_{12} was 4.1 µg/day, 9% of the participants used a dietary supplement containing vitamin B_{12} (median 0.85 µg/day) (Table 1). Six per cent of the participants had a vitamin B_{12} intake lower than the Dutch estimated average requirement (EAR) of 2.0 µg/day.

Association between vitamin B_{12} intake and vitamin B_{12} status markers

Dietary vitamin B_{12} intake was significantly correlated with total B_{12} (r= 0.13), holoTC (r= 0.11) and MMA (r= -0.12), all p<0.01, but not with tHcy (r= -0.06, p=0.12). Correlations did not alter substantially when total vitamin B_{12} intake was taken into account (dietary intake plus additional supplement intake).

The association between dietary vitamin B_{12} intake and B_{12} status was further analysed with multivariate regression analysis. Vitamin B_{12} intake was significantly associated with all biomarkers (Table 2). A doubling of vitamin B_{12} intake was associated with an approximately 9% higher total B_{12}, 15% higher HoloTC, 9% lower MMA and 2% lower tHcy status (Table 2).

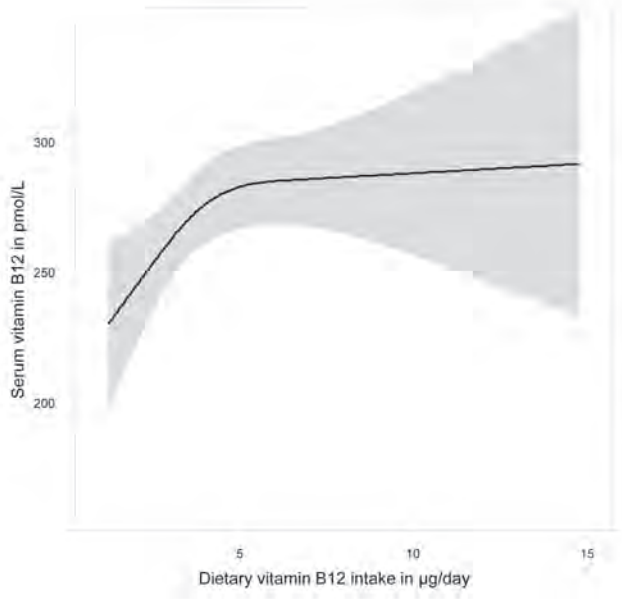
Figures 1a-d show the association between vitamin B_{12} intake and B_{12} status markers, adjusted for age, sex, smoking, alcohol intake and supplement use, as visualised with restricted cubic splines with 3 knots. These figures suggest a saturation of the biomarkers around an intake of approximately 5 µg vitamin B_{12}/day.

Table 2. Multivariate regression analysis for the association between loge dietary vitamin B12 intake and $\log_e$ biomarker (n=603)

Biomarker	Model	β (SE)	95% CI	p-value	Change for doubling B12 intake[a]
Log_e total B12	Crude	0.12 (0.04)	0.05 to 0.19	0.001	
	Adjusted[b]	0.13 (0.04)	0.06 to 0.20	0.000	9%
Log_e HoloTC	Crude	0.16 (0.05)	0.06 to 0.25	0.001	
	Adjusted[b]	0.20 (0.05)	0.10 to 0.30	<0.0001	15%
Log_e MMA	Crude	-0.14 (0.04)	-0.22 to -0.06	0.001	
	Adjusted[b]	-0.13 (0.04)	-0.21 to -0.05	0.003	-9%
Log_e tHcy	Crude	-0.03 (0.02)	-0.07 to 0.00	0.066	
	Adjusted[c]	-0.04 (0.02)	-0.07 to -0.00	0.037	-2%

Hcy, total homocysteine; holoTC, holotranscobalamin; MMA, methylmalonic acid
a a doubling of vitamin B12 intake is associated with a % change in biomarker status
b models were adjusted for age, sex, smoking, alcohol intake and supplement use
c tHcy model was adjusted for age, sex, serum levels of folic acid, smoking, alcohol intake and supplement use

a.



b.

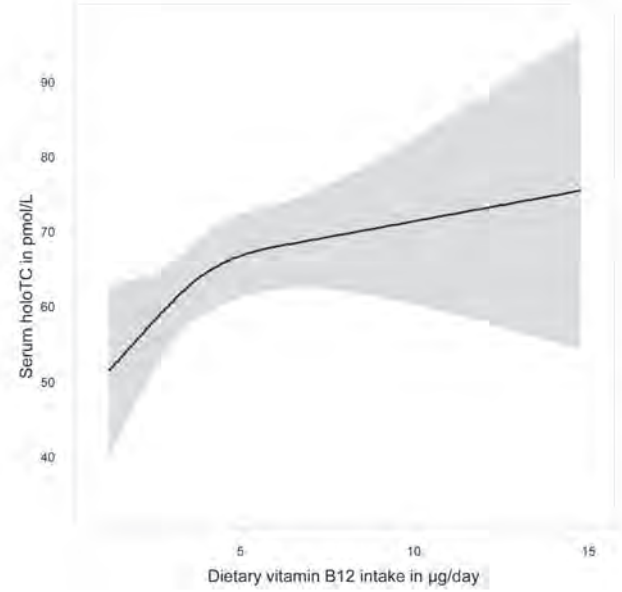
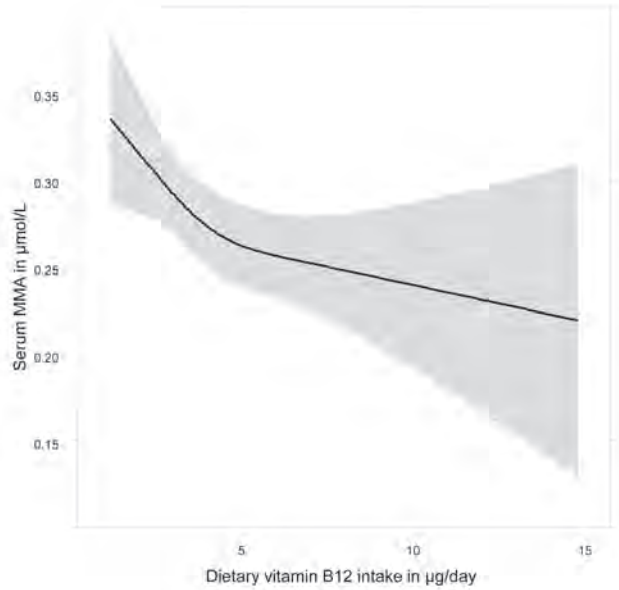


Figure 1.

a: Association between vitamin B_{12} intake and total vitamin B_{12}, adjusted for age, sex, smoking, alcohol intake and supplement use, **b:** Association between vitamin B_{12} intake and HoloTC, adjusted for age, sex, smoking, alcohol intake and supplement use.

c.



d.

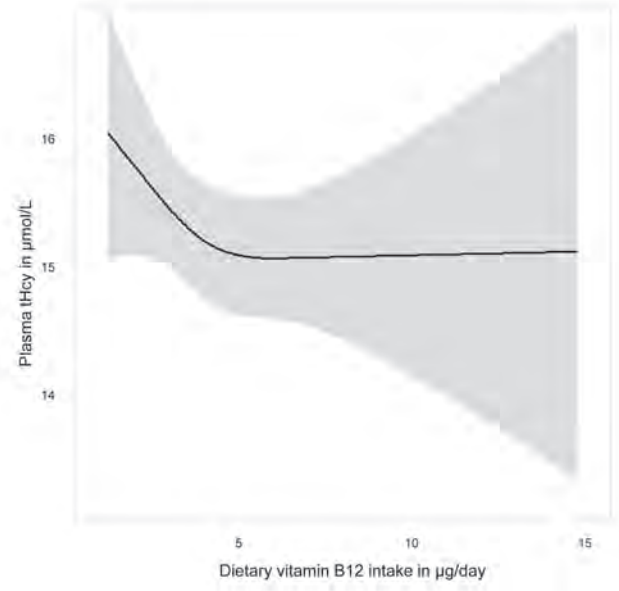


Figure 1. Continued

c: Association between vitamin B_{12} intake and MMA, adjusted for age, sex, smoking, alcohol intake and supplement use, **d:** Association between vitamin B_{12} intake and tHcy, adjusted for age, sex, smoking, alcohol intake and supplement use.

Association between the different biomarkers for vitamin B_{12} status

Correlation coefficients of the four biomarkers of vitamin B_{12} status are shown in Figure 2. B_{12} status markers were correlated with each other ($p<0.0001$), the correlation between total B_{12} and tHcy was adjusted for folate status. Adjustment for MTHFR genotype (CC, CT or TT) did not alter the correlations.

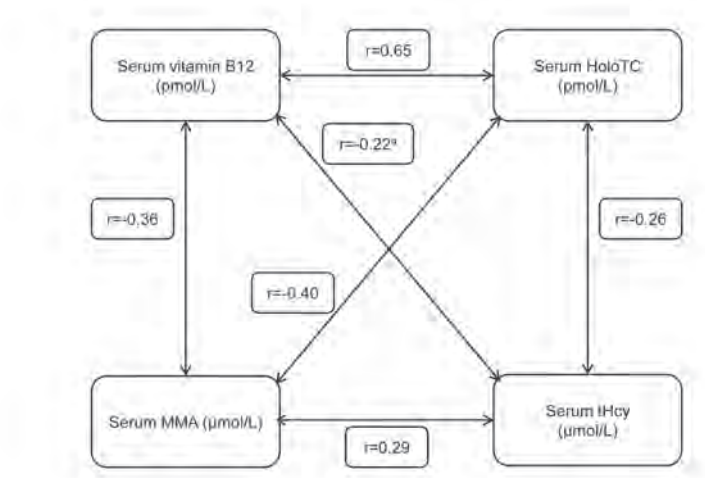


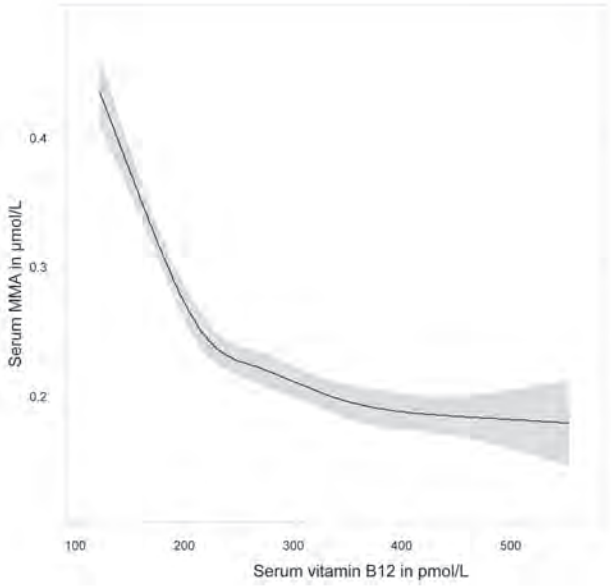
Figure 2. Spearman's correlations between biomarkers of vitamin B_{12} status (all $p<0.0001$) [a]partial correlation, adjusted for serum folic acid level.

Figures 3a-d show the association of total B_{12} with MMA and tHcy and of holoTC with MMA and tHcy, visualized with restricted cubic splines with 5 knots, without the lowest and highest 2.5 percentiles of total B_{12} and HoloTC. With total B_{12} and holoTC levels below approximately 330 and 100 pmol/L respectively, levels of tHcy and MMA start to rise, and rise steeper with total B_{12} and holoTC levels below approximately 220 and 50 pmol/L. Adjustment for serum folate did not alter the association between tHcy and total B_{12} (data not shown). The association between HoloTC and total B_{12} was linear across the whole range of measurements (data not shown).

Atrophic gastritis

We observed a prevalence of 3% for atrophic gastritis in the subsample of the B-PROOF study (n=720) when using the cut-off value of PGI/PGII ratio <1.6 as suggested by Van Asselt [2]. When using a less strict cut off value for PGI/PGII ratio of 5.0 [27], 8% of our population was diagnosed with atrophic gastritis. Participants with atrophic gastritis (PGI/PGII ratio <5.0, n=55) had significant higher levels of tHcy (mean 16.2 vs. 15.1 µmol/L, p=0.04) and MMA (mean 0.60 vs. 0.26 µmol/L, p<0.0001), lower levels of total B_{12} (mean 231 vs. 283 pmol/L, p=0.0003) and HoloTC (mean 68.4 vs. 44.3 pmol/L, p= 0.0001) and were older (mean 74.2 vs 72.5 years, p=0.0009) than participants without atrophic gastritis (n=655).

a.



b.

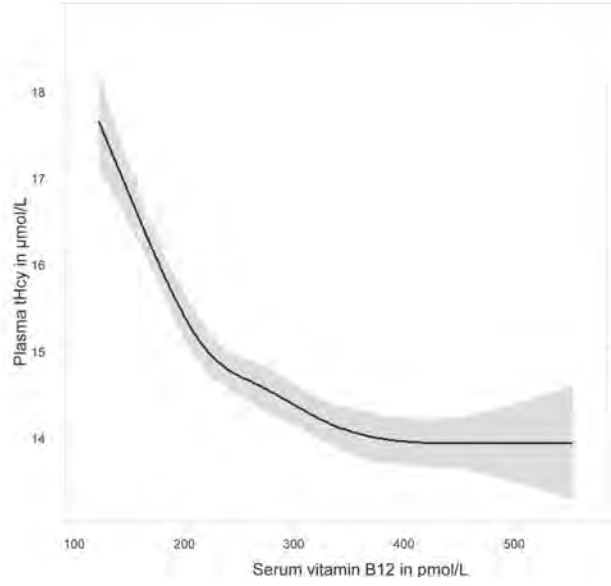
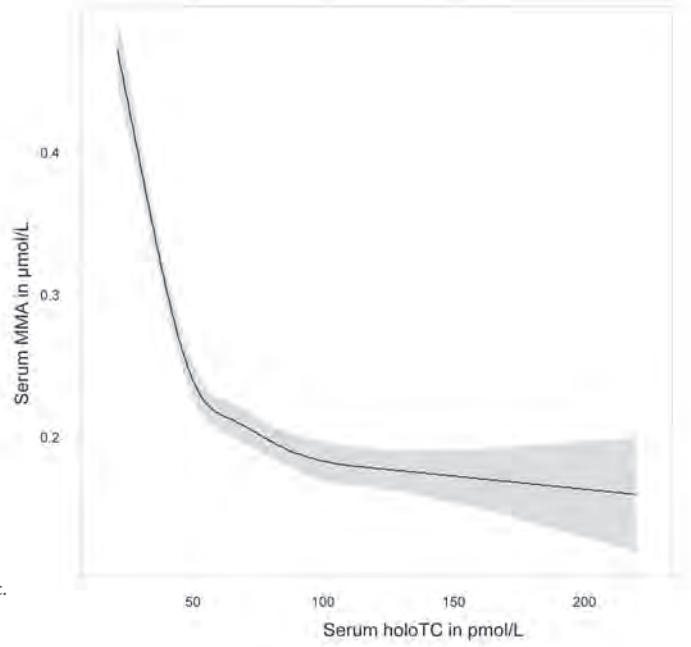


Figure 3.

a: Association between total B_{12} and MMA, adjusted for age, sex and creatinine levels. The highest and lowest 2.5% of total B_{12} are not included, b: Association between total B_{12} and homocysteine, adjusted for age, sex and creatinine levels. The highest and lowest 2.5% of total B_{12} are not included.

c.



d.

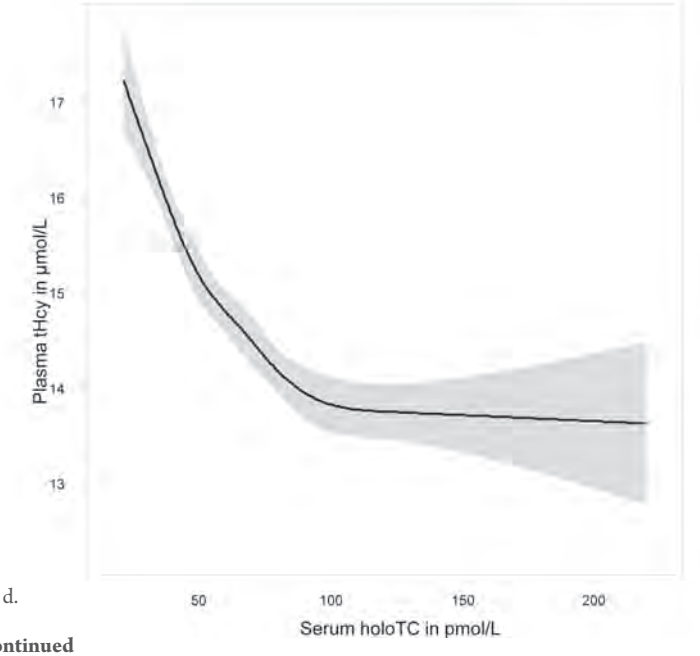


Figure 3. Continued

c**:** Association between holoTC and MMA, adjusted for age, sex and creatinine levels. The highest and lowest 2.5% of holoTC are not included, d**:** Association between holoTC and homocysteine, adjusted for age, sex and creatinine levels. The highest and lowest 2.5% of holoTC are not included.

DISCUSSION

In this study we showed that vitamin B_{12} intake was associated with all 4 biomarkers of vitamin B_{12} status. Saturation of biomarkers occurred with a habitual daily dietary intake of >5 μg of vitamin B_{12}. Spline regression showed that MMA and tHcy levels started to rise with total B_{12} levels below 330 pmol/L and HoloTC levels below 100 pmol/L.

These observations were made in a population with an adequate vitamin B_{12} intake, as 94% of the population measured reached the Estimated Average Requirements (EAR) of 2.0 μg/day. The observed median dietary vitamin B_{12} intake of 4.1 μg/day corresponds to the median intake 4.2 μg/day of the Dutch adult population, as measured in the Dutch National Food Consumption Survey of 2007-2010 [28].

The strength of the association between vitamin B_{12} intake and total B_{12} and MMA status is similar to that observed in other studies. A recent meta-analysis of 19 observational studies regarding the association between vitamin B_{12} intake and serum/plasma total B_{12} status generated an overall β of 0.10 (95% CI: 0.06-0.14) on the loge loge scale for adults and elderly, and a slightly higher overall β of 0.13 (95% CI: 0.04-0.21) for elderly only. For MMA the overall β was -0.11, based on 9 studies. These βs are similar to the βs we observed: β=0.13 for serum B_{12} and β=-0.13 for MMA. Less is published about the association of dietary vitamin B_{12} intake with holoTC and tHcy. Two studies of Bor et al., one in a healthy young population (n= 299) and one in postmenopausal women (n=98) investigated the association between vitamin B_{12} intake and biomarker status, including HoloTC and tHcy [10, 11]. Bor et al. observed higher HoloTC levels and lower tHcy levels in the upper three quintiles of vitamin B_{12} intake, although not significant for all data points. Howard et al. did not observe an association between vitamin B_{12} intake and tHcy levels in subjects with low vitamin B_{12} status [14]. Furthermore, Bor et al. observed a saturation of biomarker status of vitamin B_{12} intake between 4-7 μg/day comparing quintiles of vitamin B_{12} intake [10, 11]. In our study we observed a similar saturation level of around 5 μg/day, that is, above the current Dutch Recommended Daily Allowance (RDA) of 2.8 μg/day. These findings might indicate that the current RDA is too low to guarantee an optimal biomarker status in elderly people. Regarding the association between vitamin B_{12} intake and biomarker levels, our study clearly provides new information, as we show a continuous dose-response association between vitamin B_{12} intake and tHcy and HoloTC in a considerably large elderly population.

We visually explored the association of total B_{12} levels with tHcy and MMA with restricted cubic splines. We observed that below total B_{12} levels of approximately 330 pmol/L both tHcy and MMA levels started to rise, with a steep rise when total B_{12} levels fall below 220 pmol/L. This is in line with other studies, which observed inflections between 200 and 500 pmol/L [17, 24, 29, 30]. We furthermore explored the association of HoloTC with tHcy and MMA and observed that below HoloTC levels of approximately 100 pmol/L both tHcy and MMA levels started to rise, with a steep rise when HoloTC levels fall below 50 pmol/L. To our knowledge, such a visual representation of the continuous

association of HoloTC with MMA and tHcy has not been shown before. Important to note is that we corrected our analyses for creatinine levels, as creatinine is a marker for renal insufficiency and affects levels of tHcy and MMA [31].

The rise in MMA and tHcy below total B_{12} and HoloTC levels of 330 and 100 pmol/L respectively indicates that the enzymes methylmalonyl-CoA mutase and methionine synthase are no longer saturated by their co-factors, in other words, a state of metabolic insufficiency. This state occurs long before classical clinical vitamin B_{12} deficiency is determined (Figure 3), as traditional clinical cut-off values for total B_{12} range from 148 to 200 pmol/L[32], but corresponds well with the occurrence of subclinical vitamin B_{12} deficiency, or the presence of mild abnormal vitamin B_{12} biomarker levels without clinical signs or symptoms [31], usually defined by total B_{12} levels of <258 - 300 pmol/L [1, 33].

Cut-off values for vitamin B_{12} deficiency as suggested for HoloTC are distinctly lower, <20 pmol/L [8] or <32-35 pmol/L [23, 34], than the inflections shown in our data, confirming the sensitivity of HoloTC as an early marker for vitamin B_{12} deficiency [5]. This finding might suggest that cut-off levels for HoloTC should be raised to 50 pmol/L, but further research regarding the specificity of higher HoloTC cut-off values is desirable.

There is an active debate about the best biomarker or combination of biomarkers and cut-off values for vitamin B_{12} deficiency, and consensus has not yet been reached [4, 7, 16, 31-33, 35, 36]. Traditionally, total B_{12} is used as a marker for clinical vitamin B_{12} deficiency, with a cut-off value of ≤148 or ≤200 pmol/L [32]. Nowadays, the general idea is to combine values of at least two biomarkers, preferably a circulation biomarker (total B_{12} or HoloTC) and a metabolic biomarker (MMA, as tHcy is not specific for vitamin B_{12} deficiency) [16, 35]. For instance, current clinical practice in the Netherlands includes a cut-off value for total B_{12} of 300 pmol/L or HoloTC of 32 pmol/L for further testing of MMA levels. As subsequently measured MMA levels are >0.45 μmol/L, clinical vitamin B_{12} deficiency is diagnosed [23].

As mentioned, a distinction is made between clinical and subclinical vitamin B_{12} deficiency. There is a plea for treating people with subclinical vitamin B_{12} deficiency, as results from observational studies indicate that various health outcomes such as neural tube defects, cognitive functioning, depression, brain atrophy and bone health are already worsening at low-normal levels of vitamin B_{12} and normalizing levels of vitamin B_{12} biomarkers could theoretically improve health outcomes [33]. However, results from intervention studies are not univocal. Further research should focus on the effect of treating suboptimal vitamin B_{12} biomarker levels with vitamin B_{12}, through supplementation or food fortification, on suspected health outcomes.

Results of the B-PROOF intervention study will contribute to the evidence in this research field, as the effect of 2 year vitamin B_{12} (and folic acid) supplementation on several health outcomes, including fracture risk and cognitive function, will be investigated.

Our current results give insight in the levels of the four biomarkers and their mutual association in a large Dutch elderly population and contributes therefore to the general understanding of the distribution of vitamin B_{12} biomarkers. The inflections for total B_{12} and HoloTC as observed in our study, could be of use clinical practice and scientific research : 1) the inflections suggest a cut-off value for levels of total B_{12} and HoloTC indicating further testing of levels of MMA to determine clinical vitamin B_{12} deficiency with optimal sensitivity and specificity, 2) The inflections contribute to the determination of a cut-off value for subclinical vitamin B_{12} deficiency. This cut-off value could be used in research investigating the effect of treating subclinical vitamin B_{12} deficiency on health outcomes such as osteoporosis and cognitive function.

According to an earlier observation in Dutch elderly people [2] and other populations [37, 38] we expected atrophic gastritis to be a substantial problem in our study population, but on the contrary, we observed a low prevalence of atrophic gastritis, 3%, and 8% with a less strict cut-off value. Participants with atrophic gastritis had significantly worse outcomes on all four biomarkers. We do not have an explanation for the large difference in the observed prevalence in comparison with van Asselt et al. A study in New Zealand in elderly with comparable characteristics also observed a low prevalence of atrophic gastritis, about 7% [39]. Although measuring levels of pepsinogen I and II and addressing the ratio between these levels is a generally established method for the diagnosis of atrophic gastritis [27], the addition of serum gastrin levels or measurement of Helicobacter pylori antibodies and antibodies to parietal cells and intrinsic factor [40] might have added to our understanding of the state of atrophic gastritis in our population, but due to practical reasons this was not feasible.

This study has also some limitations. As this study population is part of an intervention study with one of the inclusion criteria being an elevated tHcy level (≥12 μmol/L), biomarker status of this population may not totally represent the biomarker status of a general elderly population. One of the four biomarkers for vitamin B_{12} deficiency is already elevated, biomarker profiles might therefore be less favourable than in the general elderly population. The use of cross-sectional data prevents us from determining causal relations.

To summarize, our study covers a large study population with data available on four biomarkers for vitamin B_{12} status, plus on vitamin B_{12} intake in a subsample of the population. The availability of data on four biomarkers is exceptional and the results of this study add to the understanding of the mutual association between the biomarkers; the inflections observed in the association of total B_{12} and holoTC with MMA and tHcy could indicate cut-off levels for further testing for B_{12} deficiency and determining subclinical B_{12} deficiency.

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4

Vitamin B12 intake and status and cognitive function in elderly people

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ABSTRACT

Current recommendations on vitamin B_{12} intake vary from 1.4-3.0 μg per day and are based on the amount needed for maintenance of hematological status or on the amount needed to compensate obligatory losses. This systematic review evaluates whether the relation between vitamin B_{12} intake and cognitive function should be considered for underpinning vitamin B_{12} recommendations in the future. The authors summarized dose-response evidence from randomized controlled trials (RCTs) and prospective cohort studies on the relation of vitamin B_{12} intake and status with cognitive function in adults and elderly people. Two RCTs and 6 cohort studies showed no or inconsistent associations between vitamin B_{12} intake and cognitive function. Random effects meta-analysis showed that serum vitamin B_{12} (50 pmol/l) was not associated with risk of dementia (4 cohort studies), global cognition z-scores (4 cohort studies) or memory z-scores (4 cohort studies). Although dose-response evidence on sensitive markers of vitamin B_{12} status (methylmalonic acid and holotranscobalamin) was scarce, 4 out of 5 cohort studies reported significant associations with risk of dementia or Alzheimer's disease or global cognition.

Current evidence on the relation between vitamin B_{12} intake or status and cognitive function does not suffice for being considered in deriving vitamin B_{12} recommendations. Further studies should consider the selection of the sensitive markers of vitamin B_{12} status.

INTRODUCTION

Dietary recommendations provide guidance on nutrient intakes that should be sufficient to fulfil requirements of nearly all apparently healthy people in a specified population. Traditionally, these recommendations were intended to prevent deficiency disorders, but today the focus is slowly changing towards optimal health also including relations between diet and prevention of chronic diseases [1, 2]. Current recommendations on vitamin B_{12} intake are similar for adults and elderly people and vary from 1.4-3.0 μg per day in Europe. They are based on the amount needed for the maintenance of hematological status and on the amount needed to compensate obligatory losses [3-5]. Relations between vitamin B_{12} intake and health related outcomes, e.g. cardiovascular diseases, cognitive function, and osteoporosis, are not yet taken into account when deriving vitamin B_{12} recommendations. To support transparent decision-making on whether these relations should be considered for setting vitamin B_{12} recommendations in the future, systematic reviews and meta-analyses are needed to objectively evaluate and integrate the available evidence [6]. Previously, 5 systematic reviews addressed the relation between vitamin B_{12} intake or status and cognitive function in a qualitative manner [7-11]. The aim of this review was to summarize dose-response evidence from randomized controlled trials (RCTs) and prospective cohort studies on the relation of vitamin B_{12} intake and status with cognitive function in adults and elderly people and to identify research gaps relevant for deriving vitamin B_{12} recommendations.

METHODS

This systematic review with dose-response meta-analyses was conducted according to standardized methodology as developed within the scope of the EURRECA (EURopean micronutrient RECommendations Aligned) Network of Excellence, which is shortly described below.

Search

We conducted a systematic literature search in the databases MEDLINE, EMBASE and Cochrane library Central through February 17, 2009, using search terms on study designs in humans AND vitamin B_{12} AND (intake OR status). The search terms both included MeSH terms and words to be found in title or abstract. The strategy was adapted for each database to fit database specific features. To be able to use the same search to identify publications on other health related outcomes both in adults and elderly and in younger population groups, no terms were added to limit the search to health outcome or study population. Moreover, by using a broad search we expected a more complete retrieval of relevant publications. The search was not limited by language. Web appendix 1 shows the full MEDLINE search strategy (available online). The initial search yielded 5,219 references after exclusion of duplicates using Endnote XII. In addition, we reviewed reference lists of 10 review articles reporting on the relation between vitamin B_{12} intake or status and cognitive function to identify potentially relevant references that were not yet collected on basis of the database search (n=134). We updated the searches on February 11, 2010 (n=560) and from February 2010 to January 2012 we checked database alerts.

Selection of studies

For the selection of relevant publications for our systematic review, we used predefined in- and exclusion criteria. In general, studies were eligible for inclusion if they were conducted in apparently healthy human subjects aged ≥18 y, and addressed cognitive function as a health outcome. We defined 4 categories of cognitive function: incident dementia, incident Alzheimer's disease (AD), global cognition, and domain-specific cognition. The specific domains of cognitive function used in this review were based on the classification of cognitive tests proposed by Wald et al. 2010 [12]: memory, speed, language and executive function. Global cognition comprised assessment methods addressing different domains of cognition e.g. a compound z-score combining z-scores of different cognitive performance tests or the mini-mental state examination (MMSE) combining aspects of orientation, memory and attention into one questionnaire [13]. Domain-specific cognition includes cognitive performance tests assessing a single domain of cognitive function.

Observational studies were included if they 1) had a prospective cohort or nested case-control design, and 2) addressed exposure by either validated dietary assessment methods or by serum/plasma concentration of markers indicating vitamin B_{12} status (vitamin B_{12}, methylmalonic acid (MMA) or holotranscobalamin (holo-TC)). Serum/plasma vitamin B_{12} is most commonly used as a marker of vitamin B_{12} status, however, the functional markers of vitamin B_{12}, holo-TC and MMA, have been suggested as more sensitive and specific. Holo-TC represents the fraction of vitamin B_{12} that is delivered to body cells and MMA is the substrate for the vitamin B_{12} dependent enzyme methylmalonyl CoA mutase, so in case of vitamin B_{12} deficiency MMA levels will increase [14-17].

Intervention studies were included if they 1) had a randomized controlled trial design, 2) studied the effects of supplements, fortified foods or micronutrient intake from natural food sources and included a placebo or untreated comparison group, and 3) had minimum intervention duration of 2 weeks. Studies were excluded if they only included patients with pre-existing disease because relations of vitamin B_{12} intake or status with cognitive function in such study populations may not be representative for the general apparently healthy population.

First, 2 reviewers (ED, SB) screened titles and abstracts of all references identified with the searches according to the in- and exclusion criteria. Secondly, 4 reviewers (ED, JvW, AS, MP) evaluated full texts of the remaining references against the same criteria. For the purpose of alignment and quality control, each reviewer screened and evaluated 10 percent of the total number of references in duplicate with another reviewer. The rare discrepancies were resolved by group consultation among all reviewers until consensus was reached. During the selection process all reasons for exclusion were registered and details are shown in Figure 1.

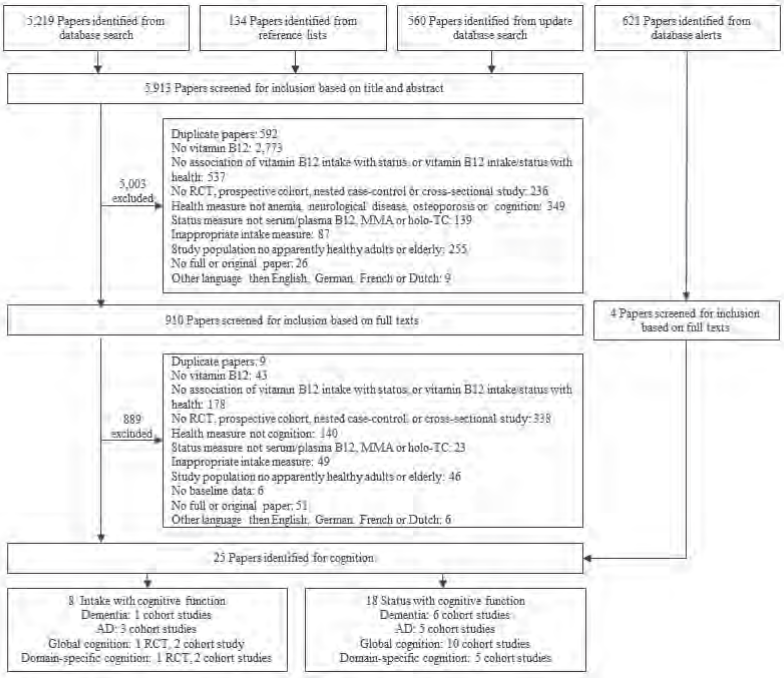

Figure 1. Study selection process for systematic review RCT, Randomized controlled trial; MMA, methylmalonic acid, holo-TC, holotranscobalamin

Data extraction

Data extraction was performed by a single reviewer (ED, JvW, or AS) using standardized data extraction forms in an Access database. A second reviewer verified the data. Disagreements were discussed and settled, if needed in consultation with a third reviewer. Data extracted included general characteristics of the study design and study population; details on measures of intake, status and cognitive function; details on data analysis and results. In addition, we extracted information concerning the validity of studies including sequence allocation (RCT), blinding (RCT), compliance (RCT), reproducibility and between-population comparability of intake, status and cognitive measures, control for confounders (at least age, sex, education, vascular disease, ApoE-4) and other forms of bias. We assessed the overall risk of bias of each individual study using standardized procedures largely based on guidance from the Cochrane Collaboration [18], resulting in one of the following judgments: low, moderate or high risk of bias.

Data-synthesis

Opportunities for meta-analysis were evaluated for comparability with regard to cognitive outcome, intake or status marker and the study population. If less than 3 comparable studies were available, results were qualitatively described. If 3 or more comparable studies were available, we carried out a dose-response meta-analysis that pools the relative risk (RR) per change in unit of exposure (binary outcomes) or the regression coefficient (β) (continuous outcomes) from multiple adjusted models. For serum/plasma vitamin B_{12} we chose to express association measures per 50 pmol/l. Hazard ratios (HR) and odds ratios (OR) were considered as RR because the outcome was relatively rare. If articles reported insufficient data (missing data, inconsistencies or any other uncertainties), we requested corresponding authors for additional information.

None of the studies on incidence of dementia or AD provided data in the desired format, but rather presented the risk of dementia or AD comparing subjects with high versus low vitamin B_{12} intake [19-21], low versus normal vitamin B_{12} status [22-25] or the number of cases and controls among subjects with and without vitamin B_{12} deficiency [26]. One author provided us with the log (RR) and its standard error (SE) upon request [22] and for 6 other studies we were able to derive the log (RR) (SE) based on reported data [19-21, 23] and data provided by the authors [25] as described in detail in web appendix 2 (available online). One author did not respond to our requests, and data were insufficient to derive the log (RR) (SE) [24].

All studies on global cognition included repeated measures of global cognition scores. Two studies did not report details on the association with vitamin B_{12} intake or status [27, 28]. We requested the authors to provide us with the missing data, but they were not able to respond to our request in time. Associations with vitamin B_{12} status were most frequently assessed by the use of linear mixed models including serum/plasma concentrations of a vitamin B_{12} status marker, a time variable and the interaction term of vitamin B_{12} status and time [29-31]. In such models, the regression coefficient for the interaction term represents the rate of change in global cognition attributable to an increase in vitamin B_{12} status additional to the deterioration in global cognition as a result of aging. Other studies presented associations between baseline serum vitamin B_{12} and serial [32] or follow-up [33] global cognition scores, associations between baseline plasma vitamin B_{12} and changes in cognitive decline during follow-up [34, 35], Spearman rank correlations between changes in plasma vitamin B_{12} and MMSE-scores [36], or mean changes in MMSE-scores by changes in serum vitamin B_{12} [37]. In 4 studies concentrations of vitamin B_{12} status markers were log-transformed [29, 32-34] and 1 study presented associations per standard deviation increase in serum vitamin B_{12} [30]. We requested corresponding authors to provide us with the regression coefficient (SE) for the interaction term (vitamin B_{12} status*time) including untransformed continuous serum/plasma vitamin B_{12} concentrations. One study already reported data in the desired format [31] and 3 authors [30, 33, 35] provided the data upon request.

We requested authors of studies only providing data on MMSE-scores [29, 32-34, 36] to repeat the analyses with MMSE expressed as a z-score, one author provided us with the requested data [33].

Studies identified for domain-specific cognition addressed several cognitive domains, however, only for the domains executive function [30, 33, 38] and memory [30, 33, 35, 38] 3 or more comparable studies were available. Although studies were comparable, there was still variation with regard to the cognitive performance tests used, the frequency of cognitive performance testing, and statistical methods used to assess the association between executive function or memory and vitamin B_{12} status. Therefore we requested corresponding authors to provide us with the regression coefficient (SE) for the association between untransformed vitamin B_{12} status measured at baseline and cognitive performance test scores at follow-up expressed as z-scores. Four authors provided these data upon request [30, 33, 35, 39], enabling us to perform a meta-analysis for the domain memory.

Statistical analyses

We calculated summary estimates of comparable studies using random effects meta-analysis. Applying the methods of DerSimonian and Laird, the between-study variance is estimated which is then used to modify the weights for calculating the summary estimate [40]. Heterogeneity between studies was evaluated using the I^2 statistic, which expresses the percentage of variation attributable to between-study heterogeneity rather than chance [41]. All statistical analyses were performed using STATA version 11.0 (College Station, TX), with statistical significance defined as a P-value <0.05.

RESULTS

In total, we identified 5,913 potentially relevant papers, of which 5,003 papers were excluded based on title and abstract. Of the remaining 910 papers, 889 were excluded based on full texts, leaving 21 papers addressing the relation between vitamin B_{12} intake or status and cognitive function. Four additional papers were identified from the database alerts (Figure 1). From the 25 included studies, 2 RCTs and 6 prospective cohort studies addressed the relation between vitamin B_{12} intake and cognitive function. Nineteen prospective cohort studies addressed the relation between vitamin B_{12} status, measured by serum/plasma vitamin B_{12} (n=17), MMA (n=3) or holo-TC (n=4) and cognitive function.

Vitamin B_{12} intake and cognitive function

Details on the studies addressing the relation between vitamin B_{12} intake and cognitive function are presented in Table 1.

Three studies involving 5,254 elderly people were included in a meta-analysis pooling relative risks for incidence of AD (431 cases) during 3.9 to 9.3y follow-up per µg increase in vitamin B_{12} intake at baseline [19-21]. Cases of AD were diagnosed based on commonly used criteria [42, 43]. The summary estimate showed no association between vitamin B_{12} intake and incidence of AD (RR=0.99, 95% Confidence interval (CI): 0.99, 1.00) with no heterogeneity between studies (I2=0%, p=0.92)

Table 1. Intake of Vitamin B12 in Relation to Incidence of Dementia, Incidence of Alzheimer's Disease (AD), Global and Domain-Specific Indicators of Cognition: RCTs and Prospective Cohort Studies (1997-2009)

	Population characteristics			Study characteristics			Exposure	Outcome			
Author, Year	**N**	**% men**	**Age (y)**	**Study design**	**Duration/ Follow-up**	**Risk of bias**	**Vitamin B12 intake (µg/d)**	**Cognitive outcome**	**Specific test/ number of cases**	**Association/effect measure**	**Results:**
La Rue 1997 (38)	122	49[a]	71.7[b,c]	Cohort	6 y	High	5.4 (2.9-11.7)[f]	Executive function Executive function Memory Memory Memory	Rey Osterrieth copy Shipley-Hartford abstraction Rey-Osterrieth recall WMS logical memory WMS visual reproduction	Spearman correlations between cognition at 6 y and baseline intake[h]	n.s. 0.20 (p<0.05) 0.19 (p<0.05) n.s. n.s.
Seal 2002 (44)	I1:10 I2:10 C:11	I1:40 I2:50 C:45	I1:82 I2:84.9 C:77.6	RCT	4 wk	Moderate	I1:10 I2:50 C:placebo	Global cognition	MMSE	Difference between treatment groups	P=0.494
Corrada 2005 (19)	579	62	69.6	Cohort	9.3 y	Moderate	6.3 (3.9–12.2)[f]	Incidence of AD	57 cases	HR (95% CI) by tertile of intake (T3/T1)[i] HR (95% CI) per unit increase in baseline intake[i]	0.84 (0.45, 1.59) 0.99 (0.98, 1.01)[b]
Morris 2005 (28)	3718	38[b]	74.4[b]	Cohort	5.5 y[e]	High	10.6[b]	Global cognition	Compound z-score of 4 tests	β (SE) for the annual change in global cognition by quintile of baseline intake[j]	n.s.
Tucker 2005 (33)	321	100	67 (7)[d]	Cohort	3 y	Moderate	9.57 (5.73)	Global cognition Executive function Memory Memory Language	MMSE Spatial copying Working memory Recall memory Verbal fluency	β for association between cognition at 3 y and baseline intake[k]	0.14, n.s. 0.37 (p<0.05) 0.12, n.s. -0.01, n.s. 0.38, n.s.

Table 1. Continued

Eussen 2006 (45)	I:54 C:57	I:23 C:22	I:82 (5) C:82 (5)	RCT	24 wk	Moderate	I:1000 C:placebo	Executive function Memory Speed	Compound z-score of 7 tests Compound z-score of 6 tests Compound z-score of 3 tests	Difference between treatment groups	n.s. p<0.05[n] n.s.
Morris 2006 (20)	1041	38[b]	72.7[b,e]	Cohort	3.9 y	Moderate	11.1 (0.5-127.2)[b,g]	Incidence of AD	162 cases	OR (95% CI) by quintile of intake (Q5/Q1)[l] HR (95%CI) per unit increase in baseline intake[l]	0.6 (0.2, 1.6) 0.99 (0.98, 1.01)[b]
Nelson 2009 (21)	3634	43	74.7 (6.7)[b]	Cohort	9 years	Low	9.6 (10.2)	Incidence of dementia	352 cases	HR (95% CI) by quintile of intake (Q5/Q1)[m] HR (95%CI) per unit increase in baseline intake[m]	0.87 (0.52, 1.46) 1.00 (0.98, 1.01)[b]
								Incidence of AD	212 cases	HR (95% CI) by quintile of intake (Q5/Q1)[m] HR (95%CI) per unit increase in baseline intake[m]	0.91 (0.52, 1.60) 1.00 (0.98,1.02)[b]

AD, Alzheimer's disease; β, regression coefficient; C, control group; CI, confidence interval; HR, hazard ratio; I, intervention group; MMSE, mini-mental state examination; n.s., not significant but no p-value provided; OR, odds ratio; RCT, randomized controlled trial; SE, standard error; wk, week; WMS, Wechsler Memory Scale; y, year

(a values are based on total cohort study population, not only participants of the study referred to here

(b values are calculated based on reported values, details of the calculations are provided in Web appendix 2, available online

(c mean, all such values, unless stated otherwise

(d mean (SD) all such values, unless stated otherwise

(e median

(f median (interquartile range)

(g mean (range)

(h La Rue et al. adjusted for age

(i Corrada et al. adjusted for age, gender, education, and caloric intake

(j Morris et al. adjusted for age, quintiles of folate intake, time, sex, education, race, vitamin E intake from food, total vitamin C intake, and time interactions with all covariates

(k Tucker et al. adjusted for age, education, body mass index, alcohol, smoking, diabetes, systolic blood pressure, baseline cognitive measures, time of second measure relative to folic acid fortification, time interval between the 2 cognitive measures, total energy intake

(l Morris et al. adjusted for age, gender, ethnicity, education, vitamin E intake, niacin intake, apolipoprotein E4 status, participation in cognitive activities

(m Nelson et al. adjusted for gender, education, body mass index, total energy, physical activity, apolipoprotein E4 status, alcohol, smoking, myocardial infarction, stroke, diabetes and the other B-vitamins.

(n Memory function improved in all treatment groups, but the improvement in the placebo group was significantly better than the improvement in the vitamin B12 group

Studies on incident dementia [21] or global cognition assessed by MMSE [33, 44] or a compound z-score [28] did not show significant associations with vitamin B_{12} intake. One RCT [45] and 2 prospective cohort studies [33, 38] addressed the association between vitamin B_{12} intake and domain-specific cognition. For the domain executive function the 2 prospective cohort studies found that higher vitamin B_{12} intakes at baseline were associated with a better cognitive performance after 3 or 6 years of follow-up [33, 38] whereas the RCT did not show beneficial effects of daily vitamin B_{12} supplementation [45]. For the domain memory results were largely inconsistent showing a positive [38], negative [45] or no [33, 38] association. The cognitive domains speed and language were only addressed in single studies and no associations with vitamin B_{12} intake were observed [33, 45].

Vitamin B_{12} status and incident dementia or AD

Table 2 presents details of the prospective cohort studies investigating the association between vitamin B_{12} status and incidence of dementia or AD. Dementia and AD were diagnosed based on similar criteria (dementia: [43, 46-48]; AD: [42, 43, 49]).

Four studies involving 2,630 elderly people (age range: 60-101 year) were included in a meta-analysis pooling relative risks for incidence of dementia (263 cases) during 2.4 to 4.5y follow-up per 50 pmol/l increase in serum/plasma vitamin B_{12} concentrations at baseline [22, 23, 26]. The summary estimate showed no association between serum/plasma vitamin B_{12} and incidence of dementia (RR=1.00, 95% CI: 0.98, 1.02) with little heterogeneity between studies (I2=9.1%, p=0.35) (Figure 2). Similar to this finding, Ravaglia et al. (2005) [24] reported that low serum vitamin B_{12} concentrations (≤250 pmol/l) did not significantly increase the risk of dementia. Although Kivipelto et al. (2009) [50] found no association between holo-TC as a continuous variable and risk of dementia, the third quartile of holo-TC was associated with a reduced risk of dementia.

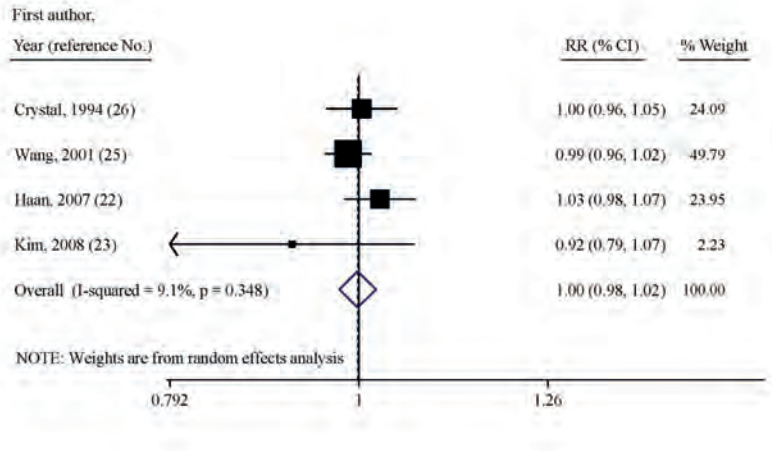

Figure 2. Forest plot of association between serum/plasma vitamin B_{12} (50 pmol/l) and risk of dementia: Meta-Analysis of 4 prospective cohort studies (n=2630, 263 cases). RR, relative risk; CI, confidence interval.

Five studies reported on the association between vitamin B_{12} status (serum vitamin B_{12} n=3, holo-TC n=2) and incidence of AD. Studies on serum vitamin B_{12} showed no significant associations [24-26]. Higher baseline concentrations of holo-TC were significantly associated with a reduced risk of AD in one study [51]. These results were not in line with findings from Kivipelto et al (2009), reporting no significant association for holo-TC as a continuous variable. However, the latter study did report a reduced risk of AD associated with the third quartile of holo-TC concentrations.

Vitamin B_{12} status and global cognition

For global cognition, we identified 4 prospective cohort studies on serum/plasma vitamin B_{12} and compound z-scores [27, 30, 31, 35], and 6 prospective cohort studies on serum/plasma vitamin B_{12} and MMSE-scores [29, 30, 32, 33, 36, 37] (Table 3).

From 4 cohort studies including 1,579 elderly people, we obtained the regression coefficient for associations between additional annual change in compound z-score [30, 31, 35] or MMSE z-score [33] and serum/plasma vitamin B_{12} concentrations (50 pmol/l). A pooled analysis of the results showed no overall association (β=0.00, 95% CI: -0.00, 0.01), with moderate heterogeneity between studies (I2=42.6%, p=0.16) (Figure 3). In line with this finding, De Lau et al. (2009) [27] observed no association for plasma vitamin B_{12} with rate of cognitive decline during follow-up.

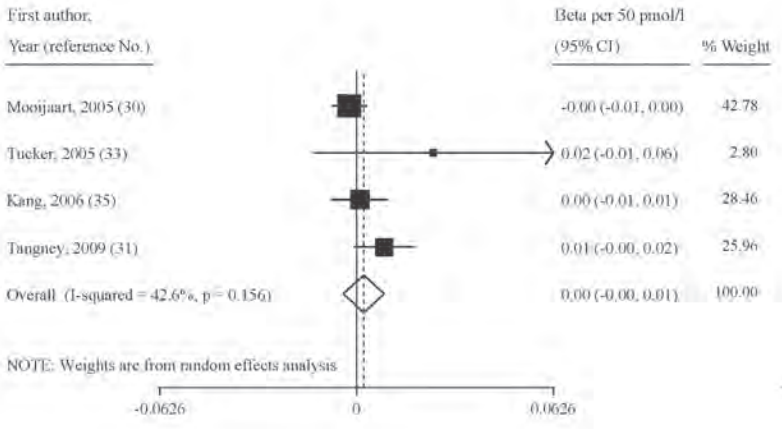

Figure 3. Forest plot of association between serum/plasma vitamin B_{12} (50 pmol/l) and additional rate of change in global cognition z-scores: Meta-Analysis of 4 Prospective Cohort Studies (n=1579).
CI, confidence interval.

Table 2. Vitamin B12 Status in Relation to Incidence of Dementia and Incidence of Alzheimer's Disease (AD): Prospective Cohort Studies (1994-2010)

	Population characteristics			Study characteristics		Exposure		Outcome			
Author Year	**N**	**% men**	**Age (y)**	**Follow-up (y)**	**Risk of bias**	**Marker of vitamin B12 status**	**Concentration**	**Incidence of dementia/ AD**	**No cases**	**Association measure:**	**Result:**
Crystal 1994 (26)	410	n.a.	75-85[b]	5	High	Serum vitamin B12	412 (74-3660) pmol/l[e]	Dementia	60	RR (95%CI) per 50 units increase in baseline status[g]	1.00 (0.96,1.03)[d]
								AD	30	RR (95%CI) per 50 units increase in baseline status[g]	0.98 (0.92,1.05)[d]
Wang 2001 (25)	370	20	75-101	3	Moderate	Serum vitamin B12	324 (510) pmol/l	Dementia	78	RR (95% CI) for low (≤250 pmol/l) versus normal baseline status[h]	1.3 (0.8, 2.1)
										RR (95%CI) per 50 units increase in baseline status[h]	1.0 (1.0, 1.1)[d]
								AD	60	RR (95% CI) for low (≤250 pmol/l) versus normal baseline status[h]	1.8 (1.0, 3.0)
										RR (95%CI) per 50 units increase in baseline status[h]	1.0 (1.0, 1.1)[d]
Ravaglia 2005 (24)	816	47	73.6 (6.3)[c]	4	Moderate	Serum vitamin B12	Hcy>15 µmol/l: 212 (73-612) pmol/l[f] Hcy≤15 µmol/l: 259 (94-708) pmol/l[f]	Dementia AD	112 70	HR (95% CI) for low (≤251 pmol/l) versus normal baseline status[i]	0.83 (0.56, .24) 0.66 (0.40, .09)
Haan 2007 (22)	1332	42[a]	60-101	4.5	Moderate	Plasma vitamin B12	334 (150) pmol/l	Dementia or CIND	80	HR (95% CI) per unit increase in sqrt baseline status[j]	1.05 (1.01, .09)
										HR (95%CI) per 50 units increase in baseline status[j]	1.03 (0.98,1.07)[o]
Kim 2008 (23)	518	43	71.8 (5.0)[d]	2.4	Moderate	Serum vitamin B12	380.7 (149.3)[d] pmol/l	Dementia	45	OR (95% CI) for low (<258 pmol/l) versus normal baseline status[k]	1.53 (0.69,3.38)
										OR (95%CI) per 50 units increase in baseline status[k]	1.08 (0.93,1.26)[d]

Table 2. Continued

Kivipelto 2009 (50)	213	25	81.0 (4.6)	6.7	Moderate	Holo-TC	105 (88) pmol/l	Dementia	83	RR (95%CI) per unit increase in baseline status[l] RR (95% CI) by quartile of baseline status (Q3/Q1)[m]	1.00 (0.99,1.00) 0.47 (0.23,0.96) 1.00 (0.99,1.00) 0.38 (0.15, .94)
								AD	61	RR (95%CI) per unit increase in baseline status[l] RR (95% CI) by quartile of baseline status (Q3/Q1)[m]	
Hooshmand 2010 (51)	271	38	70.7 (3.6)	7.4	Low	Holo-TC	91.3 (51.0)[d] pmol/l	AD	17	OR (95%CI) per unit increase in baseline status[n]	0.977 (0.958, 0.997)

AD, Alzheimer's disease; CI, confidence interval; CIND, cognitive impairment no dementia; Hcy, homocysteine; Holo-TC, holotranscobalamin; HR, hazard ratio;n.a., not available; OR, odds ratio; RR, relative risk; y, year

(a values are based on total cohort study population, not only participants of the study referred to here

(b range all such values

(c mean (SD) all such values, unless stated otherwise

(d values are calculated based on reported values, details of the calculations are provided in Web appendix 2, available online

(e mean (range)

(f geometric mean (95% confidence interval)

(g Crystal et al. unadjusted

(h Wang et al. adjusted for age, sex, education

(i Ravaglia et al. adjusted for age, sex, education, apolipoprotein E4 status, history of stroke, serum creatinine, plasma homocysteine, serum folate

(j Haan et al. adjusted for age, sex, education, baseline stroke, plasma homocysteine, red blood cell folate,

(k Kim et al. adjusted for age, sex, education, smoking, alcohol, physical activity, body weight, disability, depression, vascular risk score, vitamin intake, serum creatinine

(l Kivipelto et al. adjusted for age, sex, education

(m Kivipelto et al. adjusted for age, sex, education, body mass index, albumin, haemoglobin, creatinine, apolipoprotein E4 status, mini mental state examination-score, homocysteine, folate

(n Hooshmand et al. adjusted for age, sex, education, duration of follow-up, apolipoprotein E4 status, body mass index, mini mental state examination-score, systolic blood pressure, diastolic blood pressure, smoking, history of stroke

(o results are additionally provided by the author upon request

Table 3. Vitamin B12 Status in Relation to Global Cognition Scores: Prospective Cohort Studies (2002-2009)

	Population characteristics			Study characteristics		Exposure		Outcome		
Author Year	N	% men	Age (y)	Follow-up (y)	Risk of bias	Marker of vitamin B12 status	Concentration	Global cognition score	Association measure:	Result
Eussen 2002 (36)	189	49[a]	75-80[b]	5	High	Plasma vitamin B12	338 (323)[e] pmol/l	MMSE	Spearman rank correlation between changes in MMSE scores and status after 5y follow-up[g]	-0.069, n.s.
Kado 2005 USA (34)	370	42	74.3 (2.7)[c]	7	Moderate	Plasma vitamin B12	325 (264) pmol/l	Total score of 5 tests	RR (95%CI) of being in the worst quartile of cognitive decline after 7y comparing those in the lowest quartile of baseline status versus the rest[h]	1.42 (0.91,2.06), p=0.11
Mooijaart 2005 (30)	351	34[a]	85[d]	4	Moderate	Serum vitamin B12	315 (184)[e] pmol/l	MMSE	β (SE) for the additional annual change in MMSE per SD increase in baseline status[i] β (SE) for the additional annual change in MMSE per 50 units increase in baseline status[i]	-0.009 (0.07), p=0.89 0.012 (0.023), p=0.60[q]
								Compound z-score of 4 tests	β (SE) for the additional annual change in z-score per 50 units increase in baseline status[i]	-0.0023 (0.0028), p=0.42[q]
Tucker 2005 (33)	321	100	67 (7)	3	Moderate	Serum vitamin B12	335 (136) pmol/l	MMSE	β (SE) for association between 3y MMSE scores and log-transformed baseline status[j] β (SE) for additional annual change in MMSE per 50 units increase in baseline status[j]	-0.16 (0.22[q]), n.s. 0.041 (0.033), p=0.21[q]
								MMSE z-score	β (SE) for the additional annual change in MMSE z-score per 50 units increase in baseline status[j]	0.024 (0.020), p=0.21[q]
Kang 2006 (35)	391	0	63	4	Moderate	Plasma vitamin B12	337 pmol/l	Compound z-score of 6 tests	Mean difference in rate of cognitive decline over 4y by quartile baseline status (Q1-Q4)[k] β (SE) for additional annual change in z-score per 50 units increase in baseline status[k]	0.00 (-0.05, 0.05) 0.0010 (0.0046), p=0.8255[q]
Clarke 2007 (29)	691	40	71.9 (5.2)	10	Low	Serum vitamin B12 MMA Holo-tc	280 (106)[a] pmol/l 0.35(0.30)[a] µmol/l 73 (43)[a] pmol/l	MMSE	β (95%CI) for additional change in MMSE-score during 10y associated with a doubling of baseline status[l]	0.22 (-0.02, 0.46) -0.65 (-0.98, -0.32)[r] 0.59 (0.30, 0.88)

Table 3. Continued

Kim 2008 (37)	607	43	71.9 (5.1)	2.4	High	Serum vitamin B12		MMSE	Mean changes in MMSE scores across ascending quintiles of change in vitamin B12 status during follow-up[m]	Q1:-1.5, Q2:-1.4, Q3:-1.4, Q4:-1.3, Q5:-0.8 (F=0.532; p=0.712)
De Lau 2009 (27)	832	48[a]	72.2 (7.4)[a]	3-8	High	Plasma vitamin B12 MMA Holo-tc	244 (183-311)[f] pmol/l 0.28 (0.2- 0.34)[f] µmol/l 54.0 (38.0-74.0)[f] pmol/l	Compound z-score of 6 tests	β (SE) for annual change in z score for quintiles of baseline status[n]	Plasma vitamin B12, MMA and holo-tc: p for trend >0.1
Feng 2009 (32)	539	40	64.9 (7.2)	3.2	Low	Serum vitamin B12	396 (181) pmol/l	MMSE	β (SE) for associations between repeated MMSE-scores and log-transformed baseline status[o]	1.07 (0.35), p=0.003
Tangney 2009 (31)	516	40	80 (6)	6	Moderate	Serum vitamin B12 MMA	337 (127) pmol/l 0.279 (0.173) µmol/l	Compound z-score of 4 tests	β (SE) for the additional annual change in z-score per 50 units increase in baseline status[p] β (SE) for the additional annual change in z-score per unit increase in baseline status[p]	0.0088 (<0.005), p=0.005 -0.00016 (0.0001), p=0.004[r]

CI, confidence interval; Holo-TC, holotranscobalamin; MMA, methylmalonic acid; MMSE, Mini-Mental State Examination; n.s. not significant, but no p-value provided; SE, standard error; y, year
(a values are based on total cohort study population, not only participants of the study referred to here
(b range, all such values unless stated otherwise
(c mean (SD), all such values unless stated otherwise
(d mean, all such values unless stated otherwise
(e values are calculated based on provided values, details of the calculations are provided in Web appendix 2, available online.
(f median (interquartile range)
(g Eussen et al. unadjusted values
(h Kado et al. adjusted for age, sex, education, smoking, baseline physical function
(i Mooijaart et al. adjusted for sex, education
(j Tucker et al. adjusted for age, education, smoking, alcohol, body mass index, diabetes, systolic blood pressure, time of second measure relative to folic acid fortification, time between cognitive measures, serum creatinine
(k Kang et al. adjusted for age, education, smoking, alcohol, physical activity, body mass index, diabetes, history of high blood pressure, history of high cholesterol, postmenopausal hormone use, age at menopause, antidepressant use, aspirin use, mental health index, energy-fatigue index, assay batch, time between blood draw and cognitive interview, vitamin E supplement intake
(l Clarke et al. adjusted for sex, education, smoking, apolipoprotein E4 status, vascular disease, systolic blood pressure
(m Kim et al. unadjusted values
(n de Lau et al. adjusted for age, sex, education, smoking, alcohol, use of vitamin supplements, serum creatinine, diabetes mellitus, systolic blood pressure, depressive symptoms, intima-media thickness, plasma homocysteine , plasma folate
(o Feng et al. adjusted for age, sex, education, smoking, alcohol, physical activity, apolipoprotein E4 status, hypertension, diabetes mellitus, cardiovascular diseases, apolipoprotein E4 status *vitamin B12
(p Tangney et al. adjusted for age, sex, race, education, smoking, alcohol, frequency of cognitive activities, serum creatinine, calorie adjusted intake of saturated fat, trans unsaturated fat intake, vitamin E in food, total vitamin C, fish intake
(q results are additionally provided by the author upon request
(r higher MMA concentrations indicate a lower vitamin B12 status

Table 4. Vitamin B12 Status in Relation to Domain-Specific Cognition Scores: Prospective Cohort Studies (1997-2006)

	Population characteristics			Study characteristics		Exposure		Outcome			
Author Year	**N**	**% men**	**Age (y)**	**Follow-up (y)**	**Risk of bias**	**Marker of vitamin B12 status**	**Concentration (pmol/l)**	**Cognitive domain**	**Specific test**	**Association measure:**	**Result:**
La Rue 1997 (38)	133	49[a]	71.7[b]	6	High	Serum vitamin B12	437 (186)	Memory Memory Memory Executive function Executive function	Rey-Osterrieth Recall WMS visual reproduction WMS logical memory Rey-Osterrieth copy Shipley-Hartford abstraction	Spearman correlations between cognition at 6 y and baseline status[e]	n.s. n.s. n.s. n.s. 0.11, n.s.
Mooijaart 2005 (30)	559	34	85	4	Moderate	Serum vitamin B12	315 (184)[d]	Memory	word list	β for additional rate of change in cognitive performance for each 1-SD increase in baseline status[f]	-0.008, p=0.92
										β (SE) for cognition z-score at 4 y per 50 units increase in baseline status[f]	0.0077 (0.05), p=0.80[j]
								Memory	word list delayed recall	β for additional rate of change in cognitive performance for each 1-SD increase in baseline status[f]	0.053, p=0.19
										β (SE) for cognition z-score at 4 y per 50 units increase in baseline status[f]	0.010 (0.05), p=0.75[j]
								Speed and executive function	letter-digit coding	β for additional rate of change in cognitive performance for each 1-SD increase in baseline status[f]	-0.060, p=0.39
										β (SE) for cognition z-score at 4 y per 50 units increase in baseline status[f]	0.00085 (0.05), p=0.98[j]
									Stroop	β for additional rate of change in cognitive performance for each 1-SD increase in baseline status[f]	-0.21, p=0.66
								Speed and executive function		β (SE) for cognition z-score at 4 y per 50 units increase in baseline status[f]	

Table 4. Continued

Nurk 2005 (39)	1678	45[a]	72	6	Low	Serum vitamin B12	347[d]	Memory	Kendrick objective learning test	OR (95%CI) for memory deficit by quintiles of baseline status (Q1/Q5)[g] β (SE) for cognition z-score at 6 y per 50 units increase in baseline status[g]	1.61 (1.00, 2.64), p for trend= 0.042 0.70 (6.7)[j]
Tucker 2005 (33)	321	100	67 (7)[c]	3	Moderate	Serum vitamin B12	335 (136)	Memory	backward digit span	β for association between cognition at 3 y and log-transformed baseline status[h]	0.18, n.s.
										β (SE) for association between cognition z-score at 3 y per 50 units increase in baseline status[h]	0.017 (0.016), n.s.[j]
								Memory	word list	β for association between cognition at 3 y and log-transformed baseline status[h]	-0.20, n.s.
										β (SE) for association between cognition z-score at 3 y per 50 units increase in baseline status[h]	0.0045 (0.015), n.s.[j]
								Executive function	spatial copying	β for association between cognition at 3 y and log-transformed baseline status[h]	0.59, p<0.05
										β (SE) for association between cognition z-score at 3 y per 50 units increase in baseline status[h]	0.026 (0.016), p<0.05[j]
								Language	verbal fluency	β for association between cognition at 3 y and log-transformed baseline status[h]	0.06, n.s.

Table 4. Continued

	Population characteristics			Study characteristics		Exposure		Outcome			
Author Year	**N**	**% men**	**Age (y)**	**Follow-up (y)**	**Risk of bias**	**Marker of vitamin B12 status**	**Concentration (pmol/l)**	**Cognitive domain**	**Specific test**	**Association measure:**	**Result:**
Kang 2006 (35)	391	0	63	4	Moderate	Plasma vitamin B12	337	Memory	compound z-score of 4 tests	Mean difference (95% CI) in rate of cognitive decline over 4 y by quartile baseline status[i] β (SE) for cognition z-score at 4 y per 50 units increase in baseline status[i]	0.00 (-0.06, 0.07) 0.011 (0.012)[i]

CI, confidence interval; n.s. not significant but no p-value provided; OR, Odds Ratio; SE, standard error; WMS, Wechsler memory scale; y, year

(a values are based on total cohort study population, not only participants of the study referred to here

(b mean, all such values unless stated otherwise

(c mean (SD), all such values unless stated otherwise

(d values are calculated based on provided values, details of the calculations are provided in Web appendix 2, available online.

(e La Rue et al. adjusted for age

(f Mooijaart et al. adjusted for sex, education

(g Nurk et al. adjusted for sex, education, apolipoprotein E4 status, history of cardiovascular disease and hypertension, depression

(h Tucker et al. adjusted for age, education, smoking, alcohol, body mass index, diabetes, systolic blood pressure, baseline cognitive measures, time of second measure relative to folic acid fortification, time between cognitive measures, serum creatinine

(i Kang et al. adjusted for age, education, smoking, alcohol, physical activity, body mass index, diabetes, history of high blood pressure, history of high cholesterol, postmenopausal hormone use, age at menopause, antidepressant use, aspirin use, mental health index, energy-fatigue index, assay batch, time between blood draw and cognitive interview, vitamin E supplement intake

(j results are additionally provided by the author upon request

For MMSE-scores we did not have sufficient data available in the desired format to perform a meta-analysis and therefore results are qualitatively described. In line with the finding from the pooled analysis, 3 studies showed that the additional rate of change in MMSE-score was not significantly associated with serum vitamin B_{12} concentrations [29, 30, 33]. Moreover, Eussen et al. (2002) [36], Kado et al. (2005) [34] and Kim et al. (2008) [37] reported no significant associations between serum/plasma vitamin B_{12} concentrations and global cognition (Table 3). In contrast, Feng et al. (2009) [32] showed that higher serum vitamin B_{12} concentrations at baseline (natural log transformed, pmol/l) were significantly associated with better repeated MMSE-scores during follow-up.

Longitudinal data on MMA and holo-TC in relation to global cognition scores were scarce. Clarke et al. (2007) [29] reported the additional change in MMSE-score during 10 y related to a doubling in MMA (μmol/l) and holo-TC concentrations (pmol/l) at baseline. A doubling of MMA concentrations was associated with about 60 percent faster rate of cognitive decline and a doubling of holo-TC concentrations was associated with a 40 percent slower rate of cognitive decline. In addition, Tangney et al. (2009) [31] reported that higher MMA concentrations (μmol/l) were associated with a more rapid deterioration of global cognition assessed with a compound z-score of 4 tests. De Lau et al. (2009) [27] observed no significant association for MMA or holo-TC with rate of cognitive decline during follow-up.

Vitamin B_{12} status and domain specific cognition

Five prospective cohort studies investigated associations between serum/plasma vitamin B_{12} and memory (n=5), executive function (n=3), speed (n=1) and language (n=1) (Table 4).

For the domain memory we pooled associations between memory z-scores at 3, 4, or 6 years follow-up and serum/plasma vitamin B_{12} concentrations at baseline (50 pmol/l) from 4 studies [30, 33, 35, 39] including 3,460 elderly people. The studies of Mooijaart et al. (2005) [30] and Tucker et al. (2005) [33] both included 2 memory scores (word list and word list recall at 30 min [30] or word list and backward-digit span[33]). Therefore we performed 2 meta-analyses, one with the word list scores and one with the other 2 memory scores, but for both analyses the overall associations were similar (β=0.01, 95% CI:-0.01, 0.03), with no evidence for heterogeneity between studies (I2=0.0%, p=0.99).

In line with these results, La Rue et al. (1997) [38] reported that Spearman correlations between serum vitamin B_{12} at baseline and 3 different memory scores at 6 years follow-up were not significant (no data available).Associations between serum vitamin B_{12} concentrations at baseline and executive function at follow-up were assessed in 3 cohort studies [30, 33, 38]. La Rue et al. (1997) [38] and Mooijaart et al. (2005) [30] showed no significant associations, whereas Tucker et al. (2005) [33] observed that higher serum vitamin B_{12} concentrations (50 pmol/l) were associated with a better executive function score at 3 years follow-up. The cognitive domains language [33] and speed [30] were both assessed in single studies, but no associations with serum vitamin B_{12} were found.

DISCUSSION

Main findings

The available evidence from a limited number of RCTs and prospective cohort studies in elderly people does not support the hypothesis that vitamin B_{12} intake or status is related with dementia, AD or global cognition. For domain-specific cognition, some prospective cohort studies observed associations between vitamin B_{12} intake or serum vitamin B_{12} and executive function or memory, however, results were inconsistent. Significant associations were shown between MMA or holo-TC and risk of dementia or AD or global cognition scores indicating better cognitive function with better vitamin B_{12} status, but these findings originate from only 4 prospective cohort studies. Overall, these results suggest that current evidence on the relation of vitamin B_{12} intake or status with cognitive function does not suffice for being involved in deriving recommendations on vitamin B_{12} intake as the evidence-base for the presence of an association is limited and not supportive.

Methodological considerations of this meta-analysis

Previous systematic reviews on the relation of vitamin B_{12} intake or status with cognitive function reported a large heterogeneity between studies, mainly with regard to cognitive outcomes, cut-off levels indicating low vitamin B_{12} status and data analyses [7-11]. This heterogeneity limited previous meta-analyses. A strength of our review is that we dealt with these sources of heterogeneity by defining 4 clusters of cognitive outcomes that were separately reviewed: dementia, AD, global cognition and domain-specific cognition. The domain-specific outcomes were further categorized as measuring memory, executive function, speed or language to cover the large variation in cognitive performance tests used between studies.

To overcome the variation in cut-off levels for low vitamin B_{12} status and to allow comparison and subsequent combination of individual studies, we expressed results of individual studies in a standardized format: Relative risk (binary outcomes) or regression coefficient (continuous outcomes) per change in untransformed unit of exposure. Most of the included studies reported results based on untransformed vitamin B_{12} concentrations whereas few studies reported results based on log-transformed [32, 33] or square root transformed [22] units of exposure because the distribution of serum vitamin B_{12} concentration within the study population was skewed. We chose to use untransformed data as there is no evidence to assume that the association between vitamin B_{12} and cognitive function is not continuous across the common range of exposure [52]. Meta-analyses of associations based on log-transformed data would probably not alter our conclusions as both results from studies using log-transformed data and those using untransformed data were similar.

A common concern in meta-analyses is statistical heterogeneity between studies. Among the meta-analyses reported here, we only observed moderate statistical heterogeneity for the association between serum vitamin B_{12} and global cognition. We were not able to explain this heterogeneity with meta-regression due to the limited number of studies included, however potential sources of

heterogeneity are: age of the study populations (mean age 63-85 years), tests used to measure global cognition (MMSE, or compound z-score of 4-6 tests), duration of follow-up (3-10 years) and level of adjustment for confounders. Although most included studies adjusted for a wide range of confounders for cognitive function, residual confounding by other unmeasured or inadequately measured factors cannot be ruled out.

As the quality of included studies determines the quality of the meta-analysis, we assessed the risk of bias for each study identified in our review. Five prospective cohort studies were evaluated as having a low risk of bias, as confounders were appropriately dealt with, measures of vitamin B_{12} intake or status were adequate and no other serious risks of bias were identified [21, 29, 32, 39, 51]. The other 20 studies (2 RCTs and 18 prospective cohort studies) were evaluated as having moderate or high risk of bias as methods used for sequence generation or allocation concealment were unclear [44, 45], important risk factors were not evaluated for their confounding potential (ApoE-ε4 [19, 22, 23, 25, 27, 28, 30, 31, 33-35, 37], vascular disease [20, 50], or data were not reported in sufficient detail to be used in meta-analysis [24, 26-28, 36-38]. Due to the limited number of studies included in the different meta-analyses, we were not able to study the effect of study quality on the pooled effect measures.

Reflection on our results and considerations for future research

Observational studies

Prospective cohorts studying associations between vitamin B_{12} intake and cognitive function were limited. This is likely to be related to difficulties in interpreting values on vitamin B_{12} intake in elderly people. Although intakes of vitamin B_{12} generally exceed the current recommended amounts, the prevalence of vitamin B_{12} deficiency among elderly people in Western countries is estimated to be 20 percent [53]. The main cause for vitamin B_{12} deficiency in elderly people is food-bound malabsorption due to atrophic gastritis, a clinical condition accompanied by limited or absent secretion of gastric acid [54]. A study including elderly with this condition showed that plasma vitamin B_{12} levels were significantly correlated with vitamin B_{12} intake from supplements and fortified foods, but not with vitamin B_{12} intake from unfortified foods [55]. In addition intestinal absorption of vitamin B_{12} may be compromised by the use of acid lowering agents including proton-pump inhibitors and H2-blockers. These medicines are commonly used by elderly people, however available data on the association between vitamin B_{12} status and the use of these acid lowering agents is inconsistent [56, 57]. To deal with the potential issue of malabsorption the use of markers for vitamin B_{12} status is preferred over measures of vitamin B_{12} intake when studying associations with cognitive function in elderly people.

Prospective cohort studies on associations between vitamin B_{12} status and cognitive function mainly addressed serum/plasma vitamin B_{12} concentrations which is considered to be a less sensitive and specific marker of vitamin B_{12} status than MMA or holo-TC [14]. A recent study published after the end date of our search showed that higher holo-TC concentrations were related to better performance in executive function and psychomotor speed among non-demented elderly people [58]. Holo-TC was additionally associated with global cognition (MMSE) and had a borderline significant association

with verbal expression when including dementia subjects in the analyses. Supported by findings from studies with a case-control or cross-sectional design MMA and holo-TC seem to be more sensitive to global cognition and domain-specific cognitive performance than serum vitamin B_{12} [29, 31, 59, 60] whereas results on incidence of AD were inconsistent [61-63]. However, more prospective cohort studies are needed to confirm these observations.

RCTs

As shown in this review, the limited evidence from RCTs showed no effects of oral vitamin B_{12} supplementation on cognitive performance. RCTs on the effects of intramuscular injections with vitamin B_{12} were excluded from this review as they do not reflect dietary intake of vitamin B_{12}, however these trials did not provide evidence for a beneficial effect of supplementation on cognitive performance either [64-66]. Data from short-duration (<4 months) trials on the effect of vitamin B_{12} in combination with other B-vitamins (folic acid, vitamin B6) on cognitive performance tests did not show any effects [45, 67-70], whereas trials with a duration of 2 years did show a beneficial effect of B-vitamin supplementation on executive function/speed [71, 72]. However, de Jager et al. (2011) only observed a significant benefit in MCI patients with high homocysteine levels at baseline (≥11.3 μmol/l) [71]. Two trials including subjects with mild to moderate AD did not show an effect of 1.5-2 years B-vitamin supplementation on the rate of cognitive decline [73, 74]. More RCTs on the effect of vitamin B_{12} on cognitive function are needed that are designed according to the recommendations from previous consensus meetings regarding sample sizes, selection of study populations, study duration and doses [52, 75-79].

Measures of cognitive function

Many different mechanisms have been suggested for the potential relation between vitamin B_{12} and cognitive performance as summarized by Smith and Refsum (2009)[52]. A commonly suggested mechanism is that a low vitamin B_{12} status compromises methylation reactions in the central nervous system. Besides, low-normal vitamin B_{12} status may affect the brain via elevated levels of homocysteine, raised concentrations of MMA or changes in cytokine concentrations. Neuro-imaging studies have shown that changes in brain structures and brain volume are associated with cognitive decline [80]. In addition, low-normal vitamin B_{12} status has been associated with loss of brain tissue (atrophy) [81-83] and damage to the white matter [27, 81] in healthy elderly people. A recent trial including patients with mild cognitive impairment showed a 30 percent lower rate of brain atrophy in participants after 2 year supplementation with high doses of vitamin B6, folic acid and vitamin B_{12} as compared to placebo. In participants with elevated homocysteine levels, the rate of atrophy was even 53 percent lower after supplementation. The rate of atrophy was significantly inversely associated with change in vitamin B_{12} status as measured by serum vitamin B_{12} or holo-TC [84]. These data indicate that measures of brain atrophy alone or in combination with domain-specific tests may be more sensitive for examining associations between vitamin B_{12} intake or status and cognitive performance.

CONCLUSIONS

Current evidence on the relation between vitamin B_{12} intake or status and cognitive function does not suffice for being involved in deriving recommendations on vitamin B_{12} intake as the evidence-base for the presence of an association is too narrow. Further studies should consider the selection of sensitive markers of vitamin B_{12} status (MMA and holo-TC) as a proxy for vitamin B_{12} intake, and measures of brain atrophy alone or in combination with domain-specific tests as cognitive outcomes.

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5

Vitamin B12, folate, homocysteine and bone health in adults and elderly people: a systematic review with meta-analyses

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ABSTRACT

Elevated homocysteine levels and low vitamin B_{12} and folate levels have been associated with deteriorated bone health. This systematic literature review with dose-response meta-analyses summarizes the available scientific evidence on associations of vitamin B_{12}, folate and homocysteine status with fractures and bone mineral density (BMD).

Twenty-seven eligible cross-sectional (n=14) and prospective (n=13) observational studies and one RCT were identified. Meta-analysis on four prospective studies including 7475 people showed a modest decrease in fracture risk of 4% per 50 pmol/L increase in vitamin B_{12} levels, which was borderline significant (RR=0.96, 95% CI = 0.92 to 1.00). Meta-analysis of eight studies including 11511 people showed an increased fracture risk of 4% per µmol/l increase in homocysteine concentration (RR=1.04, 95% CI = 1.02 to 1.07). We could not draw a conclusion regarding folate levels and fracture risk, as too few studies investigated this association. Meta-analyses regarding vitamin B_{12}, folate and homocysteine levels and BMD were possible in female populations only and showed no associations. Results from studies regarding BMD that could not be included in the meta-analyses were not univocal.

INTRODUCTION

Osteoporosis is a chronic, multifactorial disorder which is characterized by low bone mass and microarchitectural deterioration of bone tissue [1]. Its major consequence is fractures. Especially hip fractures are frequently associated with institutionalization and increased mortality, and thus with an increased social and economic burden. This burden is expected to increase substantially in Europe in the coming decades due to a rise in life expectancy [2].

Elevated homocysteine concentrations and low vitamin B_{12} and folate status have been associated in several studies with lower bone mineral density (BMD) and higher fracture risk in elderly [3-11].
An elevated plasma homocysteine level (>15μmol/l) is prevalent in 30-50% of people older than 60 years [12-14]. The cause is multifactorial; a combination of environmental and genetic factors, nutrition, lifestyle and hormonal factors [15]. Vitamin B_{12} and folate are major determinants of homocysteine metabolism [16, 17], and supplementation with vitamin B_{12} and folic acid has been shown to be effective in normalizing homocysteine levels [18, 19]. Reversing elevated homocysteine levels through folic acid and vitamin B_{12} supplementation could theoretically prevent the problem of impaired bone health and osteoporosis. However, at present, no consensus is reached on the magnitude of the association between vitamin B_{12}, folate, homocysteine and bone health nor on the possible effect of vitamin B_{12} and folate supplementation on bone health.

Up until now one systematic review including a meta-analysis summarized the evidence on homocysteine and fracture risk, showing that higher homocysteine levels significantly increase the risk of fracture [20]. No meta-analyses are known on the topic of folate and vitamin B_{12} in relation to bone health. The purpose of this article is to provide a systematic overview, where possible including pooled estimates of the dose –response association, of the scientific evidence available from randomized controlled trials (RCTs), prospective cohort and cross-sectional studies addressing vitamin B_{12}, folate and homocysteine levels in association with bone health, i.e. fracture risk and BMD, in adults and elderly people.

Methods

This systematic review with dose-response meta-analyses was conducted within the scope of the EURRECA (European Micronutrient Recommendations Aligned) Network of Excellence (http://www.eurreca.org) [21]. We followed a standardized methodology which is described in short below.

Search strategy and selection of articles

We conducted systematic literature searches for 1) vitamin B_{12} 2) folate and 3) homocysteine. The electronic databases MEDLINE, EMBASE and Cochrane Library Central were searched, using search terms in 'MeSH' terms and 'title' and 'abstract' on: study designs in humans, vitamin B_{12}, folate, homocysteine, and intake or status. The full search strategy is shown in Appendix I.
To be able to use the same search to identify publications on other health related outcomes both in

adults and elderly and in younger population groups, no terms were added to limit the search to health outcome or study population. Moreover, by using a broad search we expected a more complete retrieval of relevant publications. In this review only the results on vitamin B_{12}, folate and homocysteine status (i.e. biomarkers measured in serum or plasma) in relation to bone health indicators (fracture risk and BMD) are presented. In addition to the search, reference lists of 10 review articles were checked to identify potentially relevant references that were not identified with the multi-database search. The search was not limited by language. This review contains studies up to July 2012.

We selected articles in two steps. The first selection step included screening for title and abstract by three independent investigators (JvW, ED, SB). In the second selection step, full texts of the selected abstracts were evaluated on basis of predefined inclusion criteria by four investigators (JvW, ED, AS, MP).

For the purpose of alignment and quality control 10% of the references in each selection step was screened and selected in duplicate by two investigators independently. Results were compared and discrepancies were resolved by unanimous consensus among all investigators.

Studies were eligible for inclusion if they were conducted in apparently healthy human subjects aged ≥18 y. Furthermore, studies had to report fracture incidence, fracture risk or bone mineral density (BMD) as a health outcome and had to report baseline data on the outcome measure.

Observational studies were included if they 1) had a prospective cohort, nested case-control or cross-sectional design, and 2) addressed serum/plasma concentration of markers indicating vitamin B_{12} status (serum/plasma vitamin B_{12}, methylmalonic acid (MMA) or holotranscobalamin (holoTC)), folate status (serum/plasma folate or erythrocyte folate), or homocysteine status (serum/plasma homocysteine). Intervention studies were included if they 1) had a randomized controlled trial design, 2) studied the effects of vitamin B_{12} or folic acid supplements, fortified foods or micronutrient intake from natural food sources and included a placebo or untreated comparison group, and 3) had a minimum intervention duration of six months.

Data extraction and statistical analysis

We extracted data for each of the identified studies on population characteristics, study design, assessment of vitamin B_{12}, folate and homocysteine status and fracture risk or bone mineral density.

Opportunities for meta-analysis were evaluated based on comparability of health outcome and status marker. If less than three comparable studies were available, results were qualitatively described. If three or more comparable studies were available, the results of these individual studies were expressed in a standardized format to allow comparison in the form of a continuous dose-response meta-analysis that pools the regression coefficient (β) (SE) from multiple adjusted models. We chose to express association measures for serum/plasma vitamin B_{12} per 50 pmol/L. When βs were not reported in the original article, we transformed Relative Risk (RR), Hazard Ratio (HR) or Odds Ratio (OR) to βs, using a standardized method [22]. The transformations to obtain βs and SEs and statistical analyses were performed using R statistics version 2.9.2 (www.R-project.org), with statistical significance defined as $p<0.05$. HR and OR were considered as RR because the outcome was relatively rare.. If articles reported

insufficient data (missing data, inconsistencies or any other uncertainties), we contacted corresponding authors for additional information.

We calculated summary estimates of comparable studies using random effects meta-analysis. Applying the methods of DerSimonian and Laird, the between study variance was estimated which was used to modify the weights for calculating the summary estimate [23]. Residual heterogeneity between studies was evaluated using Q-statistic and I^2 statistic.

In total, from 3 searches we identified 11837 potentially relevant articles, of which 9835 articles were excluded based on title and abstract. Of the remaining 2002 articles, 1961 articles were excluded based on full texts, leaving 41 articles. As the searches were partly overlapping, and some articles addressed more than one association this resulted in 20 unique articles, 19 observational and 1 intervention. A search update on July 2nd, 2012 resulted in an additional 8 observational studies, which makes a total of 28 included articles. All addressed the association between vitamin B_{12}, folate or homocysteine status, and fracture risk or BMD. The flow diagram of the process of screening and selection is shown in Figure 1.

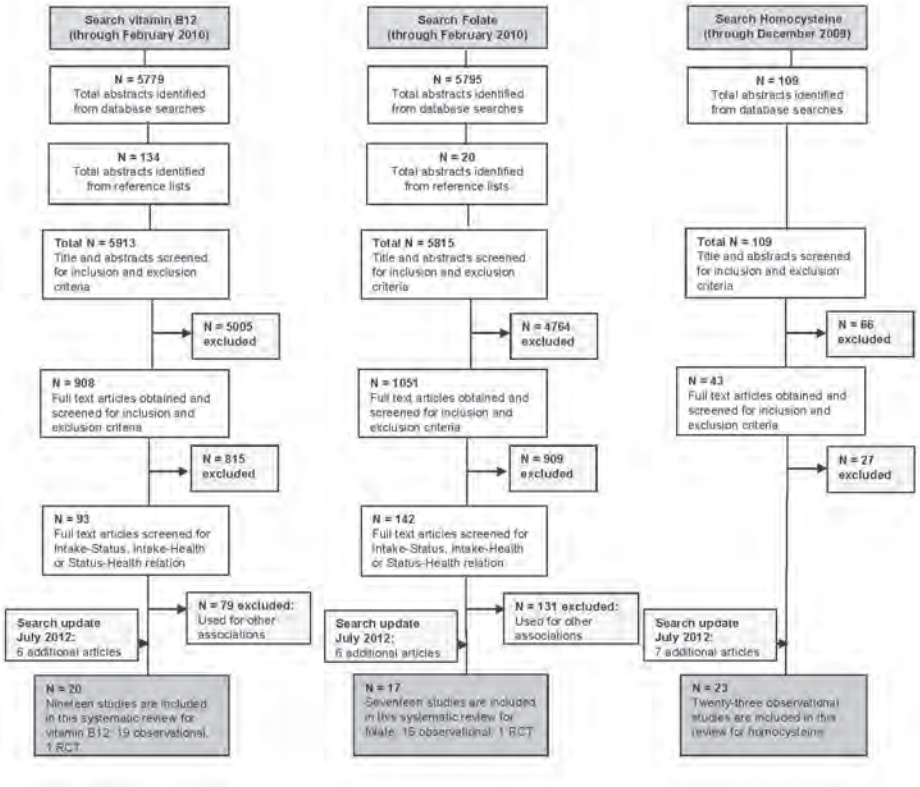

Figure 1. Flow diagram of screening and selection.

RESULTS

Fractures

Vitamin B_{12}

Four longitudinal observational studies [3, 24-26], including 7475 elderly people with 3 to 16 years of follow-up and a total of 458 cases addressed the association between serum/plasma vitamin B_{12} and fracture (Table 1). Pooled analysis of the association between 50 pmol/L increase in plasma/serum B_{12} and change in fracture risk showed an inverse association (RR= 0.96, 95% CI= 0.92 to 1.00) with no heterogeneity between studies (I^2= 0%, p= 0.84) (Figure 2). This indicates that a vitamin B_{12} increase of 50 pmol/L tends to decrease the risk of fracture with 4%.

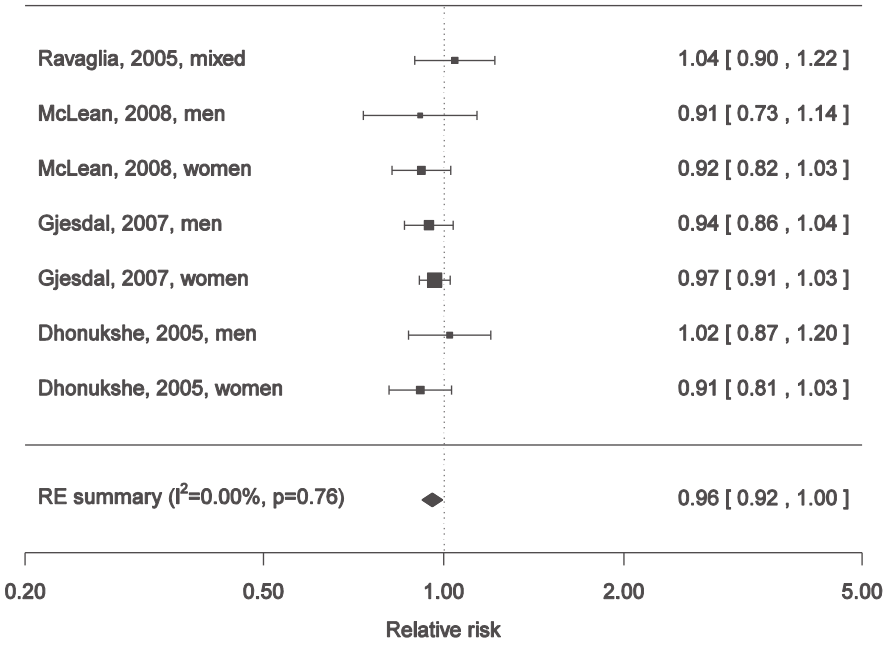

Figure 2. Forest plot of vitamine B_{12} status - fracture risk

Folate

Three longitudinal observational studies examined the association between plasma folate and fractures [24-26] (Table 2). One study showed that women, but not men, with plasma folate in the lowest quartile had a higher fracture risk (HR 2.40, 95% CI 1.50 to 3.84) compared to the highest (reference) quartile (p for trend <0.001) [24]. Ravaglia et al. (2005) showed a significant association between low folate status and fracture risk when folate was analyzed as a dichotomous variable (lowest quartile of folate status vs other 3 quartiles), but when analyzed as a continuous variable, no significant association was observed [26]. One study did not observe an association [25].

Homocysteine

Eleven longitudinal observational studies examined the association between homocysteine status and fracture incidence [3-5, 25-29] (Table 3). A meta-analysis of eight studies, including 11511 elderly people with 3 to 12.6 years of follow up and 1353 cases, showed a significantly increased fracture risk with increasing plasma homocysteine (μmol/L) (summary estimate RR 1.04, (95% CI: 1.02 to 1.07). Heterogeneity between studies was large (I^2=60.57%, p=0.0002) (Figure 3). When hip fractures (3 studies; [24, 28, 29]) and total fractures (5 studies; [3, 26, 27, 30, 31]) were analyzed separately, the relation remained significant, 1.06 (95% CI: 1.03 to 1.08, I^2=0.00%, p=0.72) and 1.04 (95% CI: 1.00 to 1.08, I^2=65.02%, p=0.011).

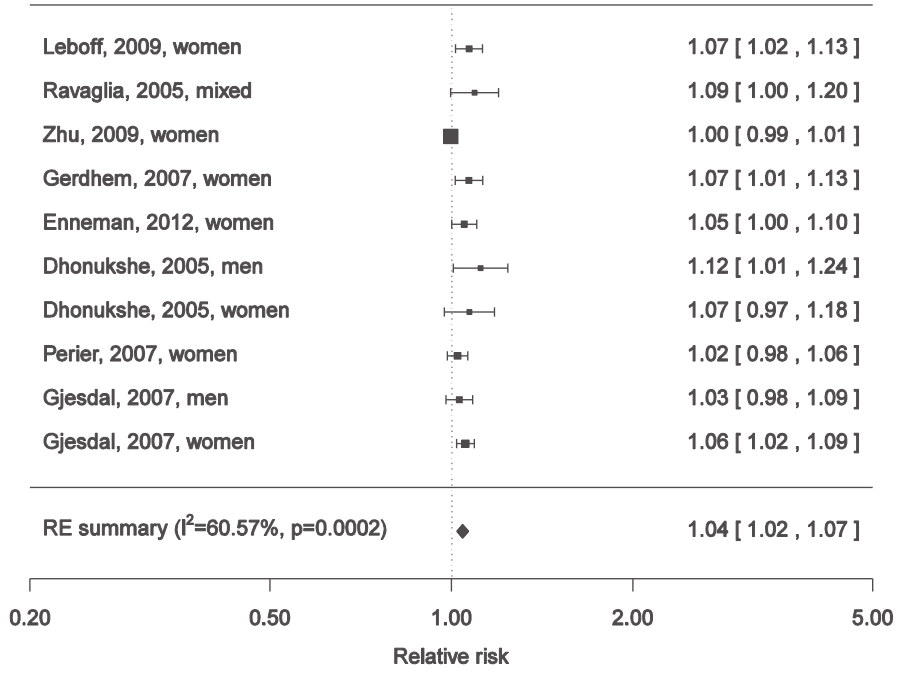

Figure 3. Forest plot of homocysteine status - fracture risk

Three studies that were not included in the meta-analysis also showed significant associations between homocysteine levels and fracture risk. These studies were not included because the necessary data could not be retrieved from the articles; either homocysteine levels were log-transformed [4, 5] or data were not shown on population homocysteine status [25]. Regardless the type of analysis, women and men in the highest homocysteine quartile had a 1.7 to 3.8 higher RR or HR than those in the lowest or the lowest three quartiles [4, 5, 25].

Table 1. Studies regarding the association between vitamin B12 and bone health

Author **Year**	**Study characteristics** **Duration of follow-up (when applicable)** **Country** ***Risk of bias***	**Population characteristics:** **N (%men)** **Age (y) ± SD**	**Vitamin B12 status pmol/L*** **Mean ± SD**	**Outcome**	**Association type**	**Results***
Dhonukshe-Rutten 2005 [3]	Cohort (3 y) The Netherlands *High risk*	1253 (48%) 75.5 ± 6.6	♀: 289 ± 99 ♂: 268 ± 89	Fracture (verified by physician or radiograph)	β (SE) for association vitB12-fracture (per 50pmol/L)	♀: -0.09(0.06) [a, 1] ♂: 0.02(0.08) [a, 1]
Gjesdal 2007 [24]	Cohort (12.6y) Norway *Low risk*	4761 (45%) 65-67 at baseline	♀: 386.4 ± 372.0 ♂: 359.3 ± 276.2	Hip fracture (verified by hospital discharge diagnoses)	β (SE) for association vitB12-hip fracture (per 50pmol/L)	♀: -0.03(0.03) [b, 2] ♂: -0.06(0.05) [b, 2]
McLean 2008 [25]	Cohort (16y) USA *Low risk*	823 (41%) 75.3 ± 4.9	Deficient (<148pmol/L) : ♀9%/ ♂14.0% Low (148-257.9): ♀24.3%/ ♂32.5% Normal (≥258): ♀66.7% / ♂53.5%	Hip fracture (verified by review medical records)	β(SE) for association vitB12-hip fracture (per 50pmol/L)	♀: -0.09(0.06) [c, 1] ♂: -0.09(0.11) [c, 1]
Ravaglia 2005 [26]	Cohort (4y) Italy *Moderate risk*	702 (47%) 73.0 ± 6.0	Geometric mean (95% CI) 249.1 (203-272)	Fracture (verified by review medical records)	β(SE) for association vitB12-fracture (per 50pmol/L)	0.04(0.08) [d, 2]
Bozkurt 2007 [38]	Cross-sectional Turkey *High risk*	178 (0%) 53.5 ± 8.0	247.7 ± 85.4	BMD: LS, FN [DXA]	Logistic regression for FN, LS and FN + LS combined for vitB12 status under the quintile value. β(SE) + p-value	LS: -2.3 (0.9) p=0.017 FN: -0.4 (0.9) p=0.669 LS + FN: 1.8 (0.8) p=0.045 [e]
Bucciarelli 2010 [37]	Cross-sectional Italy *Moderate risk*	446 (0%) 65.1 ± 9.4	(geometric mean ± SD) 399.1 ± 1.6	BMD: FN, LS, TH [DXA, Prodigy, GE, Lunar]	β for association vitB12 –TH BMD β (SE) (per 50 pmol/L)	-0.00105(0.939) [f, 2]
Cagnacci 2008 [39]	Cohort (5y) Italy *Moderate risk*	117 (0%) 54.4 ±0.5	(Mean ± SE) 548.5 ± 40.5	BMD: LS [DXA: Lunar DPX]	Regression for vitB12-BMD change β(SE) p-value	-0.003(0.012) p=0.784 [g]

Table 1. Continued

Dhonukshe-Rutten 2003 [32]	Cross-sectional The Netherlands *Moderate risk*	194 (26%) 78.3 ± 5.5	♀ 288 ± 131 ♂ 238 ± 95	BMD: whole body [DXA, Lunar DPX-L]	Multivariate regression, β for association vitB12 - BMD β (95% CI) in women	♀ $12.3 \cdot 10^{-5}$ ($0.2 \cdot 10^{-5}$ - $2.4 \cdot 10^{-4}$) [h]
Gjesdal 2006 [10]	Cross-sectional Norway *Moderate risk*	5329 (43%) middle aged: 47-50 Older: 71-75	♀ 393.4 ± 235.8 ♂ 374.6 ± 230.7	BMD: TH [DXA, Lunar EXPERT-XL]	OR (95% CI) for low BMD per category vitB12 status 1= <230 pmol/l 2= 230.0-279.9 pmol/l 3= 280.0-414.9 pmol/l 4= ≥415.0 pmol/l + p for trend	♀ ♂ 1: 0.97 (0.68-1.37) 1.22 (0.82-1.81) 2: 0.87 (0.63-1.21) 1.14 (0.80-1.62) 3: 1.02 (0.82-1.27) 0.97 (0.74-1.28) 4: 1.00 (reference) 1.00 (reference) P for trend = 0.61 P for trend = 0.25[i]
Golbahar 2004 [9]	Cross-sectional Iran *Moderate risk*	271 (0%) 60.8 ± 6.8	(geometric mean ± SD) 339.5 ± 247.6	BMD: FN, LS [DXA, Lunar DPX-L]	β(SE) for association vitB12-BMD (per 50pmol/L)	FN: 0.0002 (0.07)[2] LS: 0.0114 (0.14)[2]
Haliloglu 2010 [40]	Cross-sectional Turkey *Moderate risk*	120 (0%) 54.4 ± 1.1	Osteoporotic: 216.0 ± 135.1 Osteopenic: 190.8 ± 97.4 Normal BMD: 251.0 ± 205.8	BMD: LS [DXA, Lunar DPX-L]	ANOVA for difference in vitB12 status per BMD group compared to normal BMD group	No sign differences in vitB12 status between BMD groups
Krivošíková 2010 [35]	Cross-sectional Slovakia *High risk*	272 (0%) 41.3 ± 19.8	273.2 ± 152.7	BMD: FN, LS, trochanter, TH [DXA, Lunar DPX-L]	Stepwise multivariate linear regression, β for association vitB12 - BMD. β (SE) p-value (per 50pmol/L)	FN: -2.0 (2.73) [j,2] LS: -1.15 (1.42) [j,2] TH: -0.5 (3.03) [j,2]

Table 1. Continued

Author Year	Study characteristics Duration of follow-up (when applicable) Country *Risk of bias*	Population characteristics: N (%men) Age (y) ± SD	Vitamin B12 status pmol/L* Mean ± SD	Outcome	Association type	Results*
Morris 2005 [7]	Cross-sectional USA *Low risk*	1550 (48%) 68	Geometric mean (95% CI) Osteoporosis: 271 (243-302) Osteopenia: 309 (293-325) Normal: 310 (297-323) Serum MMA (nmol/L) Osteoporosis: 305 (276-337) Osteopenia: 251 (234-269) Normal: 241 (212-274)	BMD: Trochanter, intertrochanter, FN, Ward's triangle, TH [DXA, Hologic QDR-1000]	OR (95% CI) for mean BMD in relation to quartile categories of vitB12 and MMA status + p for trend. Category medians: B12 (pmol/L) MMA(nmol/L) Q1: 182 157 Q2: 268 206 Q3: 349 272 Q4: 495 415	Vit B12 MMA Q1: 2.0 (1.0-3.9) 1.0 (reference) Q2: 1.3 (0.6-2.7) 3.5 (1.4-8.5) Q3: 1.7 (0.8-3.3) 5.2 (2.0-13.1) Q4: 1.0 (reference) 7.2 (3.4-15.2) P for trend = 0.09 P for trend <0.001 [k] Among subjects with vitB12<220 pmol/L mean BMD increased sign with increasing vitB12 (P=0.01)
Naharci 2010 [33]	Cross-sectional Turkey *Moderate risk*	264 (100%) 77.0 ± 6.0	26.7% low (<148, group I) 39.1% borderline (148-221, group II) 34.2% normal (>221, group III)	BMD: FN, TH, trochanter, inter-trochanter [DXA, hologic QDR-4500]	Anova for differences in FN BMD between groups of serum vitB12	Sign differences FN BMD group I and II (p=0.013) group I and III (p<0.001) group II and III (p=0.003) FN BMD was positively correlated with serum vitB12 (r=0.362, p<0.001)
Ouzzif 2012 [36]	Cross-sectional Morocco *Moderate risk*	188 (0%) 57.8 ± 8.5	360.4 ± 149.2	BMD: FN, LS, TH, trochanter [DXA, Lunar prodigy]	Multivariate regression, β for association vitB12 –BMD β (SE) (per 50pmol/L) p-value	LS: -7.85 (0.25) p=0.160 [L, 2] TH: -11.65 (0.02) p=0.007 [L, 2]
Rumbak 2012 [34]	Cross-sectional Croatia *Low risk*	131 (0%) 54.0 ± 4.9	239.6 ± 97.0	BMD: FN, LS, TH, radius [DXA, Lunar-prodigy]	Stepwise multivariate regression, β for association vitB12 –BMD for pre- and postmenopausal women β (SE) p-value (per 50pmol/L)	Premenopausal: LS: -3.39 (8.91) p=0.709 [m, 2] FN: 7.45 (10.07) p=0.467 [m, 2] TH: -1.36 (7.53) p=0.862 [m, 2] Postmenopausal: LS: 7.45 (8.98) p=0.411 [m, 2] FN: 12.20 (8.97) p=0.180 [m, 2] TH: 8.81 (8.63) p= 0.314 [m, 2]

Table 1. Continued

Stone 2004 [11]	Cohort (5.9y) USA *Low risk*	83 (0%) 71.1 ± 4.4	352 ± 174	BMD: TH, FN (change) [DXA, Hologic QDR-1000]	t-test for difference in BMD change between low and normal vitB12 status	Participants with low vitB12 (≤207 pmol/l) had a more rapid decline in BMD (-1.91%/year) than part. with normal vitB12 (-0.10%/year), p<0.05
Tucker 2005 [6]	Cross-sectional USA *Low risk*	2576 (44%) 58.8 ± 9.5	Distribution per category of plasma vitB12 status 1) ♀ 4.4% / ♂4.7% ≤148 2) ♀ 6.9% / ♂7.8% >148-185 3) ♀ 25.4% / ♂28.2% >185-259 4) ♀ 63.3% / ♂ 59.3% >259	BMD: FN, LS, TH, Trochanter, Ward [DXA, Lunar DPX-L]	Differences in BMD per category of plasma vitB12 level, relative to category 1	♀: FN: no differences ♀: LS: vitB12 in cat 2 (p<0.10), 3,4 was assoc. with better BMD (p<0.05) ♀: TH: vitB12 in cat 3,4 (p<0.10) was assoc. with better BMD ♂: FN: vitB12 in cat 2,3,4 was assoc. with better BMD (p<0.05) ♂ LS: no differences ♂ TH: vitB12 in cat 2 (p<0.10), 3,4 (p<0.05) was assoc. with better BMD[n]

*Serum/ plasma vitamin B12 concentrations were converted to pmol/l if applicable, using the following equation: 1 pg/ml = 1 ng/l = 0.738 pmol/l. subsequent outcomes were also converted. where possible, subgroups were combined. BMD sites: LS = Lumbar Spine, FN = Femoral Neck, TH = Total Hip

1 β(SE) as calculated from data provided by author; 2 β(SE) as calculated from presented data

a adjusted for age, BMI, smoking, recurrent falling; b adjusted for age, BMI, smoking, coffee intake, physical activity, vit D use, educational level, estrogen use in women; c adjusted for sex, age, height, weight, estrogen use in women; d adjusted for age, sex, education, osteoporosis drugs, creatinine, tHcy; e adjusted for duration of menopause, smoking, BMI, folic acid levels, tHcy levels; f adjusted for age, BMI, logtHcy, logFolate, creatinine clearance, smoking, alcohol intake; g Adjusted for age, weight, weight change; h adjusted for weight, height, energy intake; i adjusted for smoking, BMI, creatinin, coffee intake, physical activity, use of estrogen therapy; j adjusted for age, folate, tHcy, PTH, CTx, Ca, Cr; k Adjusted for age, sex, ethnicity, BMI, smoking, physical activity, creatinin, alcohol, coffee, energy, calcium, vitamin D zinc intake; L adjusted for age, BMI, tHcy and folate; m adjusted for Age, BMI, smoking, alcohol, physical activity, tHcy, Folate; n adjusted for energy, calcium, vitamin D intake, BMI, height, smoking, age, physical activity, calcium supplement, vitamin D supplement, alcohol, osteoporosis medication, season of measurement

Table 2. studies regarding the association between folate and bone health

Author Year	Study characteristics Duration of follow-up (when applicable) Country Risk of bias	Population characteristics: N (%men) Age (y) ± SD	Folate status (nmol/L)* Mean ± SD	Outcome	Association type	Results*
Gjesdal 2007 [24]	Cohort (12.6y) Norway Low risk	4761 (45%) 65-67 at baseline	♀ 6.0 ± 3.5 ♂ 5.2 ± 2.7	Hip fracture (verified by hospital discharge diagnoses)	HR for hip fracture according to folate status <2.9 2.9-3.8 3.9-6.5 ≥6.6	♀: ♂: 1) 2.40 (1.50-3.84) 1.00 (0.48-2.12) 2) 1.15 (0.68-1.94) 0.80 (0.39-1.62) 3) 1.02 (0.68-1.54) 0.81 (0.45-1.46) 4) 1.00 (reference) [a] 1.00 (reference) [a]
McLean 2008 [25]	Cohort (16y) USA Low risk	960 (41%) 75.3 ± 4.9	Not shown	Hip fracture (verified by review medical records)	HR for hip fracture according to folate status Normal: ≥11 Low: 7-10.9 Deficient: <7	Normal: 1.00 (reference) Low: 0.76 (0.43, 1.32) Deficient: 1.38 (0.91, 2.09) [b]
Ravaglia 2005 [26]	Cohort (4y) Italy Moderate risk	702 (47%) 73.0 ± 6.0	11.7 (9.0-12.2) mean (95% CI)	Fracture (verified by review medical records)	OR (95% CI) for risk of fracture at follow-up for each increment of 1 sd in the log-transformed serum folate value	0.83 (0.59-1.19) [c]
Baines 2007 [41]	Cross-sectional Great Britain High risk	328 (0%) 67.5 (40-85) mean (range)	Osteoporosis: 8.1 ± 8.7[e] Osteopenia: 10.2 ± 4.6 Normal: 9.4 ± 6.3	BMD: os calcis/ heel bone [PIXI, GE Lunar]	ANOVA for difference between the normal, osteopenia and osteoporosis group	FA status was significantly different between osteroporotic and osteopenic group (p=0.049)
Bozkurt 2007 [38]	Cross-sectional Turkey High risk	178 (0%) 53.5 ± 8.0	24.9 ± 7.9	BMD: FN, LS [DXA]	Logistic regression for FN, LS and FN + LS combined. β(SE) + p-value for assoc. BMD-folate status under the median value	LS: -0.2(0.2) p=0.417 FN: -0.04(0.2) p=0.835 LS + FN: -0.03(0.2) p=0.896 [d]
Bucciarelli 2010 [37]	Cross-sectional Italy Moderate risk	446 (0%) 65.1 ± 9.4	(geometric mean ± SD) 3.8 ± 1.6	BMD: FN, LS, TH [DXA, Prodigy, GE, Lunar]	β for association folate –TH BMD β (SE)	0.004(0.018)[c, 2]
Cagnacci 2008 [39]	Cohort (5y) Italy Moderate risk	117 (0%) 54.4 ± 0.5	(Mean ± SE) 20.6 ± 1.4	BMD: LS [DXA: Lunar DPX]	Regression analysis for folate-BMD change β(SE) + p-value	1.602(0.803) p=0.048 [f]

Table 2. Continued

Cagnacci 2003 [8]	Cross-sectional Italy Moderate risk	161 (0%) 53.3 ± 1.04	(Mean ± SE) 21.5 ± 4.3	BMD: LS [DXA: Lunar DPX]	Regression analysis, r (p-value) for association folate-BMD	r=0.254 (p<0.002)
Gjesdal 2006 [10]	Cross-sectional Norway Moderate risk	5329 (43%) middle aged: 47-50 Older: 71-75	♀ 8.9 ± 7.1 ♂ 7.3 ± 4.6	BMD: TH [DXA, Lunar EXPERT-XL]	OR (95% CI) for low BMD per category folate status: 1= FA <3.8 nmol/l 2= FA 3.8-4.9 nmol/l 3= FA 5.0-8.4 nmol/l 4= FA ≥ 8.5nmol/l + p for trend Multivariate regression for folate – BMD β(SE) (per 50 nmol/L)	♀ ♂ 1: 1.55 (1.07-2.23) 0.81 (0.53-1.24) 2: 1.18 (0.86-1.63) 0.96 (0.67-1.38) 3: 1.24 (0.99-1.56) 1.15 (0.87-1.53) 4: 1.00 (reference) 1.00 (reference) P for trend = 0.02 P for trend = 0.26[g] Elderly women: β =0.05 (0.02) [g,2]
Golbahar 2004 [9]	Cross-sectional Iran Moderate risk	271 (0%) 60.8 ± 6.8	(geometric mean ± SD) 11.6 ± 6.5	BMD: FN, LS [DXA, Lunar DPX-L]	β for association folate –BMD β (SE)	FN: 0.008 (0.019) [h, 2] LS: 0.010 (0.018) [i, 2]
Haliloglu 2010 [40]	Cross-sectional Turkey Moderate risk	120 (0%) 54.4 ± 1.1	Osteoporotic: 12.2 ± 6.3 Osteopenic: 15.4 ± 7.4 Normal: 15.8 ± 8.3	BMD: LS [DXA, Lunar DPX-L]	ANOVA for difference in folate status per BMD group (osteoporotic, osteopenic, compared to normal BMD group)	No significant differences in folate status between BMD groups
Krivošíková 2010 [35]	Cross-sectional Slovakia High risk	272 (0%) 41.3 ± 19.8	23.8 ± 9.6	BMD: FN, LS, trochanter, TH [DXA, Lunar DPX-L]	Stepwise multivariate linear regression, β for association folate - BMD. β (SE) p-value	FN:-0.028 (0.054) p=0.606 [j, 2] LS: -0.001 (0.067) p=0.988 [j, 2] TH: -0.032 (0.060) p=0.595 [j, 2]
Morris 2005[7]	Cross-sectional USA Low risk	1550 (47%) 68	Osteoporosis: 17.2 (15.4-19.2) Osteopenia: 17.2 (16.0-18.5) Normal: 16.7 (15.3-18.3) Geometric mean (95% CI)	BMD: Trochanter, intertrochanter, FN, Ward's triangle, TH [DXA, Hologic QDR-1000]	OR (95% CI) for mean BMD in relation to quartile categories of folate status + p for trend Category median (nmol/L): Q1: 8.0 Q2: 12.4 Q3: 20.3 Q4: 38.9	Q1: 1.1 (0.5-2.3) Q2: 1.1 (0.0.5-2.9) Q3: 1.5 (0.7-3.4) Q4: 1.0 (reference) P for trend = 0.83 [k]

Table 2. Continued

Author Year	Study characteristics Duration of follow-up (when applicable) Country Risk of bias	Population characteristics: N (%men) Age (y) ± SD	Folate status (nmol/L)* Mean ± SD	Outcome	Association type	Results*
Naharci 2010 [33]	Cross-sectional Turkey Moderate risk	264 (100%) 77.0 ± 6.0	low (<7.0, group I): 0.0% borderline (7.0-10.9, group II): 9.2% normal (>10.9, group III): 90.8%	BMD: FN, TH, trochanter, intertrochanter [DXA, hologic QDR-4500]	Independent sample t-test for differences in FN BMD between group II and III of serum folate	No significant differences in BMD (all sites) between group II and III of folate status
Ouzzif 2012 [36]	Cross-sectional Morocco Moderate risk	188 (0%) 57.8 ± 8.5	15.6 ± 6.8	BMD: FN, LS, TH, trochanter [DXA, Lunar prodigy]	Multivariate regression, β for association folate –BMD β (SE) + p-value	LS: 0.007 (0.002) p=0.808[L] TH: 0.006 (0.001) p=0.834[L]
Rumbak 2012 [34]	Cross-sectional Croatia Low risk	131 (0%) 54.0 ± 4.9	22.4 ± 7.5	BMD: FN, LS, TH, radius [DXA, Lunar-prodigy]	Stepwise multivariate regression, β for association folate –BMD β + p-value	Premenopausal: LS: 3.31 (4.73) p=0.490 [m, 2] FN: 1.32 (4.90) p=0.791 [m,2] TH: 2.87 (4.35) p=0.516 [m,2] Postmenopausal: LS: -3.75 (3.47) p=0.284 [m,2] FN: -1.32 (3.15) p=0.679 [m,2] TH: 0.66 (3.89) p= 0.862 [m,2]

*Serum/ plasma folate concentrations were converted to nmol/L if applicable, using the following equation: 1 ng/ml= 2.266 nmol/L. Subsequent outcomes were also converted. where possible, subgroups were combined. BMD sites: LS = Lumbar Spine, FN = Femoral Neck, TH = Total Hip #data presented in article as µmol/l, this is presumably a typing error and should be nmol/l

1 β(SE) as calculated from data provided by author; 2 β(SE) as calculated from presented data

a adjusted for age, BMI, smoking, coffee intake, physical activity, vit D use, educational level, estrogen use in women; b adjusted for sex, age, height, weight, estrogen use in women; c adjusted for age, gender, education, osteoporosis drug, serum creatinine, tHcy; d Adjusted for duration of menopause, smoking, BMI, B12, tHcy; e adjusted for age, BMI, logtHcy, logB12, creatinine clearance, smoking, alcohol intake; f Adjusted for age, weight, weight change; g Adjusted for smoking, BMI, creatinin, coffee intake, physical activity, use of estrogen therapy; h adjusted for age, BMI, alkaline phosphatase; i adjusted for years since menopause, BMI, alkaline phosphatase, creatinine; j adjusted for age, B12, tHcy, PTH, CTx, Ca, Cr; k Adjusted for age, sex, ethnicity, BMI, smoking, physical activity, creatinin, alcohol, coffee, energy, calcium, vitamin D zinc intake; L adjusted for age, BMI, tHcy, B12; m adjusted for Age, BMI, smoking, alcohol, physical activity, tHcy, B12

Table 3. studies regarding the association between homocysteine and bone health

Author Year	Study characteristics Duration of follow-up (when applicable) Country Risk of bias	Population characteristics: N (%men) Age (y) ± SD	Homocysteine status (µmol/L) Mean ± SD	Outcome	Association type	Results
Dhonukshe-Rutten 2005 [3]	Cohort (3y) The Netherlands High risk	1253 (48%) 75.5 ± 6.6	geometric mean (10-90 percentile) ♀: 13.0 (8.6-19.7) ♂: 14.9 (10.2-22.8)	Fracture (verified by physician or radiograph)	β (SE) for association tHcy-fracture	♀: 0.07(0.05) [a, 2] ♂: 0.11(0.05) [a, 2]
Enneman 2012 [30]	Cohort (7y) The Netherlands Moderate risk	503 (0%) 68.5 (61.3-74.9) Median (range)	Median (range) 9.3 (3.5-29.7)	Fracture (verified by physician)	β (SE) for association tHcy- fracture	0.05(0.02) [b, 2]
Gerdhem 2007 [29]	Cohort (7y) Sweden Low risk	996 (0%) 75	Median (IQR) 14.1 (11.6-17.3)	Hip fracture (verified by radiograph)	β (SE) for association tHcy-hip fracture	0.07(0.03) [c, 2]
Gjesdal 2007 [24]	Cohort (12.6y) Norway Low risk	4761 (45%) 65-67 at baseline	♀: 11.6 ± 4.2 ♂: 13.1 ± 5.8	Hip fracture (verified by hospital discharge diagnoses)	β (SE) for association tHcy-hip fracture	♀: 0.05(0.02) [d, 2] ♂: 0.03(0.03) [d, 2]
Leboff, 2009 [28]	Nested case-control USA Moderate risk	800 (0%) 70.8 ± 6.2	11.2 ± 4.1	Hip fracture (verified by radiograph)	β (SE) for association tHcy-Hip fracture	0.07(0.03) [e, 2]
McLean 2004 [4]	Cohort (♀ 15y; ♀12.3y) USA Moderate risk	1999 (41%) 70.0 ± 7.0	♀: 12.1 ± 5.3 ♂: 13.4 ± 9.1	Hip fracture (verified by review medical records)	HR (95% CI) for hip fracture risk by quartiles of tHcy. Mean tHcy per quartile: ♀: Q1: 7.6 ± 1.0 ♂: 8.5 ± 0.9 Q2: 9.9 ± 0.7 11.0 ± 0.6 Q3: 12.2 ± 0.7 13.4 ± 0.9 Q4: 18.6 ± 6.4 20.8 ± 15.7 HR (95% CI) for each increase of 1 SD in log-transformed tHcy concentration	♀: ♂: 1: 1.00 (reference) 1.00 (reference) 2: 0.78 (0.45-1.33) 1.57 (0.54-5.14) 3: 1.07 (0.64-1.78) 2.07 (0.70-6.09) 4: 1.92 (1.18-3.10) 3.84 (1.38-10.70) ♀/♂Test for trend: p<0.01 ♂HR per SD 1.59 (1.31-1.94) [f] ♀ HR per SD 1.26 (1.08-1.47) [f]
McLean 2008 [25]	Cohort (16y) USA Low risk	979 (41%) 75.3 ± 4.9	73.7% normal (≤14 µmol/l) 26.3% high (>14)	Hip fracture (verified by review medical records)	HR (95% CI) for high plasma tHcy (≥14 µmol/l) vs. normal tHcy	Normal 1.00 (reference) High 1.69 (1.12-2.55) [g]

Table 3. Continued

Author Year	Study characteristics Duration of follow-up (when applicable) Country Risk of bias	Population characteristics: N (%men) Age (y) ± SD	Homocysteine status (µmol/L) Mean ± SD	Outcome	Association type	Results
Van Meurs 2004 [5]	Cohort (4.7y) The Netherlands High risk	2406 (47%) 73.9 ± 7.8	14.3 ± 5.8	Fracture (verified by physician)	RR (95% CI) for fracture for each increment of 1 SD in the natural log-transformed tHcy value.	1.4(1.2-1.6) [h]
Périer 2007 [27]	Cohort (10y) France Moderate risk	671 (0%) 61.6 ± 8.4	10.6 ± 3.5	Fracture (verified by radiograph or surgical report)	β (SE) for association tHcy-fracture	0.02(0.02) [i, 3]
Ravaglia 2005 [26]	Cohort (4y) Italy Moderate risk	702 (47%) 73.0 ± 6.0	Geometric mean (95% CI) 12.7 (11.3-15.1)	Fracture (verified by review medical records)	β (SE) for association tHcy-fracture	0.09(0.05) [j, 2]
Zhu 2009 [31]	Cohort (5y) Australia Moderate risk	1213 (0%) 75.2 ± 2.7	12.1 ± 4.6	Fracture (verified by radiograph)	β (SE) for association tHcy- fracture	-0.002(0.006) [k, 2]
Baines 2007 [41]	Cross-sectional Great Britain High risk	328 (0%) 67.5 (40-85) mean (range)	12.3 ± 5.4	BMD: os calcis/ heel bone [PIXI, GELunar]	Stepwise multivariate linear regression β(SE) + p-value for association log tHcy - BMD	-1.548(0.607) p=0.011 [L]
Bozkurt 2007 [38]	Cross-sectional Turkey High risk	178 (0%) 53.5 ± 8.0	10.4 ± 3.0[#]	BMD: FN/LS [DXA]	Logistic regression for FN, LS and FN + LS combined. β(SE) + p-value for association hcy level under the median value - BMD	LS: -0.8(0.5) p=0.140 FN: -0.5(0.6) p=0.408 LS + FN: -1.3 (0.6) p=0.032 [m]
Bucciarelli 2010 [37]	Cross-sectional Italy Moderate risk	446 (0%) 65.1 ± 9.4	(geometric mean ± SD) 10.6 ± 1.3	BMD: FN, LS, TH [DXA, Prodigy, GE, Lunar]	Multivariate linear regression β for association log tHcy – total femur BMD. β (SE) p-value	-0.050 (0.025) p=0.048 [n, 2]
Cagnacci, 2008 [39]	Cohort Italy Moderate risk	117 (0%) 54.4 ± 0.5	(Mean ± SE) 10.7 ± 0.5	BMD: LS [DXA: Lunar DPX]	Regression analysis for Hcy-BMD change β(SE) + p-value	-0.825(1.09) p=0.449 [o]

Table 3. Continued

Cagnacci 2003 [8]	Cross-sectional Italy Moderate risk	161 (0%) 53.3 ± 1.0	10.5 ± 0.9	BMD: LS [DXA: Lunar DPX]	Regression analysis, β for association Hcy-BMD	β=-0.002 [p, 1]
Gerdhem 2007 [29]	Cohort (cross sect data) Sweden Low risk	996 (0%) 75	Median (IQR) 14.1 (11.6-17.3)	BMD: FN, LS, TH [DXA: Lunar DPX-L]	t-test for difference in BMD (p-value) between highest quartile of hcy vs all others	FN: Q4 vs Q1-3: p=0.032 LS: Q4 vs Q1-3:p=0.821 TH: Q4 vs Q1-3:p=0.001
Gjesdal 2006 [10]	Cross-sectional Norway Moderate risk	5329 (43%) middle aged: 47-50 Older: 71-75	♀: 10.2 ± 4.5 ♂: 11.8 ± 3.9	BMD: TH [DXA, Lunar EXPERT-XL]	Multivariate regression, β for association tHcy-BMD (p-value) for middle aged and elderly women. (Data men not shown) OR (95% CI) for low BMD per category tHcy status + p for trend: 1: <9.0 µmol/l 2: 9.0-11.9 µmol/l 3: 12.0-14.9 µmol/l 4: ≥ 15 µmol/l	Mid. aged women: β=0.004 (p<0.001) elderly women: β=0.003 (p<0.001)[q] ♀ ♂ 1: 1.00 (reference) 1.00 (reference) 2: 1.14 (0.90-1.44) 1.01 (0.74-1.37) 3: 1.30 (0.95-1.79) 1.12 (0.79-1.60) 4: 2.19 (1.48-3.25) 1.02 (0.66-1.56) P for trend <0.001 P for trend = 0.72[q]
Golbahar 2004 [9]	Cross-sectional Iran Moderate risk	271 (0%) 60.8 ± 6.8	geometric mean (95% CI) 13.7 (7-14)	BMD: FN, LS [DXA, Lunar DPX-L]	β for association tHcy –BMD β (SE)	FN: -0.012 (0.023) [2] LS: -0.010 (0.024) [2]
Haliloglu 2010 [40]	Cross-sectional Turkey Moderate risk	120 (0%) 54.4 ± 1.1	Osteoporotic: 15.0 ± 4.6 Osteopenic: 14.2 ± 3.7 Normal: 11.2 ± 2.6	BMD: LS [DXA, Lunar DPX-L]	ANOVA for difference in tHcy status per BMD group	tHcy was sign. higher in the osteoporotic group vs normal group (p<0.05)
Krivošíková, 2010 [35]	Cross-sectional Slovakia High risk	272 (0%) 41.3 ± 19.8	(µmol/L) 14.6 ± 5.5	BMD: FN, LS, trochanter, TH [DXA, Lunar DPX-L]	Stepwise multivariate linear regression, β for association tHcy - BMD. β (SE) p-value	FN:-0.093 (0.06) p=0.100 [r, 2] LS: 0.003 (0.07) p=0.965 [r, 2] TH: -0.134 (0.06) p=0.033 [r, 2]

Table 3. Continued

Author Year	Study characteristics Duration of follow-up (when applicable) Country Risk of bias	Population characteristics: N (%men) Age (y) ± SD	Homocysteine status (µmol/L) Mean ± SD	Outcome	Association type	Results
Morris 2005 [7]	Cross-sectional USA Low risk	1550 (47%) 68	Osteoporosis: 11.5 (10.3-12.7) Osteopenia: 10.2 (9.5-10.8) Normal: 10.0 (9.6-10.5) Geometric mean (95% CI)	BMD: Trochanter, intertrochanter, FN, Ward's triangle, TH [DXA, Hologic QDR-1000]	OR (95% CI) for mean BMD in relation to quartile categories of tHcy status + p for trend Category median (µmol/L): Q1: 6.9 Q2: 8.9 Q3: 10.8 Q4: 14.8	Q1: 1.0 (reference) Q2: 0.9 (0.4-1.9) Q3: 2.0 (0.7-5.1) Q4: 2.0 (0.8-4.9) P for trend = 0.09 [s] Dose response analysis: subjects with tHcy level > 20 µmol/L had sign lower BMD than subj with tHcy level < 10 µmol/L
Ouzzif 2012 [36]	Cross-sectional Morocco Moderate risk	188 (0%) 57.8 ± 8.5	12.4 ± 4.1	BMD: FN, LS, TH, trochanter [DXA, Lunar prodigy]	Multivariate regression, β for association tHcy –BMD β (SE) + p-value	LS: -0.089 (0.003) p=0.200 [t] TH: -0.155 (0.002) p=0.021 [t]
Périer 2007 [27]	Cohort (cross-sect data) France Moderate risk	671 (0%) 61.6 ± 8.4	10.6 ± 3.5	BMD: FN, LS,TH [DXA, Hologic QDR-2000]	β for association tHcy –BMD β (SE)	LS: -0.000065 (0.004) FN: -0.006 (0.004) TH: -0.006 (0.004) [2]
Rumbak 2012 [34]	Cross-sectional Croatia Low risk	131 (0%) 54.0 ± 4.9	9.9 ± 2.0	BMD: FN, LS, TH, radius [DXA, Lunar-prodigy]	Stepwise multivariate regression, β for association tHcy –BMD. β (SE) for premenopausal and postmenopausal women	Premenopausal women [u, 2]: LS: 0.20 (0.14) p=0.176 FN: 0.17 (0.15) p=0.253 TH: 0.20 (0.14) p=0.170 Postmenopausal women [u, 2]: LS: 0.12 (0.15) p=0.439 FN: 0.20 (0.15) p=0.181 TH: 0.12 (0.14) p=0.391

Table 3. Continued

Zhu 2009 [31]	Cohort (5y) Australia Moderate risk	1213 (0%) 75.2 ± 2.7	12.1 ± 4.6	BMD: TH [DXA, Hologic Acclaim 4500A]	Change in hip BMD from 1 to 5 years per tertile of tHcy (μmol/L) ANOVA	Tertile 1 and 3 differ significantly ($p<0.05$)

BMD sites: LS = Lumbar Spine, FN = Femoral Neck # data presented in article as nmol/l, this is presumably a typing error and should be μmol/l

1 data as provided by author on our request, 2 β(SE) as calculated from presented data, 3 β(SE) as calculated from data provided by author on our request

a adjusted for age, BMI, smoking status, recurrent falling, serum creatinine; b adjusted for age and BMI; c adjusted for serum creatinine (natural log) , B12 level, folic acid level, BMI, smoking, walking speed, BMD, LnPTH; d adjusted for age, BMI, smoking, coffee intake, physical activity, vit D use, educational level, estrogen use in women; e case-control matched for age and ethnicity. Adjusted for BMI, parental history of hip fracture, treated diabetes, alcohol use, smoking, history of stroke, total calcium intake; f adjusted for sex, age, height, weight, smoking status, caffeine intake, alcohol intake, education level, estrogen use in women; g adjusted for sex, age, height, weight, estrogen use in women; h adjusted for age, sex, BMI, changes in BMI before entry in the study, smoking, fall history, serum creatinine; i adjusted for age, prevalent fractures, BMD, calcium intake, physical activity, vitamin D level, creatinine, albumin, estradiol; j adjusted for age, gender, education, serum creatinine, osteoporosis drugs; k adjusted for age, weight, hip BMD, prevalent fracture, calcium treatment;

L adjusted for weight, cysteine, smoking and height; m Adjusted for duration of menopause, smoking, BMI, folic acid levels, homocysteine levels; n adjusted for age, BMI, logFolate, logB12, creatinine clearance; o Adjusted for age, weight, weight change; p Adjusted for BMI, smoking, age; q Adjusted for smoking, BMI, creatinine, coffee intake, physical activity, use of estrogen therapy; r adjusted for age, B12, folate, PTH, CTx, Ca, Cr; s adjusted for age, sex, ethnicity, BMI, smoking, physical activity, creatinin, alcohol, coffee, energy, calcium, vitamin D zinc intake; t adjusted for age, BMI, folate, B12; u adjusted for age, BMI, smoking, alcohol intake, physical activity, duration of menopause, HRT, levels of hcy, vitB12 and folate

Bone Mineral Density

In the studies included in this review BMD was measured at various sites in the body (e.g. lumbar spine, femoral neck, radius, hip, total body). As BMD differs per site in the body, we pooled results per biomarker (serum/plasma vitamin B_{12}, folate and homocysteine) and per site for the three sites generally measured (FN, LS or total hip), thus resulting in 9 meta-analyses. Betas of the individual studies are shown in Table 1, 2, and 3. The studies included in the meta-analyses took only women into account. Only five studies regarding BMD included a male population [6, 7, 10, 32, 33], and these studies were not comparable quantitatively because differences in the presentation of results or differences in the measured BMD sites.

Vitamin B_{12}

Pooled analysis showed no association between serum/plasma vitamin B_{12} levels and BMD in women; FN: β=0.00, 95% CI: -0.13 to 0.14, I^2=0.00%, p=0.4018 [9, 34, 35]; LS: β=-2.25, 95% CI: -7.98 to 3.49, I^2=99.47%, p<0.0001 [9, 34-36]; total hip β=-2.23, 95% CI: -10.38 to 5.92, I^2=97.71%, p=0.0001 [34-37]. The studies that could not be included in the meta-analyses showed diverse results; in six out of eight studies low serum/plasma vitamin B_{12} was significantly associated with low BMD at at least one site [6, 7, 11, 32, 33, 38]. Two studies did not observe an association between vitamin B_{12} status and BMD [39, 40]. Morris et al. addressed MMA levels as well as a marker for vitamin B_{12} status, and observed a lower BMD with higher serum MMA concentrations [7].

Folate

Pooled analysis showed no association between serum/plasma folate and BMD in women; FN: β=0.00, 95% CI: -0.03 to 0.03, I^2=0.00%, p=0.8810 [9, 34, 35]; LS: β=0.01, 95% CI: 0.00 to 0.01, I^2=0.00%, p=0.7674 [9, 34-36]; total hip: β=0.00, 95% CI: 0.00 to 0.01, I^2=78.47%, p=0.0003 [10, 34-37].
From the studies that could not be compared in a meta-analysis, three studies showed significant associations between folate status and BMD or change in BMD over time [8, 10, 39]. Five studies did not observe an association between folate status and BMD [7, 33, 38, 40, 41].

Homocysteine

Pooled analyses showed no association between serum/plasma homocysteine levels and BMD in women; FN: β=-0.01, 95% CI: -0.04 to 0.02, I^2=31.48%, p=0.2116 [9, 27, 34, 35]; LS: β=-0.01, 95% CI: -0.08 to 0.05, I^2=98.44%, p<0.0001 [9, 27, 34-36]; total hip: β=-0.03, 95% CI: -0.08 to 0.02, I^2=99.87%, p<0.0001 [10, 27, 34-37]. The studies that could not be pooled showed diverse results. In five studies a high homocysteine level was significantly associated with low BMD or change in BMD over time at at least one site [7, 29, 31, 38, 41]. Three studies did not observe a significant association between homocysteine status and BMD or change in BMD [8, 39, 40].

Intervention studies:
Up until now, only one RCT (N=47) which met our inclusion criteria studied the efficacy of B-vitamin supplementation on BMD [42]. This study shows some evidence that BMD may be increased with high doses of B-vitamin supplementation in people with hyperhomocysteinemia (tHcy >15μmol/L). However, this outcome was only found in a sub-analysis of 8 hyperhomocysteinemic subjects. [42].

DISCUSSION

Our meta-analyses showed a significant association of homocysteine levels with fracture risk, a weak though significant inverse association of vitamin B_{12} levels with fracture risk. We could not draw a conclusion regarding folate levels and fracture risk, as too few studies investigated this association. Meta-analyses regarding vitamin B_{12}, folate and homocysteine levels and BMD in women found no associations. Results from studies regarding BMD that could not be included in the meta-analyses are not univocal.

To our knowledge this systematic review with meta-analyses is the most extensive systematic review on the association of vitamin B_{12}, folate and homocysteine with bone health until now. Previous non-systematic literature reviews on the association between folate, vitamin B_{12} and homocysteine with bone health reported similar results, i.e. conflicting evidence with suggestions towards the association of homocysteine levels with fracture [43-45]. These reviews did not report a systematic literatures search strategy and did not provide a quantitative cumulative result. In our review the most recent published articles have been taken into account. The search strategy we used was systematic and extensive, and we used well-defined in- and exclusion criteria.

One recent systematic review included a meta-analysis on the association between tHcy and fractures [20]. This meta-analysis is different in design than ours, as it is not a dose-response meta-analysis. To overcome the variation in cut-off levels for low vitamin B_{12} and folate status, high homocysteine status, and to allow comparison and subsequent combination of individual studies in the performed meta-analyses, we expressed results of individual studies in a standardized format. We assumed a linear, continuous dose-response association between markers of vitamin B_{12} and folate with fracture rather than a threshold effect. This assumption is generally used in meta-analyses. Furthermore, in some of the key articles addressing the association of homocysteine levels with fractures this association is present [4, 5].

A common concern in meta-analyses is heterogeneity between studies. In our meta- analyses we experienced various levels of statistical heterogeneity (no heterogeneity to large heterogeneity). The heterogeneity may be explained by the differences in mean age of the study populations (41-78 years), differences in mean status of vitamin B_{12} (190-549 pmol/L), folate (5.2-24.9 nmol/L) and homocysteine (9.3-16.5 μmol/L), differences in sex distribution of the study populations, duration of follow up (3-16 years) and level of adjustment for confounders. Although most included studies

adjusted for a wide range of confounders for fracture risk or BMD, residual confounding by other unmeasured or inadequately measured factors cannot be ruled out. For example, low vitamin D status is a risk factor for fracture [46]. From the studies included in our meta-analyses for fracture three out of nine adjusted for vitamin D status [24, 25, 27]. Outcomes do not seem to differ between studies that corrected for vitamin D status and studies that did not. Homocysteine levels are increased with renal dysfunction, often measured by serum or urine creatinine levels. Five out of eight studies in the meta-analysis regarding homocysteine and fracture risk corrected for creatinine levels [3, 24, 26, 27, 29], and outcomes did not seem to differ.

As almost all studies were performed in countries without mandatory folate fortification, or were performed before the fortification era in the USA and Australia, we do not consider folate fortification as a source of heterogeneity in our review.

The majority of studies included were longitudinal and cross-sectional observational studies. We could only include one intervention study, which had a very small study population (N=47). One intervention study which found a beneficial effect of vitamin B_{12} and folic acid supplementation on fracture risk could not be included in our systematic review, because this study investigated a population of hemiplegic patients following stroke [47]. The generalizability of these findings is confined to a highly selective patient population with a high percentage of vitamin D deficiency and a high fracture risk. As evidence from intervention studies is lacking, currently no causal effect between vitamin B_{12}, folate and homocysteine levels and bone health can be established. Consequently, it is yet unknown whether extra vitamin B_{12} and folate intake through supplementation could reverse the observed negative effects of vitamin B_{12} and folate deficiency and elevated homocysteine levels. Further evidence from an intervention study is expected soon, as a large intervention study on the effect of vitamin B_{12} and folic acid supplementation on fracture risk, BMD and bone turnover markers is currently carried out with results expected in 2013 [48].

As the quality of included studies determines the quality of the review and meta-analysis, we assessed the overall risk of bias of each individual study using standardized procedures largely based on guidance from the Cochrane Collaboration [49], resulting in one of the following judgments: low, moderate or high risk of bias. Twenty out of the 28 included studies were evaluated as having moderate (n=15) or high risk (n=5) of bias. These studies did take one or more of the predefined confounders into account, i.e. age, sex, smoking, physical activity and body weight, or the study was funded or co-funded by a commercial organization. Due to the limited number of studies included in the meta-analyses, we were not able to study the effect of the overall risk of bias, nor of its single components on the pooled effect measures. There seems to be no difference in the outcomes of studies with low risk of bias compared to studies with moderate or high risk of bias, and we therefore assume that the quality of the included studies had no effect on the outcome of this review.

The intake of folate and vitamin B_{12} are a determinant of folate, vitamin B_{12} and homocysteine status. To deal with potential malabsorption of vitamin B_{12} [50] and reduced bioavailability of folate [51], the use of biomarkers for vitamin B_{12} and folate status is preferred over measures of intake when studying associations with bone health in elderly people.
In studies addressing folate status, serum or plasma folate was measured, which is considered as an appropriate marker for folate status in epidemiological studies [52]. Homocysteine is a non-specific marker for both folate and vitamin B_{12} status [53], which makes it a relevant biomarker in this review. Regarding the metabolic interactions between vitamin B_{12}, folate and homocysteine combined with the variety in data presented in the studies, we were not able to investigate the possibility that a low vitamin B_{12} or folate status in combination with a high homocysteine level might result in a higher fracture risk in comparison to a low vitamin B_{12} or folate status or homocysteine level alone. In most studies regarding vitamin B_{12} status, status was assessed with serum or plasma vitamin B_{12}. Other, more sensitive markers for vitamin B_{12} deficiency, like MMA and HoloTC [54], were addressed only in a few studies. We could therefore not draw conclusions about the association between these biomarkers and outcomes on bone health.

There are several suggested mechanisms for the association between vitamin B_{12}, folate, homocysteine and bone health. Homocysteine may interfere with collagen cross-linking. Cross-links are important for the stability and strength of the collagen network. Interference in cross-link formation would cause an altered bone matrix, resulting in more fragile bones [55]. As collagen cross-links do not alter BMD, this may explain why a more convincing result is found regarding fractures than BMD, as suggested for example by Van Meurs et al. (2004). Vitamin B_{12} deficiency has been associated with impaired functional maturation of osteoblasts [56]. Some in vitro studies support the hypothesis of a possible favorable effect of vitamin B_{12} supplementation, although results are equivocal. Vitamin B_{12} has been shown to stimulate osteoblast proliferation and alkaline phosphatase activity [57] but Herrmann et al. were not able to show any significant and consistent effect of vitamin B_{12} or folic acid on osteoblast activity [58]. Recent publications show evidence of osteoclast stimulation in the presence of high homocysteine and low vitamin B_{12} concentrations [59-61]. Vitamin B_{12} and folate are not the only B-vitamins involved in the homocysteine metabolism. Various micronutrients, such as vitamin B2 (riboflavin), vitamin B6 (pyridoxine) and choline also affect homocysteine levels [16, 17, 62], and may consequently affect bone health. Given that vitamin B_{12} and folate are the main factors influencing homocysteine levels, and therefore the primary focus in a homocysteine lowering intervention [63], our review focused on vitamin B_{12}, folate and homocysteine.

CONSIDERATIONS FOR FUTURE RESEARCH AND CONCLUSIONS

The mechanisms involved in the association between biomarkers of B-vitamins and bone health are still unclear and therefore more fundamental research is required to establish the potential mechanisms. Subsequently, both observational and intervention studies should preferably not focus on just one biomarker in relation to the homocysteine metabolism, but take a biomarker profile into account, including serum/ plasma vitamin B_{12}, MMA, HoloTC, folate and homocysteine levels. Evidence is needed from well designed, large intervention studies to establish a causal relationship between markers of B-vitamins and bone health.

This systematic review with meta-analyses shows that elevated homocysteine levels are associated with increased fracture risk. Vitamin B_{12} status may be associated with fracture risk and evidence for an association between folate status and fracture risk is scarce. Vitamin B_{12}, folate and homocysteine levels are probably not associated with BMD, but results are not univocal.

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6

The association of vitamin B12, holotranscobalamin, methylmalonic acid, folate and homocysteine status with domain-specific cognitive function in Dutch elderly people. A cross-sectional study.

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ABSTRACT

Introduction

Elevated homocysteine levels and low levels of vitamin B_{12} and folate have been suggested to be modifiable risk factors for cognitive decline in elderly. Our aim was to evaluate the cross-sectional association of levels of homocysteine, folate, and vitamin B_{12} biomarkers (total B_{12}, holotranscobalamin (holoTC) and methylmalonic acid (MMA)) with cognitive function in specific cognitive domains in Dutch elderly.

Methods

Data were collected from 2919 elderly (≥ 65 years) participating in the B-PROOF study. A cognitive test battery was used covering four cognitive domains: attention and working memory (n=848), information processing speed (n=837), executive function (n=824) and episodic memory (n=2848). Concentrations of vitamin B_{12}, holoTC, homocysteine, MMA and folate were measured and a vitamin B_{12} biomarker combination score, the 'wellness score', was calculated. Multivariable regression analysis was used to assess the association between biomarker status and cognitive function.

Results

After adjustment for age, sex, smoking status, alcohol intake, education, GDS depression score and creatinine, levels of homocysteine (β= -0.009), folate (β= 0.002), MMA (β= -0.163) and the wellness score (β= 0.048) were significantly associated with the domain of episodic memory. Additionally, homocysteine (β= -0.015) and the wellness score (β= 0.103) were significantly associated with the domain information processing speed. No associations were observed of vitamin B_{12} nor holoTC with cognitive function in any domain. CONCLUSION: Levels of homocysteine, MMA, folate and a combination score of vitamin B_{12} biomarkers are associated with the domains episodic memory and information processing speed. Observations are in line with other observational studies.

INTRODUCTION

In general, cognitive functioning decreases with age. This decrease includes normal, age-related cognitive decline, but also more rapid cognitive decline, leading to cognitive impairment, and dementia or Alzheimer's disease. Insight in aspects that are associated with development and progression of cognitive decline may help to identify risk factors and potential therapies. Elevated homocysteine levels and low levels of vitamin B_{12} and folate have been suggested to be modifiable risk factors for cognitive decline [1]. However, intervention studies investigating the effect of vitamin B_{12} and/ or folic acid supplementation show contradictory results [2, 3].

Two major factors complicate a good comparison between studies on vitamin B_{12} status and cognitive function and may therefore contribute to the discrepancy in findings: the use of different biomarkers for vitamin B_{12} status and the large variety in tests that are used to determine cognitive function.

Research on vitamin B_{12} status and cognitive function focused mainly on total vitamin B_{12} (total B_{12}) levels. The association of levels of holotranscobalamin (holoTC) and the metabolite methylmalonic acid (MMA) with cognitive functioning has been studied in less detail, although these have been suggested as sensitive markers for vitamin B_{12} status [4, 5]. A novel approach for evaluating vitamin B_{12} status is combining four biomarkers of vitamin B_{12} status (total B_{12}, holoTC, MMA and homocysteine) into a single variable, the so-called 'wellness score'[6].

In many studies, the Mini-Mental State Examination (MMSE) is used to determine cognitive function. This test however is mainly developed as a screening instrument to alert for dementia and is not distinctive to diagnose mild or isolated cognitive deficits. The ability of the MMSE to reveal associations between exposure and cognitive function depends furthermore on the prevalence of low scores in the population [7]. Neuropsychological testing by the use of more specific cognitive tests, covering different cognitive domains, is therefore preferred to determine cognitive functioning [8]. There is however lack of a generally accepted standard test battery, which complicates comparison between studies [9].

In this study, we cross-sectionally explored the association of levels of homocysteine, folate, vitamin B_{12}, HoloTC, MMA and the wellness score with domain-specific cognitive functioning in Dutch elderly participating in the B-PROOF study.

METHODS

Participants

In this cross-sectional study, baseline data of the B-PROOF study (B-vitamins for the PRevention Of Osteoporotic Fractures) were used. The B-PROOF study is an intervention study which investigates the effect of vitamin B_{12} and folic acid supplementation on fracture risk in a general elderly population (≥65 years) with an elevated homocysteine level (≥12 μmol/l). The study population and the design of

the study have been described elsewhere in detail [10]. The study has been carried out in three study centers in The Netherlands; Erasmus MC (EMC, Rotterdam), VU University Medical Center (VUmc, Amsterdam) and Wageningen University (WU, Wageningen). Elderly people living in the areas of these three study centers were invited to participate. Participants were excluded if they recently (≤ 4 months) used intramuscular injections of vitamin B_{12} or folic acid supplements (>300 μg/ day), had serum creatinine levels >150 μmol/L, had plasma homocysteine concentrations >50 μmol/L, were diagnosed with cancer in the last five years or had mobility restrictions. The study started in August 2008 and all baseline measurements have been completed in March 2011. After checking medical history and supplement use, fasting blood samples were obtained at one of the research centers or at the homes of the elderly. Participants completed questionnaires regarding socio-demographic status, lifestyle and depressive symptoms. The questionnaires were checked with the participant to ensure that they were fully completed. Measurements were performed during a 1.5-2 hour session at the study center or at the participant's home and consisted of cognitive tests, anthropometric measurements and physical performance tests. Self-reported medical history, including current alcohol and smoking habits were investigated by a questionnaire and were reviewed by one of the co-workers with the participant.

Cognitive function and depression

We used the Mini-Mental State Examination (MMSE) as a descriptive measure for global cognitive performance [11]. An extensive cognitive test battery consisting of six standardized cognitive tests was used to address overall cognitive function as well as four specific domains: attention and working memory, information processing speed, executive function and episodic memory (Table 1). The first three domains were assessed in the subsample of WU (n=857), whereas the latter was assessed in all subjects (n=2919).

Biochemical analysis

Blood samples were obtained from participants in a fasted state or after a light breakfast in a standardized way and samples were kept frozen at -80°C until analysis. Plasma homocysteine was measured using the Architect i2000 RS analyser (VUmc, intra assay CV=2%, inter assay CV=4%), HPLC method (WU, intra assay CV=3.1%, inter assay CV=5.9%) or LC-MS/MS method (EMC, intra assay CV=5.5% at 14.1 μmol/L, inter assay CV=1.4% at 13.7 μmol/L). According to a cross-calibration, outcomes of the three centres did not differ significantly. Serum vitamin B_{12} and folate were determined with electrochemiluminescence immunoassay (Elecsys 2010, Roche, Almere, The Netherlands) (intra assay CV vitamin B_{12} 5.1% at 125 pmol/L and 2.9% at 753 pmol/L; intra assay CV folate: 5.9% at 5.7 nmol/L and 2.8% at 23.4 nmol/L)[12]. Serum holoTC was determined by the Abbott AxSYM analyser (Abbott Diagnostics, Hoofddorp, The Netherlands) (CV <8% at 20, 40 and 80 pmol/L) [13]. Serum MMA was determined using LC-MS/MS (CV = 6.7%, 5.0% and 5.0% at 0.15, 0.36 and 0.65 μmol/L. respectively). Serum creatinine was determined enzymatically with the colorimetric Roche CREA plus assay (intra assay CV=2%). DNA was isolated from buffy coats to determine MTHFR C677T genotype using the Illumina Omni-express array.

Education level was computed from self-reported highest education and classified into three levels of secondary education: <5 years (low), 5-8 years (middle) and >8 years (high). Depression was assessed with the 15-item Geriatric Depression Scale, where a score ≥5 points indicated risk of depression [14]. Smoking (never, former, and current smoker) and alcohol consumption (none, light, moderate, and excessive) were based on self-report. Classification of alcohol use was based on the number of days per week alcohol was consumed and the number of drinks per time [15]. MTHFR C677T genotype was divided into two groups: either 677TT or 677CT and 677CC genotype.

Table 1. Schematic division of cognitive tests into cognitive domains [60-63]

Cognitive test	Domain	Description	Score
Mini Mental State Examination (MMSE)	Global measure of cognitive function	Questions and tasks which provide a brief overview of basic cognitive skills.	Number of correct answers (max. 30)
Symbol Digit Modalities Test (SDMT)	Information processing speed[a]	Translate symbols into numbers using a key within limited time (1,5 minute).	Number of correct translations.
Letter Fluency Test	Executive Function[b]	Generation of words starting with specific letters within a limited time (1 minute).	Number of correct words produced.
Trail Making Test A / B (TMT)	Information Processing Speed and Executive Function	Connect an alternating sequence of numbers in ascending order.	Score is based on time required.
Digit Span Test forward/ backward	Attention and Working Memory[c]	Repeating progressively longer series of digits, forward/ backward.	Number of correct recalls forward + backward
Word Learning Test (WLT)	Episodic Memory[d]	Reproduce words of a list which is read aloud. Delayed recall: recall after 15-20min.	Number of correct reproduced words
Stroop Color Word Test	Information Processing Speed and Executive Function	Part I and II: Read names of colors and colored blocks, Part III: Name color of ink, instead of reading the conflicting word.	Score is based on time required for part III.

[a] This domain involves psychomotor speed, perceptual organization and visual scanning abilities.

[b] This domain involves cognitive flexibility: the ability to plan, organize, error correction and manage multiple tasks simultaneously.

[c] Short term working memory and attention are required to manage information and carry out a complex task.

[d] Long term memory that includes delayed recall and recognition memory.

Statistical analysis

Crude cognitive test scores were transformed into a standardized z-score (z-score = [test score – mean test score] / SD). A composite score for every domain was calculated by averaging the z-scores of tests covering that domain: Attention and Working Memory = (Z Digit Span-forward + Z Digit Span-backward) / 2; Executive function = (Z Letter Fluency-mean letters) + (-Z StroopIII/

((StroopI+StroopII)/ 2)) + (-Z Trail making B/ Trail making A) / 3; Information processing speed = (-Z SDMT nr. correct) + (-Z Trailmaking A) + ((-Z StroopI+StroopII)/ 2) / 3; Episodic Memory = (Z WLT total 1-5 + Z WLT delayed – trial 5 + Z WLT recognition total) / 3. A composite score for overall cognitive function was calculated by averaging the z-scores of each cognitive test for subjects who completed all six cognitive tests. For the Stroop Test and the Trail Making test time was the reported variable, a higher test score indicated more time needed to perform the test. Z-scores for these tests were therefore inverted. In case participants did not complete a test, no z-score could be calculated, resulting in slightly different numbers per cognitive domain.

For each participant, vitamin B_{12} status was expressed as a wellness score (w), calculated according to the formula: $w = \log_{10}(holoTC_{normalized} * B_{12normalized}) - \log_{10}(MMA_{normalized} * Hcy_{normalized})$[6]. The normalized values are the individual values expressed relative to the median value of our study population. A wellness score of -<0.5 is considered as 'transitional' or subclinically deficient, a score of <-1.5 as deficient. Continuous variables were expressed as mean ± SD, or as median (IQR) for not normally distributed data. One extreme outlier of MMA (11.1 μmol/L) was excluded from analyses considering MMA and the wellness score. The Mann-Whitney U test was used to compare homocysteine levels among the different MTHFR genotypes. Multivariate linear regression was used to describe the association between vitamin B_{12} status and overall and domain-specific cognition, based on their biological relevance and their contribution to a change in the regression coefficient of the variable of interest of at least 10% the analysis was adjusted for age, sex, smoking status, alcohol intake, education level, depressive symptoms and creatinine level. An interaction term was added to the multivariate regression model to test for interaction between vitamin B_{12} status and MTHFR genotype.

Statistical significance was defined as $p<0.05$ and all tests were two-sided. Statistical analyses were performed using SAS statistical software version 9.2 (SAS institute Inc., Cary, NC, USA).

RESULTS

Mean age was 74.1 ± 6.6 years and 50% of the population was men. Two hundred (200) participants (7%) showed depressive symptoms based on a GDS score ≥ 5. Median homocysteine level was 14.4 μmol/L (13.0 – 16.6). Median homocysteine levels were significantly higher in the MTHFR 677TT group compared to the MTHFR 677CC/CT group (14.9 μmol/L and 14.3 μmol/L respectively, p <0.001). Median total B_{12} was 266 pmol/L (208 - 342), holoTC 64.0 pmol/L (46.0 - 85.0), MMA 0.23 (0.18 - 0.30) and folate 18.7 (14.8 - 24.0) (Table 2). The distribution of the wellness score was skewed to the left ranging from -2.79 to 0.71 (Figure 1). The proportion of participants with a wellness score of -0.5 (transitional) and <-1.5 (deficient) was 17% and 2%, respectively. Median MMSE score was 28 (IQR 27-29). Mean scores of the six cognitive tests are shown in Table 3.

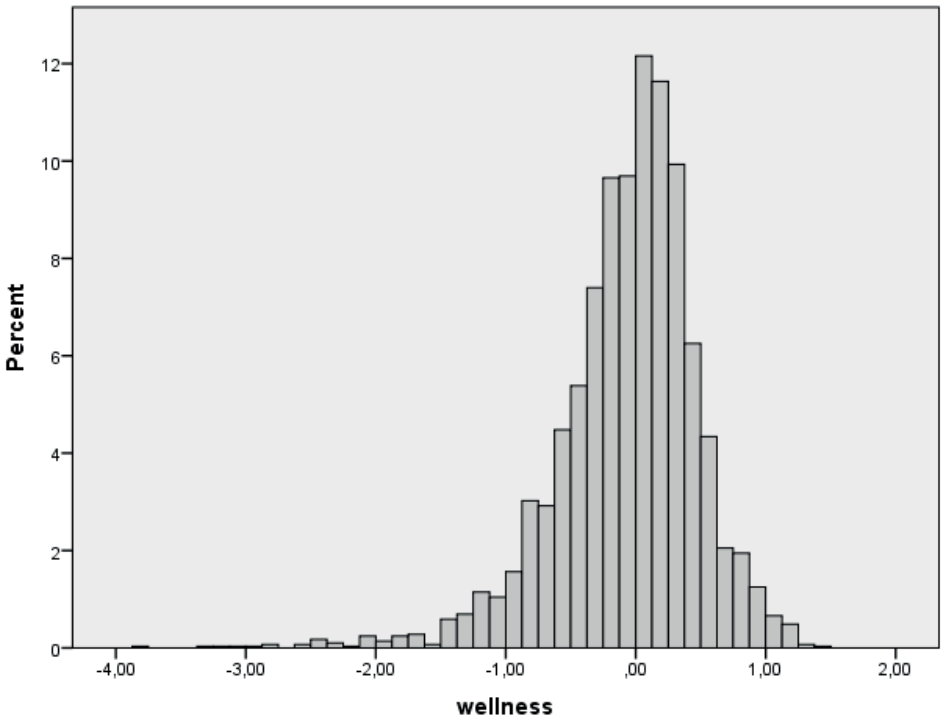

Figure 1. Distribution of the wellness score (n=2879). wellness = $\log_{10}$(holoTC/64.0 * B_{12}/266.4) - $\log_{10}$(MMA/0.23 * Hcy/14.4)

Associations between biomarkers and cognitive function

Table 3 shows the crude and adjusted multivariate linear regression analyses for the vitamin B_{12} biomarkers, folate and the wellness score, with domain-specific and overall cognitive function. In the crude regression models significant associations were observed for homocysteine and folate with overall cognitive function; for homocysteine with attention and working memory; for vitamin B_{12}, homocysteine, MMA, and the wellness score with information processing speed; homocysteine and the wellness score with executive function; and for homocysteine, MMA, and the wellness score with episodic memory.

After adjusting for age, sex, smoking status, alcohol intake, education level depressive symptoms and creatinine level, associations remained significant for homocysteine and wellness score with information processing speed (b = -0.015 (SE 0.007) and b = 0.103 (SE 0.048), respectively), and homocysteine, MMA, folate and the wellness score with the domain episodic memory (b = -0009 (SE 0.004), b = -0.163 (SE 0.057), b = 0.002 (SE 0.001) and b = 0.048 (se 0.024), respectively). No associations were observed of total B_{12} and holoTC with any cognitive domain in the adjusted models. There was no significant interaction between the vitamin B_{12} biomarkers and the MTHFR genotype observed in the regression models.

Table 2. Characteristics of the Dutch study population

Characteristic (n=2919)	Value[1]
Demographic measures	
Age (y)	74.1 ± 6.6
Men	1459 (50)
Education	
Low	1545 (53)
Middle	615 (21)
High	757 (26)
Health measures	
Weight (kg)	77.9 ± 13.3
Height (cm)	169.3 ± 9.3
Smoking status	
Current smoker	281 (10)
Former smoker	1649 (56)
Never smoker	989 (34)
Alcohol intake	
Light	1835 (70)
Moderate	715 (27)
Excessive	77 (3)
Depressive symptoms (GDS ≥5)	200 (6.9)
Biochemical measures	
Plasma Hcy (μmol/L)	14.4 (13.0 - 16.6)
Serum MMA (μmol/L)	0.23 (0.18 - 0.30)
Serum folic acid (nmol/L)	18.7 (14.8 - 24.0)
Serum vitamin B_{12} (pmol/L)	266 (208 - 342)
Serum holoTC (pmol/L)	64 (46 - 85)
MTHFR genotype	
677 CC	1200 (45.6)
677 CT	1085 (41.2)
677 TT	348 (13.2)
Cognitive measures	
MMSE score (max. 30 points)	28 (27 - 29)
Episodic Memory	
RAVLT	
Immediate recall (max. 75 words)	36.4 ± 10.1
Delayed recall (max. 15 words)	7.0 ± 3.0
Delayed recognition (max. 30 words)	27.8 ± 2.4
Attention and Working Memory*	
Digit Span	
Forward (max. 16 points)	8.1 ± 1.7
Backward (max. 14 points)	5.9 ± 1.8
Information Processing Speed*	
TMT (Part A, sec.) †	45.2 ± 18.5
Stroop ([Part 1 + Part 2] / 2, sec.) †	59.0 ± 12.0
SDMT (nr. of correct items)	46.1 ± 9.6
Executive Function*	
Letter Fluency Test (nr. of correct items)	36.4 ± 11.3
TMT (Part B / Part A) †	2.4 ± 0.8
Stroop Test (Part 3 / [Part 1 + Part 2 / 2]) †	2.0 ± 0.5

GDS, Geriatric Depression Scale; MTHFR, Methylenetetrahydrofolate Reductase; Hcy, homocysteine; holoTC, holotranscobalamin; MMA, methylmalonic acid; MMSE, Mini-Mental State Examination; RAVLT, Rey Auditory Verbal Learning Test; SDMT, Symbol Digit Modality Test; TMT, Trail Making Test.

[1] Values are presented as mean ± SD, number (%) or median (IQR) * Determined in a subsample of WU (n=857) † Higher scores indicate more time needed to complete the tests, i.e. poorer performance

Table 3. Associations of levels of vitamin B_{12} biomarkers, homocysteine and folate with cognitive function in Dutch elderly

	b (SE)[1]											
	Vitamin B_{12} (50 pmol/L)[2]		HoloTC (25 pmol/L)[2]		Homocysteine (µmol/L)		MMA (µmol/L)		Wellness score[3]		Folate (nmol/L)	
	Unadjusted	Adjusted[†]	Unadjusted	Adjusted[†]	Unadjusted	Adjusted[†]	Unadjusted	Adjusted[†]	Unadjusted	Adjusted[†]	Unadjusted	Adjusted[†]
Overall cognition (n=8_{12})	0.007 (0.007)	-0.002 (0.007)	0.004 (0.010)	-0.005 (0.009)	-0.011* (0.004)	-0.003 (0.004)	-0.083 (0.082)	0.002 (0.078)	0.048 (0.029)	-0.002 (0.028)	0.004* (0.002)	0.002 (0.002)
Attention and Working Memory (n=848)	0.015 (0.014)	-0.002 (0.013)	0.009 (0.020)	-0.010 (0.018)	-0.021* (0.009)	-0.009 (0.009)	-0.240 (0.161)	-0.085 (0.152)	0.103 (0.058)	0.001 (0.056)	0.001 (0.002)	0.001 (0.002)
Information Processing Speed (n=837)	0.037* (0.013)	0.021 (0.011)	0.025 (0.018)	0.016 (0.016)	-0.041* (0.008)	-0.015* (0.007)	-0.558* (0.148)	-0.245 (0.131)	0.238* (0.054)	0.103* (0.048)	0.005 (0.004)	0.005 (0.003)
Executive Function (n=824)	0.019 (0.011)	0.006 (0.010)	0.015 (0.016)	0.004 (0.015)	-0.014* (0.007)	-0.001 (0.007)	-0.243 (0.127)	-0.081 (0.121)	0.103* (0.046)	0.022 (0.045)	0.001 (0.003)	-0.000 (0.003)
Episodic Memory (n=2848)	0.010 (0.006)	0.001 (0.006)	0.023 (0.008)	0.004 (0.008)	-0.022* (0.004)	-0.009* (0.004)	-0.290* (0.059)	-0.163* (0.057)	0.133** (0.025)	0.048* (0.024)	0.002 (0.001)	0.002* (0.001)

[1] Regression coefficient from (multivariate) linear regression model

[2] Regression coefficients expressed per units of 50 pmol/L (vitamin B_{12}) and 25 pmol/L (holoTC)

[3] $w = \log_{10}(\text{holoTC}/64.0 * \text{B12}/266.4) - \log_{10}(\text{MMA}/0.23 * \text{Hcy}/14.4)$

[†] Adjusted for age, sex, smoking status, alcohol intake, education level, depressive symptoms and creatinine levels

* P <0.05

DISCUSSION

We assessed the association of biomarkers of vitamin B_{12} and folate status with domain-specific cognitive function in Dutch elderly in this cross-sectional study. All markers, except for total B_{12} and holoTC were associated with the domain episodic memory. Additionally, homocysteine and the wellness score were significantly associated with the domain information processing speed.

We observed an association of homocysteine, folate and MMA with cognitive function, but not for total B_{12} itself. A similar pattern is shown in several observational studies; total B_{12} is not associated with cognitive function, but levels of folate, MMA and homocysteine are [16-19]. Although some studies also show an association of total B_{12} with cognitive function in addition to homocysteine, MMA and folate [20-22], these findings may suggest that cognitive performance is not sensitive to changes in total B_{12} status, possibly because levels of total B_{12} do not adequately reflect vitamin B_{12} status. It is therefore suggested that studies investigating vitamin B_{12} status in relation with cognitive function should include holoTC or MMA as sensitive markers for vitamin B_{12} status [3]. In our study, levels of holoTC were not associated with cognitive function in any domain, in contrast with other studies, in which a significant association was observed [23-25]. HoloTC however, is not yet widely studied in association with cognitive function. We observed associations of MMA specifically with the domain episodic memory, as was observed in other observational studies [23, 26, 27]. Many cross-sectional studies have shown an inverse association between homocysteine levels and cognitive function and dementia, well summarized in several systematic reviews [1, 28, 29]. Several studies observed similar associations in the domain information processing speed [17, 30-35]. Longitudinal studies observed associations between homocysteine levels and cognitive decline [21, 24, 25, 31, 36-39], usually measured with a decline in MMSE score.

Levels of folate have been associated with cognitive function in many observational studies summarized in two reviews [1, 29]. More specifically, in our study, the association was mainly present in the domain episodic memory. This association is also prevalent among other observational studies [21, 30, 32], although in other studies this association was not observed [17, 35], mainly due to insufficient variation in folate status or overreliance on immediate versus delayed recall [29].

Besides investigating associations of separate biomarkers with cognitive function, we also combined four biomarkers of vitamin B_{12} status (total B_{12}, MMA, holoTC and homocysteine) into a single variable: the 'wellness score'. To the best of our knowledge, our study is the first to investigate the wellness score in association with domain-specific cognitive testing. One cross-sectional study used this model with MMSE score as an outcome measure, showing that a lower wellness score was associated with cognitive impairment, defined by an MMSE score <22 [40]. Although holoTC and MMA are suggested to be better biomarkers than total B_{12} and homocysteine [6, 13, 41], the four biomarkers are counted equally in the wellness score levels. Another point of consideration when using the wellness score are the normal values used to normalize the values of the biomarkers in the formula. In our study, we used

median values of our study population as normal values. We chose to do so, because our population consists of elderly with an elevated homocysteine level. The distribution of vitamin B_{12} biomarkers may therefore differ from general elderly populations. However, a comparison of results between studies will still limited when future studies will also use their own median values.

Although observational evidence of associations of homocysteine, vitamin B_{12} and folate with cognitive function and cognitive decline is abundant, evidence from randomized controlled trials (RCTs) is limited and does not support a beneficial effect of vitamin B_{12} and folic acid supplementation, as summarized in several systematic reviews [2, 3, 42-45]. One of the main reasons for the absence of a treatment effect might be the heterogeneity existing among studies, for example on dose and duration of B-vitamin supplementation, sample size and cognitive test battery.

A particular strength of our study is that we used a broad cognitive test battery which contained both easy and more difficult tests. Furthermore, each cognitive domain was represented by at least two cognitive tests, by clustering the scores into compound scores which improved the robustness of the underlying cognitive tests. All cognitive tests used in our study were sensitive tests and previously studied for test-retest reliability and validity [46]. Because the cognitive test scores in our study have been standardized, the magnitude of the beta-coefficients can be compared across all models. This shows that the association of homocysteine, MMA and the wellness score with cognitive function is mainly present within the domains Information Processing Speed and Episodic Memory. The reason that we observed significant associations within these domains might be because cognitive speed and memory are components of fluid abilities which tend to change more when ageing [47], combined with differences in sensitivity of the cognitive tests corresponding to each domain.

Recently, some studies regarding B-vitamins and cognitive function focused on brain structure, especially brain atrophy and white matter hyper intensities, as measured with Magnetic Resonance Imaging (MRI). This approach is promising as this measure is objective, and not prone to short term fluctuations, practice effects and intra/inter rater variability as may be the case for cognitive tests. Cross-sectional and longitudinal associations have been observed for homocysteine and B-vitamin levels with brain structure measurements [26, 48-50], and an intervention study showed positive effects of B-vitamin supplementation on the rate of brain atrophy in elderly with Mild Cognitive Impairment (MCI) [51, 52].

In contrast to the MRI study, our study population has a relatively high cognitive condition, as shown by a median MMSE score of 28. Participants in the B-PROOF study were furthermore included on basis of an elevated plasma homocysteine level and therefore our results cannot be directly extrapolated to the whole elderly population. On the other hand, during screening we observed that 49% of the potential participants had plasma homocysteine levels ≥12 μmol/L, indicating that our results may apply to a large part of the elderly population. The range of homocysteine levels is quite small; 95% of our population had a homocysteine level between 12.0 and 22.3 μmol/L. Due to the relatively good

cognitive condition and the limited range of homocysteine levels in our study population we may have observed a smaller effect size than we would have observed when including participants with a larger range of both cognitive function and homocysteine levels.

We observed that homocysteine levels were significantly higher among participants with the 677TT MTHFR genotype compared to the combined 677CC and 677CT genotype. This is in line with our expectations, since the MTHFR enzyme is involved in the conversion of homocysteine to methionine and the 677TT allele encodes an enzyme with reduced activity [53, 54]; individuals who are homozygote for this polymorphism show about 25% higher homocysteine levels compared to individuals with the CC genotype [55]. In our study population the prevalence of the MTHFR 677TT polymorphism was 13% (n=348). The limited sample size of the TT polymorphism population could be an explanation of the absence of an interaction between MTHFR genotype and cognitive function. Nevertheless, our results are in agreement with three other studies that did not observe an association between MTHFR genotype and cognitive function [56-58].

Since our data are cross-sectional, we cannot exclude reverse causality. It is possible that factors causing brain dysfunction and cognitive decline are also responsible for the elevation of homocysteine levels.

There are several biological mechanisms explaining why high levels of homocysteine and low levels of vitamin B_{12} and folate affect cognitive function. First, homocysteine may be neurotoxic. Furthermore, low levels of folate and vitamin B_{12} lead to low levels of S-adenosyl methionine, which impairs methylation reactions important to the maintenance of brain tissue [28]. Additionally, an elevated homocysteine level, due to its suggested cause of atherosclerosis, might cause brain atrophy and white matter hyperintensities, which are likely to increase the risk of vascular dementia [51, 59].

In summary, we observed an association of homocysteine, folate and a marker of vitamin B_{12} status, MMA with cognitive functioning in the domains episodic memory and information processing speed.

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7

Effect of daily vitamin B12 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

Importance

In the coming decades, the number of fractures is expected to rise substantially. An elevated plasma homocysteine level is a risk factor for osteoporotic fractures. Supplementation with vitamin B_{12} and folic acid lowers homocysteine levels and may therefore contribute to fracture prevention.

Objective

To determine whether vitamin B_{12} and folic acid supplementation reduces osteoporotic fracture incidence in hyperhomocysteinemic elderly.

Design, settings and patients B-proof

Is a multi-center, double-blind, randomized, placebo-controlled trial in the Netherlands, in which 2,919 participants aged ≥65 years with elevated homocysteine levels (12-50 μmol/L) were included.

Intervention

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.

Main Outcome Measure

Primary endpoint was time-to-first osteoporotic fracture. Data were analyzed according to the intention-to-treat principle, using Cox proportional-hazards models. Stratified analyses were conducted if pre-specified covariates interacted significantly with treatment. Second, per-protocol analyses were performed, including only compliant participants.

Results

Osteoporotic fractures occurred in 61 persons (4.2%) in the intervention group compared with 75 (5.1%) in placebo. 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 72% lower in the intervention group compared with placebo (HR=0.28, 95% CI 0.10-0.74). Analyses on fractures of any type showed similar results. Mortality did not differ between groups. Sixty-three vs. 42 participants in the intervention and placebo group, respectively, reported incident cancer (HR=1.55, 95% CI 1.04-2.30).

Conclusions and relevance

Combined vitamin B_{12}/folic acid supplementation had no effect on osteoporotic fracture incidence in this elderly population. Stratified analyses suggested a beneficial effect on osteoporotic fracture prevention in persons >80 years who were compliant with supplement use. However, treatment was also associated with increased cancer risk, although this possible adverse effect should be interpreted with caution. In conclusion, vitamin B_{12} and folic acid supplementation cannot be recommended at present for fracture prevention in elderly people.

Trial registration

ClinicalTrials.gov (NCT00696414).

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 (≥15μmol/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]. Two randomized controlled trials investigated the effect of B-vitamin supplementation on fracture risk [20, 21]. Among stroke survivors, a large protective effect of 2 year supplementation of 1.5 mg vitamin B_{12} and 5 mg folic acid was observed on hip fracture risk in the trial of Sato et al. [20]. 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 B6 was observed on fracture incidence among persons with high cardiovascular risk [21]. 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 and effectiveness 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.

METHODS

Study design and population

B-PROOF is a randomized, placebo-controlled, double-blind multi-center trial, of which the design and methods have been described in detail previously [22]. In brief, we included 2,919 participants aged ≥65y with elevated Hcy levels (12-50 μmol/L). Exclusion criteria were a serum creatinine level ≥150 μmol/l, cancer diagnosis in the past 5 years and severe immobility (being bedridden or using a wheelchair permanently). Participants were enrolled from September 2008 till March 2011 and were randomized to receive daily either an oral vitamin 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 [23]. Randomization was stratified for study center, sex, age (65-80y, >80y) and level of Hcy (12-18 μmol/L, >18 μmol/L). The intervention period comprised two years. As planned at the start of the study [22], 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. At baseline and 2y follow-up, a broad set of measurements was performed. The Medical Ethics Committee of Wageningen UR approved

the study protocol and the Medical Ethics Committees of Erasmus MC and VU University Medical Center (VUmc) gave approval for local feasibility. All participants gave written informed consent. The B-PROOF study is registered with the Netherlands Trial Register (NTR1333) and with ClinicalTrials.gov (NCT00696414).

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 was registered and included the use of bisphosphonates, strontium-ranelate, selective estrogen-receptor modulators, estrogens, androgens, denosumab or teriparatide. Plasma Hcy and serum creatinine, as well as the C677T polymorphism in the methylenetetrahydrofolate reductase (MTHFR) gene (rs1801133) were determined [22]. For Hcy, levels after two years of intervention 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 holotranscobalamin (holoTC) was determined by the AxSYM analyser (Abbott Diagnostics, Hoofddorp, the Netherlands) and serum methylmalonic acid (MMA) was measured by LC-MS/MS.

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. Subjects who dropped out of the study, who refused, or 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. 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 [24].

Compliance

Every six months, new tablets were sent to the participants and they were requested to return any remaining tablets. A subject was 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

Hospital admissions and other adverse events were recorded by the participants on the study calendars. In addition, all events reported to the study team by phone or otherwise were recorded. All ill-health related conditions reported by participants were considered as adverse events. In case participants reported a cancer diagnosis of any type, except for non-melanoma skin cancer, they were excluded

from further tablet use. 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.

Statistical analyses

Statistical analyses were performed before the treatment code was revealed. The primary analysis was based on the intention-to-treat (ITT) principle (adjusted model), including all subjects who agreed to start the treatment and completed the baseline measurements. 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.

The primary outcome was time-to-first osteoporotic fracture, while time-to-first fracture of any type was considered a secondary outcome. 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 Cox proportional-hazards models. Individual time of follow-up was calculated as the time until the first fracture (osteoporotic or any type), end of intervention period, date of lost-to-follow-up, or death, whichever came first.

Additionally, pre-specified per-protocol (PP) analyses were performed, including only data from subjects who were compliant to the study protocol. For drop-outs, the time until drop-out was used in these analyses.

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 excluding relapse cancer cases.

For all outcomes, interaction with treatment was tested for the pre-specified covariates sex, baseline age below and above 80y, plasma Hcy below and above 18 μmol/l, MTHFR C677T genotype and for study center. In case of significant interaction ($p<0.1$), stratified 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 level in the intervention group (p=0.03) (Table 1). Baseline anti-osteoporotic medication use was similar in the two groups (Table 1), as were numbers of new users of these medicines during the intervention period (4.2% in the intervention vs. 4.4% in the placebo group, p=0.78). Mean change in Hcy levels was -4.4 μmol/L in the intervention vs. -0.2 μmol/L in the placebo group (p<0.01) (Table 2).

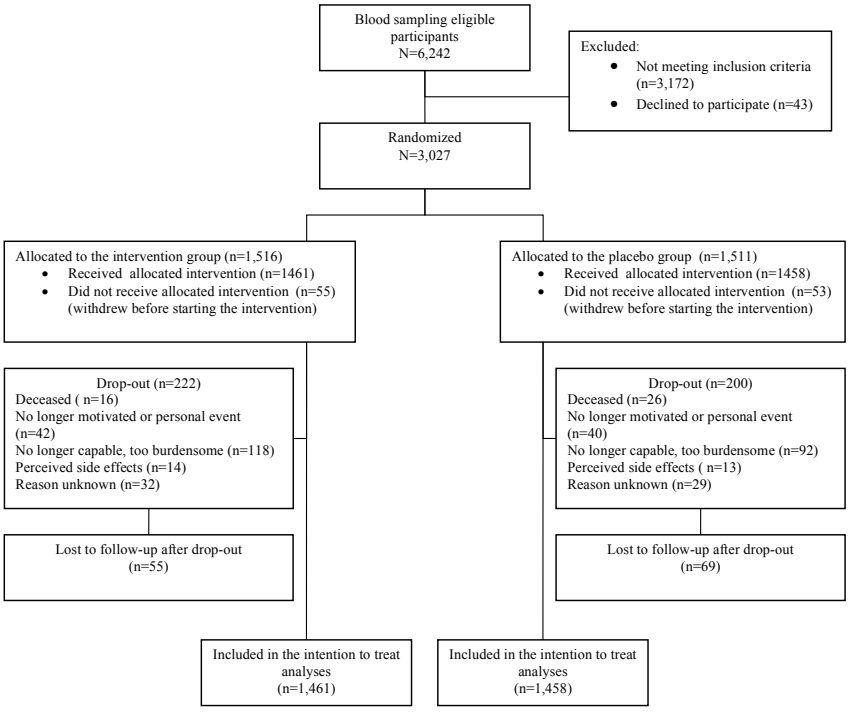


Figure 1. Screening, randomization and follow-up in the B-PROOF study

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=2919)

	Placebo group (N=1,458)	**Intervention group (N=1,461)**
Age (y)[a]	74.2 (6.4)	74.0 (6.6)
Sex (% women)[b]	49.7	50.4
Study center[b]		
WUR (%)	29.6	29.2
VUmc (%)	26.8	26.4
EMC (%)	43.6	44.4
History of fracture (% yes)[b,c]	42.9	41.3
Height (cm)[a]	169.2 (9.3)	169.4 (9.4)
Weight (kg)[a]	77.8 (13.3)	77.9 (13.3)
Current smoker (%)[b]	9.7	9.5
Alcohol use[b]		
Light (%)	66.8	68.0
Moderate (%)	29.0	28.5
Excessive (%)	4.2	3.5
Physical activity (min/day)[c]	131 [86-193]	126 [81-190]
Education[b]		
Low (%)	53.6	52.4
Intermediate (%)	21.1	21.1
High (%)	25.4	26.5
B_{12} and/or folic acid supplement use (% yes)[b,c]	15.8	15.3
Vitamin D supplement use (% yes)[b,c]	19.7	18.3
Osteoporotic medication use (% yes)[b,c]	7.1%	7.8%
Biochemical analyses:		
Homocysteine (μmol/L)[d]	14.5 [13.0-16.7]	14.3 [13.0-16.5]
Vitamin B_{12} (pmol/L)[d]	266 [204-343]	267 [213-341]
Folate (nmol/L)[d]	18.9 [14.8-24.5]	18.8 [14.9-24.7]
Methylmalonic acid (μmol/L)[d]	0.23 [0.18-0.31]	0.22 [0.18-0.30]
Holotranscobalamin (pmol/L)[d]	63.0 [45.0-84.0]	65.0 [48.0-86.0]*
MTHFR C677T[b]		
CC (%)	44.2	45.6
CT (%)	42.7	41.6
TT (%)	13.1	12.9
Creatinine (μmol/L)[a]	84.1 (18.0)	83.9 (18.6)

MTHFR=Methylenetetrahydrofolate reductase, WUR=Wageningen UR, VUmc=VU University Medical Center, EMC=Erasmus MC. * for $P < 0.05$. [a] Presented as mean (standard deviation), difference tested using t-test. [b] Presented as percentages, differences tested using Chi-squared test. [c] Data based on self-report. [d] Presented as median [interquartile range], differences tested using Mann-Whitney U test.

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)[a]	**Follow-up homocysteine (µmol/L)[a]**	**2-yr change (µmol/L)[b]**	**N**	**Baseline homocysteine (µmol/L)[a]**	**Follow-up homocysteine (µmol/L)[a]**	**2-yr change (µmol/L)[b]**	**N**	**p-value**
Intention-to-treat										
	Total sample	14.4 [13.0-16.5]	14.3 [12.4-17.0]	-0.2 (4.1)	1299	14.2 [13.0-16.4]	10.3 [8.9-12.0]	-4.4 (3.3)	1296	<0.01
	Age ≤80y	14.2 [12.9-16.1]	14.0 [12.2-16.5]	-0.3 (3.8)	1107	14.1 [12.9-16.0]	10.2 [8.8-11.8]	-4.3 (3.1)	1114	<0.01
	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	<0.01
Per-protocol										
	Total sample	14.4 [13.0-16.4]	14.3 [12.3-16.9]	-0.2 (4.0)	1231	14.2 [13.0-16.3]	10.2 [8.9-11.8]	-4.5 (3.1)	1240	<0.01
	Age ≤80y	14.2 [12.9-16.1]	14.0 [12.1-16.5]	-0.3 (3.7)	1052	14.1 [12.9-16.0]	10.2 [8.8-11.7]	-4.4 (3.0)	1076	<0.01
	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	<0.01

[a]Presented as median [interquartile range]. [b]Presented as mean (standard deviation), difference tested using t-test.

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 of cases**	**Rate/100 person-y**	**N of cases**	**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)	0.39	0.84 (0.58-1.22)[1]	0.36
Any type of fracture (n=2,919)	94	3.1	79	2.6	0.84 (0.60-1.17)	0.30	0.83 (0.59-1.16)[1]	0.28
Cancer (n=2,906)	42	1.4	63	2.1	1.56 (1.05-2.31)	0.03	1.55 (1.04-2.30)[1]	0.03
Age ≤80y (n=2,416)	36	1.4	49	1.9	1.33 (0.86-2.04)	0.20	1.30 (0.85-2.01)[2]	0.23
Age >80y (n=490)	6	1.2	14	2.9	3.66 (1.21-11.11)	0.02	3.68 (1.21-11.24)[2]	0.02
Men (n=1,450)	29	1.9	32	2.1	1.17 (0.70-1.96)	0.55	1.15 (0.69-1.94)[3]	0.58
Women (n=1,456)	13	0.9	31	2.0	2.35 (1.23-4.50)	0.01	2.34 (1.22-4.46)[3]	0.01
WUR (n=854)	8	0.8	13	1.4	1.51 (0.62-3.69)	0.37	1.49 (0.61-3.66)[4]	0.38
VUmc (n=774)	8	1.0	19	2.4	2.82 (1.18-6.71)	0.02	2.79 (1.18-6.67)[4]	0.02
EMC (n=1,278)	26	2.0	31	2.4	1.22 (0.72-2.07)	0.46	1.18 (0.70-2.01)[4]	0.53
Per-protocol analysis	N of cases	Rate/100 person-y	N of cases	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)	0.33	0.82 (0.55-1.22)[1]	0.33
Age ≤80y (n=2,263)	36	1.6	41	1.8	1.07 (0.68-1.68)	0.77	1.08 (0.69-1.71)[2]	0.73
Age >80y (n=398)	26	6.0	7	1.9	0.30 (0.11-0.82)	0.02	0.28 (0.10-0.74)[2]	0.01
Any type of fracture (n=2,661)	79	2.9	63	2.3	0.81 (0.56-1.15)	0.24	0.80 (0.56-1.15)[1]	0.23
Age ≤80y (n=2,263)	55	2.4	57	2.4	1.01 (0.68-1.51)	0.96	1.02 (0.68-1.52)[2]	0.93
Age >80y (n=398)	24	5.6	6	1.6	0.29 (0.11-0.77)	0.01	0.26 (0.10-0.71)[2]	0.01
Cancer (n=2,651)	27	1.0	43	1.6	1.66 (1.02-2.70)	0.04	1.67 (1.02-2.71)[1]	0.04

95%CI= 95% Confidence Interval, WUR=Wageningen UR, VUmc=VU University Medical Center, EMC=Erasmus MC. Stratified analyses were performed if the interaction of sex, age, homocysteine, MTHFR polymorphism, or study center with treatment was significant (p<0.10).

[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

[4] Adjusted for age, sex, and baseline levels of homocysteine and holotranscobalamin

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=0.55-1.16). 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=0.40). Cox proportional-hazard models adjusted for age, sex, study center, baseline plasma 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 0.58-1.22) (Figure 2A, Table 3). Interactions of treatment group with age, sex, Hcy concentration, study center, and MTHFR C677T genotype were not significant.

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 0.55-1.22) (Table 3). A significant interaction effect with age was observed (p=0.02). Persons >80y in the intervention group had a 72% lower probability of sustaining an osteoporotic fracture (HR=0.28, 95%CI 0.10-0.74) compared with the placebo group (Table 3). The number needed to treat was 25 (for two years).

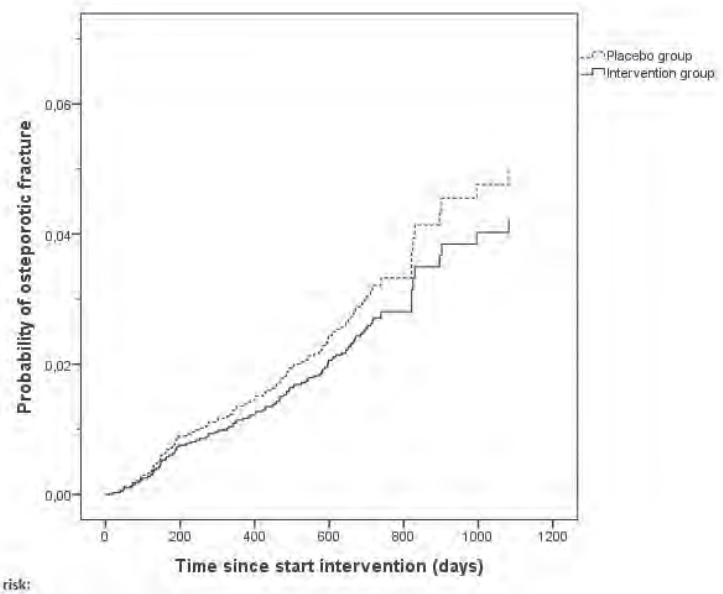
Secondary endpoint: Any type of fractures

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 effect of the intervention in both the ITT and the PP were observed (HR=0.83, 95%CI 0.59-1.16 and HR=0.80, 95%CI 0.56-1.15, respectively) (Figure 2B, Table 3). Again, interaction with age was observed in the PP analysis (p=0.02). Persons >80y in the intervention group had a 74% lower probability of a fracture of any type (HR=0.26, 95%CI=0.10-0.71) compared with placebo. Specific types of fractures are shown in eTable 1.

Adverse events

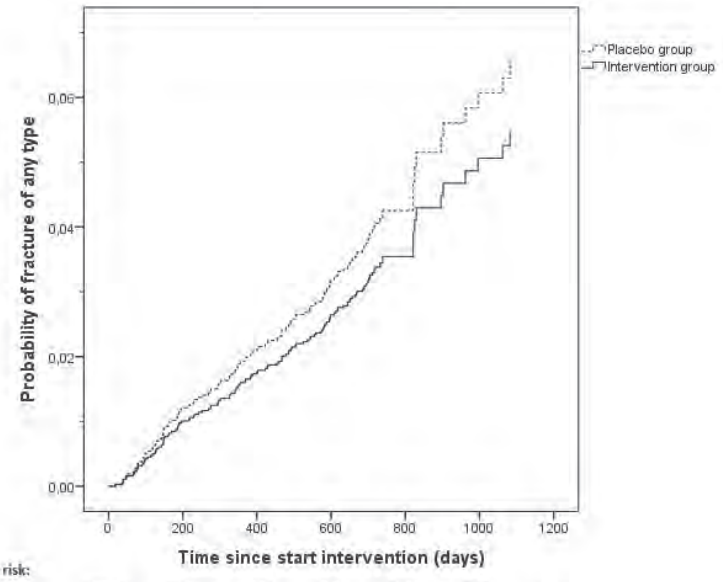
Mortality did not differ between the intervention and placebo group (n=37 vs. n=42 respectively, p=0.57, ITT). In the total number of adverse events, no difference was observed between treatment groups (p=0.84). However, 63 participants in the intervention group and 42 participants in the placebo group reported a new, subsequently verified diagnosis of cancer during the intervention period (Chi-square p=0.04). The HR was 1.55 (95%CI 1.04-2.30) (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=0.09), sex (p=0.09) and study center (p=0.09) were observed (ITT). Corresponding subgroup analyses revealed that the effect was more pronounced in participants aged >80y, more in women, and more in the VUmc study center, which recruited more women and persons >80y (Table 3). Differences mainly appeared for colorectal cancer (14 in intervention group vs. 5 in placebo) and other gastro-intestinal cancers (7 vs. 1, respectively).



No. at risk:							
Intervention	1461	1431	1431	1380	178	171	0
Placebo	1458	1421	1396	1364	200	189	0

a.



No. at risk:							
Intervention	1461	1425	1403	1369	178	171	0
Placebo	1458	1419	1389	1357	200	188	0

b.

Figure 2. Time-to- a) first osteoporotic fracture, b) first fracture of any type.

c.

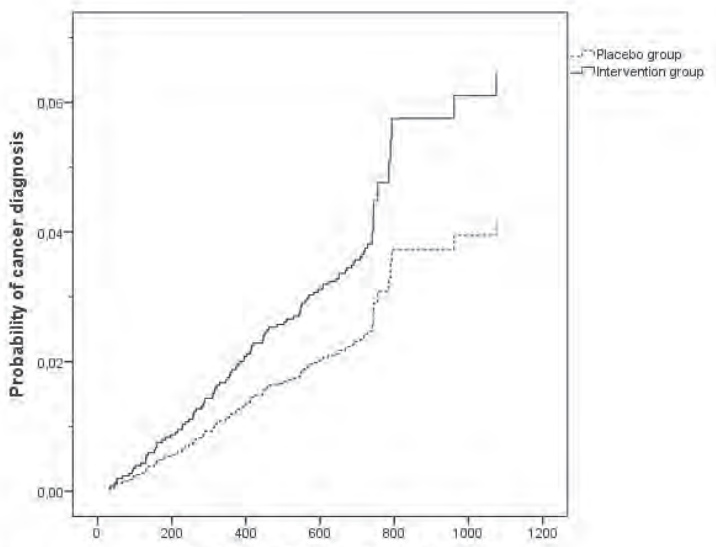


Figure 2. c) cancer diagnosis, adjusted for age, sex, study center, plasma homocysteine and serum holotranscobalamin, derived from Cox proportional-hazards analysis (Intention-to-treat)

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 level. Pre-specified interactions with subsequent subgroup analysis suggested an almost 4-fold 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.

Compared to the Sato trial [20] (a strong treatment effect on fractures) and the HOPE-2 trial [21] (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 included participants with a history of vascular disease, while B-PROOF included participants primarily based on elevated Hcy levels. Also, median Hcy levels differed substantially between the studies: 19.9 µmol/L in the Sato trial, 11.5 µmol/L in the HOPE-2 trial, 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 between Sato et al. and B-PROOF (53% vs. 50% women), fewer women participated in HOPE-2 (28%). 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 levels 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 and B-PROOF.

The fact that the age-specific effect was observed in the PP analyses and not in the ITT analyses suggests a true treatment effect. It is known that Hcy levels increase with age, and therefore baseline homocysteine levels or change in homocysteine levels might provide a possible explanation for the results. On the one hand, we did not observe significant interaction of baseline Hcy level (below and above 18 μmol/L) with treatment. Interestingly, on the other hand, Hcy levels 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 by the placebo group.

The observation of a significantly higher cancer incidence in the intervention group than in the control group was unexpected. It is important to note that B-PROOF was not designed to study cancer as a 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 0.99-1.13), or on cancer incidence at any site [25]. 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 [25]. These findings were consistent with two previous meta-analyses (RR 1.05, 95% CI 0.99-1.11 and RR 1.07, 95% CI 1.00-1.14) [26, 27]. The higher cancer risks observed in the B-PROOF trial 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 [25].

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 [28]. 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 [26, 27]. 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[25].

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 which 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 [29]. 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 [27]) 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 levels. Another strength is the high compliance with the allocated treatment.

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 levels was not observed in B-PROOF. However, stratified 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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APENDIX

Appendix eTable 1. 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

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8

General discussion

This thesis addresses the association of homocysteine, vitamin B_{12} and folate levels with cognitive function and bone health, two health outcomes of major importance for the elderly. These associations were studied using existing available scientific literature and cross-sectional observations in the B-PROOF study population at baseline. Additionally, the association between vitamin B_{12} intake and vitamin B_{12} biomarkers was studied. The effect of 2-year vitamin B_{12} and folic acid supplementation on fracture risk was assessed in the B-PROOF study, a randomized controlled trial (RCT). In this final chapter, a reflection is given on the findings in this thesis and suggestions are made for further research.

MAIN FINDINGS

Table 1 summarizes the findings from Chapter 2 to 7. Findings are presented in the context of a model which links dietary intake to biomarkers of nutritional status and subsequently to health outcomes. In general, it is shown that vitamin B_{12} intake is associated with vitamin B_{12} biomarker status. Furthermore, observational associations are shown between biomarker status and the health outcomes cognitive function and bone health. The intervention study showed prudently positive effects of vitamin B_{12} and folic acid supplementation on bone health in the subgroup of elderly aged >80 years who were compliant to the study supplement. However, these benefits should be weighed against potential risks.

METHODOLOGICAL CONSIDERATIONS

A combination of study designs was used in this thesis to obtain more insight in the role of homocysteine, vitamin B_{12} and folate levels in cognitive function and bone health: an intervention study, cross-sectional observational studies and systematic reviews with meta-analyses.

The proof of the pudding is in the eating: the B-PROOF study

After a systematic review and meta-analysis of intervention studies, an RCT is seen as a source of the highest level of evidence in research, including nutritional research [1]. In general, evidence from RCTs, such as the B-PROOF study, is needed to establish a causal relationship between exposure and outcome. In addition, adhering to the intention-to-treat principle in the analysis of the outcomes confirms the efficacy of a given intervention in a targeted population. In other words, the proof of the pudding is in the eating. Therefore, the B-PROOF study was envisaged to make a valuable contribution to the research field of homocysteine and related B-vitamins and their relation with bone health.

Table 1: Main findings of this thesis

Chapter	Intake	Biomarker Status	Health outcome	Study design	Results
Chapter 2				Study protocol of the B-PROOF study	This chapter describes the design and study population of the B-PROOF study, a two year intervention study with vitamin B12 and folic acid in 2919 Dutch elderly with elevated homocysteine levels.
Chapter 3	Dietary vitamin B12 intake	Total B12 HoloTC MMA Homocysteine		Cross-sectional study: Baseline data of the B-PROOF study	A doubling of vitamin B12 intake was associated with 9% higher total B12, 15% higher holoTC, 9% lower MMA and 2% lower homocysteine. Saturation of biomarkers occurs with dietary intakes of >5 µg B12. Levels of MMA and homocysteine started to rise when total B12 levels fell below 330 pmol/L and holoTC levels below 100 pmol/L, with a steep increase with levels of total B12 and HoloTC below 220 and 50 pmol/L respectively.
Chapter 4	Vitamin B12 intake	Total B12 HoloTC MMA	Cognitive function	Systematic review with meta-analyses	Studies showed no or inconsistent associations between vitamin B12 intake and cognitive function. Meta-analysis showed that vitamin B12 status was not associated with risk of dementia, global cognition z-scores or memory z-scores. 4 out of 5 cohort studies on MMA and holoTC reported significant associations with risk of dementia, Alzheimer's disease or global cognition.
Chapter 5		Total B12 MMA Folate Homocysteine	Bone health: Fractures and BMD	Systematic review with meta-analyses	Meta-analysis showed that serum/plasma vitamin B12 per 50 pmol/L was borderline significantly associated with a lower fracture risk (RR=0.96, 95% CI = 0.92-1.00). Homocysteine was significantly associated with a higher fracture risk (RR=1.04, 95% CI = 1.02-1.07). Meta-analyses regarding total B12, folate and homocysteine levels and BMD did not show a significant association.
Chapter 6		Total B12 HoloTC MMA homocysteine Folate Wellness score	Cognitive function	Cross-sectional study: baseline data of the B-PROOF study	Levels of total B12 and holoTC were not associated with cognitive function in any domain. Levels of homocysteine (β= -0.009), folate (β= 0.002), MMA (β= -0.163) and the wellness score (β= 0.048) were significantly associated with the domain of episodic memory. Homocysteine (β= -0.015) and the wellness score (β= 0.103) were also significantly associated with the domain information processing speed
Chapter 7	2- year vitamin B12 and folic acid supplemen-tation		Osteoporotic fracture incidence	RCT	The B-PROOF intervention did not significantly lower the risk of fracture in the total population (HR=0.84, 95% CI = 0.58-1.22). Per protocol subgroup analysis of elderly aged >80 years showed a lower risk of fracture in the intervention group (HR=0.28, 95% CI 0.10-0.74) than in the placebo group. We observed a higher cancer incidence in the intervention group (HR=1.55, 95% CI = 1.04-2.30), emerging from the occurrence of gastrointestinal cancers.

The B-PROOF study was our focus in three chapters (Chapter 3, 6 & 7). The B-PROOF study population involved elderly aged 65 years and older with an elevated homocysteine level (≥12.0 μmol/L). With this population we assumed to have recruited an appropriate study population for our intervention, since both an advanced age and an elevated homocysteine level are risk factors for fractures [2, 3]. On both age and homocysteine levels, however, we had to make concessions to our original inclusion criteria (age ≥ 70 years and tHcy ≥ 15 μmol/L, see Chapter 2) to facilitate recruitment of participants, otherwise it would not have been possible to include almost 3000 participants in the timeframe set for our research. Although we expected that in our study population still enough fractures would occur based on current fracture rates in the Netherlands, altering these inclusion criteria might have contributed to a dilution of the treatment effect in our study population.

Yet another cross-sectional study, been there, done that?

With the extensive baseline data of the B-PROOF study we were able to investigate cross-sectionally vitamin B_{12} biomarker status (Chapter 3) and cognitive function (Chapter 6). A major drawback of cross-sectional studies is, as repeatedly mentioned, the inability to establish a causal relationship between exposure and outcome. Cross-sectional studies are therefore mainly hypothesis-generating. There are already many observational studies on the association of homocysteine, vitamin B_{12} and folate status with cognitive function and also the association between vitamin B_{12} intake and status is far from unexplored. What have we contributed with these two cross-sectional studies? The answer lies in the extensive baseline assessments we performed on: vitamin B_{12} and folate intake, biomarker status and health outcomes, in this case cognitive function. Although the observational evidence seems abundant, comparability among studies is still rather low. Regarding the association between intake and status and among status markers (Chapter 3), there are only a few studies which studied all main four biomarkers of vitamin B_{12} status (total B_{12}, holoTC, MMA and tHcy). Providing information on levels of the four biomarkers and their mutual associations in such a large, relatively general elderly population contributes to the knowledge in this field of research.

The majority of evidence for the association between nutrient status and cognitive function emerges from studies using the MMSE as a single measure for cognitive function. We combined information on the four biomarkers for B_{12}, plus folate with a detailed measure on cognitive function divided in cognitive domains, again contributing to the knowledge in this research field. Herewith we adhere to our own suggestion in Chapter 4 to include MMA and holoTC as sensitive markers in research on vitamin B_{12} and cognitive function.

The icing on the evidence cake: systematic reviews with meta-analyses

We included two systematic reviews with meta-analyses in this thesis (Chapter 4 & 5). A major strength of these systematic reviews is that we included only studies which were performed in generally healthy populations. This enables us to extrapolate the findings from these reviews. Chapter 4 describes the association of vitamin B_{12} intake and status with cognitive function; homocysteine and folate were left aside in this chapter. For this thesis it would have been more appropriate to include these biomarkers as

well, but this single-nutrient approach fitted in the scope and organization of the EURRECA network, in which our systematic review took place [4]. Associations of folate with various health outcomes were considered separate from vitamin B_{12}, and homocysteine was not investigated, because of its lack of specificity for vitamin B_{12} deficiency [5]. In general our observations from chapter 6 are in line with our findings in the systematic review regarding cognitive function (Chapter 4). Total B_{12} is not associated with cognitive function, where more sensitive biomarkers like MMA are associated. However, we did not observe an association with holoTC. Additionally, although we did not consider homocysteine and folate in Chapter 4, findings are in line with existing literature; showing a stronger association of folate and homocysteine with cognitive function than total B_{12}.

The review in Chapter 5 regarding bone health describes predominantly observational studies and only one intervention study, since the available intervention studies about vitamin B_{12}, folic acid and bone health did not consider generally healthy populations [6, 7], which was a prerequisite for inclusion. Nonetheless, this review provides an extensive overview about the observational evidence in the association of levels of homocysteine, vitamin B_{12} and folate with fracture risk and BMD.

WHAT YOU SEE IS WHAT YOU GET? CONSIDERATIONS REGARDING THE ENDPOINTS IN THIS THESIS

We addressed two age-related health endpoints in this thesis: bone health and cognitive function. Furthermore, we evaluated levels of vitamin B_{12} biomarkers as both exposure (Chapter 4, 5 & 6) and outcome (Chapter 3).

B-vitamins: biomarkers

As mentioned in Chapter 3, there is no consensus about the best biomarker or biomarkers and accompanying cut-off values to determine vitamin B_{12} deficiency. This complicates the interpretation of results and the comparison with results of different studies. Currently, the general idea is that a combination of at least two biomarkers, preferably a circulating biomarker (total B_{12} or holoTC) and a functional biomarker (MMA or homocysteine) reflects vitamin B_{12} status better than the use of only one biomarker [8, 9].

A recent study of Bailey et al. emphasizes the difficulty of establishing a cut-off value for vitamin B_{12} deficiency and shows a continuous model of MMA levels against total B_{12} levels, comparable to our model as shown in Chapter 3 [10]. The authors have modeled levels of MMA against levels of total B_{12} with different mathematical models to establish an inflection using data of 12,683 adult participants of the NHANES 1999-2004 study. They conclude that the inflection was dependent on the used model, and that the use of one cut-off point for vitamin B_{12} deficiency is challenged by the presence of 3 distinct slopes. The presence of 3 slopes is in line with our findings, although the change points were different from ours: 126 and 287 pmol/L vs. 220 and 330 pmol/L in our data. Bailey et al. however, used a model with 2 knots, where we used a model with 5 knots, since this model had a better fit to our data than models with less knots.

Bailey et al. distinguished 3 groups according to total B_{12} status: a clearly vitamin B_{12} deficient group (total B_{12} <126 pmol/L), an adequate vitamin B_{12} group (total B_{12} >287 pmol/L) and a large (~33% of the participants) group with an intermediate vitamin B_{12} status. This intermediate group represents subclinical vitamin B_{12} deficiency, a suboptimal vitamin B_{12} status occurring long before classical clinical vitamin B_{12} deficiency is determined. The models shown in the study of Bailey et al. and in Chapter 3 acknowledge that the use of a single cut-off value for vitamin B_{12} deficiency does not distinguish the severely deficient group from the subclinical deficient group. The clinical relevance of subclinical vitamin B_{12} deficiency is not generally established and needs further attention to investigate the impact of this metabolic state on health in especially elderly people. Although many observational studies show negative health outcomes associated with vitamin B_{12} levels in the subclinical deficiency range, the effect of vitamin B_{12} supplementation on improving health outcomes is not established [11, 12]. All in all, it is clear that total B_{12} levels below 287-330 pmol/L deserve further attention in research.

The wellness score- which combines several markers for vitamin B_{12} status, as described in Chapter 6- may be an interesting approach in evaluating vitamin B_{12} status as it considers four biomarkers at the same time, which might reflect vitamin B_{12} status better than the level of one or two biomarkers. However, the used formula, in which all biomarkers are given the same weight and the use of reference values, could be questioned; correlations between biomarkers differ and levels of biomarkers vary per study. The implementation of different reference values in other studies jeopardizes comparison between studies. Furthermore, the costs of analyzing all four biomarkers are considerable and may be avoided when agreement is reached on cut-off values for vitamin B_{12} deficiency. Thus, the use of the wellness score seems promising, but has not been well established yet.

As described in Chapter 3, we observed a saturation of vitamin B_{12} biomarkers with dietary vitamin B_{12} intakes around 5 μg, which is well above the current Dutch RDA of 2.8 μg/day for adults and elderly. This might suggest that the current RDA for vitamin B_{12} is too low, at least for elderly, as also proposed in other studies: a cross-sectional study investigating dietary vitamin B_{12} intake and levels of biomarkers in a population of postmenopausal women [13, 14] and a systematic review regarding daily vitamin B_{12} losses and vitamin B_{12} bioavailability [15]. An increased need for dietary vitamin B_{12} may be due to the fact that many elderly have a reduced ability to absorb vitamin B_{12} from food as a result from gastric atrophy [16, 17]. However, more research is needed to confirm these observations.

Cognitive function

As mentioned earlier, the Mini Mental State Examination (MMSE) is widely used in research regarding nutritional status and cognitive function. This test however is mainly meant as a screening instrument to alert for dementia and is not distinctive to mild or isolated cognitive deficits. The ability of the MMSE to reveal associations between exposure and cognitive function depends furthermore on the prevalence of low scores in the population [18]. We measured cognitive function with an extensive test battery, and constructed cognitive domains, which is an acknowledged way to investigate cognitive

function. However, the use of the many available tests in research to measure cognitive function complicates comparison of study results. To overcome this practical problem, experts in cognitive testing should propose transparently and objectively a small set of outcomes which could be used in trials on cognitive outcomes, as suggested, among others, by Dangour and Allen [19]. Furthermore, the use of computerized tests for the assessment of short term effects on domains like attention may be considered, as they are able to capture short times with great accuracy. The use of magnetic resonance imaging (MRI) is promising in the research of B-vitamins and cognitive function. This technique can reveal associations between biomarker status and brain structure, and outcomes could be combined with performance on cognitive tests. MRI measurements may give more insight in the mechanisms by which elevated homocysteine levels and low levels of vitamin B_{12} and folate interfere with cognitive function, since effects on specific regions in the brain could be studied that are key components to the process of cognitive decline and dementia. There are several mechanisms suggested in literature as described in the Introduction, Chapter 4 and Chapter 6, in short: Hypomethylation as a result of low vitamin B_{12} and folate levels and elevated homocysteine levels causes myelin damage and disturbed neurotransmitter metabolism [20], homocysteine is suggested to be neurotoxic [21], high levels of homocysteine could cause structural vascular changes in the brain [22], leading to brain atrophy and white matter hyperintensities [23, 24] and high levels of MMA may also induce neurological damage [25].

"The toe bone is connected to the foot bone", fractures and other weaknesses

In the B-PROOF study osteoporotic fracture incidence, a clinical endpoint with high public health relevance, was our primary outcome. The occurrence of a fracture is in most cases the first clinical sign of the presence of osteoporosis. We considered all fractures osteoporotic, except for the head/ hand/ finger/ foot/ toe fractures and fractures caused by traffic accidents. The distinction between an osteoporotic fracture and a non-osteoporotic fracture may be arbitrary, as not every fracture indicates osteoporosis and osteoporosis does not always lead to a fracture. In the B-PROOF study we observed no difference in the effect of vitamin B_{12} and folic acid supplementation on osteoporotic fractures and fractures of any type.

Another indicator of osteoporosis is low bone mineral density (BMD). Contradictory, it hypothesized that elevated homocysteine levels affect bone strength without altering BMD, by interfering with collagen cross-linking [26]. The absence of an association of homocysteine, vitamin B_{12} and folate with BMD in the meta-analyses in Chapter 5 and in other observational studies support the collagen cross-link theory. In this light, the use of new techniques, such as high resolution peripheral quantitative computed tomography (hr-pQCT), may provide more insight in the mechanism by which homocysteine, vitamin B_{12} and folate affect bone health, since this method measures not only BMD, but also assesses bone micro structure and bone strength [27].

The rate of bone formation and bone resorption in the skeleton can be assessed by measuring biochemical bone turnover markers in plasma or serum. High homocysteine levels and low levels of vitamin B_{12} and folate were associated with increased bone resorption and decreased bone formation in several studies [28-31]. Intervention studies with B-vitamin supplementation did not observe effects on bone turnover markers [32-37]. It should be noted that, although measurements are relatively easy and non-invasive, the value of measuring bone turnover markers is yet to be established, since there is little consensus about which markers should be used. The International Osteoporosis Foundation therefore recently recommended that a marker of bone formation (serum procollagen type I N propeptide, s-PINP) and a marker of bone resorption (serum C-terminal telopeptide of type I collagen, s-CTX) are used as reference analytes for bone turnover markers in clinical studies [38]. Moreover, levels of bone turnover markers cannot be interpreted straightforwardly and possible treatment-induced changes are not fully understood yet.

It is furthermore suggested that homocysteine could affect bone health via decreased methylation capacity, since methylation is important for gene expression. Decreased methylation capacity is reflected by a lower ratio between S-adenosylmethionine (SAM) and S-adenosyl homocysteine (SAH). A lower SAM/SAH ratio has been shown to be correlated with lower bone strength in rats [39]. In the Rotterdam study, in elderly women, lower methylation capacity was associated with lower BMD, but not with an increased fracture risk [40]. Furthermore, fracture risk may also be affected by elevated homocysteine levels via poor physical performance, since this association is observed in several observational studies [41-43]. Within the B-PROOF study, physical performance and handgrip strength were indeed inversely associated with homocysteine levels, but only in women [44]. We do not have an explanation for the sex-specificity of this effect, but this interaction of physical performance with sex has been observed in another study as well [43].

Consider the endpoints bone health and cognitive function and the conclusions emerging from our systematic reviews in Chapter 4 and 5, an intervention study such as the B-PROOF study is a logical next step. An intervention study could reveal whether B-vitamin supplementation is indeed the panacea it is suggested to be in observational studies.

B-PROOF, ONE OF A KIND

The B-PROOF study is the first RCT in a general elderly population investigating the effect of vitamin B_{12} and folic acid on fracture risk as a primary outcome. The only two other RCTs regarding this topic are inconclusive: the study of Sato et al. observed a large treatment effect in Japanese post stroke hemiplegia patients [6] and the HOPE-2 trial did not observe a treatment effect in a Western population with a high cardiovascular risk from countries with and without mandatory folic acid fortification [7] (Chapter 7).

The absence of a significant treatment effect in our study could have various reasons. First of all, it is possible that there is no effect of vitamin B_{12} and folic acid supplementation on fracture risk. It has been postulated, provoked by inconclusive findings from homocysteine lowering trials, that not an elevated homocysteine level itself is deleterious, but that it is a biomarker for 'something else', for example a low general nutritional status, well-phrased by Raisz as homocysteine being a 'culprit or innocent bystander' [45].

We observed a strong treatment effect in participants aged 80 years and older who were compliant to the study supplementation. This finding suggests that vitamin B_{12} and folic acid supplementation is genuinely beneficial for fracture prevention in elderly with an elevated homocysteine level. What could then have contributed to the absence of a significant overall effect?

It could be possible that the study duration was not long enough. The intervention period was two years, with a one year extension for a subgroup consisting of almost 400 participants. In interventions on the effect vitamin D on fracture risk however, study duration ranged from 12 to 60 months [46]. Comparable sample size calculations and outcome estimations have been used in the vitamin D supplementation study of Chapuy et al. [47] which showed a significant fracture incidence reduction after 18 months of follow-up. Furthermore, Sato et al. achieved beneficial results after intervening for two years [6]. In addition, the treatment effect observed in elderly aged >80 years in the B-PROOF study suggests that a two year intervention is long enough.

It is very well plausible that the study population was not sensitive enough. We observed less fractures than expected based on fracture incidence in Dutch elderly. It might be that our study population, except from having an elevated homocysteine level, is healthier than a random sample of Dutch elderly aged 65 years and older. This possible selection bias could be due to the fact that people who are willing to participate in a two year intervention study are the healthier part of the general population. People who beforehand perceive an intervention study as too burdensome and are therefore not willing to participate might be the people who have a higher a priori fracture risk and would theoretically benefit more from an intervention study such as the B-PROOF study. In addition, although homocysteine levels were elevated, the range was relatively small: 95% of our population had a homocysteine level between 12.0 and 22.3 μmol/L. Regarding earlier observational evidence on associations of homocysteine levels with fracture risk [3, 48], it could well be that a threshold-effect exists. In both studies the association was present in the highest quartile of homocysteine levels compared to the lowest quartile [48] or lowest 3 quartiles combined [3]. Mean homocysteine levels in the highest quartile were in both studies higher than our median homocysteine level: 20.8 μmol/L (men) and 18.6 μmol/L (women) [48]; in the study of Van Meurs et al. quartiles were defined in a sex-specific and age-specific manner for five-year categories, mean values per quartile are therefore not provided in the article, but since mean homocysteine level ranged from 11.9 to 15.9 μmol/L in the total cohorts investigated and were higher in older age groups [3], one could but conclude that the highest quartile included people with homocysteine levels above our range of homocysteine levels.

The observation that in the intervention group more participants reported cancer incidence than in the control group was against our expectations, as meta-analyses, including up to 50,000 participants did not show negative effects of folic acid supplementation on cancer incidence [49-51]. We are still refining our analyses and we can neither confirm nor deny that the supplementation of vitamin B_{12} and folic acid had a negative effect on cancer progression in our study population. We should note however, that we observed no difference in mortality between the intervention groups.

The ambiguous role of folate, and especially synthetic folic acid in cancer development, and more specifically colorectal cancer development, has been topic of research in the last decade and there seems to be a dual role of folic acid in cancer: it may protect against the initiation of cancer, but facilitate the growth of neoplastic cells [52], questioning the value of folic acid supplementation.

When we designed our intervention study we chose a folic acid dose well below the safe upper level of 1 mg. In the B-PROOF study we included vitamin B_{12} in the supplement because of its homocysteine-lowering capacity, the generally low vitamin B_{12} status in elderly and the positive results from the Sato trial. The duration of our intervention, i.e., two years, is a time span very limited for a possible initiation, development and discovery of a cancer, especially colorectal cancer. It is therefore more plausible that folic acid supplementation in our study might have accelerated growth of already present tumors, but in this stage we can only speculate.

Although folic acid is highly effective in lowering homocysteine levels, focusing on ways to lower homocysteine levels without the use of folic acid, such as supplementation with vitamin B6, B_{12} and possibly B2 might have more positive health effects, although further research is warranted.

In general, RCTs are needed to provide an evidence base for nutrient recommendations, which is still scarce in the case of homocysteine, vitamin B_{12} and folic acid in relation to bone health and cognitive function.

Besides our speculations about the absence of an overall treatment effect in the B-PROOF study one could furthermore cautiously question the possibility that the absence of a significant health effect from homocysteine lowering trials is inherent in such a trial. The difficulty of obtaining evidence from RCTs in nutrition research has been acknowledged before, in general [53] and for homocysteine lowering RCTs [54]. Blumberg et al. emphasized why nutritional interventions are different from drug interventions, from which the need for evidence-based proof originates, the most important being: I) medical interventions are designed to cure a disease not produced by their absence, while nutrients prevent dysfunction that would result from an inadequate intake; II) drug effects can be tested against a non-exposed group, whereas it is impossible or unethical to attempt a zero intake group for nutrients; III) drug effects are generally expected to be large and with a limited scope of action, where nutrient effects are smaller, mainly within the range of biological variability and have a wide range of biochemical functions [53]. These differences complicate the establishment of evidence from RCTs that a nutrient

affects a health outcome. In addition, one has to take the specific shortcomings of homocysteine lowering RCTs in account, i.e., although there seems to be a linear association, the possibility that homocysteine levels are not elevated enough to show a treatment effect; a too short intervention period; the contrast between intervention and placebo group being too small (as point II mentioned above) and the potential adverse effects of folic acid [55]. Taken together these considerations it is very well plausible that the characteristics of the RCT study design itself contribute to the null findings in homocysteine lowering trials. We should therefore not ignore the vast body of observational research evidence regarding homocysteine, vitamin B_{12} and folate.

Lost in translation? Where are we and where are we going to? Implications and outlook

We can conclude from the results of this thesis that further research is necessary, but what will be the next steps in research regarding homocysteine, vitamin B_{12} and folate in association with bone health and cognitive function?

To start with, the B-PROOF study is a source of very relevant information. Besides the outcomes and associations described in this thesis, many other measurements were performed. Serum and plasma samples are available for further analysis of biomarkers, for example levels of vitamin B2 and B6, or metabolites in the homocysteine metabolism. Genetic aspects beyond the consideration of a single SNP, such as common genetic polymorphisms for homocysteine levels [56] could be studied in relation to fracture incidence.

With respect to bone health, we measured, besides fracture incidence, physical performance, fall incidence and BMD, the latter in a subsample. Associations of homocysteine with these outcomes and the effect of vitamin B_{12} and folic acid supplementation could be investigated. In addition, plasma/serum samples could be used for the determination of bone turnover markers. This would provide insight in levels of bone turnover markers in elderly and in changes in bone turnover markers induced by B-vitamin supplementation. Regarding cognitive function, the effect of 2-year supplementation of vitamin B_{12} and folic acid will be studied. We furthermore performed brain MRI measurements in 225 participants at the end of the intervention, which enables us to investigate differences in brain measures between participants in the intervention and placebo group. Last but not least, it would be highly relevant to follow the B-PROOF participants over a longer period regarding the occurrence of malignancies. Supplementation of a combination of 400 μg folic acid and 500 μg vitamin B_{12}, as supplemented in the B-PROOF study, is currently not recommended for elderly, awaiting further analysis of our data.

In this thesis, we have considered bone health and cognitive function as two separate health outcomes, which is common in research investigating associations between exposures and health outcomes. Nevertheless, these health outcomes are intertwined, since physical performance affects cognitive function and vice versa.

A suggestion could be to combine these and other health outcomes related to homocysteine, vitamin B_{12} and folate into a 'global health index'. Such a suggestion for the use of global indices as a primary health outcome in nutrition research has been proposed by Heaney [57]. Heaney acknowledges the association of a single nutrient with many health outcomes. A global index combining these health outcomes could improve power to detect differences that are relatively small, but summed up may be relevant and detectable. Although this approach clearly needs further substantiation, combining physical and cognitive performance into a single health outcome would be an interesting next step in research regarding homocysteine, vitamin B_{12} and folate status and health in elderly people.

To conclude this thesis, our literature reviews and observational data confirm an association of levels of homocysteine, vitamin B_{12} and folate with cognitive function and fracture risk in elderly. Supplementation with vitamin B_{12} and folic acid did not lower the risk of fracture in the total study population. Though beneficial effects on fracture incidence emerged from elderly aged >80 years, these benefits should be weighed against potential risks.

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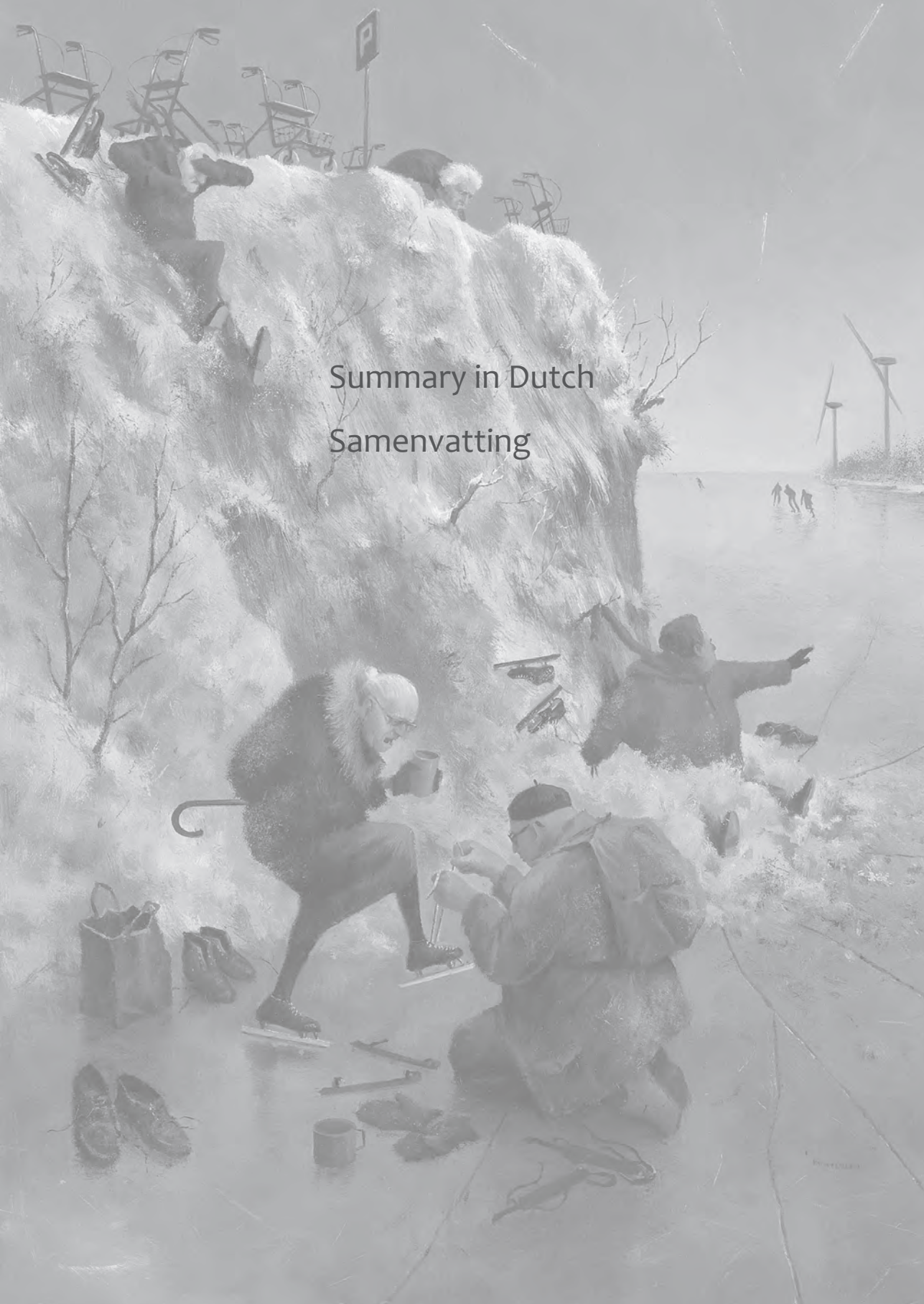

Summary in Dutch

Samenvatting

SAMENVATTING

Een verhoogd homocysteine gehalte in het bloed blijkt een risicofactor te zijn voor onder andere hart- en vaatzieken, cognitieve achteruitgang en fracturen. Omdat vitamine B_{12} en foliumzuur het homocysteine gehalte verlagen, zou suppletie met vitamine B_{12} en foliumzuur het risico op deze verschillende leeftijd gerelateerde aandoeningen kunnen verlagen en dientengevolge van substantieel belang kunnen zijn voor de volksgezondheid.

Dit proefschrift richt zich op twee gezondheidsuitkomsten die in de wetenschappelijke literatuur vaak worden geassocieerd met een verhoogd homocysteine gehalte en lage gehaltes aan vitamine B_{12} en foliumzuur, namelijk: osteoporose en cognitieve achteruitgang bij ouderen.

De inhoud van dit proefschrift is voornamelijk gebaseerd op het B-PROOF onderzoek. Het B-PROOF onderzoek is een grote, gerandomiseerde, dubbelblinde interventiestudie, uitgevoerd in drie verschillende onderzoekscentra: Wageningen University, Wageningen, VU medisch centrum, Amsterdam en Erasmus medisch centrum, Rotterdam. Het B-PROOF onderzoek is opgezet om het effect te onderzoeken van suppletie van vitamine B_{12} en foliumzuur gedurende twee jaar op het risico op fracturen bij ouderen (65 jaar en ouder) met een verhoogd homocysteine gehalte (≥12 μmol/L). Verder is de beschikbare wetenschappelijke literatuur over de associatie van vitamine B_{12}, foliumzuur en homocysteine met cognitief functioneren en botgezondheid systematisch beschreven en waar mogelijk kwantitatief vergeleken in een meta analyse om een overzicht te geven van de beschikbare informatie. Deze reviews zijn geschreven in de context van het EURRECA (EURopean RECcomendations Alligned) Netwerk. Het EURRECA netwerk had als doel de methoden voor het afleiden van aanbevolen hoeveelheden voor micronutriënten binnen Europa te harmoniseren. Hierbij werd speciale aandacht gegeven aan aanbevelingen voor kwetsbare populatiegroepen zoals oudere mensen.

De opzet van het B-PROOF onderzoek is uitgebreid beschreven in **Hoofdstuk 2**. Bij aanvang van de studie zijn bloedmonsters verzameld en hebben de deelnemers (N=2919) vragenlijsten ingevuld over hun gezondheid en leefstijl. Verder ondergingen de deelnemers een uitgebreide screening met metingen op het gebied van antropometrie, cognitief functioneren en fysiek functioneren. Dit heeft geresulteerd in een uitgebreide dataset die is gebruikt om verschillende cross-sectionele associaties te beschrijven.

In **hoofdstuk 3** is bij aanvang van de B-PROOF studie in een subgroep van de onderzoekspopulatie de associatie onderzocht van de inname van vitamine B_{12} met 4 biomerkers voor vitamine B_{12} status (serum B_{12}, holotranscobalamine (holoTC), methylmalonzuur (MMA) en homocysteine). Serum B_{12} en holoTC zijn zogenaamde circulatie biomerkers, die een directe weergave zijn van de vitamine B_{12} status. MMA en homocysteine zijn zogenaamde metabole biomerkers, waarbij een verhoogde status een indicatie is voor een vitamine B_{12} tekort. Wij zagen dat een tweemaal zo hoge inname van vitamine B_{12} was geassocieerd met een 9% hogere serum B_{12} status, een 15% hogere holoTC status, een 9% lagere MMA status, en een 2% lagere homocysteine status. De biomerkers leken verzadigd bij een vitamine B_{12} inname boven de 5 microgram per dag; dat wil zeggen, de status veranderde niet boven een inname

van 5 microgram per dag. Verder bleek de status van de verschillende biomerkers met elkaar samen te hangen: de status van MMA en homocysteine zijn hoger bij serum B_{12} waarden lager dan 330 pmol/L en bij holoTC waarden lager dan 100 pmol/L, met een sterke verhoging bij serum B_{12} en holoTC waarden onder de, respectievelijk, 220 en 50 pmol/L. Deze waarden geven een aanwijzing vanaf welke biomerker waarden er mogelijk sprake is van een vitamine B_{12} tekort.

Het systematische literatuuronderzoek in **Hoofdstuk 4** liet geen, of inconsistente associaties zien van vitamine B_{12} inname met cognitief functioneren. Verder bleek serum vitamine B_{12} niet geassocieerd te zijn met het risico op dementie, globaal cognitief functioneren of geheugen. Studies die MMA en HoloTC als biomarker onderzochten, rapporteerden significante associaties met dementie, de ziekte van Alzheimer en globaal cognitief functioneren.

De meta-analyse van de beschikbare wetenschappelijke literatuur, beschreven in **hoofdstuk 5**, liet zien dat serum/plasma vitamine B_{12} (per 50 pmol/L) net niet significant geassocieerd was met een lager risico op fracturen (RR=0.96, 95% CI = 0.92-1.00) en dat homocysteine significant was geassocieerd met een hoger risico op fracturen (RR=1.04, 95% CI = 1.02-1.07). Meta-analyses naar de associatie tussen vitamine B_{12}, foliumzuur, homocysteine en botdichtheid (BMD) lieten geen significante associatie zien.

In **hoofdstuk 6** is de associatie bekeken van de eerder genoemde vitamine B_{12} biomerkers en foliumzuur met cognitief functioneren bij aanvang van de B-PROOF studie. Serum B_{12} en holoTC waarden waren in geen enkel cognitief domein geassocieerd met het cognitief functioneren. De homocysteine status (β= -0.009), foliumzuur status (β= 0.002), MMA status (β= -0.163) en de 'wellness-score' – een combinatiescore voor vitamine B_{12} biomerkers – (β= 0.048) waren significant geassocieerd met het domein 'episodisch geheugen'. Verder waren homocysteine status (β= -0.015) en de wellness-score (β= 0.103) significant geassocieerd met het domein 'snelheid van informatieverwerking'.

Hoofdstuk 7 beschrijft het resultaat van het B-PROOF onderzoek. Dagelijkse suppletie van 400 microgram foliumzuur en 500 microgram vitamine B_{12} gedurende twee jaar verlaagde het risico op fracturen niet in de algemene B-PROOF populatie (HR=0.84, 95% CI = 0.58-1.22) (intention-to-treat). Subgroup-analyse (per-protocol) in ouderen ouder dan 80 jaar liet wel een significant lager risico op fracturen zien in de interventiegroep ten opzichte van de placebogroep (HR=0.28, 95% CI 0.10-0.74). In de interventie groep bleek de incidentie van kanker hoger dan in de placebogroep (HR=1.55, 95% CI = 1.04-2.30) en hoewel dit mogelijk een toevalsbevinding is, kan op basis van dit resultaat de mogelijkheid van een (versnelde) kankergroei als negatieve bijwerking van de vitamine B_{12} en foliumzuur suppletie niet uitgesloten worden.

De conclusie van het onderzoek in dit proefschrift is dat zowel de observationele data als het systematische literatuuronderzoek een associatie bevestigen van homocysteine, vitamine B_{12} en foliumzuur status met cognitief functioneren en risico op fracturen in ouderen. Suppletie met vitamine B_{12} en foliumzuur verlaagde het risico op fracturen echter niet in de algemene B-PROOF populatie.

Dankwoord

DANKWOORD

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Janneke

About the author

CURRICULUM VITAE

Janneke Petra van Wijngaarden was born on January 27, 1981 in Emmeloord, the Netherlands. In 1999 she completed secondary school at Zuyderzee College in Emmeloord and started her study Nutrition and Health at Wageningen University. As part of her studies she conducted a minor thesis at Communication Sciences at Wageningen University entitled 'The specific role of the general practitioner in nutrition communication'. Her major thesis at the division of Human Nutrition was entitled 'the effect of in-school noodle consumption on out-school food consumption in children aged 6-8 year in rural North Vietnam', and included 4 months of field work in rural North Vietnam.

After obtaining her master's degree in 2005, she started working as a research assistant on several projects at the division of Human Nutrition, and Communication Sciences at Wageningen University. From 2006 to 2008 she worked as Scientific Knowledge Manager at Numico Research (currently Danone Research) in Wageningen. In May 2008 Janneke started her PhD project on the B-PROOF study, of which the results are partly described in this thesis. She spent most of her time on the B-PROOF study, in which she set up many of the measurements and managed this multi-center intervention study for the Wageningen University center, in which over 2000 potential participants were screened and more than 800 people participated. She joined the educational programme of the Graduate School VLAG and was involved in teaching and supervising students at BSc and MSc level.

Currently, Janneke is appointed as a postdoctoral researcher at the division of Human Nutrition of Wageningen University.

LIST OF PUBLICATIONS

Publications in peer-reviewed journals

van Wijngaarden JP, Dhonukshe-Rutten RAM, van Schoor NM, van der Velde N, Swart KM, Enneman AW, van Dijk SC, Brouwer-Brolsma EM, Zillikens MC, van Meurs JB, Brug J, Uitterlinden AG, Lips P, de Groot CPGM. 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. BMC Geriatrics, 2011 Dec 2;11:80

van Wijngaarden JP, Doets EL, Szczecińska A, Souverein OW, Duffy ME, Dullemeijer C, Cavelaars AEJM, Pietruszka B, van 't Veer P, Brzozowska A, Dhonukshe-Rutten RAM, de Groot CPGM. Vitamin B12, folate and homocysteine and osteoporosis in adults and elderly people: a systematic review with meta-analyses. the Journal of Nutrition and Metabolism, 2013, article ID 486186

Doets EL, **van Wijngaarden JP**, Szczecińska A, Dullemeijer C, Souverein OW, Dhonukshe-Rutten RAM, Cavelaars AEJM, van 't Veer P, Brzozowska A, de Groot CPGM. Vitamin B12 intake and status and cognitive function in elderly people. Epidemiologic Reviews, 2013, 35(1);2-21

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Submitted publications

van Wijngaarden JP, Dhonukshe-Rutten RAM, Brouwer-Brolsma EM, Enneman AW, Swart KMA, van Dijk SC, in 't Veld PH, van Schoor NM, van der Velde N, de Jonge R, Lips P, Uitterlinden AG, de Groot CPGM. Associations of vitamin B12 intake and related biomarkers in a Dutch elderly population.

van Wijngaarden JP/ Swart KMA/ Enneman AW*, Dhonukshe-Rutten RAM, Van Dijk SC, Ham A, Brouwer-Brolsma EM, Van der Zwaluw NL, Sohl E, Van Meurs JBJ, Zillikens MC, Van Schoor NM, Van der Velde N4, Brug J, Uitterlinden AG, Lips P, De Groot CPGM. Effect of daily vitamin B12 and folic acid supplementation on fracture incidence in elderly with an elevated plasma homocysteine level: B-PROOF, a randomized controlled trial. (*these authors contributed equally to the contents of the manuscript)

OVERVIEW OF COMPLETED TRAINING ACTIVITIES

With the educational activities listed below the PhD candidate has complied with the educational requirements set by the Graduate School VLAG (Food Technology, Agrobiotechnology and Health Sciences).

Description	Organiser and location	Year
Discipline specific activities		
Courses and workshops		
Systematic review and meta-analysis	PoE, Soesterberg	2009
Workshop Micronutrient Bioavailability: priorities and challenges for setting dietary reference values	ILSI/EURRECA, Barcelona, Spain	2009
Good clinical practice, wet-en regelgeving voor klinisch onderzoek	Clinical Trial Service, Ede	2011
Advanced Topics in Clinical Trials	NIHES, Rotterdam	2011
Conferences and meetings		
Annual meeting NWO nutrition	NWO, Deurne	2009, 2011, 2012
Wageningen Nutritional Sciences Forum	Division of Human Nutrition, WUR, Arnhem	2009
EURRECA annual meeting	EURRECA, Barcelona, Spain/ Copenhagen, Denmark	2009, 2010
IANA conference	IANA, Albuquerque, USA	2010
FASEB Summer Research Conference on folate, vitamin B12 and one-carbon metabolism	FASEB, Carefree, USA	2010
4e nationale Voedingscongres	Alliantie Voeding, Gelderse Vallei, Ede	2011
Annual conference VoedingNederland	Voeding Nederland, Nieuwegein	2011
IAGG conference	IAGG, Bologna, Italy	2011
EUGMS conference	EUGMS, Malaga, Spain	2011
Geriatriedagen	Nederlandse Vereniging voor Gerontologie, Den Bosch	2011, 2012
NCHA conference	NCHA, Den Haag	2013
Ageing and Cognition conference	IfaDo Dortmund, Germany	2013
General courses		
VLAG PhD week	VLAG, Bergeijk	2008
Preparation PhD research proposal	VLAG, Wageningen	2008
Project- and time management	WGS, Wageningen	2008
Afstudeervak organiseren en begeleiden	Docenten Ondersteuning, Wageningen	2008
PhD Competence assessment	VLAG, Wageningen	2009
Masterclass regression analysis	VLAG, Wageningen	2010
Masterclass 'How to write a world class paper'	WUR library, Wageningen	2010
Masterclass multilevel analysis	VLAG, Wageningen	2011

Scientific writing	WGS, Wageningen	2011
How to present	Mennen Training & Consultancy, Wageningen	2011
Optional courses and workshops		
Oldsmobiles	Division of Human Nutrition, Wageningen	2008-2013
PhD Study Tour Nordic countries	Division of Human Nutrition	2009
PhD study Tour Mexico, USA	Division of Human Nutrition	2011
presentation Alzheimer Café 'Dementie en voeding'	Alzheimer stichting, Elst Gld	2011

COLOPHON

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