Vitamin B_{12}: a novel indicator of bone health in vulnerable groups

Rosalie AM Dhonukshe-Rutten

PROMOTOR

Prof. Dr. W.A. van Staveren

Hoogleraar in de voeding van de oudere mens, Afdeling Humane Voeding, Wageningen Universiteit

CO-PROMOTOR

Dr. Ir. C.P.G.M. de Groot

Universitair Hoofddocent, Afdeling Humane Voeding, Wageningen Universiteit

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Dr. H.J. Blom

University Medical Center Nijmegen

Prof. Dr. Ir. G.J. Schaafsma

Wageningen Universiteit

Dit onderzoek is uitgevoerd binnen de onderzoekschool VLAG

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Rosalia Antonia Maria Dhonukshe-Rutten

PROEFSCHRIFT

Ter verkrijging van de graad van doctor
op gezag van de Rector Magnificus
van Wageningen Universiteit,
Prof. Dr. Ir. L. Speelman,
in het openbaar te verdedigen
op maandag 18 oktober 2004
des namiddags te vier uur in de Aula

Dhonukshe-Rutten, Rosalia Antonia Maria

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Thesis Wageningen University – with references – with summary in Dutch and Hindi

ISBN 90-8504-107-4

Abstract

Background: A number of modifiable predictors for osteoporosis and fractures have been identified, including nutritional factors, such as vitamin D deficiency and low calcium intake. Cobalamin deficiency has been suggested to affect bone metabolism. Pernicious anaemia, which can result in cobalamin deficiency, has been identified as a risk factor for osteoporosis. Also, it is known that homocystinuria patients are often diagnosed with osteoporosis. Both moderate hyperhomocysteinemia and vitamin B_{12} deficiency are highly prevalent in old age and may play a role in diseases characteristic for old age.

Objectives: 1) To show the associations of vitamin B_{12} and homocysteine with measures of bone health in three different populations. 2) To assess the effect of supplementation with 1000 μg crystalline cobalamin, carried either by a milk product or a capsule, on cobalamin status in mildly cobalamin deficient Dutch elderly people

Results: Data analyses in three different populations supplied the following information. Macrobiotic-fed adolescents: adolescents (9-15 y) with a low BMD had a significantly less favorable vitamin B_{12} status (adjusted mean, SD: 344 ± 24 pmol/L) and MMA status (adjusted mean, P5, P95: 0.31 [0.26, 0.35] μmol/L) than adolescents with a normal BMD, with levels of respectively 442 (18) and 0.20 (0.16, 0.23) μmol/L. Free-living elderly: An increased Hcy level appeared to be a strong and independent risk factor for osteoporotic fractures in elderly men and women of the Longitudinal Aging Study Amsterdam (n=1267, mean age: 76 yrs). Relative risks (95% CI) for the highest Hcy quartile versus lowest three homocysteine quartiles were 4.6 (1.4-14.5) in men, and 1.8 (0.8-3.7) in women. Frail elderly people: Osteoporosis (defined by BMD T-score < -2.5) occurred almost five times more often in frail elderly women with a marginal vitamin B12 status and seven times more often in women with a deficient vitamin B_{12} status than in women with a normal status in the VFit-study. Intervention study with vitamin B_{12}: Crystalline cobalamin added to milk is an effective alternative for cobalamin capsules in improving cobalamin status.

Conclusions: We found a relevant association between vitamin B_{12} metabolites and bone health in various studies with different study designs and diverse populations. Since these observed associations broaden the scope for randomized clinical trials, we conducted an intervention study in which we showed that milk enriched with vitamin B_{12} is an as effective treatment as cobalamin capsules.

Voor onze ouders/For our parents

Contents

Introduction

1

Along with the growing number of elderly people, there is an increased prevalence of illness, which is accompanied with an intensification in functional impairment, disability and lower quality of life [1-3]. Specific for old age – but starting earlier in life – is bone demineralization leading to osteoporosis. Counteracting this process at least partly is feasible by addressing modifiable lifestyle factors. In this thesis, we focus on the role of nutrition in bone health and particularly on vitamin B_{12} status, of which deficiency is highly prevalent in old age. New insights in the metabolism of vitamin B_{12} pointing to a relation of vitamin B_{12} status with osteoporosis are emerging.

Before describing these new insights, food sources and functions of vitamin B_{12} will be described along with the prevalence and diagnosis of its deficiency, and consequences and treatment of vitamin B_{12} deficiency will be reviewed. Since bone health can be measured in several ways, the most important methods and the ones used in this thesis will be described. Finally methods to prevent deficiencies and counteract bone loss by modifiable (lifestyle) factors will be addressed.

VITAMIN B_{12}

Vitamin B_{12} is mainly found in foods of animal origin. Therefore, people consuming a vegetarian or lactovegetarian diet have a lower vitamin B_{12} intake than nonvegetarians. Plant foods do not provide vitamin B_{12} unless the plant has either been exposed to vitamin B_{12}-producing bacteria, or contaminated with vitamin B_{12}-containing substances (soil, insect parts, etc), or fortified with vitamin B_{12} (e.g. fortified ready-to-eat breakfast cereals). Food rich in vitamin B_{12} are dairy products, meat, liver, fish, eggs, and shellfish. Unlike folic acid, vitamin B_{12} is only slightly destroyed with cooking.

Functions of vitamin B_{12} in enzymatic pathways

Vitamin B_{12} plays an important role in DNA synthesis and thus in neurologic functions. In humans, vitamin B_{12} exerts its physiologic effect on two major enzymatic pathways. In the first enzymatic pathway, methionine synthase requires the cofactor methylcobalamin for the conversion of homocysteine to methionine. This reaction is essential for the conversion of dietary methyltetrahydrofolate to tetrahydrofolate (Figure 1A). If this pathway is impaired, methyltetrahydrofolate accumulates along with an increase in serum homocysteine. In the second pathway, adenosylcobalamin is a cofactor for the enzyme L-methylmalonyl coenzyme A (CoA) mutase in the conversion of methylmalonyl CoA to

succinyl-CoA (Figure 1B). The diminished activity in this pathway results in an accumulation of serum methylmalonic acid (MMA) [4;5].

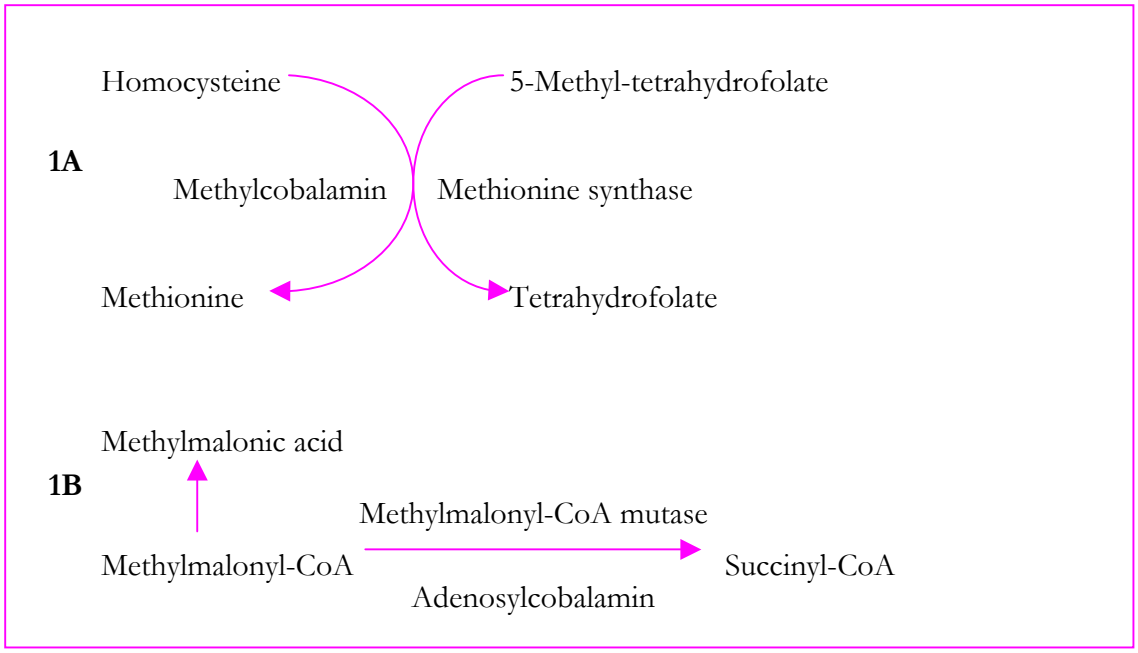

Figure 1A, 1B Vitamin B_{12}-dependent metabolic pathways

Development of vitamin B_{12} deficiency

Normally, nonvegetarian humans maintain a large vitamin B_{12} reserve. Vitamin B_{12} is the best stored of all vitamins, with enough stores to last 3-5 years in a normal replete subject. Yet, elderly people may develop a vitamin B_{12} deficiency. Among them it has been shown to be a common and clinically important problem [6-8]. Based on low serum vitamin B_{12} levels and elevated methylmalonic acid (MMA) levels, approximately 24% of the Dutch elderly people have a mild vitamin B_{12} deficiency [9]. The majority of older adults with vitamin B_{12} deficiency appear to have food-bound vitamin B_{12} malabsorption due to gastrointestinal changes. Decreased absorption of protein-bound vitamin B_{12} in elderly is most likely due to a high prevalence of atrophic gastritis. Atrophic gastritis is accompanied by low acid-pepsin secretion by the gastric mucosa, which in turn results in a reduced release of free vitamin B_{12} from food proteins [10].

Pernicious anemia, which was considered as the most common classical cause of cobalamin deficiency, occurs less often than cobalamin malabsorption. In pernicious anemia, vitamin B_{12} deficiency can be developed in several ways: achlorhydria (reduction in secretion of intrinsic factor and HCl by parietal cells), low serum pepsinogen I

concentrations, and high serum gastrin concentrations (caused by hyperplasia of gastrin-producing cells) [11].

Vegetarians have more often a vitamin B_{12} deficiency or elevated levels of homocysteine and methylmalonic acid than nonvegetarians due to a lower vitamin B_{12} intake [11-20].

Diagnosis of vitamin B_{12} deficiency

The diagnosis of vitamin B_{12} deficiency is complicated by the limitations of the current vitamin B_{12} assay techniques, whereby a low vitamin B_{12} concentration does not always indicate vitamin B_{12} deficiency. The Schilling test has virtually disappeared for the diagnosis of vitamin B_{12} deficiency, because it can only be used for the diagnosis of pernicious anemia or malabsorption of crystalline vitamin B_{12} and not for other causes of vitamin B_{12} deficiency. Besides this, the absorption of crystalline vitamin B_{12} may differ from the absorption of vitamin B_{12} in food. Moreover, it is complicated to perform and interpret [21].

Given its role in the two pathways, lack of hydroxycobalamin leads to an accumulation of methylmalonic acid (MMA) and homocysteine (Hcy) in plasma. Plasma Hcy concentration is elevated, in over 90% of patients with either vitamin B_{12} deficiency or clinical folate deficiency. As a consequence, Hcy concentration is correlated with MMA concentration [21;22]. In presence of kidney problems (most easily diagnosed with raised serum creatinine concentrations) plasma MMA and Hcy concentrations are elevated [23]. Plasma Hcy concentrations are different between men and women [23], viz. higher in men. Accordingly, Klee strongly advises to incorporate the assessment of methylmalonic acid as the first choice when evaluating the vitamin B_{12} status [21]. Overall, MMA and Hcy are good metabolic indicators of vitamin B_{12} deficiency at tissue level.

Consequences of vitamin B_{12} deficiency

Vitamin B_{12} deficiency is associated with gastrointestinal, hematological, neurologic and psychiatric manifestations. Several physiological disorders can take place, such as megaloblastic anemia, growth retardation, psychomotor retardation, neural tube effects, neurologic problems (cognitive impairment), cardiovascular diseases, or abnormalities in bone marrow or in the small intestine [22]. Gastrointestinal symptoms of vitamin B_{12} deficiency are expressed as sore tongue, appetite loss, constipation or diarrhea [24]. Elevated levels of Hcy and MMA may explain neurologic manifestations or vascular diseases.

Treatment of vitamin B_{12} deficiency

Although functional vitamin B_{12} deficiency may cause irreversible neurologic damage [20;25-27], biochemical vitamin B_{12} deficiency is well treatable. Parenteral injections with high doses of vitamin B_{12}, first weekly and then monthly, have shown to be effective in normalizing the vitamin B_{12} status. Parenteral injections can, however, be painful and difficult to provide in patients who have a tendency to bleed or who are very thin. Next to that, parenteral injections are costly if given by health professionals [28-30]. For this reason studies with oral supplementation of vitamin B_{12} are being conducted and found to be a good alternative for parenteral injections. Although the optimal dose and duration of intervention still needs to be defined, several studies with a high dose of oral cyanocobalamin supplementation were effective to improve the vitamin B_{12} status in elderly people [31-38].

Elderly people often use a high number of medicines for treatment of several diseases or symptoms. For many elderly people it is a burden to take these medicines everyday [39]. Therefore it would be helpful if nutritional supplements could be added to food, i.e. functional foods.

Before unfolding the thoughts and theories behind the association of vitamin B_{12} status with bone health, first some essentials of the latter are described.

DEFINITION OF OSTEOPOROSIS AND ITS CONSEQUENCES

Osteoporosis is a multifactorial disease and defined as "a systemic skeletal disease characterized by low bone mass and microarchitectural deterioration of bone tissue, with a consequent increase in bone fragility and susceptibility to fractures" [40]. Osteoporotic fractures create a major burden to public health. Fractures are associated with increased morbidity and mortality, impaired quality of life and high costs for society [41-45]. It is estimated that annually in The Netherlands more than 15.000 people of 65 years and older are admitted to hospital because of a hip fracture [46]. The incremental cost for a Dutch person with a hip fracture is estimated to be € 10.000 in the first year after a hip fracture compared to a matched counterpart without fracture [43].

Indicators of bone health

In this thesis, we focus on bone health, and not only on osteoporosis which occurs mainly in elderly people. Several approaches can be used to describe or to assess bone health. Hence we describe the most important techniques and assessments of bone health that we used in this thesis.

Bone mineral density & bone mineral content

Noninvasive techniques have been developed, each with its own characteristics. They are based on the attenuation of ionizing radiation when passing through the body. Techniques used in this thesis to quantify bone mineral density (BMD) and bone mineral content (BMC) are dual-energy X-ray absorptiometry (DEXA) and quantitative ultrasound (QUS).

BMD data are used more often than BMC data in epidemiological studies and have been found useful to predict future fracture risk [47]. BMD reflects the BMC status. BMD is an areal density measurement (g/cm2) derived by dividing BMC (g) by the scanned bone area. Therefore BMD is not a measure of true density because this method does not provide information about the depth of bone in the scan path nor does it distinguish between osseous and nonosseous areas within the bone envelope [48]. Three-dimensional information is needed for factual data of BMD. The size adjustments for BMD are determined by using predefined indexes - in fact mainly by height, which gives one-dimensional information. These adjustments may fail to correct BMD fully for bone and body size. Therefore it would be better to use BMC data [48].

The World Health Organisation (WHO) has given four general categories based on DEXA-measurements and are actually only applicable to women to diagnose osteoporosis [49]:

Normal. A value for BMD or bone mineral content (BMC) within 1 SD of the young adult reference mean.

Low bone mass (osteopenia). A value for BMD or BMC more than 1 SD below the young adult mean but less than 2.5 SD below this value.

Osteoporosis. A value for BMD or BMC 2.5 SD or more below the young adult mean.

Severe osteoporosis (established osteoporosis). A value for BMD or BMC more than 2.5 SD below the young adult mean in the presence of one or more fragility fractures.

Quantitative ultrasound is a mechanical vibration which passes through bone. This gives quantitative information on mechanical properties of cortical and trabecular bone which

in turn are important determinants of whole bone stiffness, bone strength and fracture risk [50-52].

Bone turnover markers

Bone metabolism is a dynamic and continuous remodeling process that is normally maintained in a tightly coupled balance between resorption of old or injured bone and formation of new bone. Bone remodeling, also called bone turnover, can be measured in blood or urine and falls into three categories: a) bone formation markers: enzymes or proteins that are secreted by cells involved in the remodeling process, b) bone resorption markers: breakdown products generated in the resorption of old bone, and c) byproducts produced during the synthesis of new bone (Delmas *et al.* [53] and Watts [54]).

The most important markers for bone formation are alkaline phosphatase (ALP) and osteocalcin and are determined in blood. Osteoblasts (bone-forming cells) are rich in ALP. The latter is suggested to play an important role in the mineralization of newly formed bone. Osteocalcin is the major noncollagen protein of bone matrix. It is involved in bone remodeling via a negative feedback mechanism and it is considered to be a specific marker of osteoblast function as its levels correlate with bone formation rates.

Most biochemical markers of bone resorption are degradation products of bone collagen. The hydroxypyridinium crosslinks of collagen, pyridinoline (PYR) and deoxypyridinoline (DPD), are formed during the extracellular maturation of fibrillar collagens and are released upon the degradation of mature collagens. Both markers are measured in urine.

Causes, prevention and treatment of osteoporosis

Biological, environmental and genetic factors as well as lifestyle factors play an important role in the onset and development of osteoporosis. Twin and family studies suggest that 60% to 80% of the variance in peak bone mass is determined by genetic factors [55]. Many gene polymorphisms have been proposed in a review to be involved in bone variation [56] such as the gene polymorphism of vitamin D receptor (VDR) [57][58], collagen 1α1 (COL1A1) [59;60] and estrogen receptor (ER) [61;62].

Age is another important predictor of bone health. Bone loss commences around the age of forty years and is on average between 0.5% and 1% per year in men, and in women the rate of bone loss accelerates around the menopause to about 2% per year [63].

Prevention and treatment of osteoporosis are directed towards nutrition and lifestyle factors or medical intervention. Most medical research concentrates on hormone replacement therapy (HRT), which has a consistent, favorable effect on bone density. It

decreases bone resorption and would thus prevent postmenopausal bone loss. Regrettably, estrogen therapeutic treatments have disadvantages such as an increased risk of breast and endometrial cancer [64]. Bisphosphonates, such as risedronate, alendronate and ibandronate, are thought to have a great antiresorptive activity and to decrease fracture risk. Adverse effects are mostly esophageal and gastrointestinal side effects, but these appear to occur occasionally [65]. Raloxifene treatment, a nonhormonal agent that binds to the estrogen receptor and that exhibits estrogen-agonist effects on bone, increases bone density and is suggested to have a positive impact on vertebral fractures. Adverse effects, such as hot flashes, thrombosis and influenza, may cause discontinuation of the treatment [66]. Moreover, most of these medicines should be taken for many years and according to strict rules [67]. After discontinuation of treatment, bone loss may resume again [68]. A promising intervention in the prevention of hip fractures is the hip protector. Due to low compliance, the effect of hip protectors is low and therefore it remains questionable whether hip protectors are useful [69].

Although a low percentage (20%-40%) of the bone mass can be explained by nutritional and lifestyle factors, they still can have a large impact on bone health. Here, we portray the role of nutrition. Extensive reviews on lifestyle factors, such as physical activity and smoking have been given among others by New [70] and Law & Hackshaw [71].

Dietary therapy of vitamin D and calcium and especially the combination of these two is promising as most of the times it shows beneficial effects on BMC, BMD, bone turnover and a reduced risk on fractures without serious adverse side effects [70;72;73]. Vitamin D has also a beneficial effect on the risk of falling [74], which can be explained by the active vitamin D metabolite which binds to a highly specific nuclear receptor in muscle tissue, leading to improved muscle function [75;76].

It is important to consider both vitamin D supply and calcium intake in order to evaluate the need of either vitamin D or calcium therapy or the combination of the two. A low calcium intake increases PTH secretion and consequently increases serum 1,25-dihydroxyvitamin D and thus leads to an increased catabolism of 25-hydroxyvitamin D (25(OH)D), thereby decreasing 25(OH)D and inducing or aggravating vitamin D deficiency. The reverse may also be true: a high calcium intake may suppress serum PTH and thus may decrease bone turnover and bone loss. Estimations of the threshold value of 25(OH)D deficiency vary between 30 and 100 nmol/L. This may be due to laboratory differences, but also due to the different dietary calcium intakes across the countries. Vitamin D requirements may be higher in countries where calcium intake is low [77;78].

Evidence for associations between protein intake and bone mass or fractures rates is diverse and includes positive, negative and no associations [79-83]. Dietary protein

increases renal calcium excretion and increases circulating levels of insulin-like growth factor-I (IGF-I). This growth factor is thought to play an important role in bone formation. Theoretically, to optimize the impact of dietary protein on the skeleton, one would want to minimize the impact of protein on renal calcium losses but not impair its impact on promoting the production of IGF-I. This has been nicely shown in a trial with effective calcium and vitamin D supplementation and where higher protein intake was associated with favorable changes in bone mineral density [84]. Yet, an optimum protein intake has to be defined.

Other dietary factors, such as vitamin K, vitamin A, caffeine and fluoride are subjects of current bone research as well and are under debate whether important and truly associated with bone health [85-92].

VITAMIN B_{12}: A NOVEL INDICATOR OF BONE HEALTH?

Vitamin B_{12} and homocysteine status have been suggested to play a role in osteoporosis. Although little seems to be known in this area, evidence for an association between vitamin B_{12} and bone emerges from *in vitro* studies. In the first *in vitro* study of Carmel *et al.* [93] it was shown that the alkaline phosphatase content in calvarial cells from chicken embryos was not only cobalamin-dependent, but also dose-dependent. In another *in vitro* study, experiments were elaborated and again it was shown that vitamin B_{12} had a stimulating influence on osteoblast proliferation and alkaline phosphatase activity in human bone marrow stromal osteoprogenitor cells and in rat osteoblastic osteosarcoma cells [94]. Osteoblast proliferation was already stimulated at a low concentration of vitamin B_{12}; higher concentrations did not further increase proliferation. Therefore, it can be speculated that vitamin B_{12} at a minimum concentration is required to exert a permissive effect on osteoblast proliferation.

A few human studies have described an association of vitamin B_{12} with BMC and BMD in humans. A study of Van Dommelen & Klaassen, performed in 1964, reported that serum levels of total alkaline phosphatase were lower in patients with cobalamin deficiency than in matched controls, and the alkaline phosphatase values rose when cobalamin was replenished [95]. Similar observations were present in patients with pernicious anemia. The severity of the megaloblastic anemia seemed to affect serum levels of skeletal alkaline phosphatase. Serum osteocalcin levels and skeletal alkaline phosphatase rose in most patients after cobalamin therapy. These levels remained unchanged in the control group [93].

Studies on the effects of a vegetarian diet on bone health are numerous but there is no consensus yet on the benefits or drawbacks of a vegetarian diet. Early studies showed that bone mineral mass was higher in the vegetarian group compared with their omnivorous counterparts [96;97]. These positive findings can be most probably subscribed to the different lifestyle of the vegetarians (no smoking and higher physical activity levels) compared to nonvegetarians. Postmenopausal vegetarians have a higher risk of osteopenia [98] or a lower bone mineral density [99] than their omnivorous counterparts. Parsons *et al.* showed that macrobiotically-fed children had a lower bone mineral content than control subjects, although these differences were not reflected by differences in bone turnover markers in the two groups [100;101]. Vitamin B_{12} intake and total body fat predicted BMD with vegetarian women in another study [102].

In a prospective study with a follow-up time of two years, low vitamin B_{12} levels were associated with increased hip bone loss in elderly women [103]. Goerss *et al.* [104] found a two-fold higher risk on osteoporotic fractures in subjects with pernicious anemia in a retrospective cohort study.

Up till now no well-designed randomized clinical trials have been conducted in order to investigate the effect of vitamin B_{12} replacement on bone health. One case study reported the reversal of severe osteoporosis with vitamin $B_{12,}$ etidronate, calcium and vitamin D therapy in a patient with pernicious anemia. This report could not ascribe the dramatic increase of bone mineral density to etidronate treatment alone. In another intervention study, without a placebo group, vitamin B_{12} injections were given to pernicious, osteoporotic Dutch patients (n=15). Alkaline phosphatase, osteocalcin and carboxy terminal propeptide of type I collagen (PICP) concentrations were increased and reflected a stimulation of osteoblastic activity [105]. A positive effect on BMD of spine and hip was also observed. Yet, it is difficult to judge this study as no control group was included and calcium supplements were introduced after four weeks of vitamin B_{12} supplementation. Still, increases in bone markers were observed already in the first weeks of vitamin B_{12} treatment.

It might be that this mechanism is interrelated with another explanation of the mechanism underlying the association of Hcy with bone as suggested by McKusick [106]: Hcy might interfere with collagen cross-linking. In a study with homocystinuria patients it was shown that these patient had lower amounts of collagen cross-links in serum than normal controls [107]. Interference in cross-link formation would result in an altered bone matrix resulting in more fragile bone.

RATIONALE AND OUTLINE OF THIS THESIS

The aim of the studies described in this thesis is to further investigate the association of vitamin B_{12} and homocysteine status with outcome measures of bone health. Furthermore we assessed whether foods fortified with vitamin B_{12} are as effective as vitamin B_{12} capsules to improve the vitamin B_{12} status in vitamin B_{12} deficient elderly people.

Chapter 2 explores whether associations of vitamin B_{12} and MMA status with bone mineral density and bone mineral content are present in children who followed a macrobiotic diet or an omnivorous diet.

In **Chapter 3** the association between the vitamin B_{12} status and bone mineral content and bone mineral density will be evaluated in frail elderly people.

Chapter 4 elaborates on the association between the homocysteine status and fractures in elderly people in two large prospective studies: i.e. the Longitudinal Aging Study Amsterdam (LASA) and the Rotterdam Elderly study.

Chapter 5 gives more information on the association of the vitamin B_{12} status and homocysteine status with fractures in the LASA study, but it also reveals on the association of the biochemical parameters with broadband ultrasound attenuation and bone turnover markers in the LASA study.

Chapter 6 evaluates the effectiveness of vitamin B_{12} fortified milk supplements and vitamin B_{12} capsules in vitamin B_{12} deficient elderly people.

In the General Discussion (Chapter 7) the main findings of our studies are summarized, a reflection on methodological considerations, underlying mechanisms and future directions will be given.

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Low bone mineral density and bone mineral content are associated with low cobalamin status in adolescents

2

Rosalie AM Dhonukshe-Rutten[1], Marijke van Dusseldorp[2], Jörn Schneede[3], Lisette CPGM de Groot[1], Wija A van Staveren[1]

[1]Division of Human Nutrition, Wageningen University, Wageningen, The Netherlands; [2]Food and Chemical Risk Analysis, TNO Nutrition and Food Research, Zeist, The Netherlands; [3]Department of Clinical Chemistry, University of Umeå, Umeå, Sweden

European Journal of Nutrition 2004 Aug 30; Epub ahead of print

ABSTRACT

Background Cobalamin deficiency is prevalent in vegetarians and has been associated with increased risk of osteoporosis.

Aim of the study To examine the association between cobalamin status and bone mineral density in adolescents formerly fed a macrobiotic diet and in their counterparts.

Methods In this cross-sectional study bone mineral density (BMD) and bone mineral content (BMC) were determined by DEXA in 73 adolescents (9-15 y) who were fed a macrobiotic diet up to the age of 6 years followed by a lacto-(-ovo-)vegetarian or omnivorous diet. Data from 94 adolescents having consumed an omnivorous diet throughout their lives were used as controls. Serum concentrations of cobalamin, methylmalonic acid (MMA) and homocysteine were measured and calcium intake was assessed by questionnaire. Analysis of covariance (MANCOVA) was performed to calculate adjusted means for vitamin B_{12} and MMA for low and normal BMC and BMD groups.

Results Serum cobalamin concentrations were significantly lower (geometric mean (GM) 246 pmol/L vs. 469 pmol/L) and MMA concentrations were significantly higher (GM 0.27 μmol/L vs. 0.16 μmol/L) in the formerly macrobiotic-fed adolescents compared to their counterparts. In the total study population, after adjusting for height, weight, bone area, percent lean body mass, age, puberty and calcium intake, serum MMA was significantly higher in subjects with a low BMD ($p=0.0003$) than in subjects with a normal BMD. Vitamin B_{12} was significantly lower in the group with low BMD ($p=0.0035$) or BMC ($p=0.0038$) than in the group with normal BMD or BMC. When analyses were restricted to the group of formerly macrobiotic-fed adolescents, MMA concentration remained higher in the low BMD group compared to the normal BMD group.

Conclusions In adolescents, signs of an impaired cobalamin status, as judged by elevated concentrations of methylmalonic acid, were associated with low BMD. This was especially true in adolescents fed a macrobiotic diet during the first years of life, where cobalamin deficiency was more prominent.

INTRODUCTION

Cobalamin deficiency has been suggested to affect bone metabolism [1]. Likewise, pernicious anemia, an autoimmune disorder resulting in cobalamin deficiency, has been identified as a risk factor for osteoporosis [2;3]. Furthermore, a case report demonstrated that a patient with pernicious anemia and osteoporosis had a marked improvement of bone mineral density after combined cobalamin and etidronate therapy [4]. A high urinary methylmalonic acid (MMA) concentration, a metabolic marker of functional cobalamin deficiency, was observed in 55% of the children of a small macrobiotic community [5]. Similarly, low cobalamin concentrations were observed in 26% of a vegetarian group and in 78% of a vegan group [6]. It is conceivable that cobalamin deficiency in these cases may contribute to the risk of reduced bone mass in subjects following these diets, although data about this relation are scarce [7].

Since 1985, we have been studying the nutritional status of Dutch children consuming a macrobiotic diet. A macrobiotic diet consists mainly of cereals, pulses and vegetables, and occasionally some fish. The consumption of meat, dairy products and vitamin D supplements is normally avoided. In a previous study, our group reported that infants who followed a macrobiotic diet had very low dietary intakes of cobalamin and calcium [8]. In addition, plasma cobalamin concentrations were low [9] and many infants had a marked vitamin D deficiency [10].

A follow-up study was carried out in 1995 and included most of the formerly investigated macrobiotic children. We here report on these children, at that time young adolescents (9-15 y), and investigated the potential influence of a macrobiotic diet in early childhood on bone mass in later life. After the age of six years, most of these subjects had switched to a lacto-(-ovo-)vegetarian or even omnivorous diet at the advice of the former investigators. Hereafter, we refer to these adolescents, who previously received a macrobiotic diet, as 'the macrobiotic group'. The macrobiotic group had a 3-8% lower bone mass as compared to age-matched controls who had consumed the omnivorous diet throughout their lives [11].

In an earlier report we found that a substantial portion of the macrobiotic adolescents still had signs of an impaired cobalamin status [12] as indicated by their low serum concentrations of cobalamin and/or serum elevated concentrations of methylmalonic acid (MMA), a sensitive and specific marker of functional cobalamin deficiency [13]. Our findings of a higher prevalence of osteoporosis in elderly women with a low cobalamin status compared to those with a normal cobalamin status [14] and the high prevalence of an inadequate cobalamin status among the macrobiotic group motivated us to test the

hypothesis whether the low BMD frequently found in adolescents previously fed a macrobiotic diet was associated with signs of impaired cobalamin status.

MATERIALS AND METHODS

Subjects and design

The subjects included in this study have been characterized in detail previously [11]. In summary, bone mass was measured in 195 adolescents aged 9-15 y between May and July 1995. The macrobiotic adolescents were recruited from an existing group of macrobiotic families affiliated with the Division of Human Nutrition, Wageningen University. Ninety-three adolescents (50 boys and 43 girls), born from macrobiotic mothers and receiving a macrobiotic diet from birth till about the age of 6 years, were included in the study and are referred to as the "macrobiotic group or macrobiotic subjects". The control group consisted of 102 adolescents (42 boys and 60 girls) aged between 9 and 15 years, having consumed an omnivorous diet throughout their lives. We excluded twenty-five subjects from the present study as they either did not give their consent to blood sampling or the amount of serum obtained from them was insufficient for the biochemical analysis of cobalamin status parameters. Thus, complete data of 170 adolescents (76 macrobiotic subjects and 94 controls) were available for further statistical analyses. All subjects were Caucasian, in good health and without any medication known to affect bone or calcium metabolism. Socio-economic status was determined by the Attwood scores, a five-point scale based on occupation and highest level of education attained by both parents. The external Medical Ethical Committee of the Division of Human Nutrition and Epidemiology of Wageningen University approved the study and written informed consent was obtained from the subjects and their parents.

Anthropometry

Subjects were weighed (in underwear) to the nearest 0.1 kg using a digital scale (ED-60T, Berkel, Rotterdam, The Netherlands). Standing height was measured (without shoes) to the nearest 0.1 cm using a microtoise. Pubertal stage was determined by one investigator, according to the Tanner method, using the development of breast in girls and that of pubic hair in boys [15]. One subject refused observers' assessment and therefore a self-assessment value was used.

Lean body mass and fat mass were determined using a dual energy X-ray absorptiometer (DEXA, model DPX-L, Lunar Radiation Corp., Madison, WI) with software version 1.31.

All the measurements were obtained from the total body DEXA scan. In data analysis percentage lean body mass ([non-bone lean body mass/body weight]*100) was used as a measure of body composition in data analysis.

Bone measurements

Bone mineral content (BMC, g), bone area (BA, cm^2) and resulting bone mineral density (BMD, g/cm^2) were determined using DEXA. A spine phantom, provided by the manufacturer, was scanned weekly throughout the study period and gave coefficients of variation of 0.65% for L1-L4 BMC and 0.73% for L1-L4 area bone density (BMD, g/cm^2). In vivo precision was assessed using repeated scans of six adults, which gave coefficients of reproducibility for BMD ranging from 0.6% (for the total body) to 3.2% (for the trochanter).

Biochemical measurements

Non-fasting blood specimens were taken (90% taken from mid May-mid June 1995, followed by other 10% in the subsequent month). Samples were allowed to clot and were then centrifuged (1190 x g for 10 min at 4°C). Serum was separated from blood cells within 60 min and separated serum samples were stored at -80°C till further analysis. Serum concentrations of cobalamin and folate were measured by using a microparticle-based enzyme immunoassay (IMx system; Abbott Laboratories, North Chicago, IL). The coefficient of variation (CV) for intra- and interassay of cobalamin were below 5%. The intra-assay CVs of the folate assay remained between 3% and 6% and the interassay CVs ranged between 6% and 10% depending on the folate concentration. MMA was measured by capillary electrophoresis, with intra- and interassay coefficients of variation below 12% at low physiological concentrations [16]. Concentrations of total homocysteine (tHcy) were assayed by a method based on HPLC and fluorescence detection, with a between-day CV < 4% [17]. 1,25-dihydroxyvitamin D ($1,25(OH)_2D$) was measured by immunoassay (IDS, Bolden Business Park, Bolden, UK). Intra- and interassay coefficients of variation were 5-8% and 9% respectively.

Lifestyle parameters

Current calcium intake (mg/d) was estimated using a validated food frequency questionnaire [18], to which several questions were included regarding non-dairy sources of calcium that are present in a macrobiotic diet. The reference period of the questionnaire was one month before, and food intake was estimated in terms of

standardized household portion sizes. Daily calcium intake was computed using the values from the Dutch Food Composition Table released in 1993 [19].

Physical activity was assessed by asking every subject about the time spent on physical activity (sports) during and after the school time. The total number of minutes spent per week on sporting activities was calculated.

Statistical analyses

Means and standard deviations (SD) were calculated for demographic data and lifestyle factors. For serum cobalamin, MMA, tHcy and folate concentrations, geometric means (GM) and 5th and 95th percentiles were calculated from lognormal transformed data because of skewed distributions. Differences in demographic data, lifestyle factors, biochemical and bone composition variables between the macrobiotic and control group (separately for boys and girls) were investigated using the two-sample Student's *t* test.

The 25th percentile of the control subjects was used as cut-off for definition of 'low' or 'normal' total body BMC and 'low' or 'normal' total body BMD. Analysis of covariance (MANCOVA) was performed to calculate adjusted means of vitamin B_{12}, MMA and Hcy for the low and normal BMC and BMD groups. Sex, weight, height, percent lean body mass, age and (stage of) puberty were added as covariates. Calcium intake was added as a covariate to the regression models as well, since it was an independent predictor of total BMD. Bone area was also added to the regression model to calculate the adjusted means for the different BMC groups. Boys (n=76) and girls (n=91) were analyzed together because there was no interaction between the variables of interest and sex. Also no different results between boys and girls were observed when the data were analyzed separately instead of together with sex as a covariate.

To exclude a possible group effect, separate MANCOVA analyses were also performed for the macrobiotic cohort alone.

The investigated associations were corroborated with multiple regression analyses (SAS GLM procedure). Regression models were constructed with (continuous) BMD as the dependent variable; weight, height, percent lean body mass, calcium intake, age and (stage of) puberty (treated as a discrete variable) were added simultaneously as covariates; and serum concentrations of cobalamin and MMA as the (independent) variables of interest. These multiple regression analyses were also used in order to know which variables were needed to adjust for in the MANCOVA analysis.

Data were analyzed using SAS system release 8.0 (SAS Institute Inc., Cary, NC, USA). In all analyses, a probability of 0.05 was considered significant.

Table 1 Characteristics of Dutch adolescents participating in a study on cobalamin status and bone mass[1,2]

	Boys		Girls	
	Macrobiotic	Control	Macrobiotic	Control
n	39	39	37	55
Age (y)	12.7 ± 2.2[a]	11.7 ± 1.5	11.9 ± 1.6	11.7 ± 1.7
Height (m)	1.57 ± 0.14	1.53 ± 0.12	1.51 ± 0.12	1.52 ± 0.11
Weight (kg)	42.7 ± 11.4	41.2 ± 9.2	38.9 ± 9.4	41.3 ± 8.6
% lean body mass	84.4 ± 3.0[c]	79.4 ± 7.5	78.2 ± 5.3[b]	74.2 ± 6.9
Pubertal stage†	2.6 ± 1.4	2.3 ± 1.1	2.2 ± 1.3	2.4 ± 1.2
Physical activity (min/wk)	280 ± 148	279 ± 94	234 ± 165	260 ± 102
Socio-economic status	1.9 ± 0.5[a]	2.3 ± 0.8	2.2 ± 0.7	2.3 ± 0.8
Calcium intake (mg/d)	665 ± 421[c‡]	1056 ± 380	518 ± 321[c‡]	1030 ± 348‡
Cobalamin (pmol/l)*	212 (103-370)[c]	484 (259-813)	286 (115-580)[c]	458 (219-850)
MMA (µmol/l)*	0.30 (0.11-0.94)[c]	0.15 (0.05-0.34)	0.25 (0.09-0.76)[c]	0.17 (0.07-0.28)
Folate (nmol/l)*	18.0 (9.5-26.0)[b]	14.7 (8.4-22.0)	18.7 (10.0-27.0)[c]	14.5 (9.1-26.0)
Total homocysteine (µmol/l)*	8.3 (5.3-15.5)[b]	7.0 (4.2-10.8)	7.6 (4.7-16.7)	7.2 (4.6-14.5)
Vitamin D (pmol/l)	138 ± 40	133 ± 40	152 ± 31	149 ± 44
Total body BMD (g/cm²)	0.97 ± 0.09	0.98 ± 0.06	0.93[a] ± 0.09	0.97 ± 0.08
Total body BMC (g)	1770 ± 554	1681 ± 418	1510 ± 432	1633 ± 395

1 Unless otherwise indicated, values are mean ± SD of raw data

2 Two-sample Student's t test: Significance of difference between macrobiotic and control subjects (separately for boys and girls): [a]p<0.05, [b]p<0.01, [c]p<0.001

* Values are geometric mean (P5-P95); percentiles estimated from lognormal distribution

† Range pubertal stage (1 to 5)

‡ n=34, 34 and 54, respectively for macrobiotic boys and girls, and control girls

RESULTS

Descriptive characteristics, lifestyle parameters, and blood and bone parameters for macrobiotic and control subjects are shown in **Table 1**. Macrobiotic boys were on average one year older than the control boys. Yet, weight was similar for macrobiotic and control adolescents, whereas %lean body mass was significantly higher in macrobiotic adolescents than in the control adolescents. Socio-economic status and physical activity

level were similar for all groups. In both sexes, calcium intake was significantly lower in macrobiotic than in control subjects. Serum cobalamin concentrations were significantly lower, and concentrations of MMA and folate were significantly higher in macrobiotic subjects compared to controls. Total homocysteine concentrations were only significantly higher in macrobiotic boys as compared to controls. Serum 1,25-dihydroxyvitamin D showed no significant differences between the groups.

Table 2 shows that the adjusted mean (SD) of serum vitamin B_{12} concentration for the subjects in the group with low total body BMD was significantly lower (p=0.0035) than the adjusted mean for the subjects in the normal BMD group, 344 (24) pmol/L vs. 442 (18) pmol/L, respectively. After dividing the subjects into two groups of total body BMC, the adjusted means of vitamin B_{12} concentration were again significantly different between the low and normal BMC (p=0.0038). Moreover, the adjusted mean MMA concentration, was higher for the subjects in the low BMD group than in the normal BMD group (p=0.0003). Adjusted mean concentration of MMA was not significantly different between the low and normal BMC groups (p=0.32). Also, no significant differences in Hcy values were found between the low and normal BMD (p=0.25) or BMC (p=0.59) groups (data not shown).

Table 2 Adjusted mean (SD or P5, P95) concentrations of vitamin B_{12} and MMA for different groups of BMD and BMC in omnivorous- and previously macrobiotic-fed Dutch adolescents (n=161) and separately for previously macrobiotic-fed Dutch

	Low BMD	Normal BMD	Low BMC	Normal BMC
All subjects, n	**60**	**101**	**50**	**111**
Vitamin B_{12} (pmol/L)	344 (24)*	442 (18)	323 (31)*	442 (18)
MMA (µmol/L)	0.31 (0.26, 0.35)*	0.20 (0.16, 0.23)	0.26 (0.21, 0.32)	0.22 (0.19, 0.26)
Only macrobiotic-fed adolescents, n	**36**	**32**	**28**	**40**
Vitamin B_{12} (pmol/L)	269 (23)	279 (25)	286 (32)	266 (24)
MMA (µmol/L)	0.40 (0.32, 0.49)*	0.22 (0.13, 0.32)	0.26 (0.14, 0.39)	0.35 (0.26, 0.45)

[1] Adjusted for: bone area (only in BMD groups), weight, height, percent lean body mass, age, puberty, sex, total physical activity and calcium intake.

* The concentration of vitamin B_{12} or MMA is statistically different between the low and normal BMC or BMD ($p < 0.05$).

Table 2 also shows the adjusted means of vitamin B_{12} and MMA for the macrobiotic group only. The mean (P5, P95) MMA concentrations were significantly different ($p = 0.0148$) between the low and normal total body BMD, 0.40 (0.32, 0.49) and 0.22 (0.13, 0.31) μmol/L, respectively.

Results of multiple regression analyses demonstrated that serum MMA was inversely associated with BMD at total body (β=-0.018, p=0.02), after adjusting for weight, height, %LBM, age, puberty, calcium intake and sex. No significant associations of serum cobalamin or tHcy with bone mineral density were found, but there was a tendency of a cobalamin association with total body BMD (β=0.018, p=0.06) as was the case for MMA.

In the separate regression analysis of the macrobiotic adolescents a significant inverse relation was observed between serum MMA and total body BMD (β = -0.025, p=0.04), after adjusting for weight, height, %LBM, age, puberty, calcium intake and sex. No associations between cobalamin or tHcy with BMD were found.

Similar results were found for the association between MMA and total body BMC (β=-0.017, p=0.02) and also between vitamin B_{12} and total body BMC (β=0.016, p=0.06).

DISCUSSION

The present study shows that serum cobalamin concentrations were significantly lower and MMA concentrations significantly higher in previously macrobiotic-fed adolescents as compared to the controls. Moreover, irrespective of the dietary group, adjusted vitamin B_{12} concentrations were significantly lower in subjects of the low BMD or BMC group compared to subjects of the normal BMD or BMC group. Adjusted MMA concentrations were significantly higher in the low BMD group than in the normal BMD group.

Most studies only take the bone mineral density into account when investigating bone health or osteoporosis. Analyzing BMC as a dependent variable is preferred above the use of bone mineral density (BMD, g/cm^2) or bone mineral area density (BMAD, g/cm^3) by some groups because no assumptions are made about the relationships between BMC and BA, and potential size-related artefacts are avoided [20]. The similarity in associations found between vitamin B_{12} or MMA with BMC, strengthen our findings of the associations with BMD.

The association between MMA and total body BMD remained present in the separate analysis of macrobiotic subjects (boys and girls), whereas this association was not found in control subjects. This leads to the suggestion that the observed association was confined to the vitamin B_{12} status and not concomitant to other characteristics of the

macrobiotic group. We assume that other dietary and lifestyle factors were similar for all macrobiotic-fed adolescents. This implies that these and other (unknown) factors that may be different between macrobiotic-fed adolescents and control subjects cannot disturb the association between MMA and BMD. The association between MMA and BMD in the total study group is mostly driven by the macrobiotic subjects in whom impaired cobalamin status was prevalent. Our results with respect to cobalamin status and bone mass are in line with earlier published data [2;3;7].

Remarkably, the concentrations of vitamin B_{12} and MMA in the separate analysis of macrobiotic subjects were somewhat more favorable in the low BMC group than in the normal BMC group. The nonexpected reversed concentrations of vitamin B_{12} and MMA in the low and normal macrobiotic groups were probably only found coincidentally due to the low number of subjects, and furthermore, these concentrations were not significantly different from each other.

We have previously reported [12] that a substantial number of the formerly strict macrobiotic-fed adolescents still showed signs of an inadequate cobalamin status, even after they had changed their diet. About 41% of the macrobiotic subjects had cobalamin values below the 5th percentile of the control group (<229 pmol/l). Similarly, 41% of the macrobiotic subjects had serum MMA concentrations above the 95th percentile of the control group (>0.29 μmol/l). The inadequate cobalamin status is most likely the result of a low present dietary cobalamin intake in combination with low body stores of cobalamin in general as a result of long-term insufficient cobalamin supply during pregnancy and childhood. The macrobiotic diet is known to contain only very small amounts of cobalamin. Although our formerly strict macrobiotic-fed subjects had changed their diet at an average age of 6 years, the amounts of cobalamin supplied by the modified diet were obviously not sufficient to restore a normal cobalamin status in later life.

To keep sufficient power we did not perform gender-specific analyses of our data. The serum cobalamin concentrations were higher in girls than in boys, which could explain why elevated MMA concentrations were less common in girls than in boys. Serum MMA is assumed to be a better functional indicator of cobalamin status than serum cobalamin concentration itself [21], which may explain the lack of associations between BMD and serum cobalamin in our data. However, a larger sample size would have most likely given significant associations.

As opposed to MMA, not only vitamin B_{12}, but several B-vitamin deficiency states and a great number of confounders may influence homocysteine concentrations [22]. The vegetarian diet is rich in folate and low in methionine. We have also observed higher serum folate concentrations in the macrobiotic-fed adolescents than in the controls.

These factors may obscure potentially existing interactions between homocysteine and low cobalamin intake. Although there is a strong correlation between homocysteine and cobalamin concentrations [23], homocysteine status may be a less strong functional indicator of cobalamin status in vegetarians, as the homocysteine status is influenced by the intake of folic acid and methionine. Thus, an association of homocysteine status with bone mass may be less likely found in people following a vegetarian diet.

We did not observe an association between vitamin D status and BMD. This may be due to similar vitamin D concentrations for macrobiotics and controls and because the production of 1,25-dihydroxyvitamin D is under tight feedback control [24;25]. Therefore, 25-hydroxyvitamin D would have been a more reliable indicator of vitamin D status. Also, in contrast to cobalamin status, it is possibly easier to restore vitamin D status. In our first study of these macrobiotic children we frequently observed vitamin D deficiency at the age of 6-18 mo [26]. This vitamin D deficiency could have had a negative influence on BMC and BMD in earlier childhood and it is possible that the catch-up growth of BMC and BMD is slower than that of the vitamin D status.

The mechanism of cobalamin-dependent changes in bone mass has been investigated in 1964 by Van Dommelen and Klaassen [27] and by Carmel et al [1]. Van Dommelen and Klaassen reported that serum concentrations of total alkaline phosphatase were lower in patients with cobalamin deficiency than in matched controls; these values rose after cobalamin replenishment. Carmel et al [1] demonstrated that serum concentrations of skeletal alkaline phosphatase and osteocalcin were decreased in cobalamin-deficient patients and returned to normal after therapy with vitamin B_{12}. Osteocalcine is a marker of osteoblast activity and alkaline phosphatase is a marker for bone formation. Furthermore, *in vitro* studies of calvarial cells from chicken embryos showed that the alkaline phosphatase content was cobalamin-dependent [1]. These findings indicate that osteoblast function depends on an adequate supply of cobalamin.

The strengths of our study are the use of a combination of two biochemical markers (cobalamin and MMA) to measure cobalamin status, and the adjustment for important covariates like height, weight, percent lean body mass, age, puberty and calcium intake. In addition, a separate analysis was carried out for the macrobiotic group alone to exclude influence of other group effects. This implies that other dietary and lifestyle factors that can differ between macrobiotic and control subjects, such as vitamin D status, fiber intake, and physical activity, are unlikely to have confounded the association between cobalamin status and bone mass.

In conclusion, our data suggest that signs of low functional cobalamin status are associated with low bone mineral density especially in adolescents who were fed a strict

macrobiotic diet during the first years of life. These findings do not give a clarification on cause and consequence of the low vitamin B_{12} status and low BMD and BMC. A causal relation may nevertheless exist between vitamin B_{12} status and bone health in these adolescents, as the low vitamin B_{12} status is by and large a prolonged condition and might consequently be a causal factor for low bone health. Further research is strongly advocated to study whether these results can be extrapolated to the elderly population. We have recently shown an association of vitamin B_{12} status with bone mineral content and bone mineral density in frail elderly women [14] and an association between homocysteine status with fractures in a Dutch population [28] which was corroborated in an American study [29]. It is important to study the causality of these associations.

ACKNOWLEDGEMENTS

This work was supported by the Dutch Prevention Fund/Netherlands Organization for Health Research and Development (grant no: 28-1052-1/2) and by the Dutch Dairy Foundation for Nutrition and Health.

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Vitamin B_{12} status is associated with bone mineral content and bone mineral density in frail elderly women but not in men

3

Rosalie AM Dhonukshe-Rutten[1], Martine Lips[1], Nynke de Jong[1], Marijke JM Chin A Paw[1], Gerrit J Hiddink[2], Marijke van Dusseldorp[3], Lisette CPGM de Groot[1] and Wija A van Staveren[1]

[1]Division of Human Nutrition and Epidemiology, Wageningen University, Wageningen; [2]Dutch Dairy Foundation for Nutrition and Health, Utrecht; [3]TNO Food and Nutrition Research, Zeist
All in the Netherlands

Journal of Nutrition 2003; 133:801-807

ABSTRACT

Background Subclinical vitamin B_{12} deficiency is common in the elderly. Encouraged by early indications, we investigated the plasma vitamin B_{12} status in association with bone mineral content (BMC) and bone mineral density (BMD) in frail elderly people.

Materials and Methods Data of 194 free-living Dutch frail elderly (143 women and 51 men) were available. BMC and BMD were measured by dual energy X-ray analysis. Biochemical analyses were performed on plasma or serum including vitamin B_{12}, methylmalonic acid, homocysteine, 25-hydroxy vitamin D and parathyroid hormone.

Results Women had higher plasma vitamin B_{12} (288 and 238 pmol/L, respectively) and lower plasma homocysteine levels (15.8 and 21.3 μmol/L, respectively) than men. Of the total explained variance of BMC and BMD in women (46 and 22%, respectively), 1.3–3.1% was explained by plasma vitamin B_{12}, in addition to weight and height or energy intake. In men, the variance of BMC and BMD was explained by weight, smoking and/or height (total R^2 was 53 and 25%, respectively), but not by plasma vitamin B_{12}. Osteoporosis occurred more often among women whose vitamin B_{12} status was considered marginal or deficient than in women with a normal status, i.e., the prevalence odds ratios (after adjustment for weight, age and calcium intake) (95% confidence intervals) were 4.5 (0.8;24.8) and 6.9 (1.2;39.4), respectively.

Discussion These results suggest that vitamin B_{12} status is associated with bone health in elderly women. Future studies on bone health should take into account a possible role of vitamin B_{12} status in different populations.

INTRODUCTION

Osteoporosis is a major problem in our contemporary society. Osteoporosis increases morbidity and dependence, which in turn decrease the quality of life and create a burden on health care costs [1]. This problem will increase with the growing number of elderly people and will result in a higher number of osteoporotic fractures [2].

Most research on the treatment of osteoporosis is focused on hormone replacement therapy (HRT), which decreases bone resorption and should prevent postmenopausal bone loss. Unfortunately, estrogen therapeutic treatments have disadvantages such as an increased risk of breast and endometrial cancer [3]. By contrast, dietary therapy of vitamin D and calcium is promising because it appears to have beneficial effects on bone mineral content (BMC) and bone mineral density (BMD) without serious adverse side effects [4;5].

Several nutrients positively influence bone-forming cells. Calcium and phosphorus mineralize the matrix formed by osteoblasts. Previous studies in which elderly people received a calcium supplement showed a reduced bone loss. This effect was especially observed in persons with low calcium intake [6]. The combination of vitamin D and calcium causes a greater decrease in the function of the parathyroid and bone turnover and induces a higher gain of bone mineral density. This in turn decreases the incidence of hip fractures and other nonvertebral fractures in high risk populations [7]

It is hypothesized that, in addition to calcium and vitamin D, vitamin B_{12} also influences BMC and BMD. In vitro studies have demonstrated that vitamin B_{12} has a significant effect on osteoblast proliferation. Also alkaline phosphatase activity was increased in osteoblastic cells after stimulation with vitamin B_{12}. A minimum concentration of vitamin B_{12} may be necessary for a positive effect on osteoblast proliferation [8]. A few human studies have described an association of vitamin B_{12} with BMC and BMD in humans. A study of Carmel *et al.* [9] reported that alkaline phosphatase and osteocalcin rose significantly in vitamin B_{12}–deficient patients after treatment with vitamin B_{12}, whereas they remained unchanged in the control group. Parsons *et al.* [10] found a lower bone mass in Dutch macrobiotic-fed adolescents than in control subjects. A macrobiotic diet could induce vitamin B_{12} deficiency [11;12]. Goerss *et al.* [13] described an increased risk of osteoporotic fractures in subjects with pernicious anemia; in another study with vegetarian women, BMD was predicted by vitamin B_{12} intake and total body fat [14]. These studies were conducted in subjects with specific health conditions.

Because subclinical vitamin B_{12} deficiency is common in elderly people, it may play a role in bone health. Therefore, we investigated vitamin B_{12} status in relation to BMD and BMC in a group of frail elderly people. If vitamin B_{12} is related to bone health, then a

decrease in the prevalence of vitamin B_{12} deficiency may help to prevent osteoporosis in elderly people.

SUBJECTS AND METHODS

Study population

Data were derived from a previous study in which frail elderly people were recruited to participate in a 17-wk trial to study the effects of nutrient-dense foods and physical exercise on nutritional and health status [15]. The study population consisted of 217 free-living Dutch frail elderly people. Frailty in this study was defined according to the following criteria: requirement of health care services (such as home care or meals-on-wheels), no regular exercise, body mass index (BMI) ≤25 kg/m^2 or recent weight loss. Other characteristics of the study population were age ≥70 y; no use of multivitamin supplements and ability to understand the study procedures. If participants used a single supplement they were allowed to participate.

Complete data collected at baseline were available for 194 subjects. The research protocol was approved by the external Medical Ethical Committee of the Division of Human Nutrition and Epidemiology of the Wageningen University. All subjects gave their written informed consent.

Data collection

Anthropometry and bone composition

Body weight was measured to the nearest 0.05 kg on a digital scale (ED-6-T; Berkel, Rotterdam, Netherlands) and height to the nearest 0.001 m with a wall-mounted stadiometer. Subjects were wearing light underclothes. BMI was calculated as weight in kilograms divided by height in meters squared.

Body composition was determined by dual energy X-ray analysis (Lunar DPX-L, whole body scanner; Radiation, Madison, WI). For all subjects, the fast scan mode was used. The total body scan gave information on fat mass, lean mass, total BMC, BMD of the whole body and T-scores. T-scores reflect the BMD values compared with a young healthy adult reference group and are expressed in SD. T-scores are used for classification in diagnostic categories when screening for osteoporosis [16]. The categories are: *normal* (more than -1 SD), *osteopenia* (between -1 SD and -2.5 SD) and *osteoporosis* (≤ -2.5 SD).

Table 1 General characteristics, body composition and biochemical values for a group of Dutch frail elderly women and men[1]

	Women	Men
General characteristics		
n	143	51
Age (y)	77.9 ± 5.3	79.3 ± 5.9
Weight (kg)	63.6 ± 8.7[a]	73.1 ± 8.2
Height (m)	1.61 ± 0.06[a]	1.74 ± 0.07
Energy intake (MJ/day)	7.0 ± 1.5[a]	8.8 ± 2.1
Calcium intake (g/day)	923 ± 308	1002 ± 376
PASE[2,3]	58.6 [31.4;99.6]	58.6 [27.2;107.0]
Body composition		
n	143	51
Lean tissue mass (kg)	38.4 ± 3.6[a]	51.7 ± 5.9
Body fat mass (kg)	23.1 ± 6.8[a]	18.9 ± 5.7
Bone mineral content (kg)	2.0 ± 0.3[a]	2.8 ± 5.0
Bone density (g/cm^2)	1.00 ± 0.09[a]	1.14 ± 0.12
Biochemical parameters		
n	112	46
Vitamin B_{12} (pmol/L)	288 ± 131[a]	238 ± 95
MMA[2] (µmol/L)	0.29 [0.16;0.56]	0.33 [0.16;0.82]
Hcy (µmol/L)	15.8 ± 5.4[b]	21.3 ± 13.5
PTH (pmol/L)	6.3 ± 2.5	6.6 ± 3.5
25(OH)Vitamin D (nmol/L)	36.2 ± 18.5	39.3 ± 16.8

[1] Values are means ± SD

[2] Median [P10 = 10th percentile; P90 = 90th percentile]

[3] Range PASE: 0–400 (a higher score indicates a higher activity).

[a,b] Values in a row are significantly different between women and men: [a] $P < 0.001$. [b] $P < 0.05$.

Abbreviations: PASE: Physical Activity Scale for Elderly

Questionnaires

A questionnaire filled out by an interviewer obtained information on age, sex, marital status, education, living conditions, disease, number of fractures in the last 5 y, medicine

and supplement use. Supplement intake was not included in the dietary intake. Physical activity was assessed using the validated Physical Activity Scale for Elderly (PASE) [17;18], slightly adjusted for Dutch elderly people [17]. A 3-d estimated dietary record was used to obtain the energy and calcium intake. Details of the questionnaires used are given by De Jong *et al.* [15].

Blood sampling and laboratory analysis

Blood samples from fasting subjects were collected between 0700 and 0900 h at home for all indicators, except for homocysteine (Hcy). For Hcy analysis, 0.5 mL EDTA-treated blood from nonfasting subjects was collected in our research center at 1200 h and put on ice immediately before further processing. All samples were measured within one run by HPLC-fluorometry with a CV of 3.5%. Of the fasting blood samples, 1.5 mL EDTA-treated blood was preserved for analyses of vitamin B_{12} and folate by ion-capture IMx (Abbott Laboratories, Abbott Park, IL). Between-run CV were <5% and <10%, respectively. Methylmalonic acid (MMA) was measured in 0.5 mL plasma by stable-isotope-dilution capillary gas chromatography-mass spectrometry. The between-assay CV was 9%. 25-Hydroxy vitamin D was analyzed in a 0.5 mL serum sample for vitamin D with a between-run CV of 5–10% [19]. The cut-off value for vitamin D deficiency is <30 nmol/L [20]. For parathyroid hormone (PTH) analysis, 0.2 mL plasma was determined in duplicate with a chemiluminescence immunometric assay kit (Nichols Institute Diagnostics, San Juan Capistrano, CA).

Data analysis

All statistical analyses were performed by SAS System for Windows, release 6.12 (SAS Institute, Cary, NC). Means and SD, percentages or medians (and their 10th and 90th percentiles) for the total study population were calculated. All analyses were performed for men and women separately, which is the general practice in studies related to BMC and BMD because men and women have a different body composition.

Three different approaches were used to investigate the association of vitamin B_{12} status with BMC and BMD. The first approach used Scheffé's ANOVA procedure to examine differences in mean plasma vitamin B_{12} and MMA concentration among groups of subjects having osteoporosis, osteopenia or normal bone health (based on T-scores).

In the second approach forward, backward and stepwise multiple regression analyses with variables possibly related to BMC or BMD were conducted to determine which variables were independent and significant predictors of BMC and BMD for men and women.

Forward, backward and stepwise multiple regression analyses revealed the same significant variables explaining the variance of BMC and BMD. The newly composed models with only the variables that were significantly related to BMC and BMD in the forward, backward and stepwise analyses are shown.

In the third approach, prevalence odds ratios (POR) for osteoporosis were calculated. First, subjects with osteopenia or normal bone health were categorized in the *normal bone health* group for the remaining analyses. This was done because only a small, nonsignificant difference was found in plasma vitamin B_{12} concentration between the two groups. Then, three categories of vitamin B_{12} concentration were created on basis of the tertiles of vitamin B_{12} concentration in the *normal bone health group*: vitamin B_{12}–deficient (≤ 210 pmol/L), marginal vitamin B_{12} concentration (210 < plasma vitamin B_{12} ≤320 pmol/L) and normal vitamin B_{12} status (> 320 pmol/L). Because there are no generally accepted cut-off points to categorize people with a deficient, marginal or normal vitamin B_{12} status, we decided to use these tertiles instead of the cut-off points used or proposed in the literature. The prevalence of osteoporosis was calculated for each of these categories. Prevalence of osteoporosis in the marginal and deficient vitamin B_{12} groups was compared with the normal vitamin B_{12} group by calculating the odds ratio with multiple logistic regression, i.e., the POR.

We decided a priori to include the continuous variables weight and age as confounders in the adjusted logistic regression model. Weight instead of lean mass and fat mass was chosen because it had the highest correlation with BMD ($r = 0.54$). Based on the literature [21], age was considered to be a confounding factor, although the coefficient of the two vitamin B_{12} categories changed only slightly after adjustment for age. Calcium intake was included in the adjusted logistic regression model because the ß coefficient for vitamin B_{12} changed > 5% when calcium intake was entered after weight (data not shown).

POR for osteoporosis for women and men with normal, intermediate or high MMA or homocysteine status were also calculated but did not differ significantly. The three categories of MMA and homocysteine concentration were also created on basis of the tertiles of MMA and homocysteine concentrations in the *normal bone health group*.

Of the participants, 11% had been using supplements in the past year before the interview took place. Eleven participants had been using a B complex, one woman used a vitamin D supplement and six participants used a calcium supplement. To exclude an effect of supplement use on our results, all analyses were repeated with only the participants who had not been using supplements. No differences were observed between these results and our main results (all participants), i.e., the results were the same for both analyses (including the significant results). In addition, we repeated the analyses and adjusted for

supplement use. This alteration also did not change our results. Therefore, we show here only the results of all participants without exclusion of or adjustment for supplement use. The level of significance was $P < 0.05$ for all analyses.

RESULTS

General characteristics

The mean age of 194 participants included in the analyses of the study was 78 y. Most of the participants (74%) were women. The physical activity score was similar for men and women (**Table 1**). Almost 70% of the participants were living alone. The majority of the study population reported one or more diseases (89%) and use of medicine(s) (73%). Of the participants, 11% were single supplement users. Only 11% currently smoked. Self-reported fractures in the last 5 y tended ($P = 0.233$) to be more frequent in women (16%) than in men (7%).

Body composition and biochemical measurements

All body composition variables were significantly lower in women than in men, except for body fat mass, which was significantly higher. Apart from a higher vitamin B_{12} and lower Hcy concentration in women than in men, there were no significant differences in biochemical variables between men and women. Forty-four percent had a vitamin D deficiency. Due to practical reasons, biochemical values were not available for all participants (**Table 1**).

Vitamin B_{12} and MMA concentrations in the different screening categories for osteoporosis (approach 1)

According to the WHO screening categories [16] for osteoporosis, 36 participants were classified as having osteoporosis, 6 men and 30 women. Nineteen men and 69 women were considered to have osteopenia and 25 men and 45 women were classified as normal. Women in the osteoporosis category had a significantly lower vitamin B_{12} concentration than women in the normal and osteopenia categories. The MMA concentration was slightly higher in the osteoporosis category for women than in the other two categories. In men, no significant differences were found among the three categories for vitamin B_{12} and MMA (**Table 2**). Hcy concentrations were not different in the three screening categories for osteoporosis (data not shown). In addition, no significant differences were observed in vitamin D concentrations among the three screening categories for osteoporosis.

Table 2 Vitamin B_{12} and methylmalonic acid (MMA) concentrations in the different screening categories for osteoporosis in a group of Dutch frail elderly men and women[1]

	n	Women		n	Men	
		Vitamin B_{12} (pmol/l)	MMA (µmol/L)		Vitamin B_{12} (pmol/l)	MMA (µmol/L)
Normal	33	317 ± 131[a]	0.29 [0.18;0.53]	24	250 ± 98	0.31 [0.15;0.58]
Osteopenia	54	301 ± 140[a]	0.27 [0.15;0.69]	18	221 ± 100	0.38 [0.16;0.73]
Osteoporosis	25	221 ± 84[b]	0.32 [0.15;0.65]	6	245 ± 77	0.35 [0.16;2.73]

[1] Incomplete data of n = 38.Values for vitamin B-12 are mean ± SD and values for MMA are median [P_{10};P_{90}]

[a,b] Means or medians within a column with different superscript letter differ, P < 0.05

Relationship between bone composition and biochemical, anthropometric and lifestyle variables (approach 2)

The relationship of BMC and BMD with biochemical, anthropometric and lifestyle variables was explored using multiple regression models. For both women and men, BMC and BMD were explained mainly by weight (**Table 3** for women and **Table 4** for men). A relatively small proportion of the variance of BMC and BMD was accounted for by vitamin B_{12} and energy intake in women, as indicated by the adjusted R^2, which ranged from 1.3 to 3.1%. BMC and BMD had a total R^2 of 46 and 22%, respectively. In men, BMC (R^2 = 53%) and BMD (R^2 = 25%) were explained positively by weight and height (only for BMC), and negatively by smoking.

Forward, backward and stepwise regression analyses did not reveal calcium intake as a significant variable for explaining the variance of BMC and BMD in women and men. Therefore calcium intake was not chosen in the multiple regression models (**Tables 3** and **4**). Moreover, including the variable calcium intake in the models (**Table 3**) did not improve our models. Vitamin B_{12} did not significantly explain a part of the variance of BMC (P = 0.18) and BMD (P = 0.09), nor did calcium intake for BMC (P = 0.21) and BMD for men (P = 0.80) in women. Including calcium intake in the models (**Table 4**) also did not improve our models. Calcium intake did not contribute to the total explained variance of BMC (P = 0.31) and BMD (P = 0.54) in men.

Table 3 Variables related to bone parameters in multiple regression models for a group of Dutch frail elderly women[1]

	BMC		BMD	
	β (95% c.i.) (g)	Adj-R^2 (%)[a]	β (95% c.i.) (g/cm²)	Adj-R^2 (%)[b]
Weight (kg)	17 (10;23)	37.1	$4.4 \cdot 10^{-3}$ ($2.5 \cdot 10^{-3}$;$6.0 \cdot 10^{-3}$)	17.7
Height (cm)	16 (8;25)	7.1		
Energy intake (MJ)			$1.3 \cdot 10^{-2}$ ($0.2 \cdot 10^{-2}$;$2.3 \cdot 10^{-2}$)	1.4
Vitamin B_{12}	0.36 (0.01;0.72)	1.3	$12.3 \cdot 10^{-5}$ ($0.2 \cdot 10^{-5}$;$2.4 \cdot 10^{-4}$)	3.1
Intercept	-1780 (-2985;-576)		0.6 (0.5;0.7)	

1 The evaluated variables in regression analyses were: weight, height, age, plasma vitamin B-12, plasma MMA, plasma tHcy, serum vitamin D, plasma PTH, energy intake, calcium intake, smoking and PASE. Only the variables that contributed significantly to the variance of BMC and BMD were selected. Each column represents one multiple regression model in which only the variables were selected that were significantly related to BMC and BMD in the forward, backward and stepwise analyses. The coefficient ß (95% confidence interval) represents changes per variable unit. The adj-R^2 represents the explained variance of BMC or BMD per added variable. *n* = 111. Abbreviations: BMC, bone mineral content; BMD, bone mineral density; CI, confidence interval; PASE, Physical Activity Scale for Elderly

a Total R^2 = 45.6 %

b Total R^2 = 22.2 %

Table 4 Variables related to bone parameters in multiple regression models for a group of Dutch frail elderly men[1]

	BMC		BMD	
	β (95% c.i.) (g)	Adj-R^2 (%)[a]	β (95% c.i.) (g/cm²)	Adj-R^2 (%)[b]
Weight (kg)	24 (6.8;40.2)	46.3	$5.1 \cdot 10^{-3}$ ($1.3 \cdot 10^{-3}$; $8.8 \cdot 10^{-3}$)	19.5
Currently smoking (1=yes/0=no)	-322 (-594;-62)	2.2	$88.7 \cdot 10^{-3}$ ($9.0 \cdot 10^{-3}$;-$168.5 \cdot 10^{-3}$)	5.7
Height (cm)	22 (4;39)	4.7		
Intercept	-2592 (-497;-214)		0.8 (0.5;1.1)	

1 The evaluated variables in regression analyses were: weight, height, age, plasma vitamin B-12, plasma MMA, plasma tHcy, serum vitamin D, plasma PTH, energy intake, calcium intake, smoking and PASE. Only the variables that contributed significantly to the variance of BMC and BMD were selected. Each column represents one multiple regression model in which only the variables were selected that were significantly related to BMC and BMD in the forward, backward and stepwise analyses. The coefficient ß (95% confidence interval) represents changes per variable unit. The adj-R^2 represents the explained variance of BMC or BMD per added variable. *n* = 45. Abbreviations: see Table 3.

a Total R^2 = 53.2 %

b Total R^2 = 25.2 %

Table 5 Crude and adjusted prevalence odds ratios for osteoporosis for a group of Dutch frail elderly women with normal, marginal or deficient vitamin B_{12} status[1]

Women	Normal vitamin B_{12} status	Marginal vitamin B_{12} status	Deficient vitamin B_{12} status
n	34	43	35
Crude PR	1.0	4.8 (1.0-23.9)	9.5 (1.9-46.1)
+ weight	1.0	5.9 (1.1-32.1)	10.5 (2.0-56.5)
+ weight + calcium intake	1.0	4.6 (0.8-25.5)	7.1 (1.3-40.7)
+ weight + calcium intake + age	1.0	4.5 (0.8-24.8)	6.9 (1.2-39.4)

[1] Normal vitamin B-12 status: > 320 pmol/l; marginal vitamin B-12 status: between 210 and 320 pmol/l; deficient vitamin B-12 status: < 210 pmol/l. Values are crude and adjusted prevalence odds ratios (95% confidence interval)

Prevalence odds ratios for osteoporosis (approach 3)

Participants were divided into three vitamin B_{12} groups: normal, marginal and deficient. The prevalence of osteoporosis was 6% in women in the normal vitamin B_{12} group (n = 34). In the marginal (n = 48) and deficient vitamin B_{12} groups (n = 30), prevalences in women were 25 and 37%, respectively. The overall mean prevalence for women was 22%. The prevalence of osteoporosis in men was 10% in the normal group (n = 10), 40% in the marginal (n = 14) and 5% in the deficient group (n = 22). The overall mean prevalence for men was 13%.

Compared with the normal vitamin B_{12} group, the POR for osteoporosis in women was 4.5 [95% confidence interval (CI): 0.8;24.8] times higher in the marginal vitamin B_{12} group and 6.9 (95% CI:1.2;39.4) times higher in the deficient vitamin B_{12} group after adjustment for weight, calcium intake and age (**Table 5**). The POR for osteoporosis in men were not significantly increased in the marginal and deficient vitamin B_{12} groups compared with the normal vitamin B_{12} group. The POR of increased MMA or Hcy were not increased significantly in men or women (data not shown).

DISCUSSION

Our results highlight an association of vitamin B_{12} status with BMC and BMD in elderly women. To the best of our knowledge, this is the first report exhibiting this association.

Plasma vitamin B_{12} concentration explained a small but significant part of the variance of BMC and BMD in women but not in men. Only a small part of BMC and BMD is determined by modifiable factors. Thus, an increase or decrease in vitamin B_{12} might be relevant for BMD, which is related to bone fractures. Indeed, the POR for osteoporosis were higher in the marginal and deficient vitamin B_{12} groups than in the normal vitamin B_{12} group in women.

Population

Although only frail elderly people >70 y old were eligible for participation, one exception was made (one woman was 66 y old). Compared with the apparently healthy Dutch elderly, the health profile of our frail elderly was poorer. Their mean body weight (66 kg) was lower than that reported in the SENECA study (70 kg) [22], in which prevalences of self-reported disease (89%), osteoporosis (22%) and vitamin D deficiency (44%) were much higher than prevalences among apparently healthy Dutch elderly, namely, 54% for diseases [23], 7% for osteoporosis [24] and 29% for vitamin D deficiency [25]. Because of their poorer nutritional status, an association of vitamin B_{12} status with BMC and BMD might be detected earlier in these frail elderly people.

Methods

In this study, BMC and BMD were measured by dual-energy X-ray absorptiometry. This method is considered to be accurate and precise in comparison to in vivo or in vitro multiple component methods [26]. We used the fast scan mode. Data from others did not indicate a difference between fast and medium scan modes [27].

The results of BMC and BMD were interpreted together, with size-correction for BMD by predefined indices. According to Prentice et al. [28], such correction may cause misinterpretation when identifying determinants of bone mass and fracture risk. Still, many research groups use BMD as a valuable tool for assessing fracture risk and clinical management. We used both BMC and BMD as dependent variables; therefore, we assume that it is justified to use BMD as well in this context.

Classification by T-scores of total body mineral density gives a good indication of bone health [29;30]because of a high correlation between total body BMD and BMD of other regions, such as femur. In this study, these criteria were used for both women and men, because there are no specified and defined criteria for men. The use of T-scores, however, remains questionable for men because the scores were originally designed to classify female subjects.

There are indications that diabetes or rheumatoid arthritis might be associated with bone health [31;32]. It is not likely that these diseases interfered with our results because diabetes (n = 11) and rheumatoid arthritis (n = 35) were evenly distributed among the three screening categories for osteoporosis. Alcohol intake has also been associated with osteoporosis [33]. Because alcohol intake was very low in our study, we could not examine its relationship to BMC and BMD.

Data on energy intake were obtained from 3-d dietary records. This method was selected because of its extensive and structured approach to assess energy intake in elderly people. Although it may underestimate true dietary intake in elderly people [34], its influence on our results is assumed to be mainly of a systematic nature because many factors affect underreporting; therefore, it should not affect our results. In addition, underreporting should be similar for all participants.

The physical activity level was determined with the Physical Activity Scale for Elderly. The PASE has been validated by the doubly-labeled water method and confirmed as a reasonably valid method with which to classify healthy elderly people into categories of physical activity [17].

Results of vitamin B_{12} status in relation to (indicators of) bone health

The results supporting our hypothesis of vitamin B_{12} in relation to (indicators of) bone health emerge from the three different approaches applied in this study. The first indication of a relationship between vitamin B_{12} and bone health evolved from differences we observed in vitamin B_{12} levels among three screening categories for osteoporosis in women. In men, no significant differences in vitamin B_{12}, MMA and Hcy concentrations were found.

In our second approach, plasma vitamin B_{12} explained the variance of BMD (3.1%) in women in our multiple regression models. Peak bone mass is determined up to 60–80% by genetic factors, age and sex; other modifiable factors can explain only the remaining 20–40%. A small increase or decrease in these factors may have an influence on fracture incidence, e.g., a decrease of BMD of 0.05 g/cm^2 is associated with an odds ratio of 1.5 for hip fracture [35]. Barr *et al.* [14] examined a similar relationship and found that total body fat and vitamin B_{12} intake explained 24% of spinal BMD in premenopausal vegetarian and nonvegetarian women.

The multiple regression models for BMC showed similar results. Again plasma vitamin B_{12} levels explained a small but significant part of the variance (1.3%) of BMC in women, but not in men. Before our study, we decided to stratify by gender. Biologically, the gender

variable is an effect modifier of body composition. In general, data from studies of body composition are analyzed separately for women and men because of the large differences. These differences are natural and independent of our study. In addition, different variables explained the variance of BMC and BMD for women and men. These variables are also of different magnitude. This strengthens our opinion that the gender variable acts as a biological effect modifier of body composition. However, no significant interactions (indicating effect modification) ($P = 0.17$ and $P = 0.37$) were observed between gender and plasma vitamin B_{12} in the regression of BMC and BMD, respectively. This implies that there are no direct indications of vitamin B_{12} influencing BMC or BMD differently in men and women. It is likely that the small sample size of men precluded the ability to observe an association between vitamin B_{12} status and BMC or BMD in men.

In men, BMC was explained by weight, height and smoking. It has been shown before that weight and height have a positive influence on BMC. Smoking has a negative influence on bone density and fracture risk [36-38]. Our results are consistent with these earlier outcomes.

Calcium intake did not contribute to the total explained variance of BMC and BMD in the complete model, nor did vitamin B_{12} concentration. This might have been due to the strong correlation ($r = 0.39$, $P < 0.0001$) between calcium intake and vitamin B_{12} concentration in women. Therefore, vitamin B_{12} concentration could be a marker for calcium intake. On the other hand, the POR for osteoporosis in women was significantly higher in the deficient group even after adjusting for weight, calcium intake and age. This implies that vitamin B_{12} concentration might in fact be associated with bone health. The observed association might not be causal, yet vitamin B_{12} might be a marker for another (unknown) factor.

According to our third approach, the prevalence of osteoporosis in the marginal and deficient vitamin B_{12} groups was higher than that in the normal group for women but not for men. The POR for osteoporosis in women was significantly higher in the deficient group even after adjusting for weight, calcium intake and age.

No other studies have reported the association of plasma vitamin B_{12} concentration with BMC and BMD in elderly people. In addition to that, little is known about the association in other groups, such as patients with pernicious anemia, which is regarded as a risk factor for osteoporosis [39]. In our study, plasma vitamin B_{12} seemed to be associated with BMC and BMD in women. There might be a similar clinical effect in men that we were not able to detect. This may be due to the low number of participating men, especially the low number of men ($n = 6$) classified as osteoporotic. Due to the design of the original study, it was not necessary to recruit more men. Furthermore, it might be that men are less

sensitive to an effect of vitamin B_{12} deficiency on their bone health. This is speculative because we do not know how vitamin B_{12} affects BMC and BMD. Kim et al. [8] found that alkaline phosphatase activity (associated with osteoblasts) was increased by vitamin B_{12} in a concentration-dependent manner in an in vitro study. They suggested that clinical vitamin B_{12} deficiency might be associated with defective functional maturation of osteoblasts. Mulder and Snelder [39] showed that supplements of vitamin B_{12} and calcium had a positive effect on the BMD of the spine and hip in pernicious, osteoporotic patients ($n = 15$).

In our study neither significant differences in the MMA concentrations among the three screening categories for osteoporosis (**Table 3**) nor significant POR for women with different MMA status were found. This might be explained by differences in variation and distribution of the concentrations of vitamin B_{12} and MMA. MMA had a narrower concentration range than vitamin B_{12}; as a consequence, significant results were more difficult to detect. The correlation between vitamin B_{12} and MMA in our study was -0.33 ($P < 0.001$). Forty-eight of 158 people had a deficient vitamin B_{12} concentration (<210 pmol/L). Of these 48 people, 32 had also a high concentration for MMA when a cut-off value >0.35 μmol/L was used [15]. This indicates that MMA is indeed a good marker for vitamin B_{12} deficiency. According to the review by Klee [40], many groups now recognize MMA and Hcy tests as the most sensitive and specific markers of functional vitamin B_{12} deficiency. Usually, MMA increases as a result of deficient or subnormal vitamin B_{12}.

In conclusion, our study showed that plasma vitamin B_{12} is associated with (indicators of) bone health in frail elderly women. This could not be shown, however, in frail elderly men. These results might indicate that the role of vitamin B_{12} is most obvious in persons with marginal or poor bone health. In women, the prevalence of osteoporosis was higher in the marginal and deficient vitamin B_{12} groups than in the normal vitamin B_{12} group. In the future, different populations with different vitamin B_{12} status should be investigated. The cause-effect relationship between vitamin B_{12} and bone health should be examined. With this improved understanding of the relationship of vitamin B_{12} to BMC and BMD, further research should be focused on the effect of extra (supplemental) vitamin B_{12} on bone health.

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Homocysteine levels and the risk of osteoporotic fracture

4

Joyce BJ van Meurs[1], **Rosalie AM Dhonukshe-Rutten[4]**, Saskia MF Pluijm[5], Marjolein van der Klift[1], Robert de Jonge[3], Jan Lindemans[3], Lisette CPGM de Groot[4], Albert Hofman[2], Jacqueline CM Witteman[2], Johannes PTM van Leeuwen[1], Monique MB Breteler[2], Paul Lips[5], Huibert AP Pols[1,2] and André G Uitterlinden[1,2,3]

From the Departments of [1]Internal Medicine; [2]Epidemiology and Biostatistics, [3]Clinical Chemistry, Erasmus Medical Center, Rotterdam; [4]Division of Human Nutrition, Wageningen University, Wageningen; and [5]the Institute for Research in Extramural Medicine and Department of Endocrinology, Vrije Universiteit Medical Center, Amsterdam. All in the Netherlands

New England Journal of Medicine 2004; 350(20):2033-41

ABSTRACT

Background Very high plasma homocysteine levels are characteristic of homocystinuria, a rare autosomal recessive disease accompanied by the early onset of generalized osteoporosis. We therefore hypothesized that mildly elevated homocysteine levels might be related to age-related osteoporotic fractures.

Methods We studied the association between circulating homocysteine levels and the risk of incident osteoporotic fracture in 2406 subjects, 55 years of age or older, who participated in two separate prospective, population-based studies. In the Rotterdam Study, there were two independent cohorts: 562 subjects in cohort 1, with a mean follow-up period of 8.1 years; and 553 subjects in cohort 2, with a mean follow-up period of 5.7 years. In the Longitudinal Aging Study Amsterdam, there was a single cohort of 1291 subjects, with a mean follow-up period of 2.7 years. Multivariate Cox proportional-hazards regression models were used for analysis of the risk of fracture, with adjustment for age, sex, body-mass index, and other characteristics that may be associated with the risk of fracture or with increased homocysteine levels.

Results During 11,253 person-years of follow-up, osteoporotic fractures occurred in 191 subjects. The overall multivariable-adjusted relative risk of fracture was 1.4 (95 percent confidence interval, 1.2 to 1.6) for each increase of 1 SD in the natural-log–transformed homocysteine level. The risk was similar in all three cohorts studied, and it was also similar in men and women. A homocysteine level in the highest age-specific quartile was associated with an increase by a factor of 1.9 in the risk of fracture (95 percent confidence interval, 1.4 to 2.6). The associations between homocysteine levels and the risk of fracture appeared to be independent of bone mineral density and other potential risk factors for fracture.

Conclusions An increased homocysteine level appears to be a strong and independent risk factor for osteoporotic fractures in older men and women.

INTRODUCTION

Osteoporosis is a major health problem that is characterized by low bone mineral density, deterioration of bone microarchitecture, and an increased risk of fracture [1]. Osteoporotic fractures are associated with increased morbidity and mortality and with substantial economic costs [2-4].

It has been hypothesized that the metabolism of homocysteine is involved in osteoporosis. Homocystinuria, a rare autosomal recessive disease characterized by markedly elevated levels of plasma homocysteine, has several clinical manifestations involving the eyes, the vasculature, and the central nervous system. The presence of homocystinuria is associated with the early onset of generalized osteoporosis [5;6]. The underlying pathophysiological mechanism for the occurrence of early osteoporosis in patients who have homocystinuria is not completely understood. However, in vivo and in vitro studies support the concept that a homocysteine-associated disturbance in collagen cross-linking in bone is involved [7-11].

In the general population, a mildly elevated plasma level of homocysteine, termed hyperhomocysteinemia, is a common condition. Hyperhomocysteinemia is recognized as a major risk factor for atherosclerotic and thromboembolic disease [12] as well as for cognitive impairment, including that seen in Alzheimer's disease [13;14]. Although a previous study suggested the possible involvement of increased plasma homocysteine levels in age-dependent bone loss [15], the role of moderately elevated plasma homocysteine levels in diseases of the skeletal system — in particular, osteoporotic fracture — is unknown. To examine the influence of homocysteine on osteoporosis, we studied the relationship between circulating levels of homocysteine and the incidence of fracture in two independent, prospective studies of three groups of men and women 55 years of age or older.

METHODS

Study subjects

We analyzed data from two independent samples, one sample from the Rotterdam Study consisting of two cohorts of subjects, and the other sample from the Longitudinal Aging Study Amsterdam (LASA). The Rotterdam Study is a prospective, ongoing population-based, cohort study of persons 55 years of age or older residing in the Ommoord district of the city of Rotterdam, in the Netherlands. The study was designed to investigate

chronic, disabling diseases. The rationale and design of the study have been described previously [16]. The baseline examination included 7983 subjects. The medical ethics committee of the Erasmus Medical Center approved the Rotterdam Study.

Two independent, nonoverlapping samples of subjects were included in the present study. At baseline (1991–1993), a random sample of 562 subjects was studied (cohort 1), and at a follow-up visit (1995–1996), a second sample of 553 subjects was studied (cohort 2). Cohort 2 was originally recruited for a study of age-related changes in the brains of elderly persons [17], and the exclusion criteria were dementia, blindness, and the presence of standard contraindications to the use of magnetic resonance imaging. The subjects in cohort 2 ranged in age from 60 to 90 years and were randomly selected, with stratification according to age (in five-year groups) and sex.

LASA is an ongoing cohort study of the predictors and consequences of changes in autonomy and well-being in older persons in the Netherlands. The procedures used in sampling and data collection have been described in detail elsewhere [18]. Briefly, a sample of persons 55 to 85 years of age, stratified according to age, sex, and level of urbanization of residence (number of addresses per square kilometer), was drawn from the population registers of 11 municipalities. At the baseline examination (in 1992 or 1993), 3107 subjects participated. The present study was performed with a subsample of 1291 persons who were interviewed at the time of the second collection of data (in 1995 or 1996) and who were 65 years of age or older on January 1, 1996. The medical ethics committee of the Vrije Universiteit Medical Center approved the study.

All the subjects in the Rotterdam Study and LASA who participated in the present study gave written informed consent.

Assessment of fractures

In the Rotterdam Study, general practitioners monitored the subjects for incident fractures, which were reported by means of a computerized system. Events were classified independently by two research physicians according to the International Statistical Classification of Diseases and Related Health Problems, 10th Revision (ICD-10-CM) [19]. An expert in osteoporosis reviewed all coded events for final classification. For this study, follow-up ended on January 1, 2002.

In LASA, fractures that occurred between the second examination (in 1995 or 1996) and the third examination (in 1998 or 1999) were recorded prospectively on a calendar.

Table 1 Baseline characteristics of study subjects in the two study cohorts of the Rotterdam Study and the LASA Study*

Characteristic	The Rotterdam Study†		The LASA Study†
	Cohort 1 (N=562)	Cohort 2 (N=553)	(N=1291)
Women – no. (%)	351 (62)	278 (50)	663 (51)
Age (yr)	70.3 ± 8.8	73.6 ± 7.9	75.6 ± 6.6
Body mass index (kg/m²)	26.5 ± 3.9	26.3 ± 3.6	26.8 ± 4.2
Current smoker (%)	24	21	18
Fall in previous year (%)	21	NA	32
Homocysteine level	15.9 ± 5.7	11.9 ± 4.3	14.7 ± 6.0
Serum creatinine level‡	82.6 ± 20.4	89.1 ± 20.2	93.6 ± 22.0
Bone mineral density§			
Lumbar spine BMD	1.09 ± 0.20	1.11 ± 0.21	0.97 ± 0.19
Femoral neck BMD	0.85 ± 0.13	0.83 ± 0.15	0.70 ± 0.13
Follow up time (years)	8.1 ± 3.7	5.7 ± 1.9	2.7 ± 0.7
Lost to follow-up (%)	2.3	3.8	4.3
Incidence of fracture (no fractures/1000 person-yr)	18.2	15.8	16.4

* Plus-minus values are means ± SD. LASA denotes the Longitudinal Aging Study Amsterdam, and NA is not available.

† In the Rotterdam Elderly Study, homocysteine levels were determined in serum with the use of high-performance liquid chromatography for cohort 1 and in sodium citrate-treated plasma with the use of an immunoassay for cohort 2. In LASA, homocysteine levels were determined in EDTA-treated plasma with the use of an immunoassay.

‡ To convert the values for creatinine to milligrams per deciliter, divide by 88.4.

§ Measurements of bone mineral density were available for 474 subjects in cohort 1 of the Rotterdam Elderly Study, 475 subjects in cohort 2, and 515 subjects in LASA. The measurements used for subjects in cohort 2 of the Rotterdam Elderly Study had been obtained approximately 2.4 years before the study began. In the Rotterdam Elderly Study bone mineral density was measured with the use of a Lunar densitometer, and in LASA with the use of a Hologic densitormeter. Bone mineral density was measured at the lumbar spine in vertebrae L1-L4 in the Rotterdam Study and in vertebrae L2-L4 in LASA.

Information about fractures was noted retrospectively for respondents who did not participate in the follow-up. All reported fractures were verified by a physician.

To ensure sufficient statistical power, a fracture in any skeletal location was documented as an outcome measure. All fractures that were considered to be nonosteoporotic (i.e.,

fractures due to cancer or to an accident, such as a motor vehicle accident, and all hand, foot, skull, and facial fractures) were excluded. The period of follow-up was calculated as the time from enrollment in the study to the first fracture, death, or the end of the planned follow-up period, whichever occurred first. For subjects lost to the study during follow-up, the follow-up period was calculated as the time from enrollment to the date of the last contact with the subject.

Measurement of bone mineral density

Bone mineral density was measured by dual-energy x-ray absorptiometry at the femoral neck and lumbar spine (vertebrae L2–L4 in the Rotterdam Study and L1–L4 in LASA) [20;21]. In the Rotterdam Study, bone mineral density was measured with the use of a Lunar DPX-L densitometer (Lunar), and in LASA, measurements were made with the use of a Hologic QDR 2000 densitometer (Hologic).

Measurement of homocysteine levels

Blood samples were placed on ice immediately, processed within 60 minutes, and kept frozen until homocysteine levels were measured. For cohort 1 of the Rotterdam Study, serum samples were obtained from subjects who were not fasting, and total homocysteine levels were determined as a fluorescence derivative with the use of high-pressure liquid chromatography [22;23]. For cohort 2 of the Rotterdam Study, plasma samples treated with sodium citrate were obtained from subjects who were not fasting, whereas in the LASA group, EDTA-treated plasma samples were obtained in the morning, after subjects had eaten a light breakfast. Total homocysteine levels were measured with the use of a fluorescence polarization immunoassay on an IMx analyzer (Abbott Laboratories).

Potential confounders

Height and weight were measured while the study subjects were wearing lightweight clothing and no shoes. Data on the body-mass index before the present study were available for cohort 2 of the Rotterdam Study (over a period of approximately 2.4 years) and for subjects in LASA (over a period of approximately 3.0 years), and we calculated the change in the body-mass index between the most recent previous visit and enrollment in the present study. Current smoking status and the number of falls in the preceding year were assessed with the use of a questionnaire.

Levels of serum creatinine were measured with the use of standard laboratory procedures. In the Rotterdam Study, the presence of type 2 diabetes mellitus was defined by the

current use of antidiabetic medication or by a nonfasting or post-load plasma glucose level above 11 mmol per liter. Peripheral arterial disease was evaluated as described previously [24].

In cohort 1 of the Rotterdam Study, dementia was diagnosed with the use of the Mini–Mental State Examination and the Geriatric Mental State Schedule [25], and dietary intake of calories, protein, calcium, 25-hydroxyvitamin D, folate, and vitamins B_6 and B_{12} during the preceding year was assessed with the use of a food-frequency questionnaire [26]. In LASA, the presence of diabetes and peripheral arterial disease was assessed with the use of a detailed questionnaire concerning self-reported chronic disease [27]. Cognitive impairment was diagnosed with the use of the Mini–Mental State Examination; a score below 24 was considered to be positive for cognitive impairment. Levels of serum 25-hydroxyvitamin D were measured with the use of a competitive protein-binding assay (Nichols Institute Diagnostics).

Statistical analysis

The distribution of the plasma homocysteine levels was skewed toward higher values. Therefore, we used natural-log–transformed values, which provided the best-fitting model for analyses in which the plasma homocysteine levels were treated as a continuous variable. In order to compare the homocysteine levels in the three cohorts with one another, sex-specific standard-deviation scores were calculated separately for each subject in each cohort. The standard-deviation score was calculated with the formula $(hcys_i – hcys_m) \div SD$, where $hcys_i$ is the natural-log–transformed homocysteine level in the individual subject, $hcys_m$ the mean natural-log–transformed homocysteine level in the cohort, and SD the standard deviation of the natural-log–transformed homocysteine level in the cohort. This calculation allowed us to determine the increase in the risk of fracture for each increment of 1 SD in the natural-log–transformed homocysteine level.

The relation between the risk of fracture and various homocysteine levels was evaluated with a quartile-based analysis. The quartiles were defined in a sex-specific and age-specific manner for each of the five-year categories.

Cox proportional-hazards regression analysis was used to estimate the risk of fracture. Data were either pooled or were analyzed for each study sample. When all subjects were included, the analysis was adjusted for the study cohort. All estimated risks of fracture were adjusted for age and sex. Additional analyses were adjusted for body-mass index, smoking status, presence or absence of a history of recent falls, and serum creatinine levels. In further analyses, we also adjusted for recent changes in the body-mass index and for dietary intake of calories, protein, calcium, 25-hydroxyvitamin D, folate, and vitamins

B_6 and B_{12} (or for serum 25-hydroxyvitamin D), and the presence or absence of diabetes mellitus, dementia (or cognitive impairment), and peripheral arterial disease.

Population attributable risks were calculated with the use of the formula {P(RR–1) ÷ [P(RR–1) + 1]} x 100, where P is the percentage of the population exposed and RR is the relative risk. We calculated the 95 percent confidence interval by determining the 95 percent confidence interval for log (P[RR–1]) on the basis of the standard errors for P and RR, with the use of the delta method, and transforming back to the 95 percent confidence interval for the population attributable risk.

Table 2 Distribution of types of incident fracture according to study cohort and the quartile of homocysteine level*

Type of fracture	Rotterdam Study		LASA	Total	Homocysteine level	
	Cohort 1	Cohort2			Quartile 1-3	Quartile 4
	number (percent)					
Hip	22 (27)	11 (22)	18 (31)	51 (27)	36 (29)	15 (23)
Wrist	21 (25)	14 (28)	18 (31)	53 (28)	37 (29)	16 (25)
Upper humerus	10 (12)	3 (6)	8 (14)	21 (11)	15 (12)	6 (9)
Vertebrae	7 (8)	7 (14)	2 (3)	16 (8)	8 (6)	8 (12)
Extremities*	19 (23)	10 (20)	8 (13)	37 (19)	25 (20)	12 (18)
Rib and sternum	3 (4)	3 (6)	3 (5)	9 (5)	3 (2)	6 (9)
Pelvis	1 (1)	2 (4)	1 (2)	4 (2)	2 (2)	2 (3)
Total	83 (100)	50 (100)	58 (100)	191 (100)	126 (100)	65 (100)

* Extremities include arm, leg and ankle fractures

RESULTS

Baseline characteristics

Selected baseline characteristics of the study subjects in the three cohorts are shown in **Table 1**. The three cohorts differed significantly with regard to mean age and sex ratio. Mean homocysteine levels were different in all three cohorts; the levels increased with age and were higher in men than in women in all three cohorts (see **Appendix**).

Table 3 Results of multivariate analyses of the relationship between homocysteine levels and the risk of fracture in the three cohort studies

Analysis	Continuous analysis*		Quartile-specific analysis†	
	No. of fractures/ No. of subjects	RR (95%CI)	No. of fractures/ No. of subjects	RR (95%CI)
Adjusted for age and sex	191/2406	1.3 (1.1-1.5)	191/2406	1.9 (1.4-2.6)
Multivariate 1‡	180/2332	1.4 (1.2-1.6)	180/2332	2.0 (1.4-2.8)
Multivariate 2§	180/2332	1.4 (1.2-1.6)	180/2332	2.0 (1.4-2.7)

* Homocysteine levels were analyzed as a continuous measure. The relative risk (RR) is for each increment of 1 SD in the natural-log-transformed homocysteine value. CI denotes confidence interval

† The homocysteine level was analyzed as a dichotomous variable. The relative risk is for subjects in the highest quartile of homocysteine levels as compared with subjects in the three lower quartiles.

‡ This analysis included adjustments for age, sex, body mass index, changes in body mass index before entry in the study, smoking status, presence or absence of a history of recent falls, and serum creatinine level. Data for changes in previous body mass index were not available for cohort 1 of the Rotterdam Study, and data for recent falls were not available for cohort 2.

§ This analysis included adjustments for age, sex, body mass index, smoking status, presence or absence of a history of recent falls, diabetes mellitus, dementia (in the Rotterdam Study) or cognitive impairment (in LASA), peripheral arterial disease, and serum creatinine level.

Homocysteine levels and fracture risk

During 11,253 person-years of follow-up, 191 subjects (135 women and 56 men) sustained an osteoporotic fracture; a majority were hip and wrist fractures (**Table 2**). High homocysteine levels were associated with an increased risk of fracture (**Table 3**). After adjustment for age and sex, the overall relative risk of fracture for each increment of 1 SD in the homocysteine level was 1.3 when all subjects were pooled. The risk of fracture with increasing homocysteine levels was similar in all three groups of subjects (data not shown). The risk was similar in men and women: 1.4 (95 percent confidence interval, 1.1 to 2.8) in men, and 1.3 (95 percent confidence interval, 1.1 to 1.5) in women.

Because the three cohorts differed with regard to age and sex distribution (Table 1), the subjects were grouped in sex- and age-specific quartiles according to the homocysteine level. In all three cohorts, subjects in the highest quartile had an increase in the risk of fracture so that the risk was twice as high as the risk in each of the lower three quartiles.

We subsequently analyzed the homocysteine levels divided into the highest quartile (risk group) and the lower three quartiles combined (reference group) (Table 3). The absolute cutoff values used to define the risk groups are described in the Appendix. Subjects in

whom homocysteine levels were above the cutoff value had a risk of fracture that was two times as high as that for subjects with lower values. The risk estimates were similar in all three cohorts. The frequency of nontraumatic vertebral fracture was doubled in the highest quartile (risk group) (Table 2), although this trend did not reach statistical significance (P=0.26). **Figure 1** shows the cumulative incidence of fracture in the three cohorts according to the age-specific quartile of homocysteine levels.

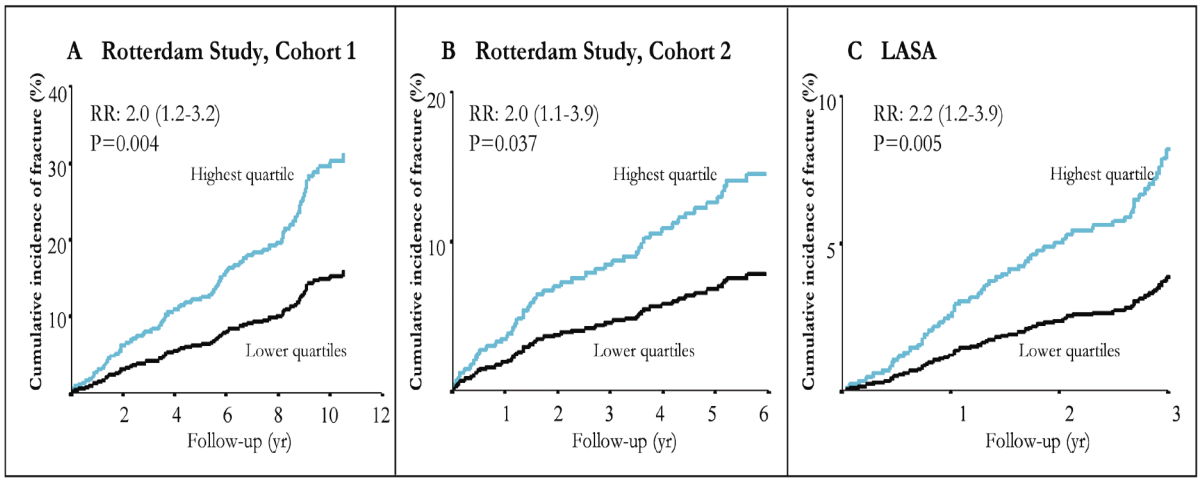

Figure 1 Cumulative incidence of fracture among study subjects with homocysteine levels in the highest age and sex-specific quartile as compared with all other subjects

RR denotes relative risk, and CI confidence interval

Homocysteine levels and bone mineral density

As shown in **Figure 2**, after adjustment for age and sex, homocysteine levels were not associated with bone mineral density at either the femoral neck or the lumbar spine. When we included bone mineral density in the multivariate regression model, the risk estimates were not substantially changed.

Possible confounding variables

The association between homocysteine levels and the risk of fracture was not reduced after adjustment for the body-mass index, changes in the body-mass index, smoking status, recent falls, serum creatinine levels, and the presence or absence of diabetes mellitus, peripheral arterial disease, and dementia or cognitive impairment (Table 3). In addition, the observed association between homocysteine levels and the risk of fracture in cohort 1 of the Rotterdam Study was not substantially reduced after adjustment for

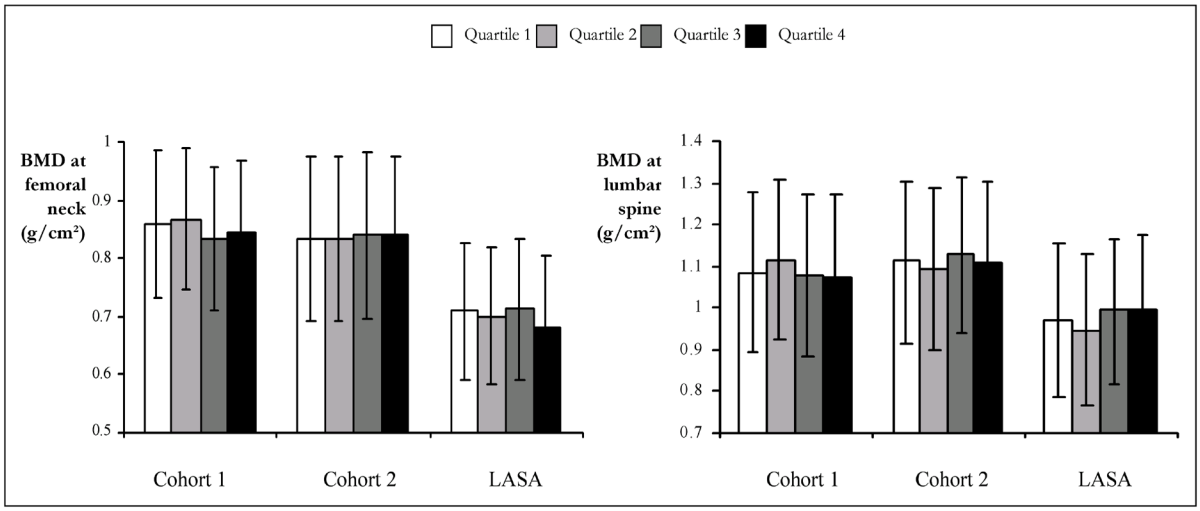

Figure 2 Means (±SD) bone mineral density (BMD) according to age- and sex-specific quartiles of homocysteine levels

dietary intake of calories, protein, calcium, and vitamins (data not shown). The same analysis could not be performed for cohort 2 of the Rotterdam Study or for LASA, because data on dietary intake were not available for those cohorts. Instead, serum levels of 25-hydroxyvitamin D were used as a measure of nutritional status for subjects in LASA, and adjustment for this covariable did not alter the risk estimates.

Population attributable risk

Table 4 shows the population attributable risks for the independent risk factors for fracture in the total study population. The risk of fracture that was attributable to a homocysteine level in the highest age-specific quartile was estimated at 19 percent. The association of a high homocysteine level with the occurrence of incident fractures was similar to the association of the risk of fracture with low bone mineral density, cognitive impairment, and recent falls.

DISCUSSION

Our analyses of data from three cohorts of subjects in two independent studies show a strong association between increased homocysteine levels and the risk of osteoporotic fracture. The age- and sex-adjusted risk of fracture increased by 30 percent for each increase of 1 SD in the homocysteine level. A serum homocysteine level in the highest quartile doubled the risk of fracture. The magnitude of this effect is similar to that

previously observed for the increase in the risk of cardiovascular disease and dementia according to homocysteine level [28].

A novel aspect of this study is an examination of the relationship between the risk of fracture and homocysteine levels in a general, older population. The association appears to be consistent within this population, since we found similar risk estimates in the two cohorts of the Rotterdam Study and in LASA. The association appears to be independent of age, sex, and other risk factors for fracture, such as smoking, recent falls, dementia, diabetes mellitus, peripheral arterial disease, and nutritional deficiency. In view of the inherent limitation of measuring dietary intake by means of a questionnaire, nutritional deficiency cannot be completely ruled out as a confounder.

Table 4 Relative risks and population attributable risks for independent risk factors for incident fracture*

Factor	Relative Risk (95% CI)	Population Attributable Risk (95% CI)
		%
Age >75	2.3 (1.7-3.1)	31 (25-48)
BMD, lowest quartile	1.6 (1.1-2.3)	13 (2-25)
Current smoking	1.6 (1.1-2.3)	10 (4-23)
Fall in preceding year†	1.9 (1.2-2.7)	20 (10-35)
Dementia and cognitive impairment†	2.5 (1.5-4.1)	15 (7-30)
Homocysteine level, highest quartile	1.9 (1.4-2.6)	19 (10-29)

* All relative risks were adjusted for age and sex, except for an age over 75 years. CI denotes confidence interval, and BMD bone mineral density

† Only data from cohort 1 of the Rotterdam Study and LASA were used to calculate the population attributable risk

The calculated population attributable risk of the effects of increased homocysteine levels is considerable. A homocysteine level in the highest age-specific quartile conferred a 19 percent attributable risk in our population. The population attributable risks were similar for well-known risk factors for fracture, such as low bone mineral density, cognitive impairment, and recent falls, in the study population. A recent report showed that in the Rotterdam Study, the population attributable risks of myocardial infarction associated with hypercholesterolemia and hypertension — two well-known risk factors — were 18

percent and 14 percent, respectively [29]. Thus, a high homocysteine level appears to have an effect whose size is similar to that of established risk factors for fractures and for cardiovascular disease.

There were considerable differences in the techniques used to assess homocysteine levels in the three study cohorts. For cohort 1 of the Rotterdam Study, serum samples were obtained, and for cohort 2, sodium citrate–treated plasma samples were obtained. In LASA, EDTA plasma samples were used. Furthermore, different methods were used to determine the homocysteine levels. These differences in method are known to influence the measurement of homocysteine levels [30-32]. Together with differences in the age and sex distribution among the three cohorts, the differences in method may explain the considerable variation in the mean homocysteine levels among the three cohorts. Despite these differences, an association between the homocysteine level and the risk of fracture was consistent in each cohort. Thus, this association appears to be independent of the method of measuring homocysteine levels. However, because of the large differences among the three cohorts, we refrained from using a single cutoff value for the presence of hyperhomocysteinemia.

According to a long-standing hypothesis, the mechanism underlying the association between the homocysteine level and the risk of fracture may involve interference by homocysteine in collagen cross-linking [7]. Homocysteine has been shown to interfere specifically with the formation of collagen cross-links and fibrils in solution [10]. In addition, lower amounts of collagen cross-links have been found in serum from patients who have homocystinuria — that is, persons with very high levels of circulating homocysteine — than in normal controls [11]. Because collagen cross-links are important for the stability and strength of the collagen network, interference in the formation of cross-links results in an altered bone matrix, which then results in fragile bone. Thus, increased homocysteine levels could lead to an increase in the risk of fracture through interference in collagen cross-linking. We therefore speculate that homocysteine interferes with the development of the microarchitecture of bone independently of the amount of mineral in the bone. This notion was corroborated by the fact that we did not find evidence of a relationship between homocysteine levels and bone mineral density.

The association between elevated homocysteine levels and the risk of fracture should be confirmed in other large population studies. Proof of a causal relationship between increased homocysteine levels and bone disease could be established by intervention studies aimed at lowering the serum homocysteine level. Whereas randomized, controlled trials have shown that folic acid–based vitamin supplements can effectively reduce homocysteine levels [33] and reduce the rate of coronary restenosis [34], additional studies are needed to assess whether the use of such therapy will reduce the risk of fracture.

ACKNOWLEDGEMENTS

We are indebted to the participants in the Rotterdam Study and to the general practitioners and field workers at the research center in Ommoord for their essential contribution to this study; to Frank van Rooij for providing data on dietary intake of vitamin B; to the participants in LASA for their contribution to this study; to Jan Poppelaars and Els Lommerse and the field workers for their help in collecting the data; and to our colleagues at the Metabolic Laboratory of Vrije Universiteit Medical Center for their assistance in the determination of homocysteine levels.

This research was supported by grants from the Nederlandse Organisatie voor Wetenschappelijk Onderzoek (925-01-010, 903-46-178, and 014-90-001); the Praeventiefonds, The Hague, the Netherlands (28-25510); and the Dutch Dairy Association. The Rotterdam Study and the Longitudinal Aging Study Amsterdam are supported by the Ministry of Health, Welfare, and Sports.

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Appendix Distribution of homocysteine levels with the use of a 75th percentile cut-off values, according to age

	Rotterdam Cohort 1			Rotterdam Cohort 2			LASA		
	n	Homocysteine level	Cut-off value	n	Homocysteine level	Cut-off value	n	Homocysteine level	Cut-off value
Age		**µmol/liter**			**µmol/liter**			**µmol/liter**	
Men									
55-59	20	14.3 ±2.1	15.5						
60-64	43	14.2 ± 2.9	16.3	44	11.7 ± 4.2	13.0			
65-69	51	17.1 ± 4.9	19.9	60	11.2 ± 2.9	12.5	163	14.1 ± 5.5	15.8
70-74	38	16.3 ± 4.6	17.9	49	12.3 ± 3.7	14.7	140	15.2 ± 5.4	17.4
75-79	30	16.3 ± 4.2	18.6	51	12.1 ± 3.6	14.6	131	15.4 ± 4.7	17.7
80-84	20	19.3 ± 5.5	22.8	52	14.5 ± 3.9	17.0	132	17.3 ± 7.3	18.9
≥ 85 yr	9	24.8 ± 11.7	38.6	19	16.3 ± 7.9	20.5	63	18.5 ± 11.0	21.4
Women									
55-59	42	12.6 ± 2.4	15.1						
60-64	80	13.3 ± 3.2	14.7	52	9.6 ± 2.6	11.8			
65-69	54	14.6 ± 5.3	16.7	51	10.2 ± 3.2	11.1	165	12.7 ± 4.8	14.4
70-74	73	16.6 ± 5.0	19.0	57	11.1 ± 4.2	12.2	171	12.6 ± 4.3	14.6
75-79	42	16.5 ± 5.9	18.3	45	11.2 ± 3.9	13.2	143	13.8 ± 4.6	16.2
80-84	30	17.5 ± 6.6	18.7	38	13.2 ± 5.8	14.3	116	15.3 ± 6.2	17.9
≥ 85 yr	30	21.3 ± 10.9	24.1	35	13.4 ± 4.8	15.4	68	16.2 ± 5.7	19.3

* Plus-minus values are means ± SD. In cohort 1 of the Rotterdam Study, homocysteine levels were measured in serum; in cohort 2, in sodium citrate-treated plasma; and in the LASA study, in EDTA-treated plasma.

Homocysteine and vitamin B_{12} status relate to bone turnover markers, broadband ultrasound attenuation and fractures in healthy elderly people

5

Rosalie AM Dhonukshe-Rutten[1], Saskia MF Pluijm[2], Lisette CPGM de Groot[1], Paul Lips[2,3], Johannes H Smit[4,5], Wija A van Staveren[1]

[1]Department of Human Nutrition, Wageningen University, Wageningen; [2]EMGO Institute, VU University Medical Center, Amsterdam; [3]Endocrinology, VU University Medical Center, Amsterdam; [4]Department of Sociology, VU University, Amsterdam; [5]Department of Psychiatry VU University Medical Center, Amsterdam
All in the Netherlands

Provisionally accepted for publication

ABSTRACT

Introduction Hyperhomocysteinemia may contribute to the development of osteoporosis. Vitamin B_{12} is closely correlated to homocysteine. The relation of homocysteine (Hcy) and vitamin B_{12} with bone turnover markers, broadband ultrasound attenuation (BUA) and fracture incidence was studied in the Longitudinal Aging Study Amsterdam (LASA).

Materials and Methods Subjects were 615 men and 652 women with a mean (SD) age of 76 (6.6) yr. Blood samples were obtained in 1995/1996. Plasma Hcy was measured with IMx, serum vitamin B_{12} with competitive immunoassay (IA) luminescence, serum ostocalcine (OC) with immunoradiometric assay (IRMA), urinary excretion of deoxypyridinoline (DPD) with competitive IA and corrected for creatinine concentration. BUA was assessed in the heel bone. A prospective follow-up of fractures was done until 1999. Analysis of covariance was performed to calculate mean values of BUA, OC and DPD/Cr_{urine} based on specified categories of Hcy and vitamin B_{12} and adjusted for several confounders. The relative risk (RR) of any fracture was assessed with Cox regression analysis.

Results 14% of the men and 9% of the women had a high Hcy (≥15 μmol/L) and low vitamin B_{12} (≤ 200 pmol/L) concentration. Women with low vitamin B_{12} and high Hcy concentration had lower BUA, higher DPD/Cr_{urine} and higher OC concentrations than their counterparts. In men, no differences were found between the different Hcy and vitamin B_{12} categories in adjusted means of BUA, OC or DPD/ Cr_{urine} 28 men and 43 women sustained a fracture during the 3-year follow-up period. The adjusted RR for fractures (95% CI) for men with high Hcy and/or low vitamin B_{12} concentration was 3.8 (1.2-11.6) compared to men with normal Hcy and vitamin B_{12} concentration. Women with high Hcy and/or low vitamin B_{12} concentrations had an adjusted RR for fractures of 2.8 (1.3-5.7).

Conclusions High Hcy and low vitamin B12 concentrations were significantly associated with low BUA, high markers of bone turnover and increased fracture risk.

INTRODUCTION

Osteoporosis is a multifactorial disease with an increasing incidence with aging. The burden of osteoporosis is caused by fractures of the distal radius, vertebrae, hip and other bones. This burden on society is increasing because of the rapidly increasing number of elderly people. Fractures are associated with considerable morbidity and mortality, impaired quality of life and high costs for society [1-5].

A number of modifiable risk factors for osteoporosis and fractures have been identified, including nutritional factors, such as vitamin D deficiency and low calcium intake [6-8]. It is well known that homocystinuria patients are often diagnosed with osteoporosis [9]. High homocysteine levels can be modified by dietary folate, vitamin B_{12} and vitamin B6. Both moderate hyperhomocysteinemia and vitamin B_{12} deficiency are highly prevalent in old age [10-12] and may play a role in diseases that are characteristic for old age. *In vivo* and *in vitro* studies support disturbance of cross-linking of collagen in bone by homocysteine as a possible explanation [13;14]. Recently, a relationship was observed between homocysteine levels and the incidence of fracture in three subcohorts from two independent prospective population-based studies of older men and women. A serum homocysteine level in the highest quartile doubled the risk for fractures when compared to the three lowest quartiles, and this increased risk appeared to be independent of age, sex and other risk factors for fracture, such as smoking, recent falling, diabetes mellitus and peripheral arterial disease [15]. Similar findings were observed in McLean's study [16].

In one of these prospective studies, the Longitudinal Aging Study Amsterdam (LASA), data on bone mineral density, broadband ultrasound attenuation and bone turnover markers were also collected. Broadband ultrasound attenuation (BUA) can predict bone strength [17] risk for hip fracture or any fracture [18].

The hypothesis of the study reported in this paper is that high serum homocysteine, possibly in combination with low serum vitamin B_{12}, is associated with fractures, broadband ultrasound attenuation, the bone formation marker osteocalcine and the bone resorption marker deoxypyridinoline (which was corrected for creatinine). This would indicate that the metabolite homocysteine could influence bone strength and cause collagen disturbances in bone as has been suggested by Lubec *et al* [13]. Therefore the main objective of our study was to examine the association of homocysteine and vitamin B_{12} status, and especially the combined effect of these two, with fracture incidence, broadband ultrasound attenuation and bone turnover markers.

MATERIALS AND METHODS

Study sample & procedures

The current study was performed within a subsample of The Longitudinal Aging Study Amsterdam (LASA) [19]. For LASA a random sample of elderly men and women (55-85 years), stratified by age, sex, level of urbanisation, and expected 5-year mortality was drawn from the population registers of 11 municipalities in three areas of The Netherlands. Data collection took place in 1992/1993, in 1995/1996, in 1998/1999 and in 2001/2002. The data collected in 1992/1993 and 2001/2002 are not used in this study. The data which were collected in 1995/1996 were used for the cross-sectional data analysis to examine the relation of homocysteine status and vitamin B_{12} status with broadband ultrasound attenuation and the bone turnover markers DPD/Cr_{urine} and osteocalcine. The fracture data that were collected from 1995/1996 to 1998/1999 were used for the prospective cohort analysis to examine the relation of homocysteine status and vitamin B_{12} status with fractures.

In the second cycle in 1995/1996, medical data were obtained from 1509 participants who were 65 years or older on 1 January 1996 (**Figure 1**). Respondents were subsequently invited to the VU University Medical Center (VUMC) or a health care center near their homes where BUA measurements were performed and blood and urine samples were collected (n=1321). Information on incident fractures and falls was obtained for 1289 respondents between the second cycle of data collection in 1995/1996 and the third cycle of data collection in 1998/1999. More sampling and data collection procedures have been described in detail elsewhere [20;21].

Informed consent was obtained from all respondents. The study was approved by the Medical Ethics Committee of the VU University Medical Center (VUMC) and conducted according to the principles of the Helsinki declaration.

Outcome measurements

Quantitative ultrasound measurements

Quantitative ultrasound data were obtained using the CUBA Clinical instrument (McCue Ultrasonics, Winchester, UK). Broadband ultrasound attenuation (BUA) (dB/MHz) was measured twice in both the right and left calcaneus. Mean BUA value was calculated from these four measurements [18].

Ascertainment of fractures

Data on fractures that occurred between the second examination (1995/96) and the third examination in 1998/1999 were prospectively reported by the participant on a calendar. Eighty-two percent of all reported fractures were verified by a physician or by radiographs. To reach sufficient power, every osteoporotic fracture was noted as an outcome measure. Fractures caused by an (motor vehicle) accident *(n=10)* and fractures of the head, hand, fingers, foot, toes, ankle and vertebrae (n=20) were excluded. Duration of follow-up was calculated as the time from the first examination in 1995/1996 to the first occurrence of a fracture in weeks.

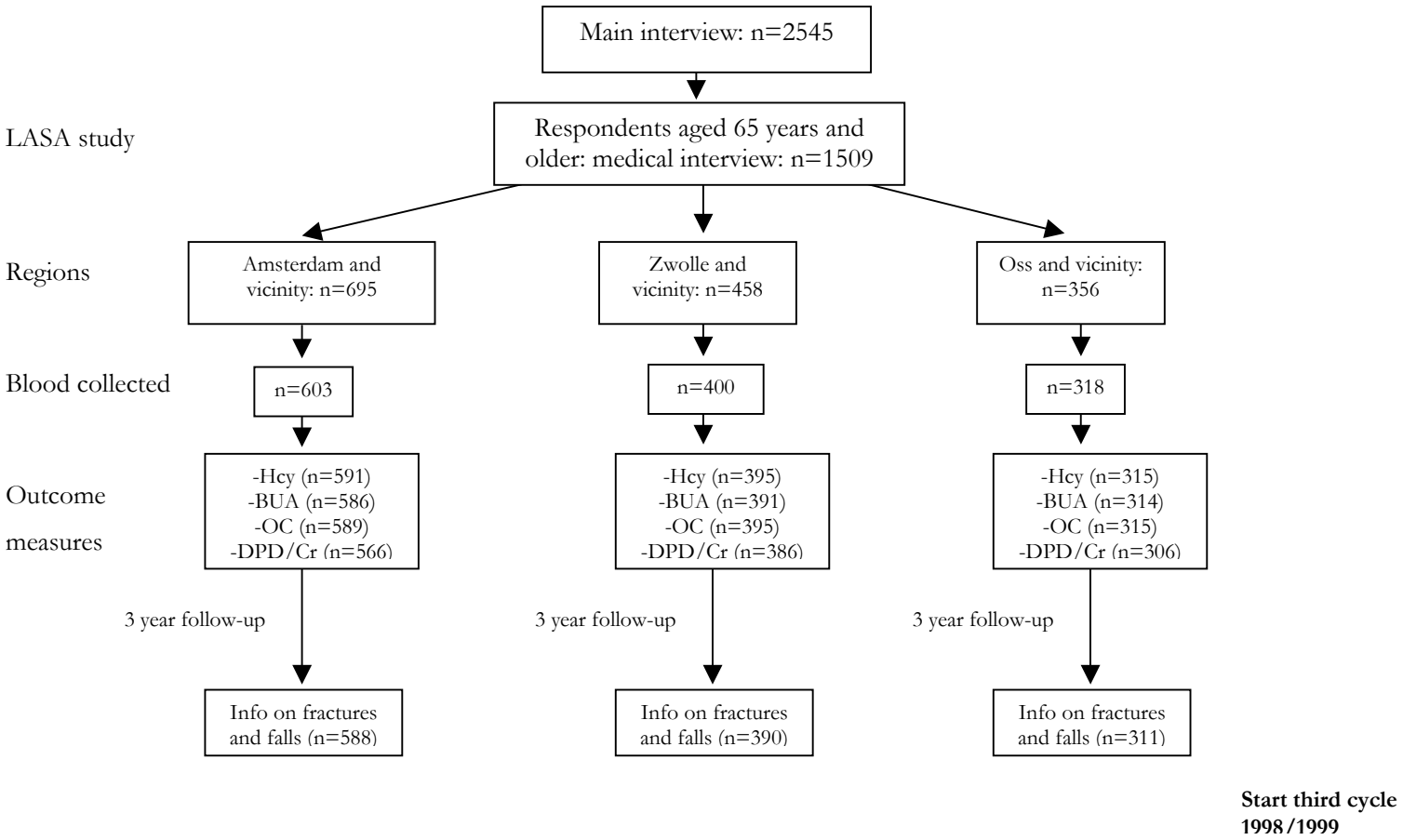


Figure 1 Study design of the additional study on falls and fractures within the Longitudinal Aging Study Amsterdam (LASA); Hcy = homocysteine; BUA = Broadband ultrasound attenuation; OC = osteocalcine; DPD/Cr = deoxypyridinoline corrected for creatinine

Assessment of falls

Participants were asked to record fall events on a weekly 'fall calendar' during three years. A fall was defined as 'an unintentional change in position resulting in coming to rest at a lower level or on the ground'. A 'recurrent faller' was defined as a subject who fell at least two times within six months during the three year fall follow-up [22].

Biochemical assays

Blood was collected in 1995 and 1996 after an overnight fast. EDTA plasma samples were analyzed for total homocysteine (tHcy) with the Abbott IMx analyzer at the Laboratory of Clinical Chemistry of the VUMC. The IMx method uses fluorescence polarization immunoassay (FPIA) technology.

Serum levels of vitamin B_{12} were determined at the Endocrine Laboratory of the VUMC with a competitive immunoassay luminescence on the automated ACS 180 System (Bayer Diagnostics, Mijdrecht, The Netherlands). Serum OC was measured with an immunoradiometric assay (Biosource Diagnostics, Fleuris, Belgium).

Overnight urinary excretion of deoxypyridinoline (DPD) was determined at the Endocrine Laboratory of the VUMC. DPD was determined with a competitive immunoassay on the automated ACS 180 System (Chiron Diagnostics, Emeryville, USA). The values were corrected for creatinine concentration (Cr) in the same urine sample.

Levels of serum 25-hydroxy-vitamin D were measured with a competitive protein-binding assay (Nichols Institute Diagnostics).

Potential confounders

Baseline information on age and sex was derived from the municipal registries. During main interview of the second data collection, body weight, body height, current smoking (yes/no), and mobility were assessed in a face-to-face interview. Body weight was measured without clothes and without shoes using a calibrated bathroom scale. Height was measured using a stadiometer. Body mass index (BMI) was calculated as body weight in kilograms divided by height in meters squared. The level of mobility was assessed with three physical performance tests[23], including a walking test, a chair stands test and a cardigan test. For each test, a score of 1 to 4 points was assigned corresponding to the quartile of the time needed. The more time was needed, the lower the score. Participants who were not able to perform a test obtained a score of zero points. The scores of the three tests were summed up to a physical performance score (range 0-12).

Cognition was measured using the Mini-Mental State Examination (MMSE), which is a questionnaire used as a screening test for general cognitive functionining. The highest possible score is 30; a score of 23 or less indicates the presence of a cognitive impairment [24].

Statistical analysis

Because of the substantial differences in fractures, BUA and bone markers between men and women, all analyses with osteoporosis measures as outcome were stratified by gender. The distributions of Hcy, OC and DPD/Cr_{urine} were normalised by transformation to their natural logarithm.

Men and women were divided into different categories of Hcy and vitamin B_{12} concentration. For these different categories the adjusted means of the outcome measurements BUA, OC and DPD/Cr_{urine} were calculated by performing analysis of covariance.

The risk of fracture with homocysteine and vitamin B_{12} levels were evaluated with a quartile-based analysis and with predefined cut off points for Hcy (> 15 μmol/l) and vitamin B_{12} (< 200 pmol/L) levels. The quartiles were defined in a sex-specific and age-specific manner for each of the five-year categories. The relative risk (RR) (95% CI) of any fracture (except non-osteoporotic fractures and fractures caused by an accident) was assessed with Cox regression analysis. For calculation of risk of fracture on basis of cut off points, the group with normal Hcy and vitamin B_{12} levels was used as the reference group; the other group had therefore high Hcy and/or low vitamin B_{12} levels.

Potential confounders were included in the multivariate models to control for confounding and to enhance precision. Because age is a well known determinant for markers of bone turnover and fracture risk, all analyses were adjusted for age.

RESULTS

Respondent characteristics

The mean age of the LASA participants in this substudy was around 75 years. 46% of the men and 29% of the women had a Hcy concentration higher than 15 μmol/L [25;26]. A low serum vitamin B_{12} level (< 200 pmol/L) occurred in 22% of the men and in 16% of the women. The combination of elevated Hcy and low vitamin B_{12} concentration occurred in 14% of the men and in nine per cent of the women. The median serum

osteocalcine concentration was approximately 2 nmol/L and the median urinary DPD/creatinine excretion was approximately 5 nmol/mmol for both men and women. Twenty-eight men and 43 women sustained an osteoporotic fracture during the 3 year follow-up period. Twenty-five percent of the subjects were recurrent fallers according to the definition (**Table 1**).

Table 1 Characteristics of 630 elderly men and 668 women from the LASA study

	Women (n=651)§		Men (n=602)§	
	Mean	**SD or P10, P90**	**Mean**	**SD or P10, P90**
Age (yr)	75.4	6.5	75.7	6.6
Homocysteine level (μmol/L)*	13.0	8.6, 19.7	14.9	10.2, 22.8
Vitamin B_{12} (pmol/L)	289	99	268	89
BMI (kg/m²)	27.6	4.7	26.0	3.4
Physical performance score (0-12)	6.8	2.8	7.0	2.8
Serum creatinine level (μmol/l)	85.5	32.8	105.5	35.9
BUA (dB/MHz)	61.4	17.4	81.0	18.4
DPD/Creatinine (nmol/mmol)*	5.6	3.4, 9.3	4.6	2.9, 7.5
Osteocalcine (nmol/L)*	2.1	1.1, 3.6	1.8	1.0, 3.1
	n	%	n	%
Fractures	43	6	28	4
Recurrent fallers (≥2 falls within 6 months)†	181	25	166	25
Smoking	100	13	185	26
Cognitive impairment (MMSE≤23)‡	132	17	135	19

* Geometric mean

§ A slight variation in the number of subjects per variable is present due to missing data

† Recurrent falling: at least two times within half a year during 3 year fall follow-up

‡ MMSE: Mini-Mental State Examination

Association of Hcy status and vitamin B_{12} status to fracture risk (prospective cohort study)

High plasma Hcy levels were associated with an increased fracture risk. After adjustment for age, the overall relative risk for fracture for each increment of one SD in Hcy level was 1.4 (95% CI 1.0-2.0) for men and 1.1 (95% CI 0.8-1.6) for women. After multivariate

adjusting for several confounders, the relative risks increased slightly. In **Table 2**, relative risks are shown for the upper quartile (age- and sex-specific) of plasma Hcy concentration compared to the combined lower three quartiles (as the reference group). Men with a Hcy concentration in the upper quartile had a more than 2-fold increased risk to suffer a fracture after adjusting for age and after multivariate adjusting. In women, the increased RRs were not significant.

Table 2 Multivariate Cox proportional hazards regression models for the risk of fractures by homocysteine and vitamin B_{12} status in 1252 men and women from the LASA study

		Variables adjusted for			
		Age		Multivariate*	
		Men	Women	Men	Women
Hcy† Highest quartile versus lowest three quartiles[a]	Number of cases/total number of subjects	21/593	37/631	21/585	36/624
	RR [95% CI][a]	2.4 [1.0-5.6]	1.5 [0.7-2.9]	2.6 [1.1-6.5]	1.7 [0.8-3.5]
Vitamin B_{12}‡ Lowest quartile versus highest three quartiles[b]	Number of cases/total number of subjects	21/592	34/618	20/575	33/609
	RR [95% CI][b]	0.7 [0.3-1.8]	2.2 [1.1-4.3]	0.8 [0.3-2.0]	2.2 [1.1-4.4]
High Hcy and/or low vitamin B_{12}[c]	Number of cases/total number of subjects	21/592	34/618	20/565	33/599
	RR [95% CI][c]	3.6 [1.2-10.7]	2.4 [1.2-4.8]	3.8 [1.2-11.6]	2.8 [1.3-5.7]

[a] The serum homocysteine level was analysed by using log-transformed age- and sex-specific homocysteine quartiles[15]. The RRs were estimated for the highest homocysteine quartile compared to the lowest three quartiles representing the reference group.

[b] The serum vitamin B12 level was analysed by using age- and sex-specific vitamin B12 quartiles. The RRs were estimated for the lowest vitamin B12 quartile compared to the highest three quartiles representing the reference group.

[c] RRs were estimated for the group with subjects having a high homocysteine concentration and/or low vitamin B12 concentration compared to the group with a normal homocysteine (≤ 15 μmol/L) and vitamin B12 concentration (≥ 200 pmol/L).

* The multivariate analysis included adjustments for age, BMI, smoking status, recurrent falling. Hcy relative risks were also adjusted for serum creatinine level.

† Subjects with a serum creatinine level higher than 200 μmol/L were excluded from these analyses.

‡ Subjects with a vitamin B12 level higher than 800 pmol/L were excluded from these analyses.

Similar analyses were performed to investigate the relative risk of low serum vitamin B_{12} concentration for fracture. Women with a serum vitamin B_{12} concentration in the highest

three quartiles were protected from a fracture compared to women in the lowest quartile (Table 2). After adjusting for age, women in the lowest vitamin B_{12} quartile had a RR for fractures of 2.2 (95% CI 1.1-4.3) compared to those in the highest three quartiles. No significant relationship between vitamin B_{12} concentration and fracture risk was observed in men. RRs for fracture incidence for each decrease of one SD in vitamin B_{12} level were similar as to the RRs after dichotomizing the vitamin B_{12} concentration (data not shown).

After multivariate adjusting, men with both a high plasma Hcy concentration (> 15 μmol/L) and/or a low serum vitamin B_{12} concentration (< 200 pmol/L) had a 3.8 (95%CI 1.2-11.6) times higher risk to suffer a fracture than men with both a normal plasma Hcy concentration and serum vitamin B_{12} concentration. In women, the relative risk for a fracture was 2.8 (95%CI 1.3-5.7) in those women with a high Hcy concentration and/or a low vitamin B_{12} concentration after multivariate adjusting (Table 2).

Association of Hcy status and vitamin B_{12} status to broadband ultrasound attenuation (cross-sectional study)

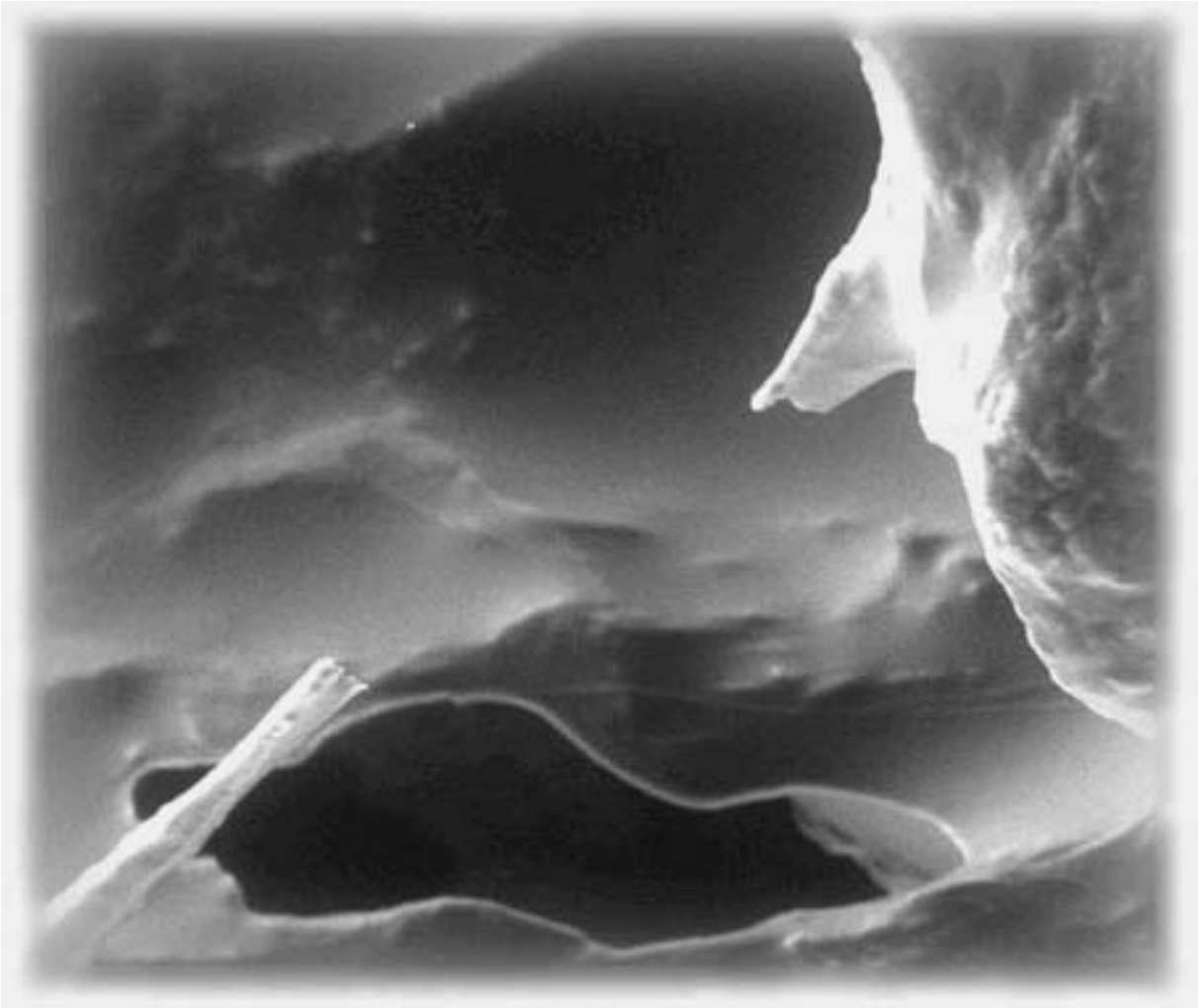
Figure 2 shows the mean BUA for different Hcy and vitamin B_{12} groups adjusted for age, BMI and creatinine. Women with a low serum vitamin B_{12} concentration and high plasma Hcy concentration had a significantly lower adjusted mean BUA value than women with normal vitamin B_{12} and Hcy concentrations ($p = 0.004$) and also compared to women with a low vitamin B_{12} and normal Hcy concentration ($p = 0.04$). In men, no significant differences were observed in adjusted BUA levels in the different categories for vitamin B_{12} and Hcy concentration.

Association of Hcy status and vitamin B_{12} status to bone turnover markers

After adjusting for age and BMI, median urinary DPD/creatinine excretion was significantly higher in women with a low serum vitamin B_{12} and high plasma Hcy concentration than in women with a normal vitamin B_{12} and Hcy concentration ($p = 0.0003$) and also compared to women with a normal vitamin B_{12} and high Hcy concentration ($p = 0.04$) (**Figure 3**). In men, again no significant differences were observed in urinary DPD/creatinine excretion for different Hcy and vitamin B_{12} groups.

Figure 4 shows that after adjusting for age, BMI and serum creatinine concentration, women with a normal vitamin B_{12} and Hcy concentration had a significantly lower mean osteocalcin concentration than women with a low vitamin B_{12} and high Hcy concentration ($p = 0.015$) and women with a low vitamin B_{12} and normal Hcy concentration ($p = 0.01$).

See next page for Figures 2, 3 and 4



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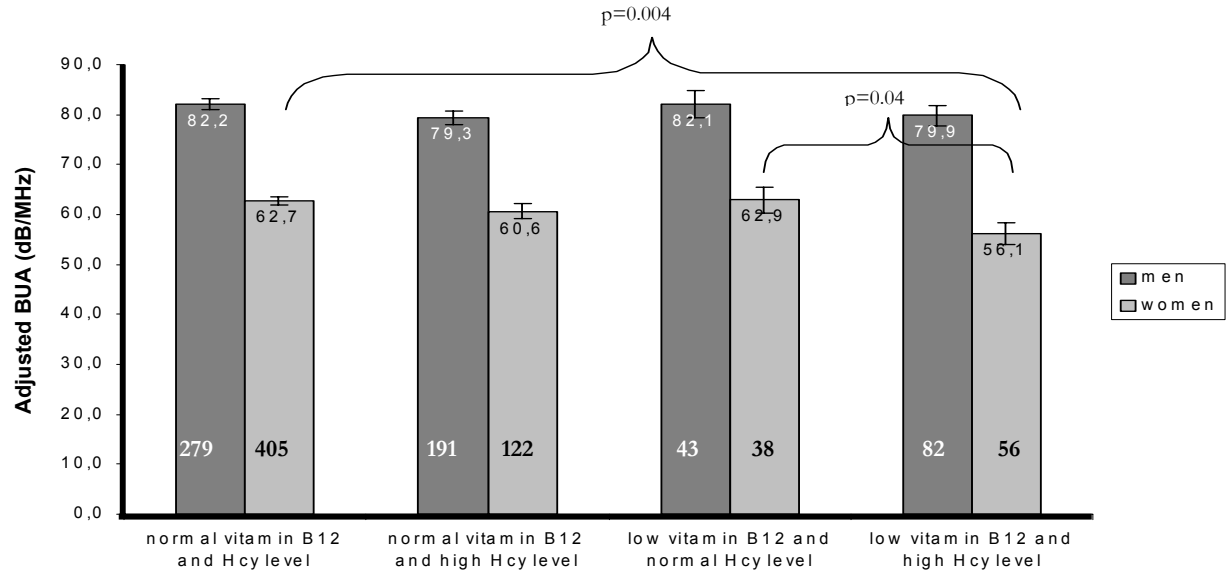

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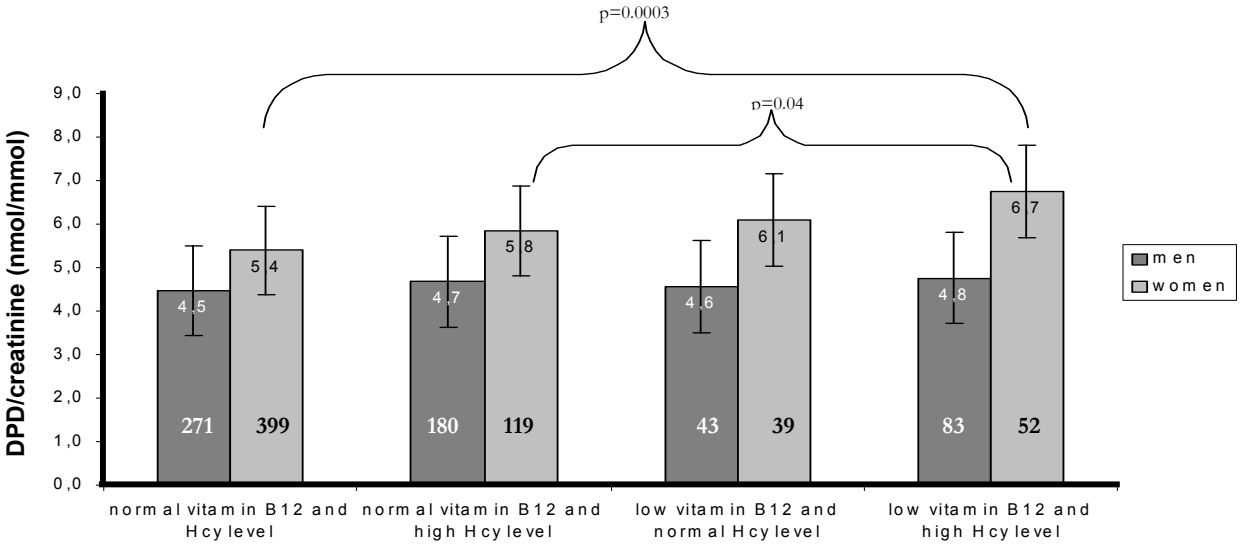

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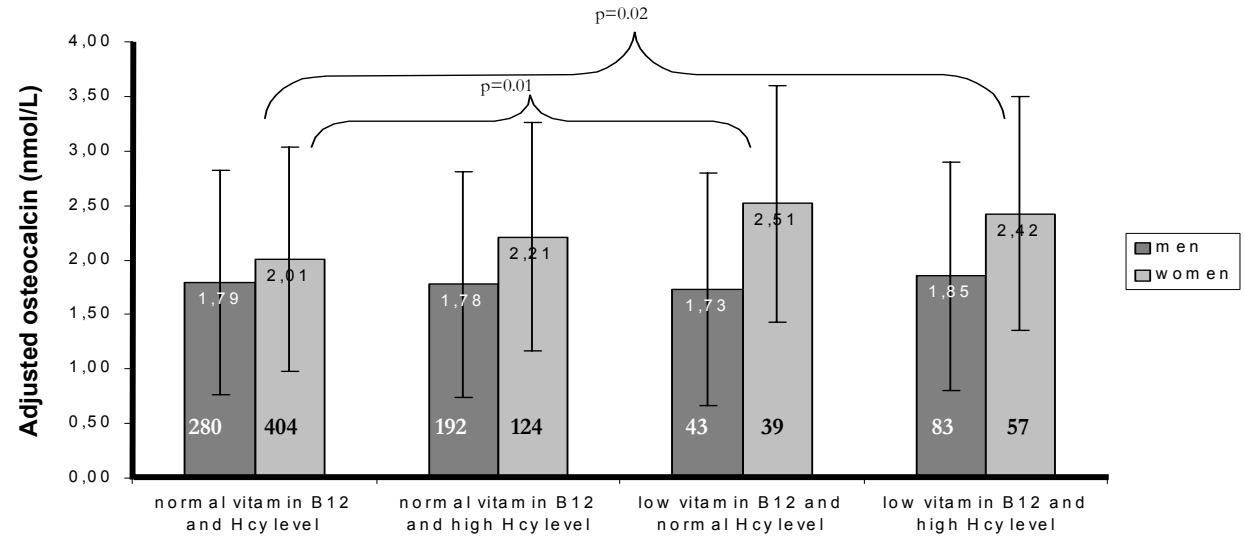

Figure 2, 3 and 4 **Adjusted mean BUA (2), DPD/cr (3) and osteocalcine (4) for different groups of homocysteine and vitamin B_{12} status are given at the top of each bar. Adjusted for age, BMI and creatinine serum level (except for DPD/cr). Subjects with vitamin B_{12} levels > 800 pmol/l or creatinine serum level > 200 pmol/l were excluded from analyses. Number of subjects is given at the bottom of each bar**

Association of Hcy status and vitamin B_{12} status to recurrent falling

Because fractures are usually caused by a fall, we evaluated whether Hcy or vitamin B_{12} status was associated with falling. Neither Hcy status nor vitamin B_{12} status was related to recurrent falling (OR was for both Hcy and vitamin B_{12} 1.0 [0.9-1.1]).

DISCUSSION

Hyperhomocysteinemia increases the risk of osteoporotic fractures according to two different prospective population-based cohort studies, the Rotterdam Elderly Study and the Longitudinal Aging Study Amsterdam [15]. In this study, the presence of either a low vitamin B_{12} or high Hcy concentration or having both conditions was associated with a three times higher risk for fractures in men as well as in women. After inclusion of potential confounders, the relative risks changed but remained significant. This implies that the increased relative risks, due to a high Hcy concentration and/or low vitamin B_{12} concentration, was independent of other common risk factors for fractures. The risk to suffer from a fracture was more than two times in men with a Hcy level in the highest quartile, whereas surprisingly no significant higher relative risks were observed in women. A test for trend was also not observed in women ($p=0.53$). Even though a higher number of men had vitamin B_{12} deficiency, vitamin B_{12} deficiency alone was not associated with a higher relative risk for fractures. A test for trend was also not observed in men ($p=0.24$). The association between vitamin B_{12} status and fractures was, however, high in women. The impact of a low vitamin B_{12} status is apparently more severe in women than in men, whereas a high Hcy status is more severely associated with fractures in men than in women.

The results of the second part (the cross-sectional study) showed that in women the combination of a low vitamin B_{12} and high Hcy status was associated with low broadband ultrasound attenuation and also with elevated bone turnover markers: DPD/creatinine and osteocalcine.

Fractures are most often caused by a fall. The Hcy and vitamin B_{12} status was not associated with recurrent falling. Therefore, falling cannot be an intermediate step in the association of Hcy or vitamin B_{12} status with fracture incidence. In other words, falling is not a consequence of a low vitamin B_{12} status or high Hcy status, but the Hcy and vitamin B_{12} status is directly related to fractures.

The major strength of our study is the use of two types of data: prospective data and observational data. Hcy status in this study was similarly associated with fractures in

longer prospective studies such as two cohorts of the Rotterdam Elderly Study with a follow-up time of 6 and 8 years [15] and the Framingham Heart Study with a follow-up time of 11.7 years [16]. Our cross-sectional results can only show the association but they cannot ascertain a causal relationship. A prospective study would have been more suitable to also explore the association between Hcy and B_{12} with bone turnover markers.

As has been suggested by McKusick [14], the mechanism underlying the association of Hcy with fracture risk, BUA and bone turnover markers might involve interference of Hcy with collagen cross-linking. Later Lubec *et al* [13] indeed showed lower amounts of collagen cross-links in serum of homocystinuria patients compared to those in normal controls. However, homocystinuria is often accompanied by several other medical conditions [27] which could influence collagen cross-links as well. This study gives further evidence for McKusick's statement because women with elevated Hcy concentrations had higher DPD/cr_{urine} concentrations than women with a normal Hcy concentration. Interference in cross-link formation would result in an altered bone matrix resulting in more fragile bone.

BUA as well as DPD/cr_{urine} are good predictors of bone density and fracture risk [18;28;29]. Our observation that Hcy status and vitamin B_{12} status are associated with BUA in women, support the proposed mechanism that vitamin B_{12} deficiency and (as a consequence) elevated Hcy levels may play an important role in the cross-linking of collagen.

As a matter of fact osteocalcine, a noncollagenous protein [30], is not related to collagen cross-linking. Nevertheless, we found an association of Hcy status and vitamin B_{12} status with osteocalcin. This suggests that bone formation may also be affected by elevated Hcy levels and low vitamin B_{12} status. We are not aware of other studies that examined the association of homoysteine status and vitamin B_{12} status with osteocalcin. Therefore, more studies are needed to confirm and further explore this finding. Osteocalcin influences mineralization [31] and serum osteocalcine has been associated with BMD and bone loss [32]. In this study we did not find an association of Hcy status [15], vitamin B_{12} status or the combination of Hcy and vitamin B_{12} status (data not shown) with BMD of the spine and hip site. In another population we have shown a higher prevalence of osteoporosis (defined by BMD T-score < -2.5) in vitamin B_{12}-deficient frail elderly women than in their counterparts with a normal vitamin B_{12} status [33]. Stone *et al* [34] recently showed that low vitamin B_{12} levels were associated with increased hip bone loss in older women, but unfortunately no information was available on homocysteine and methylmalonic acid levels. The explanation for the association between vitamin B_{12} concentration and osteoporosis is less clear than for the association between Hcy and osteoporosis. *In vitro* experiments showed that alkaline phosphatase activity increased

when vitamin B_{12} was added in a concentration-dependent manner to osteoblastic cells [35]. In a clinical study alkaline phosphatase and osteocalcin concentration rose significantly in vitamin B_{12}-deficient patients after treatment with vitamin B_{12}, whereas they remained unchanged in the control group [36].

From our results it cannot be concluded whether the elevation of Hcy levels or the vitamin B_{12} deficiency is causally responsible for the low broadband ultrasound attenuation and increased fracture risk. Elevated Hcy levels are highly correlated with low folic acid levels and low vitamin B_{12} levels [37;38]. Unfortunately, we do not have data on folic acid levels in our participants. Therefore our study does not provide conclusive evidence on the dominance of Hcy status or vitamin B_{12} status.

Studies on gene polymorphisms which have only an effect on Hcy status[39] might give an indication on the role of Hcy with regard to osteoporosis outcome measures. Abrahamsen *et al* showed a relation between the polymorphism of the methylenetetrahydrofolate reductase (MTHFR) gene and fracture incidence in the Danish Osteoporosis Prevention Study. Postmenopausal women with the TT genotype had a higher hazard ratio for fractures than those with the CC or CT genotype [40]. Similar odds ratios were found in the Danish twin study [41]. Unfortunately the association between Hcy concentrations and fracture risk was not assessed in this study, although data were available. In a Danish case-control study contradictory results were found: increased odds ratios of fracture for postmenopausal women with the wild-type C-allele [42]. A lower BMD was found in Japanese [43] and Danish postmenopausal women [40] with the MTHFR TT genotype than women with the CC or CT genotype. McLean *et al* [44] found contradictory results in the association of CC, CT and TT individuals with BUA and BMD after dividing the individuals in low and normal folate status.

What could explain the difference in results found between men and women? We found an association between Hcy status and fracture risk in men, but not in women; while we found an association between the vitamin B_{12} status and fractures in women, but not in men. It is known that hormone replacement therapy suppresses Hcy levels in women [45-48]. We evaluated whether serum estradiol levels confounded the association between Hcy and fractures. Also because estradiol levels were not correlated to Hcy or vitamin B_{12}, the levels were not associated with fractures and they did not confound the association of Hcy status or vitamin B_{12} status with fractures (data not shown).

Our study has some limitations. First, the respondents of this study are the more healthy older people of a population sample, because the frailest respondents of the LASA study could not visit the hospital or healthcare center for blood sampling and ultrasound measurement. Figlin *et al* [49] demonstrated a trend towards a higher incidence of folate

deficiency and vitamin B_{12} deficiency in dependent persons compared to persons who were living independently. It is likely that the prevalence of vitamin B_{12} deficiency was also higher in these non-responders. Therefore, underestimation of the associations might have occurred. Second, although the sample size was relatively large, the power to detect significant differences was still limited for most outcome measures. We cannot exclude the possibility that there was an association present between the Hcy status or vitamin B_{12} status and BMD, because BMD was only measured in a part of this cohort study (n= 515). Third, cutoff points for vitamin B_{12} deficiency are still not generally defined and accepted. Several studies have used 150 pmol/L as a cutoff point (see review of Baik and Russell [50]), although nowadays more investigations choose a higher cutoff point for defining mild vitamin B_{12} deficiency in combination with elevated methylmalonic acid (MMA) concentrations. For defining vitamin B_{12} deficiency, 258 pmol/L was used in the Framingham Heart Study [51] and 260 pmol/L was used in combination with 0.32 μmol/L for MMA in the Dutch part of the SENECA study [12]. In the Dutch study a prevalence of 24% was found for vitamin B_{12} deficiency. Therefore we decided to use 200 pmol/L as a cut off point for vitamin B_{12} deficiency, which was found in about twenty percent of the participants in our study.

In conclusion, the results of this large community based study show that high plasma levels of Hcy and low serum levels of vitamin B_{12} and especially the combination of these two parameters are related to high fracture risk, to low broadband ultrasound attenuation and to increased bone turnover markers. Large-scale trials of folic acid and vitamin B_{12} supplements have shown an effective reduction in Hcy levels [52]. A causal relation between Hcy status and collagen cross-links could be further explored by measuring the urinary DPD/cr excretion or serum cross-links concentration following folic acid and vitamin B_{12} supplementation. In addition, long-term intervention trials with B vitamin supplementation should be performed in order to evaluate fracture risk and its relation to Hcy and vitamin B_{12} status. Furthermore, *in vitro* studies should be performed to increase complementary understanding of the underlying mechanism.

ACKNOWLEDGEMENTS

This study was based on data collected from the Longitudinal Aging Study Amsterdam (LASA), which is largely funded by the Ministry of Health, Welfare, and Sports of the Netherlands. It was partly supported by the Praeventiefonds, The Hague, The Netherlands (grant no 28-25510) and by the Dutch Dairy Association, Zoetermeer, The Netherlands.

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Effect of supplementation with cobalamin carried either by a milk product or a capsule in mildly cobalamin deficient Dutch elderly people

6

Rosalie AM Dhonukshe-Rutten[1], Moniek van Zutphen[1], Lisette CPGM de Groot[1], Simone JPM Eussen[1], Henk J Blom[2], Wija A van Staveren[1]

[1]Department of Human Nutrition, Wageningen University, Wageningen; [2]Laboratory of Pediatrics and Neurology, University Medical Center Nijmegen, Nijmegen
All in the Netherlands

Submitted for publication

ABSTRACT

Background A high prevalence of cobalamin deficiency occurs in the elderly population, which may be treated orally or with injections. Little is known about the relative bioavailability of crystalline cobalamin added to food products.

Objective To assess the effect of supplementation with 1000 µg crystalline cobalamin, carried either by a milk product or a capsule, on cobalamin status in mildly cobalamin deficient Dutch elderly people.

Design Two double-blind randomized controlled intervention studies, covering each a 12-week supplementation period, were carried out in parallel. Mildly cobalamin deficient elderly (n = 112) were separately recruited for the milk and capsule trial. Allocation to the placebo or cobalamin carrier was carried out independently in both trials.

Results In the cobalamin group receiving fortified milk the mean ± SD increase in serum cobalamin was 250 ± 96 pmol/L, the median (P_{10}, P_{90}) decline in plasma MMA was -0.19 (-0.71, -0.05) µmol/L and the decline in plasma Hcy amounted to -4.0 (-6.4, -0.2) µmol/L. All changes were significantly different from those in the placebo milk group ($p < 0.01$). Likewise in the cobalamin capsule group the mean increase in serum cobalamin was 281 ± 136 pmol/L, the median decrease in plasma MMA was -0.18 (-1.09, -0.02) µmol/L and in plasma Hcy it was -1.8 (-8.8, 1.0) µmol/L. All changes were significantly different from the group receiving placebo capsule ($p < 0.01$). No differences in effect were observed between groups receiving cobalamin fortified milk or cobalamin capsules ($p > 0.40$).

Conclusion Crystalline cobalamin added to milk is an effective alternative for cobalamin capsules in improving cobalamin status.

INTRODUCTION

Many studies have shown a high prevalence of cobalamin deficiency in the elderly population, mostly ranging from 12% up to 40% [1-4]. This deficiency may cause neuropsychiatric damage, including cognitive impairment, and hematologic abnormalities, even in mild cobalamin deficiency [1;2;5]. Therefore cobalamin deficiency in old age is considered a substantial problem for public health, which should be managed adequately.

For defining (mild) cobalamin deficiency, different indicators and cut off points have been used. Nowadays plasma methylmalonic acid (MMA) and homocysteine (Hcy) concentrations are used along with the serum cobalamin concentration to assess cobalamin deficiency. Homocysteine, however, is also increased in folate and vitamin B6 deficiency. MMA is therefore the preferred indicator given its higher sensitivity and specificity [6].

High doses of cobalamin, e.g. from intra-muscular injections or oral supplements, are needed to treat cobalamin deficiency [7-9]. Unfortunately injections may be painful and are difficult to provide in people who have a tendency to bleed or who are very thin. Moreover, injections are costly when given by health professionals [10;11]. Of the alternatives, supplements with 1000 μg vitamin B_{12} per day might be required, because only high doses produce successful long-term results as indicated by Elia and Lane & Rojas-Fernandez [10;12]. Such high doses cannot be derived from diet alone, which provides an average of about 5.5 μg/d [13]. This amount [4] meets the American and Dutch Recommended Daily Allowance (2.4 μg/d and 2.8 μg/d, respectively). The dose of 1000 μg/d cobalamin is generally considered as safe. A Safe Upper Level for cobalamin intake has not been set up yet, but a supplemental intake of 2000 μg/d can be used as a safe guidance [14]. No treatment-related adverse events following doses of 1000 to 2000 μg/d for periods ranging from 6 weeks to 12 months have been reported [8;9;15]. Though supplements may be advised, an extra pill every day may affect compliance for regular medication especially in the elderly because they already use a lot of medicines daily [16]. An alternative would be to add cobalamin to food. It is likely that such fortified foods may have other beneficial characteristics, such as the supply of energy and other nutrients which may be low in the diet of elderly people [17].

In view of fortification of cereals with folate, which is mandatory in the USA since January 1998, there are several summons to add cobalamin to the food fortification program [18;19] as folate fortification alone may lead to an increased risk of masked cobalamin deficiency particularly in elderly people [20-22]. However, little is known about the bioavailability of crystalline cobalamin added to a food product. Only Russell *et al*

studied absorption percentages of 0.25 μg cobalamin from water (55%), milk (65%) and bread (55%) in non-cobalamin deficient older adults [23]. The impact that high doses of crystalline cobalamin in fortified foods, which would fit in the diet of Dutch elderly people, may have on cobalamin status in elderly people with a mild cobalamin deficiency is not yet clear. Therefore the aim of our study was to assess the effect of supplementation with 1000 μg crystalline cobalamin for 12 weeks, carried either by a milk product or a capsule, on cobalamin status in mildly deficient Dutch elderly people, compared to a group receiving a placebo product or capsule.

SUBJECTS AND METHODS

In our study two double-blind randomized controlled trials were carried out independently and covered a 12-week supplementation period each. In one of these trials a milk product carried the high cobalamin dose; in the other a capsule did so. In both trials a placebo group, consuming either a milk drink without extra cobalamin or a placebo capsule, served as control. Neither the subjects nor the investigators knew during the study which supplements served as a placebo and which as an intervention. The two capsule groups were part of a larger study in which three capsule groups were studied: placebo, vitamin B_{12}, or vitamin B_{12} and folic acid treatment. By evaluating the change of plasma MMA and serum cobalamin the improvement in cobalamin status after 12 weeks was assessed. Next to these parameters, plasma homocysteine and red blood cell folate were evaluated as secondary outcome measures.

Subjects

Men and women aged 70 years or older were enrolled voluntarily for both trials by mail, with the consent of the staff of their sheltered housing residence. Subjects with history of cobalamin deficiency, use of high cobalamin (> 50 μg/day) or folate (> 200 μg/day) supplementation or injections, history of gastrointestinal surgery, presence of renal dysfunction (serum creatinine > 120 μmol/l), anemia or cancer were excluded. Only subjects with mild cobalamin deficiency were included in the trials: their serum cobalamin concentration had to be between 100 and 300 pmol/l [6] and their plasma MMA concentration ≥ 0.30 μmol/l [1], which was checked after a blood sampling screening visit for which participants were allowed to consume a prescribed light breakfast one hour before sampling. All subjects gave their written informed consent. The medical ethics committee of Wageningen University approved the research protocols.

Trial design

Before the start of the trials the eligible subjects participated in a run-in period of 2 weeks. In this period all subjects received a placebo milk drink or capsule and were matched on sex, MMA concentration and age to either receive at random the placebo or the cobalamin carrier. In the matching procedure, priority was given to MMA. The MMA concentration of the matched pairs did usually not differ more than 0.02 μmol/L. As a consequence, within one matched pair an age difference of 10 years occurred. Allocation of the placebo or cobalamin carrier was carried out independently for both trials. After the run-in period, baseline blood samples and anthropometric data were collected after an overnight fast (no food after 20:00hrs), although a prescribed light breakfast was allowed the next morning until one hour before the blood sampling. After this blood sampling the subjects received either the placebo or the cobalamin carrier. The milk was provided in 500 ml containers along with 125 ml cups. Every morning the subjects had to pour out 125 ml milk in a cup and finish it. The capsules were provided in medicine boxes on which the days and week were indicated, so each box contained 7 capsules. Every day one capsule had to be taken. Each subject had a diary in which he/she indicated whether he/she consumed the milk or capsule each day. Compliance was assessed by review of completed diary forms and counting of the remaining capsules. The diary of the subjects who received the milk drink contained an additional column after every four days, in which the subjects could write how much milk approximately was left in the container. We calculated 'noncompliance' as the amount of milk which was not consumed or the number of capsules which were not consumed. In both trials, compliance (%) was calculated by using the following formula:

100 - (noncompliance/total amount of milk or capsules to be consumed) * 100

During the 12-week period the participants were asked to maintain their regular diet and to avoid consumption of cobalamin rich food products. A list of products rich in cobalamin, mainly liver products, was provided to participants from the 'milk trial'. After 12 weeks, a second blood sample was collected for each subject, again with allowance of a light breakfast until one hour before blood sampling.

Cobalamin carrier

The milk was manufactured by NIZO food research (Ede, the Netherlands). To semi-skimmed milk either 8000 μg/L of crystalline cyanocobalamin (fortified product) or 25-μg/L carmine-extract with E-number E120 (placebo product) was added. Subsequently the products were homogenized at 150/30 bar, sterilized at 140 ºC for 5 sec and aseptically filled in 500 ml polyethylene containers. The containers were closed with an

aluminum seal. The carmine-extract was added to the placebo milk to achieve a similar color as the fortified milk. Carmine-extract is known not to have any flavor characteristics. Therefore, no differences in flavor and color between the two milk products were observed. The energy content of both milks was 194 kJ/100 ml. One cobalamin assay was performed during the study on one sample of both types of milk. The cobalamin-fortified milk contained 7000 μg/L, whereas the placebo milk contained 3.7 μg/L cobalamin.

The capsules were manufactured by Dutch BioFarmaceutics (Helmond, the Netherlands) and contained AVICEL PH102 as a filler (placebo) and 1000 μg crystalline cyanocobalamin. The capsules had identical appearance, smell and taste. On several capsules a cobalamin assay was performed, the mean concentration was 936 ± 34 μg.

The milk containers and the capsules were coded so that neither the investigator nor the participants were aware of the contents.

Laboratory methods

Serum cobalamin, red blood cell folate (RBC folate), plasma MMA and plasma homocysteine (Hcy) were measured at baseline and after 12 weeks. Blood hemoglobine (Hb), hematocrite (Ht), mean cell volume (MCV) and polymorphonuclear hypersegmentation were measured at baseline.

Cobalamin and RBC folate were analyzed on the same day as collection took place. Blood samples for cobalamin determination were placed in the dark immediately after collection. Blood samples for measurement of RBC folate were put on 7 ºC within four hours after blood collection. An automated chemilumiscent immunoassay analyzer (Access 2, Beckman Coulter, Mijdrecht, the Netherlands) was used to measure serum cobalamin and RBC folate concentrations. The interassay coefficient of variation (CV) was 6.3% for the cobalamin assay and 5.9% for the RBC folate assay (Stichting Huisartsenlaboratorium Oost, Velp, the Netherlands).

Hb, Ht and MCV were measured with the Beckman-Coulter hematology analyzer. The reference value for low Hb is < 7.5 mmol/L, for low Ht < 0.38 L/L and for macrocytosis > 100 fL [24]. Polymorphonuclear hypersegmentation was checked by microscopy and defined as hypersegmentation when five-lobed neutrophils/four-lobed neutrophils ≥ 0.17 [25].

Blood samples for measurement of plasma MMA and Hcy were collected in EDTA-treated tubes and placed in ice water immediately. Plasma was separated within 30 min by centrifugation (2600 x g for 10 min at 4 ºC) and stored at –20 ºC until further analysis.

Plasma MMA concentrations were measured with the use of the LC-MS-MS method, with a CV of 5% (personal communication, University Medical Center St Radboud, Nijmegen). Total plasma Hcy concentration was measured by HPLC with fluorimetric detection (CV 7%) at the Division of Human Nutrition, Wageningen University, The Netherlands.

Statistical analysis

Where necessary, baseline data were log transformed to normalize the distribution and geometric means were calculated. Baseline characteristics between treatment groups were compared by one-way analysis of variance (ANOVA) and chi-square analysis for categorical variables. Levene's test was used to test for equal variances. Compliance between groups was compared with Kruskal-Wallis.

Changes from baseline to the end of the 12-week study were analyzed with a paired Student's t-test or with Wilcoxon signed-rank test. Differences in mean change in cobalamin concentration between groups were analyzed with independent sample Student's t-test. Mean changes of MMA, Hcy and RBC folate concentrations were compared between the cobalamin groups with Mann-Whitney-U test. Mann-Whitney-U test was used because it was not possible to normalize the skewed changes. Mann-Whitney-U test and Student's t-test were also used to analyze if there was a difference in effect between cobalamin fortified milk and cobalamin capsules and the results were corroborated by two-way ANOVA (cobalamin x carrier). Data were analyzed using SAS system release 8.0 (SAS Institute Inc., Cary, NC, USA). In all analyses, a probability of 0.05 was considered significant.

RESULTS

The flow of selection of participants in both randomized trials is shown in **Figure 1**. Of the total number of elderly subjects who were interested to participate in one of the studies (n = 1079), 615 were screened for cobalamin deficiency. Others had second thoughts (n = 322) or were excluded based on the exclusion criteria in the health questionnaire (n = 142). After screening, 112 persons were identified as mildly cobalamin deficient and were randomly assigned to one of the treatment groups, which consisted of a cobalamin carrier or a placebo. Seven persons subsequently withdrew for health reasons. Compliance was not significantly different between treatment groups (p = 0.09): 90% of subjects consumed > 90% of their supplements.

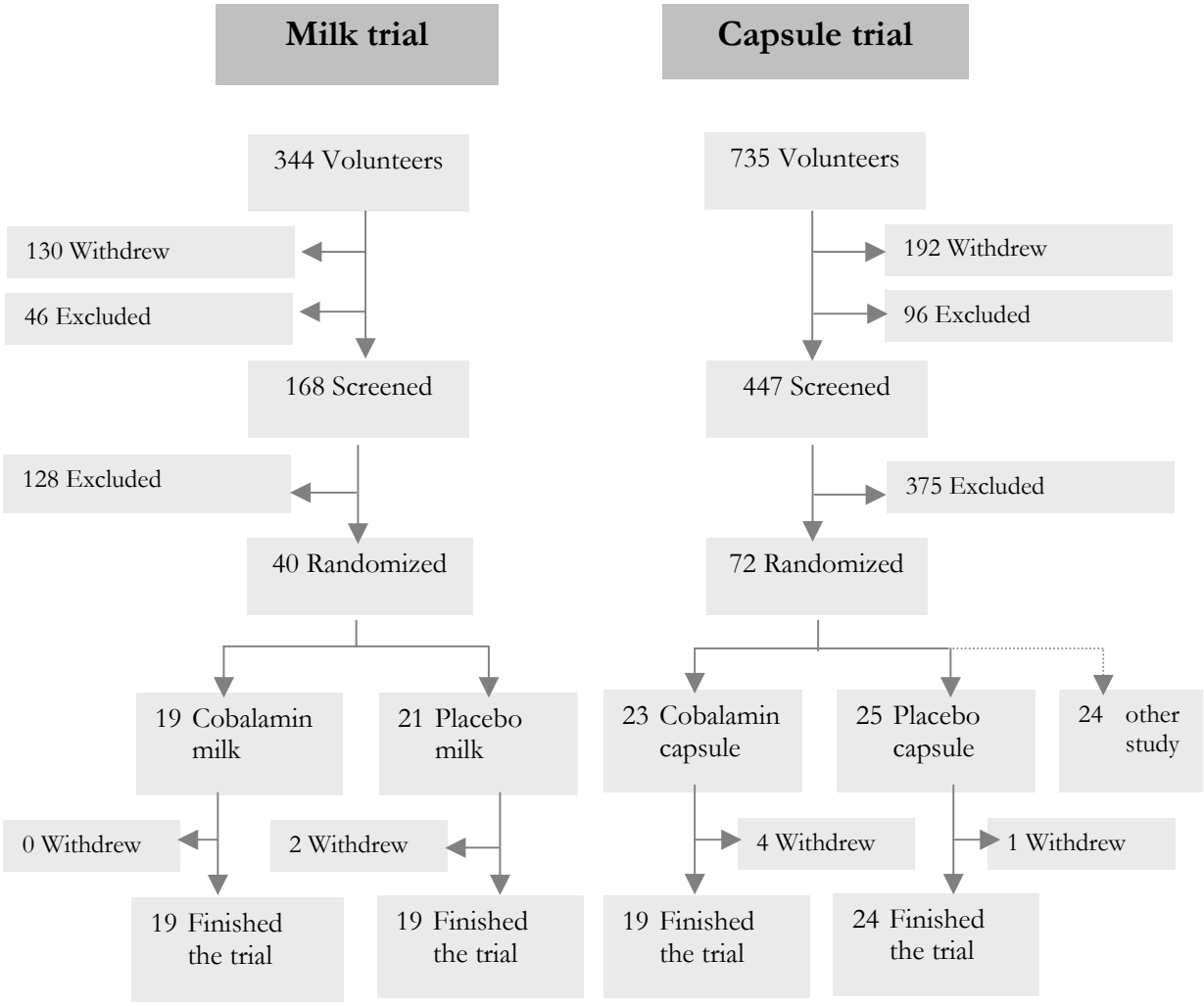

Figure 1 Participants flow and follow up of mildly cobalamin deficient Dutch participants by treatment group

Baseline Hb, Ht and MCV values were similar for all treatment groups. Only three subjects had low Hb, nine subjects had low Ht and one subject had macrocytosis. Polymorphonuclear hypersegmentation was found in 85% of the subjects and these subjects were equally distributed among the treatment groups except for the cobalamin capsule group where 59% of the subjects were diagnosed with hypersegmentation.

Most probably due to successful randomization, there were no significant differences between any of the baseline characteristics among the treatment groups (**Table 1 and 2**). Cobalamin status of one subject in the fortified milk group was improved in the period between screening and baseline measurement, so this subject could no longer be defined as cobalamin deficient at baseline and was therefore excluded from further analyses.

Table 1 Baseline characteristics of mildly cobalamin deficient Dutch participants by treatment group[1]

Characteristic	Milk group		Capsule group	
	Cobalamin (*n* = 19)	Placebo (*n* = 19)	Cobalamin (*n* = 19)	Placebo (*n* = 24)
Age (y)	81 ± 5.6	82 ± 3.7	82 ± 5.4	82 ± 4.7[3]
Women [*n* (%)]	13 (68)	13 (68)	15 (79)	18 (75)
Height (m)	1.70 ± 0.11	1.65 ± 0.09	1.65 ± 0.11[2]	1.65 ± 0.10[3]
Weight (kg)	74.4 ± 11.8	70.5 ± 11.4	70.3 ± 11.2[2]	74.2 ± 10.3[3]

1 Data are presented as mean ± SD unless otherwise indicated.
2 *n* = 18
3 *n* = 22
No significant differences were present between the groups

In the cobalamin carrier groups all biochemical changes from baseline to 12 weeks were significant, except for RBC folate concentrations in the cobalamin capsule group ($p = 0.10$) (Table 2). There were no significant biochemical changes in the placebo groups, except for an increase in MMA concentration in the milk group ($p = 0.04$).

A comparison of changes induced by the milk cobalamin carrier versus the milk placebo revealed significant differences, whereby in the milk cobalamin group MMA ($p < 0.001$) and Hcy concentrations ($p = 0.001$) had decreased and cobalamin ($p < 0.001$) and RBC folate concentrations ($p = 0.02$) had increased. A comparison of changes induced by the capsule cobalamin carrier versus the placebo revealed also significant differences, whereby the capsule cobalamin group had decreased MMA ($p < 0.001$) and Hcy concentrations ($p = 0.01$), and increased cobalamin concentrations ($p < 0.001$). RBC folate concentrations were also increased, but not significantly ($p = 0.12$).

No significant differences in effect were observed between the cobalamin fortified milk and the cobalamin capsules. Changes in cobalamin, MMA, Hcy and RBC folate were comparable between both intervention groups ($p>0.40$). These results were corroborated by two-way ANOVA (no data shown).

The cobalamin fortified milk and capsules normalized the cobalamin status of all subjects, except of five of them. Two subjects in the milk trial did not reach a cobalamin concentration of 300 pmol/L. These two subjects had low baseline cobalamin concentrations (84 and 101 pmol/L). The MMA concentration of another subject in the milk trial was reduced from 0.71 μmol/L to 0.33 μmol/L. In the capsule trial, two

Table 2 **Mean (SD) for cobalamin (Cb) concentration and median (P_{10}, P_{90}) for methylmalonic acid (MMA), homocysteine (Hcy) and red blood cell (RBC) folate concentrations in mildly cobalamin deficient participants per group at baseline, after 12 weeks and its change**

	Milk group						
	Cobalamin			Placebo			P [1]
Blood parameter	Baseline (*n* = 19)	Week 12 (*n* = 19)	Change (*n* = 19)[2]	Baseline (*n* = 19)	Week 12 (*n* = 19)	Change (*n* = 19)	
Cb (pmol/L)	182 (60)	432 (134)	**250 (96)**	195 (55)	207 (68)	12 (50)	<0.0001
MMA (µmol/L)	0.39 (0.25, 0.96)	0.22 (0.17, 0.28)[a]	**-0.19 (-0.71, 0.05)**	0.38 (0.25, 0.63)	0.44 (0.25, 0.76)[a]	0.04 (-0.08, 0.15)	<0.0001
Hcy (µmol/L)	16.0 (9.5, 21.1)	11.9 (8.4, 18.3)[a]	**-4.0 (-6.4, 0.2)**	14.8 8.5, 24.2)	15.1 (8.6, 29.4)	-0.3 (–3.1, 3.5)	0.0012
RBC folate (nmol/L)	539 (362, 908)	664 (444, 856)[a]	**68 (-228, 301)**	684 (414, 1538)	589 (331, 1810)	-59 (-337, 272)	0.0203
	Capsule group						
	Cobalamin			Placebo			P [1]
Blood parameter	Baseline (*n* = 19)	Week 12 (*n* = 19)	Change (*n* = 19)[2]	Baseline (*n* = 24)	Week 12 (*n* = 24)	Change (*n* = 24)	
Cb (pmol/L)	171 (51)	453 (165[a])	**281 (136)**	206 (64)	206 (65)	1 (37)	<0.0001
MMA (µmol/L)	0.38 (0.27, 1.31)	0.23 (0.15, 0.29)[a]	**-0.18 (-1.09, -0.02)**	0.38 (0.25, 060)	0.34 (0.27, 0.54)	0.00 (-0.11, 0.06)	<0.0001
Hcy (µmol/L)	17.6 (12.1, 24.8)*	13.3 (11.5, 19.1)[a]*	**-1.8 (-8.8, 1.0)***	14.3 (10.8, 21.9)*	14.2 (11.0, 20.4)*	-0.1 (-1.2, 2.6)*	0.0104
RBC folate (nmol/L)	600 (405, 867)	666 (453, 843)	**45 (-36, 159)**	746 530, 1243)	734 (591, 1339)	-15 (-272, 163)	0.1233

[1] Comparison of changes in cobalamin and placebo group for each carrier with Student's t-test or Mann-Whitney U test.

[a] Significantly different from baseline, $p < 0.05$.

* Hcy measurements were not available for two to three samples per group due to less plasma.

[2] The changes (in bold) between the cobalamin carriers are comparable as they are not significantly different from each other. Cb: $p = 0.41$, Student's t-test.; MMA: $p = 0.80$; Hcy: $p = 0.54$; RBC folate: $p = 0.41$, all Mann-Whitney-U test.

subjects had minor improvements (59 and 42 pmol/L) in cobalamin concentrations; their baseline values were 102 and 123 pmol/L, respectively. More importantly, the subject with a cobalamin concentration of 123 pmol/L had an increased MMA concentration of

0.46 μmol/L at baseline to 0.60 μmol/L after 12 weeks. His compliance could however not be checked, because diary and medicine boxes were not handed in.

DISCUSSION

We performed a double-blind placebo-controlled supplementation study in mildly cobalamin deficient elderly people who received daily 1000 μg crystalline cobalamin either carried by milk or a capsule. After 12 weeks the cobalamin status in both intervention groups improved to the same extent. Therefore, we conclude that high doses of crystalline cobalamin added to milk are as easily absorbed as crystalline cobalamin in capsules.

Our subjects were considered compliant in consuming the fortified milk drinks and capsules. The cobalamin status improved in all subjects who received a cobalamin carrier, except for one subject whose MMA concentration increased. Yet, compliance of this subject is doubtful. After excluding this subject from the analyses, conclusions did not change. In four other subjects cobalamin status did not normalize, although their cobalamin status improved. We presume that for three of these subjects the supplementation period was too short and that their vitamin B_{12} status would have normalized if the supplementation period had been longer. For the fourth subject it is not clear why cobalamin concentration and MMA concentration did not improve sufficiently, as favorable changes in RBC folate concentration and homocysteine concentration did occur. We do not expect that his cobalamin status would have improved more if he was supplemented with both cobalamin and folate instead of with cobalamin alone. For all other subjects, complementary folate supplementation could have been useful to lower the Hcy concentrations to a larger extent [26], though cobalamin supplementation alone appeared to be sufficient to normalize the cobalamin status in this study [27].

Our cut-off points for defining mild cobalamin deficiency are deliberately chosen based on literature and laboratory experience, but are debatable because there are no universally accepted limits for defining (mild) cobalamin deficiency. The lower limit for normal serum cobalamin ranges from 150 to 300 pmol/L [1;2;4;6], while the upper limit for normal plasma MMA ranges from 0.27 to 0.38 μmol/L [1-4]. This variation reflects differences in analytical methods, statistical analysis and differences in the composition of control populations. In future studies, holo-transcobalamin may be used as an additional indicator of true cobalamin deficiency [28], because early changes in blood cobalamin homeostasis may be detected [29]. Still, universally accepted cut-off points for holo-transcobalamin need to be defined for cobalamin deficiency.

The high prevalence of hypersegmentation (85%) is in line with the vitamin B_{12} deficiency for which was screened. Neutrophil hypersegmentation has been suggested in the past to point into the direction of vitamin B_{12} or folate deficiency [30] but may also indicate megaloblastic and pernicious anemia [24]. It can be routinely performed and may be more convenient when assays for vitamin B_{12} status are not available.

Cause of cobalamin deficiency was not assessed in this study, because we expected all mildly cobalamin deficient elderly people to benefit from the crystalline cobalamin dose. This improvement in cobalamin status does not assist in differentiating pernicious anemia from causes of malabsorption of cobalamin in food, because such an improvement would occur in all cases. Although the regular pathway of absorption (with intrinsic factor) in perniciously anemic subjects may be disturbed, approximately 1% of the oral dose will be absorbed by passive diffusion [11;31]. Still, the cause of cobalamin deficiency should be investigated and hence a permanent solution could be given instead of temporary treatment. As pernicious anemia is only present in 1-1.9% of the elderly [1;32;33] and given the low prevalence of low values of Hb and Ht in our subjects, pernicious anemia was not or minimally present in this study. In the remainder of our subjects, either deficient dietary intake or malabsorption of food-bound cobalamin are possible causes. A study among 103 Dutch elderly showed that only 6% had low dietary intakes [4]. Therefore it is more likely that most of our subjects have food-bound cobalamin malabsorption. Causes of food-cobalamin malabsorption are atrophic gastritis (which may cause a decrease in gastric acidity or hypochlorhydria), *Helicobacter Pylori* infection or gastric infection by anaerobic bacteria [34;35].

A few studies assessed either changes in biochemical parameters or studied the absorption (or matrix) of cobalamin after supplementation with radioactive crystalline cobalamin. As expected from these biochemical studies in elderly people [7-9;36] the cobalamin status improved after 12 weeks of supplementation with cobalamin capsules. All studies that assessed the absorption of crystalline cobalamin used very low doses. Therefore sufficient passive diffusion was minor. It appeared that the carrier of cobalamin is of importance because the absorption of 0.56 μg crystalline cobalamin was inhibited by egg white and egg yolk, when measured with fecal and urinary excretion in a 'normal volunteer' [37]. In a more recent study 0.25 μg crystalline cobalamin was administered in three different carriers to 16 non-cobalamin deficient older adults. Here, similar absorption percentages (measured with whole body gamma-ray counter / spectrophotometer) were observed for the carriers: 55% in water, 65% in milk 65% and 55% in bread 55% [23]. These absorption studies are difficult to compare with our study. Instead of studying the absorption of one low crystalline cobalamin dose with a radioactive marker, we administered a high crystalline cobalamin dose in milk and capsules. Cobalamin status

improved to the same extent for each carrier, although content of cobalamin in milk was somewhat 60 μg lower than in the capsules.

As mentioned before, compliance was good for both products. However, the compliance of a person voluntarily enrolled in a clinical trial may not reflect the compliance in routine medical care. A cobalamin fortified food could be helpful and could replace the capsule. The amount of 125 ml milk daily fits in the dietary pattern of many Dutch elderly, since the mean consumption of milk and milk products is above 350 ml a day and 97% of the elderly use them [38]. Fortified milk may therefore be a good alternative carrier for cobalamin capsules with the advantage of supplying energy and other nutrients at the same time. Further studies are required to assess if lower dose cobalamin fortified foods may prevent cobalamin deficiency, especially in the elderly.

ACKNOWLEDGEMENTS

We gratefully thank the participants for their enthusiastic involvement and interest. We acknowledge the directors and staff of the participating residences for their willingness to let their inhabitants participate and their hospitality at any time. Wilma Staring and Lucy Okma are acknowledged for their practical assistance and Arno van Rooij for his technical assistance. The Dutch Dairy Association is greatly acknowledged for their financial support.

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General Discussion

7

Before the start of this thesis, few studies were conducted on the association between vitamin B_{12} status and bone health. This new research area is receiving more attention since the beginning of the 21st century. The few *in vitro* studies and small clinical studies performed in the past decades and the obviously low bone mass and low vitamin B_{12} status observed in macrobiotic-fed adolescents challenged us to investigate further the association in detail. To provide a sound platform for future controlled trials examining the effects of vitamin B_{12} on bone health, we obtained more evidence on this association by re-analyzing available data from cross-sectional and observational prospective and population-based studies.

In line with our expectations, we found a relevant association between vitamin B_{12} metabolites and bone health in various studies with different study designs and diverse populations. An overview of our main findings is presented in Table 7.1. Since these observed associations broaden the scope for randomized clinical trials, we conducted an intervention study in which we assessed the relative bioavailability of vitamin B_{12} fortified milk against vitamin B_{12} capsules.

In this discussion we reflect on the most important findings of this thesis and their implications by taking into account the methodologies associated with our own studies and related literature.

METHODOLOGICAL CONSIDERATIONS

Study populations

One of the major strengths of our approach was that three different populations were studied: adolescents, free-living elderly people and frail elderly people. In all three populations we found significant associations between vitamin B_{12} status and bone health.

The adolescents (Chapter 2) represented a group of adolescents who had been following a strict macrobiotic diet early in life and a group of adolescents following an omnivorous diet. Low vitamin B_{12} status in macrobiotic adolescents was most likely due to their low vitamin B_{12} intake [1].

Low vitamin B_{12} status in elderly people is mostly ascribed to malabsorption, rather than to an inadequate dietary supply as the intake meets the American and Dutch Recommended Daily Allowance (2.4 μg/d and 2.8 μg/d, respectively) [2;3]. Though our studies focused on the majority of those who reached old age, the free-living people – including apparently healthy and frail elderly people (Chapter 4 & 5 and Chapter 3,

Table 7.1 Overview of the main findings and conclusions on the association of vitamin B_{12} status with bone health

Research question	Study	Results	Conclusions
Is an inadequate cobalamin status associated with low bone mineral content (BMC) or bone mineral density (BMD) in **adolescents**?	Cross-sectional study of 167 adolescents who were formerly fed a macrobiotic diet and their counterparts (Chapter 2)	Adjusted geometric means (GMs) of cobalamin status were significantly worse in adolescents with a low BMC and low BMD than compared to those with a normal BMC and BMD. Adjusted GMs of methylmalonic acid (MMA) remained significantly higher in adolescents with a low BMD than in adolescents with a normal BMD when studied in the macrobiotic group alone.	An association between the vitamin B_{12} status and BMD is present and this is probably not confined to the macrobiotic diet alone (page 129).
Is vitamin B_{12} status related to BMD and BMC in **frail elderly people**?	Cross-sectional study of 143 women and 51 men (Chapter 3)	In women, 3.1% of the total variance (46%) of BMC was explained by the vitamin B_{12} status. Women with a deficient vitamin B_{12} status had a prevalence odds ratio of 6.9 for osteoporosis in comparison to women with a normal vitamin B_{12} status.	An association of vitamin B_{12} status and bone health was only found in women and not in men (page 131).
Does a relationship exist between homocysteine levels and the incidence of fracture in **free-living elderly** of 55 years or older?	Two prospective population-based studies: Longitudinal Aging Study Amsterdam (LASA) and the Rotterdam Study (Chapter 4)	The overall multivariable-adjusted relative risk of fracture 1.4 (95%c.i. 1.2-1.6) for each increase of 1 SD in the natural-log-transformed homocysteine level. A homocysteine level in the highest age-specific quartile was associated with an increase by a factor of 1.9 in the risk of fracture (95%c.i. 1.4-2.6) (page 131).	The risk of fracture that was attributable to a homocysteine level in the highest age-specific quartile was 19%.
What is the relation of homocysteine and vitamin B_{12} levels with bone turnover markers, broadband ultrasound attenuation (BUA) and fracture incidence in **free-living elderly** of 65 years or older?	Prospective study and cross-sectional study: LASA (Chapter 5)	In women, a low vitamin B_{12} and high Hcy concentration was associated with lower adjusted BUA and higher adjusted bone turnover markers. In men, no associations were found. Women with high Hcy and/or low vitamin B_{12} concentrations had an adjusted RR for fractures of 2.9 (95% c.i. 1.4-6.0) compared to those with a normal status. In men, the RR for fractures was 3.5 (95% c.i. 1.1-10.8) (page 132).	High plasma levels of Hcy and low serum levels of vitamin B_{12} and especially the combination of these two levels are related to high fracture risk, to low broadband ultrasound attenuation and to increased bone turnover markers.

respectively), we presume that the associations we found also exist in institutionalized elderly. Among them a high risk of hip fractures [4] concurs with a high prevalence of vitamin B_{12} deficiency as found in Spanish [5] and in Dutch institutionalized elderly people [6-8]. Such combination of unfavorable features makes it worth to screen for vitamin B_{12} deficiency and subsequently treat these institutionalized elderly to normalize their vitamin B_{12} status.

It must be noted that studies in a more heterogeneous group, such as the free-living elderly people rather than institutionalized elderly people, offer a broader range of parameters of interest. This provides the possibility of adjusting for confounders in our studies. We confirmed the associations between vitamin B_{12} status and bone health in the free living elderly people as found in adolescents and frail elderly people, also after adjusting for several confounders.

These findings reveal that, in terms of bone health, concerns related to vitamin B_{12} status are not confined to one specific population but to all the populations mentioned above.

Study design

Most of the findings presented in this thesis are based on cross-sectional data (Chapter 2, 3 and 5). Though cross-sectional data may give clues to possible relations, the main drawback of a cross-sectional design is that exposure and outcome are measured at the same time, and, therefore, causal inference is not possible. Yet, a causal relation may nevertheless exist between the vitamin B_{12} status and bone health, because low vitamin B_{12} status is by and large a prolonged condition, especially in (previously) macrobiotic-fed adolescents (Chapter 2), and might therefore affect bone health. In order to preclude the effect of other factors already proven to be related to bone health, such as physical activity, vitamin D and calcium intake or other unknown variables, we conducted a sub-analysis including only the macrobiotic group. This group is assumed to be a more homogenous group for factors such as diet, physical activity and other unknown variables. Again, we found a significantly higher MMA concentration in the group with a low BMD than in the group with a normal BMD.

The sequence of decline in vitamin B_{12} status and bone health in free-living elderly people is less certain (Chapter 3, 4 and 5). These populations are considered more heterogeneous than the adolescents, implying that in addition to vitamin B_{12} status more factors may have influenced their bone health than in the younger age group. Prospective data provide a better insight in the sequence of changes. In Chapter 4 and 5, in which data collected from prospective, population-based cohorts have been used, large relative risks for osteoporotic fractures were found when subjects had a high homocysteine level or a low

vitamin B_{12} level. Although not completely proven, indications for causality are stronger than in case of a cross-sectional design.

Though many design issues can be discussed in relation to our final study with improved vitamin B_{12} status resulting from two different products carrying vitamin B_{12} (Chapter 6), we will only touch the two most important issues, namely type of study and duration of intervention.

Type of study: We conducted two randomized trials in parallel. Because of practical reasons we did not perform one combined intervention study including both cobalamin carriers and placebo treatments. Furthermore inclusion of a group receiving fortified milk and vitamin B_{12} capsules would have resulted in intakes of 2000 μg cobalamin per day, which would have been incongruous. An attempt to alter this would have affected dose, design and compliance. A cross-over trial design was not possible for any of the parallel trials due to the long wash-out period required in view of the low excretion of vitamin B_{12}.

Duration of intervention: The intervention lasted for twelve weeks in both studies. Other reports suggest that significant and relevant changes in vitamin B_{12} status could be found only after 12 weeks or more [9-12]. In contrast, a recent dose-response study conducted within our research group at Human Nutrition (Wageningen University) relevant changes were already observed after two months of intervention (Eussen, submitted). Thus it turned out that intervening with high dose vitamin B_{12} products for three months is more than sufficient to improve the vitamin B_{12} status of mildly vitamin B_{12} deficient elderly people.

Vitamin B_{12} status

So far there exists no consensus for the best method possible for assessing vitamin B_{12} status. Over the past 10 years it became clear that vitamin B_{12} status is properly evaluated by a combination of biochemical measures in blood: cobalamin levels and methylmalonic acid (MMA) levels, which is highly specific for vitamin B_{12} deficiency, as has been described in Chapter 1. Valuable information can also be derived from homocysteine (Hcy) levels, despite the fact that these data are influenced by many other factors such as age, sex, lifestyle factors, genetics, drugs and folic acid status [13]. Holo-transcobalamin II might be an even better marker of vitamin B_{12} status, because it is considered to provide early indications of changes in cobalamin homeostasis [14]. This is because holo-transcobalamin II carries the biologically available cobalamin and promotes specific uptake of its cobalamin by all cells [15].

Vitamin B_{12}, MMA and Hcy levels were investigated in our studies for determining the association between vitamin B_{12} status and bone health. Not each parameter showed similar associations with bone health. In the adolescents study (Chapter 2), we found an association of vitamin B_{12} and MMA with bone mineral density (BMD) and bone mineral content (BMC), but not between Hcy and BMD or BMC. This can be explained by the high serum folate concentration – especially observed in the macrobiotic-fed adolescents – which may have a suppressing effect on Hcy levels [16]. Thus, an association of homocysteine status with bone mass may less likely be found in people who are following a vegetarian diet rich in folate that causes less variation in Hcy levels.

In case of the frail elderly people (Chapter 3) we have mainly focused on the cobalamin levels since multiple regression analyses indicated a significant contribution of serum vitamin B_{12} status to the explained variance of BMD and BMC. Surprisingly, neither MMA nor Hcy status were found to be associated with BMD or BMC. This might be due to the smaller range of these two indicators: less people were diagnosed with high levels of MMA or Hcy. Here, serum vitamin B_{12} status turned out to be a strong predictor of osteoporosis. Although vitamin B_{12} status explained only 3% of the explained variance of BMD, we should keep in mind that 60% to 80% of the variance in peak bone mass is determined by genetic factors [17]. More important is the observation of the prevalence odds ratios for having osteoporosis. This odds ratio was seven times higher in women with severe vitamin B_{12} deficiency than for women with a vitamin B_{12} status within the normal ranges. No such associations were observed in men, probably due to the low number of men (n = 51) who participated in our study.

In the prospective studies in free-living elderly people (Chapter 4 and 5) we mainly studied homocysteine status and vitamin B_{12} status, because data on MMA levels were only available for ≈ 50% of the participants of the LASA study. The highest Hcy quartile (age-specific and sex-specific) was associated with a more than 2-fold risk of fractures in men compared to men in the lower three Hcy quartiles. These risks were not confirmed in women by quartile comparisons, neither did a test for trend. In women, the lowest vitamin B_{12} quartile was associated with a two-fold risk of fractures compared to women with normal vitamin B_{12} levels. We were unable to explain the differences observed between men and women. Hormone replacement therapy or a different hormonal status in women as compared to men might be the key factor in the observed different associations between men and women. However estradiol levels did not alter the associations in men and women in our study. Unfortunately, more information on hormonal status was not available. Previous studies with hormone replacement therapy point towards a lowering effect on homocysteine status [18-22]. In contrast to the differences in effect between the sexes, the combination of Hcy and vitamin B_{12} status

showed clear and high fracture risks in both men and women. Thus it seems recommendable to investigate the combination of the indicators rather than the two indicators separately. Subjects with both unfavorable levels had severe vitamin B_{12} deficiency. As data on folate levels were not available, we were not able to differentiate between vitamin B_{12} deficiency and folate deficiency. Elevated Hcy levels are highly correlated with low folate levels and low vitamin B_{12} levels [23;24]. Therefore our study does not provide conclusive evidence on the dominance of vitamin B_{12} status over Hcy/folate status.

Studies on gene polymorphisms that have an effect only on Hcy status [25], such as MTHFR gene polymorphism, might give an insight in the role of Hcy in osteoporosis outcome measures. Miyao *et al.* became the first group to investigate the MTHFR gene polymorphism in relation to bone [26]. A lower BMD was found in Japanese [26] and Danish postmenopausal women [27] with the MTHFR TT genotype as compared to women with the CC or CT genotype. Abrahamsen *et al.* showed also that women with the TT genotype had more than 2-fold risk for fracture than those with the CC or CT genotype [27]. In another Danish (twin) study, the odds ratio for fracture was 1.5 per number of T-alleles [28]. Unfortunately the association between Hcy concentrations and fracture risk was not assessed, although data on Hcy were available in this study. McLean *et al.* [29] found an association of CC, CT and TT individuals with BUA and BMD after dividing the individuals in low and normal folate status. For example, TT individuals with low folate status had lower mean BUA and Ward's area BMD compared to CC and CT individuals, but a higher neck BMD was observed within the normal folate status group. Contradictory results were, however, found in another Danish case-control study showing increased odds ratios for fracture in postmenopausal women with the wild-type C-allele [30]. These studies suggest a possible gene-nutrient interaction of folate status with the C677T mutation on bone phenotypes that needs thorough investigation.

In Chapter 6 we used vitamin B_{12} and MMA levels as primary outcome measures for reflecting the vitamin B_{12} status and homocysteine and folate as secondary outcome measures. Both vitamin B_{12} and MMA levels improved significantly after treatment with vitamin B_{12} in capsules or fortified milk. Homocysteine levels decreased in both groups and folate levels improved only significantly in the group which received vitamin B_{12} fortified milk. It would have been interesting to know whether serum holo-transcobalamin II corroborated the improvement of vitamin B_{12} status in our study. Information on holo-transcobalamin II levels - which promotes the cellular uptake of cobalamin - may give a conclusive answer on the required duration of vitamin B_{12} treatment. It is expected that improvement of vitamin B_{12} status would have been

confirmed or even be earlier observed with elevations in holo-transcobalamin II levels [14;31].

While evaluating vitamin B_{12} status in population-based studies, measurement of plasma MMA and plasma Hcy levels should be included and in intervention studies plasma MMA and serum holo-transcobalamin II levels should be measured. In addition, vitamin B_{12} levels can be measured to corroborate the results and serum creatinine levels should be measured for excluding subjects with impaired renal function.

Indicators of bone health

The outcome measures in the association studies were bone mineral content (BMC), bone mineral density (BMD), broadband ultrasound attenuation, the bone turnover markers: urinary deoxypyridinoline excretion and serum osteocalcin, and fractures.

As described in Chapter 1, BMC data are more appropriate than BMD data. Though until now it has been widely accepted to use BMD data above BMC data. In this thesis, we present both BMD and BMC data allowing for comparisons with literature and acknowledging the relevance of BMC. For the latter we found similar associations with vitamin B_{12} status as with BMD data. These BMD data were used to define osteoporosis which turned out to be more prevalent in vitamin B_{12}-deficient frail elderly women than in their counterparts with a normal vitamin B_{12} status (Chapter 3). Information on bone loss was not available in our studies, but Stone *et al.* [32] showed an interesting association of low vitamin B_{12} levels with increased hip bone loss in older women. Unfortunately, no information was available on homocysteine and methylmalonic acid levels to strengthen these findings. Another study showed that subjects in the lowest folate quartile had a lower BMD than in the highest folate quartile. Although vitamin B_{12} and homocysteine concentrations were not independently related to BMD and results were not shown, it must be noted that homocysteine and vitamin B_{12} concentrations in this study were considered as normal [33].

Broadband ultrasound attenuation (BUA) was measured in the LASA study (Chapter 5). This method is easy to apply, inexpensive and free of radiation. It has been suggested that broadband ultrasound attenuation not only depends on bone density but also on bone structure and elasticity [34]. Therefore, this measure can be used as a predictor of fractures [35;36]. The concordance in the observed association of the vitamin B_{12} status with broadband ultrasound attenuation and with fractures, suggests that the broadband ultrasound attenuation method would be useful for future studies investigating this association in other populations. In our study with free-living elderly people we observed that women with low vitamin B_{12} and high homocysteine status had lower mean BUA

values, after adjustment for several confounders, compared to women with a normal vitamin B_{12} and homocysteine status.

A range of bone turnover markers is available nowadays. In the LASA study, urinary deoxypyridinoline excretions and osteocalcin concentrations were chosen as measures of bone turnover. DPD/creatinine (urinary excretion of deoxypyridinoline corrected for creatinine), a bone resorption marker, is considered a good predictor of bone density and fracture risk [35-37]. Osteocalcine is a noncollagenous protein [38] and bone formation marker. We are not aware of any studies assessing the association between any of the bone turnover markers and vitamin B_{12} status. We showed that women with a low vitamin B_{12} status and a high homocysteine status had significantly higher osteocalcin levels than women with a normal vitamin B_{12} and homocysteine status. Another interesting bone turnover marker is undercarboxylated osteocalcin. The degree of carboxylation of osteocalcin seems to influence bone mineralization. [39]. Low levels of undercarboxylated osteocalcin may reflect poor nutritional status and in particular vitamin D and vitamin K status in elderly people [40;41]. We do not have data on undercarboxylated osteocalcin status in our studies, but we examined whether vitamin D status influenced our investigated associations (Chapter 3 and 4). After dividing the frail elderly people (Chapter 3) in three categories for osteoporosis (normal, osteopenia and osteoporosis), vitamin D concentrations remained similar for all categories. Adjusting for vitamin D status in the LASA study (Chapter 4) did not alter the risk estimates. Data on vitamin K status were not available in our studies. From our studies it cannot be concluded whether vitamin B_{12} status is a culprit or a bystander, only that it is somehow related to bone health. We should, however, keep in mind that the cause of a low vitamin B_{12} status in elderly people is the malabsorption of vitamin B_{12} and that it is not due to low vitamin B_{12} intake. This may imply that, instead of low nutritional status, vitamin B_{12} status itself is truly associated with bone health.

Of most clinical relevance for osteoporosis is the occurrence of fractures, which are hard endpoints. Therefore, the assessment of fractures is considered a more useful instrument to assess the association of bone health with vitamin B_{12} status than all the above-mentioned instruments. The ascertainment of fractures but also falls in the LASA study was based on self-report by means of a three-months calendar. A physician verified all fractures. In case of doubt, radiographs were obtained in the hospital and checked by a clinician. Although several efforts were undertaken, such as contact by telephone with participants who did not return the calendar every three months, misclassification in this ascertainment might have been occurred [42]. Consequently, the strengths of the association between the vitamin B_{12} status and fractures may have been somewhat underestimated. We observed a two-fold higher relative risk for suffering from an

osteoporotic fracture when subjects were in the highest quartile of homocysteine levels compared to the lower three homocysteine quartiles. These findings were confirmed in the Framingham Study where men in the highest quartile of homocysteine had a four times higher risk and women a 1.9 times higher risk to suffer from a hip fracture than those in the lowest homocysteine quartile [43]. It is, however, possible that this higher hazard ratio for risk of hip fracture is due to age rather than homocysteine, because these ratios were not estimated with the use of age-specific quartiles but only with adjustment for age.

In summary, we found associations of the vitamin B_{12} status with BMD, BMC, broadband ultrasound attenuation, bone turnover markers and fractures in three different populations. As we observed associations of vitamin B_{12} and homocysteine status with broadband ultrasound attenuation and the bone turnover marker DPD/cr, we suggest that this status could interfere with the microarchitecture of bone and not only with the amount of mineral in bone. With respect to this speculation, it is most valuable and easy to incorporate measurements of bone turnover markers related to the collagen network, measurements of broadband ultrasound attenuation and assessment of fractures in future population-based studies.

SUGGESTED MECHANISMS

Our findings of the association of vitamin B_{12} status and bone health based on several populations are novel. We elaborated briefly on possible mechanisms in the Introduction (Chapter 1) and here we return to these mechanisms. In the eighties, clinical vitamin B_{12} deficiency was suggested to be associated with defective functional maturation of osteoblasts [44] and it has been shown that vitamin B_{12} had a stimulating influence on osteoblast proliferation and alkaline phosphatase activity [45]. The mechanism behind this proliferation of osteoblasts and increased activity of alkaline phosphatase is not known. Nevertheless, an association of vitamin B_{12} status and Hcy status with osteocalcin was observed in our study. This suggests that low vitamin B_{12} status and elevated Hcy levels may affect bone formation.

This mechanism might be interrelated with another explanation underlying the association of Hcy with bone which has been proposed earlier in the sixties [46]: Hcy might interfere with collagen cross-linking due to structural similarity of Hcy to D-penicillamine. D-penicillamine is known to interfere with stable collagen cross-linking by binding to the precursor aldehydes [46]. Cross-links are important for the stability and strength of the collagen network. Interference in cross-link formation would result in an altered bone

matrix further resulting in more fragile bone. This interference was confirmed in two homocystinuric, mentally retarded patients who had an abnormal collagen profile, although two other, not mentally retarded, patients had a normal collagen profile [47]. Similar results were found in another study where three homocystinuric patients had lower cross-link contents and higher solubility of dermal collagen as compared to age-matched controls [48]. The higher solubility implies a defect in the intra-molecular cross-link. In a larger study with homocystinuric patients it was shown that these patients had lower amounts of collagen cross-links in serum than normal controls [49]. Though homocystinuria is often accompanied by several other medical conditions [50], which could influence collagen cross-links as well, our study provides further evidence for the interference of cross-links in bone. We observed that women with elevated Hcy concentrations had higher DPD/cr concentrations than those with a normal Hcy concentration.

In contrast to the disturbed cross-linking in collagen formation in bone, an enhanced dose-dependent collagen production and accumulation of smooth muscle cells because of homocysteine has been observed in the cardiovascular research field [51]. Collagen is a critical component of atherosclerotic lesions. Uncontrolled collagen accumulation leads to arterial stenosis [52]. Aortic calcification is a common feature among women with osteoporotic vertebral fractures and the degree of aortic calcification is inversely related to bone mineral density. Moreover, low bone mineral content or density appears to be a risk factor for increased mortality in later life, especially for cardiovascular disease [53-57]. Although the current evidence linking osteoporosis and cardiovascular disease is only superficial, it must be noted that many risk factors co-occur for both cardiovascular disease and low bone mineral density as reviewed by McFarlane *et al.* [58].

It is clear that these mechanisms, and especially the interference of homocysteine on collagen synthesis or disturbance, need further investigation at the cellular level with the incorporation of *in vitro* studies that include identification of (novel) target genes affected by vitamin B_{12} and homocysteine in (differentiating and proliferating) osteoblasts and smooth muscle cells (hallmarks for atherosclerosis) combined with gene profiling.

FUTURE DIRECTIONS

This thesis provides a sound basis for future (randomized controlled) trials for disentangling whether vitamin B_{12} status is one of the true underlying cause of low bone health. We observed similar associations between vitamin B_{12} status and bone health in thee different populations. We propose three types of studies in free-living elderly people

with (mild) vitamin B_{12} deficiency in order to investigate whether vitamin B_{12} (and folate) supplementation can truly exert a positive influence on bone health:

Large and long-term randomized clinical trials, with at least two to three years follow-up, with emphasis on bone mineral density, bone mineral content, broadband ultrasound attenuation and fractures

Small and short randomized clinical trials with emphasis on bone turnover markers as outcome measures

Ongoing studies in which vitamin B_{12} and folate treatment is provided and data on fracture incidence is collected

Large and long-term randomized clinical trials should preferably be performed with a placebo-controlled design. The most important endpoint in this trial would be the incidence of fractures. Though other studies have shown that biochemical vitamin B_{12} deficiency can be reversed within an intervention period of three months, it is still unclear whether it is possible to reverse functional vitamin B_{12} deficiency and, if so, whether it is possible within this specific time period. Moreover, the process of new bone formation can vary in length from under three months to more than a year. Therefore, a longer period of intervening is preferred and possibly with a lower dose than during a short period of intervening. A follow-up period of at least two to three years will be required to deduce the efficacy of vitamin B_{12} treatment on the reduction of fracture occurrence. Obviously a longer follow-up period is preferred. This type of design would directly support an inference of efficacy.

The Declaration of Helsinki, however, urges to test new methods against the best current prophylactic, diagnostic and therapeutic methods [59]. This type of study is called 'comparator study' or 'study of equivalence'. An advantage of this design is that fewer patients will be disadvantaged because the control wing is an active drug. This design would be preferred above placebo-controlled trials. Such an equivalent trial would comprise very large samples when vitamin B_{12} treatment is evaluated against an established treatment either alone or in combination, such as vitamin D and calcium supplementation. Confidence intervals around the estimates for the two agents need to be narrow enough to reveal a biologically or clinically important difference between them [60]. The null hypothesis would be that no difference in outcome measures exists between the established treatment and vitamin B_{12} treatment. The alternative hypothesis would be that vitamin B_{12} treatment is better than the established treatment. Even when large sample sizes are used, a finding of no significant difference between groups means that the two agents are equivalent – equivalently efficacious or equivalently inefficacious. It is hard to know its absolute efficacy, because no placebo control is present in such a trial.

The incidence of fracture is the most relevant outcome measure, but bone mass and remodeling activity are also valuable outcome measures because increased BMD and lowered remodeling rates reduce fracture risk independent of each other. These endpoints would result in shorter trials and smaller samples. Various studies have been performed with vitamin B_{12} treatment, folate treatment or the combination of these two in elderly people, either deficient or with a normal vitamin B_{12} or folate status. In these studies, measurements of bone turnover markers such as (undercarboxylated) osteocalcin and (bone) alkaline phosphatase (bone formation markers) and tartrate-resistant acid phosphatase (TRAP) [61], aminoterminal propeptides (PINP) cross-linked carboxyterminal (ICTP) and aminoterminal (NTX) telopeptides of type I collagen [62] (bone turnover markers) could be performed in spare serum samples.

Unfortunately, at present the perfect bone turnover marker does not exist. A valid interpretation of bone turnover marker values remains complicated and not always reliable. Therefore a better and more complete answer to the effect of vitamin B_{12} (and folate) treatment on bone health could be given by ongoing (cohort) studies in which vitamin B_{12} and folate treatment is provided. These studies are presently mainly performed in the cardiovascular field, in which retrospectively (or prospectively when the study is still in its beginning phase) data on fracture incidence can be collected.

Besides studying elderly people with (mild) vitamin B_{12} deficiency, there is scope for assessing the effect of vitamin B_{12} treatment (in combination with folate treatment) on bone health in several populations with vitamin B_{12} deficiency in developing countries. In these countries vitamin B_{12} intake is often low and prevalence of vitamin B_{12} deficiency is high (e.g. in India [63]), also the bone mineral density is lower [64] and the absolute number of fractures is higher [65;66] compared to developed countries. Certainly, other variables, such as genetics, dietary intake, physical activity and other environmental circumstances are different and should be taken into account as well.

EPILOGUE

It has become clear that an association between the vitamin B_{12} status and bone health does exist in several populations, but causality remains unclear. Even though genetic studies have suggested that 60% to 80% of the variance in peak bone mass is determined by non-modifiable factors [17], a complex of nutrients and food constituents interact and affect bone status. Plasma vitamin B_{12} levels explained 3% of the variance in BMD in frail elderly women (Chapter 3). It is not feasible to infer from this explained variance a lower preventable number of fractures. Still, it is clear that on the population level improvement

of vitamin B_{12} status could substantially contribute to fewer fractures and a lower burden on public health and health care costs. The calculated population attributable risk of the effects of increased homocysteine levels in free-living elderly is indeed considerable (Chapter 4). A homocysteine level in the highest age-specific quartile conferred a 19 percent attributable risk in our population. This population attributable risk (PAR) can be regarded as an index for the number of preventable fractures. This PAR is similar to that of well-known risk factors for fracture in the study population, such as low bone mineral density and recent falls.

Trials are essential to study causality into further detail and to give conclusive answers on the number of preventable fractures. Furthermore, genetic factors are likely to interact with these dietary exposures and will increase the complexity of the association between vitamin B_{12} status and bone health. With advances in both nutrition and genetics, an improved understanding of these interactions will contribute to recommendations for both bone growth and for prevention of bone loss and osteoporosis in the ageing population. Improving vitamin B_{12} status in elderly people seems possible by consuming food fortified with vitamin B_{12} which is as effective as vitamin B_{12} capsules. However, further evaluation of such fortification should include dose, feasibility, potential benefits, adverse effects, stability of the fortificant, identification of any degradation products, and bioavailability in normal subjects and in those with atrophic gastritis. The required dose for the general elderly population may need to be lower than that for elderly people with (mild) vitamin B_{12} deficiency.

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Summary

Along with the growing number of elderly people, there is an increased prevalence of illness, which is accompanied with an intensification in functional impairment, disability and lower quality of life. Specific for old age – but starting earlier in life – is bone demineralization leading to osteoporosis. In this thesis, we focus on the role of nutrition in bone health and particularly on vitamin B_{12} status. Vitamin B_{12} deficiency is highly prevalent in old age. New insights in the metabolism of vitamin B_{12} pointing to a relation of vitamin B_{12} status with osteoporosis are emerging.

VITAMIN B_{12}

Vitamin B_{12} is mainly found in foods of animal origin. Therefore, people consuming a vegetarian or lactovegetarian diet have a lower vitamin B_{12} intake and may develop vitamin B_{12} deficiency. The majority of older adults with vitamin B_{12} deficiency appear to have food-bound vitamin B_{12} malabsorption due to gastrointestinal changes. In humans, vitamin B_{12} exerts its physiologic effect on two major enzymatic pathways. Diminished activity in both pathways results in an accumulation of serum methylmalonic acid (MMA) and homocysteine (Hcy). Vitamin B_{12} deficiency is associated with hematological manifestations, growth and psychomotor retardation, neural tube effects, neurologic problems (cognitive impairment), cardiovascular diseases, or abnormalities in bone marrow or in the small intestine.

Although functional vitamin B_{12} deficiency ultimately may cause irreversible neurologic damage, biochemical vitamin B_{12} deficiency is well treatable with parenteral injections, oral supplementation with capsules or fortified food products.

BONE HEALTH

Osteoporosis is a multifactorial disease and defined as "a systemic skeletal disease characterized by low bone mass and microarchitectural deterioration of bone tissue, with a consequent increase in bone fragility and susceptibility to fractures". Osteoporotic fractures create a major burden to public health. Fractures are associated with increased

morbidity and mortality, impaired quality of life and high costs for society. Annually, in The Netherlands, more than 15.000 people of 65 years and older are admitted to hospital because of a hip fracture. The incremental cost for a Dutch person with a hip fracture is estimated to be € 10.000 in the first year after a hip fracture.

The most important techniques and assessments of bone health are measurements of bone mineral density (BMD), bone mineral content (BMC), bone turnover markers which show the level of the bone remodeling process, and fractures. Biological, environmental and genetic factors as well as lifestyle factors play an important role in the onset and development of osteoporosis. Age is another important predictor of bone health. Prevention and treatment of osteoporosis are directed towards nutrition and lifestyle factors or medical intervention. Medical research concentrates on hormone replacement therapy (HRT), which has a consistent, favorable effect on bone density. Regrettably, estrogen therapeutic treatments have disadvantages such as an increased risk of breast and endometrial cancer. Although a low percentage (20%-40%) of the bone mass can be explained by nutritional and lifestyle factors, they still can have a large impact on bone health. Dietary therapy of vitamin D and calcium and especially the combination of these two is promising as most of the times it shows beneficial effects on BMC, BMD, bone turnover and a reduced risk on fractures without serious adverse side effects. Other dietary factors, such as vitamin B_{12}, vitamin K, proteins, fluoride and caffeine are subject of bone research as well.

VITAMIN B_{12}: A NOVEL INDICATOR OF BONE HEALTH

Vitamin B_{12} and homocysteine status have been suggested to play a role in osteoporosis. Few studies were conducted on the association between vitamin B_{12} status and bone health. This new scientific topic is receiving more attention since the beginning of the 21st century. The few *in vitro* studies and small clinical studies performed in the last decades challenged us to investigate this association in further detail. As to provide a stronger basis for future controlled trials in which effects of vitamin B_{12} on bone health will be examined, we obtained more evidence from cross-sectional and observational prospective and population-based studies on this association.

In this thesis we have shown a relevant association between vitamin B_{12} metabolites and bone health in various studies with different study designs and diverse populations.

Three populations were studied: previously macrobiotically fed adolescents and their counterparts, free-living elderly people and frail elderly people. The study with adolescents as subjects gave us the first indications of a possible association between

vitamin B_{12} status and bone health. We showed that the vitamin B_{12} status was less favorable in adolescents with a low bone mass or bone mineral density (Chapter 2). These findings were confirmed by a study in frail elderly people (Chapter 3) and particularly in elderly women. Women with a vitamin B_{12} deficiency had seven times more often osteoporosis (OR, 6.9; 95% confidence interval [c.i.], 1.2 to 39.4) than women with a vitamin B_{12} status within normal ranges. These results were corroborated by our findings in free-living elderly people (Chapter 4). Here, data from three large prospective cohort studies were combined from two independent prospective studies: the Longitudinal Aging Study Amsterdam (LASA) and the Rotterdam Elderly Study. We clearly showed that free-living elderly people with high homocysteine levels had a higher fracture risk. A homocysteine level in the highest age-specific quartile resulted in a two-fold increase in fracture risk (RR, 1.9; 95% c.i. 1.4 to 2.6). In Chapter 5 we elaborated on the LASA study more into detail. Hence, we found that either a high homocysteine concentration, a low vitamin B_{12} concentration or the combination of both was accompanied with a high fracture risk: 2.9 (95% c.i. 1.4 to 6.0) in women and 3.5 (1.1 to 10.8) in men. The occurrence of fracture is considered as a barometer and of most clinical relevance for osteoporosis. Although not fully proven, indications for causality in these prospective studies are stronger than in a cross-sectional design.

Finally, we assessed whether fortified vitamin B_{12} milk was as effective as vitamin B_{12} capsules for improving the vitamin B_{12} status in vitamin B_{12} deficient elderly people (Chapter 6). Indeed, we did not observe significant differences in the changes of the vitamin B_{12} status between the two groups who were supplemented with the vitamin B_{12} carrier (either fortified milk or a vitamin B_{12} capsule).

Our findings of the association of vitamin B_{12} status and bone health based on several populations are new. Clinical vitamin B_{12} deficiency was suggested to be associated with defective functional maturation of osteoblasts (bone-forming cells). It has been shown that vitamin B_{12} had a stimulating influence on osteoblast proliferation. The mechanism behind this is not known. Another explanation of the mechanism underlying the association of Hcy with bone is the interference of homocysteine with collagen cross-linking. Cross-links are important for stability and strength of the collagen network in bone. This interference may lead to an altered bone matrix resulting in more fragile bone.

It is clear that these mechanisms, and especially the interference of homocysteine on collagen synthesis or disturbance, need more investigation on the cellular level with the use of *in vitro* studies including identification of (novel) target genes that are directly or indirectly affected by vitamin B_{12} and homocysteine in (differentiating and proliferating) osteoblasts and smooth muscle cells (which are hallmarks for atherosclerosis) and its gene expression. On the individual and population level, three possible studies in free-living

elderly people with vitamin B_{12} deficiency are proposed in order to investigate whether vitamin B_{12} (and folate) supplementation can truly exert a positive influence on bone health via reduced homocysteine levels: 1) Large and long-term randomized clinical trials, with at least two to three years follow-up, with emphasis on bone mineral density, bone mineral content, broadband ultrasound attenuation and fractures, 2) Small and short randomized clinical trials with emphasis on bone turnover markers as outcome measures, 3) Ongoing studies with folate and vitamin B_{12} treatment and retrospective data collection on fracture.

Besides studying elderly people with (mild) vitamin B_{12} deficiency, there is scope for assessing the effect of vitamin B_{12} treatment (in combination with folate treatment) on bone health in several populations with vitamin B_{12} deficiency in developing countries. In these countries vitamin B_{12} intake is often low and prevalence of vitamin B_{12} deficiency is high (e.g. in India), also the bone mineral density is lower and the absolute number of fractures is higher compared to developed countries. Certainly, other variables, such as genetics, dietary intake, physical activity and other environmental circumstances are different and should be taken into account as well.

CONCLUSIONS

It has become clear that an association between the vitamin B_{12} status and bone health does exist in several populations, although causality remains unclear. Even though sex, age and non-modifiable genetic factors explain 60% to 80% of the variance in bone mass, a complex of nutrients and food constituents can have a large impact on bone status.

Although, it is not feasible to infer from our information a lower preventable number of fractures, it is clear that on the population level the improvement of vitamin B_{12} status could substantially contribute to less fractures and a lower burden on public health and health care costs. Trials are essential to study this into further detail and to give conclusive answers. With respect to designing these kind of trials, we showed that fortified vitamin B_{12} food products are as effective as vitamin B_{12} capsules.

Samenvatting

Met het snel stijgende aantal ouderen in de samenleving neemt het belang van inzicht in een optimale voeding voor de oudere mens toe. Een adequate voeding is namelijk één van de elementen die er toe bijdraagt om de latere levensjaren in goede gezondheid door te kunnen brengen. Naar verwachting zal de bevolking in het jaar 2030 voor circa vijfentwintig procent uit 65-plussers bestaan tegenover 14% in 2003. Door deze vergrijzing neemt de prevalentie van ziekten toe. Dit gaat gepaard met lichamelijk ongemak, hoog medicijngebruik en psychologische en sociale veranderingen. Voedingsinterventies laten zien dat het mogelijk is de biochemische lichamelijke status in verschillende groepen ouderen te verbeteren. Verbetering in de functionele status met behulp van voedingsinterventies wordt echter niet altijd waargenomen en behoeft meer onderzoek.

VITAMINE B_{12}

Mensen krijgen vitamine B_{12} voornamelijk binnen via de voeding. Dit vitamine zit in dierlijke producten zoals vlees, melk en eieren. Fruit en groenten bevatten geen vitamine B_{12}. Wie gezond en gevarieerd eet, krijgt voldoende vitamine B_{12} binnen. Mensen die vegetariër zijn, hebben daarom soms een lage inname van vitamine B_{12}. Bij oudere mensen is er iets anders aan de hand. Door het verouderen verandert het maag-darmkanaal. Hierdoor kan vitamine B_{12} niet goed worden vrijgemaakt uit het voedsel en zodoende wordt vitamine B_{12} niet goed meer opgenomen in het lichaam. Dit noemen we malabsorptie. Doordat vitamine B_{12} niet meer in voldoende mate opgenomen kan worden in het lichaam, ontstaat er op den duur een tekort aan vitamine B_{12}. Dit tekort zou uiteindelijk kunnen leiden tot verhoogde risico's op hart- en vaatziekten, bloedarmoede, zenuwklachten en verminderde cognitieve vermogens.

Het is mogelijk om het vitamine B_{12}-tekort op te heffen door middel van een injectie waarin vitamine B_{12} zit. Deze methode wordt doorgaans gebruikt in de huisartsenpraktijk. Een injectie met vitamine B_{12} is echter vaak pijnlijk voor de patiënten en ook erg duur. Een andere manier om het vitamine B_{12}-tekort op te heffen is door middel van (multi)vitamine-preparaten, oftewel pillen waarin vitamine B_{12} zit. Veel oudere mensen gebruiken echter al een groot aantal medicijnen. Het is vaak een zware last voor oudere

mensen om zoveel medicijnen te gebruiken, waardoor elke extra pil/medicijn als bezwaarlijk wordt gezien.

BOTGEZONDHEID

Osteoporose is een ziekte die door verschillende factoren kan ontstaan. Iemand met osteoporose heeft een lage botmassa en een lage botdichtheid waardoor de kans op een breuk hoog is. Een botbreuk, fractuur, is geassocieerd met een verhoogd risico op het voorkomen van ziekten en sterfte. Ook gaat de kwaliteit van leven veelal sterk achteruit bij mensen die iets gebroken hebben, vanwege een vermindering van lichamelijke functies. In Nederland worden meer dan 150000 ouderen boven de 65 jaar in het ziekenhuis opgenomen vanwege een heupfractuur. De kosten hiervan zijn erg hoog.

Biologische, genetische en omgevingsfactoren spelen een belangrijke rol bij het ontstaan van osteoporose. De preventie en behandeling van osteoporose is veelal gericht op medische interventie, verbetering van voeding en leefstijlfactoren. Een hormoonbehandeling, een van de medische interventies, heeft echter ook als nadeel dat het een verhoging van het risico op borstkanker met zich meebrengt. Het is gebleken dat vitamine D en calcium (dat met name in zuivelprodukten zit) positieve effecten hebben op de botmassa. Ook is dan de kans op fracturen lager dan bij mensen met een lage inname van calcium en een laag bloedgehalte van vitamine D. Vitamine D wordt in de huid aangemaakt met behulp van zonlicht. In Nederland is de inname van calcium bij veel mensen optimaal door het hoge gebruik van zuivelprodukten, maar de vitamine D voorziening is marginaal. De Gezondheidsraad beveelt een vitamine D supplement aan voor ouderen boven de 70 jaar.

VITAMINE B_{12}: EEN NIEUWE INDICATOR VOOR BOTGEZONDHEID?

In het verleden is gesuggereerd dat vitamine B_{12} een rol speelt bij botgezondheid. Tot enkele jaren terug zijn er maar een paar studies uitgevoerd waarin deze rol van vitamine B_{12} is onderzocht. In dit proefschrift is in verschillende onderzoekspopulaties onderzocht of vitamine B_{12} daadwerkelijk gerelateerd is met botgezondheid. Deze nieuwe inzichten zouden een basis kunnen vormen voor interventie-onderzoeken waarin gesuppleerd wordt met vitamine B_{12} en bekeken wordt of het een positief effect heeft op de botgezondheid.

In **Hoofdstuk 2** is allereerst onderzocht of er een relatie bestaat tussen vitamine B_{12} status en botmassa en botdichtheid in kinderen die een streng vegetarisch dieet hebben gevolgd. Naast deze groep kinderen zijn er ook kinderen in het onderzoek meegenomen die wel vlees en zuivelprodukten aten. In deze studie zagen we duidelijk dat de kinderen met een lage vitamine B_{12} status een lagere botmassa of botdichtheid hadden dan kinderen met een normale vitamine B_{12} status. Een lage vitamine B_{12} status kwam doordat de kinderen een lage inname hadden van produkten waar veel vitamine B_{12} in zit.

In **Hoofdstuk 3** vonden we soortgelijke resultaten bij fragiele ouderen. Fragiele ouderen zijn oudere mensen die veelal meer zorg behoeven en ook vaker lichamelijk ongemak ondervinden dan andere (zelfstandig wonende) ouderen. In deze groep zagen we dat bij vrouwen met een tekort aan vitamine B_{12} in het bloed zeven keer zo vaak osteoporose voorkwam dan vrouwen met een normale vitamine B_{12} status.

In **Hoofdstuk 4** zijn de gegevens van drie verschillende studies samengevoegd. Een grote groep van meer dan 1200 mensen uit de LASA-studie (Longitudinal Aging Study Amsterdam), en twee groepen uit de ERGO-studie (Erasmus Rotterdam Gezondheid en Ouderen). Deze oudere mensen woonden, op het moment van onderzoek, allemaal nog zelfstandig. Bij deze oudere mensen is de concentratie van homocysteine in het bloed onderzocht. Homocysteine is een stofwisselingsproduct in het bloed dat zich opstapelt als er een tekort aan vitamine B_{12} en foliumzuur is. In deze studie zagen we dat mensen met een hoog homocysteine gehalte in het bloed een twee keer zo hoge kans hadden op een fractuur dan de rest. In **Hoofdstuk 5** wordt nog dieper ingegaan op de LASA-studie. We hebben namelijk gekeken of een combinatie van een laag vitamine B_{12} en een hoog homocysteine gehalte in het bloed nog sterker is geassocieerd met fracturen. Dit bleek inderdaad het geval te zijn.

In **Hoofdstuk 6** hebben we gekeken of het mogelijk is om het vitamine B_{12} tekort bij oudere mensen op te heffen met behulp van een melkdrank verrijkt met vitamine B_{12}. Eerst is een grote groep ouderen onderzocht op hun vitamine B_{12} status. De mensen met een tekort aan vitamine B_{12} in hun bloed zijn ingedeeld in twee groepen. Eén groep kreeg melk mét vitamine B_{12} en de andere groep kreeg melk zónder vitamine B_{12}. Hieruit bleek dat het zeer goed mogelijk is om het tekort op te heffen met een melkdrank waarin extra vitamine B_{12} zit. Deze gegevens hebben we vergeleken met een andere studie waarin gekeken werd of een capsule met vitamine B_{12} het tekort kan opheffen. De resultaten waren vergelijkbaar met die van de verrijkte melk. Verrijkte melk kan een goede manier zijn om het vitamine B_{12} tekort op te heffen bij mensen die liever geen capsules slikken, bijvoorbeeld bij ouderen die veel medicijnen gebruiken.

In de algemene discussie (**Hoofdstuk 7**) geven we aan wat de volgende stappen zouden kunnen zijn voor nieuw onderzoek om verder vast te stellen of vitamine B_{12} daadwerkelijk een positieve invloed kan hebben op de botgezondheid. In dit proefschrift is er vastgesteld dat de vitamine B_{12} status gerelateerd is met botgezondheid. Dit betekent nog niet dat suppletie van vitamine B_{12} de botgezondheid verbetert. Hiervoor zijn interventiestudies nodig. Een valide studieopzet is een groot langlopend onderzoek waarbij wordt onderzocht of vitamine B_{12} het aantal fracturen kan verminderen. Voor zo'n onderzoek zijn veel deelnemers nodig en deze deelnemers moeten aan veel criteria voldoen. Het is moeilijk en erg duur om zo'n onderzoek op te zetten. Een goedkopere oplossing is een aantal kleinere studies waarin gekeken wordt of bepaalde botparameters positief beïnvloed kunnen worden. Dit is echter minder overtuigend. Een andere mogelijkheid is om in onderzoeken die nu lopen retrospectief (terugblikkend) te onderzoeken of er minder fracturen voorkwamen in de groep deelnemers die vitamine B_{12} (en eventueel ook foliumzuur) kregen dan in de controlegroep die géén vitamine B_{12} kregen (deze deelnemers kregen een zogenoemde placebo).

Dankwoord

Elke AIO denkt wel eens dat hij/zij er helemaal alleen voor staat. Tijdens mijn AIO-onderzoek is gelukkig het tegendeel gebleken. Dankzij de betrokkenheid, gezelligheid en wetenschappelijke bijdrage van veel mensen is dit proefschrift tot stand gekomen en kijk ik met plezier terug op deze bijzondere fase in mijn leven. Ik heb veel herinneringen aan de afgelopen vier, vijf jaar en ben in vele opzichten nog gegroeid. Ik ben daar veel mensen dankbaar voor want zonder hen was ik niet zo ver gekomen als waar ik nu ben en mijn proefschrift al helemaal niet. Graag zou ik iedereen willen bedanken voor zijn of haar interesse in mij en mijn proefschrift. Een aantal mensen wil ik hieronder in het bijzonder noemen.

Allereerst wil ik stilstaan bij mijn (co-)promotoren. Professor Van Staveren, Wija, hartelijk dank voor het vertrouwen dat je in me gesteld hebt. Al hadden we soms een andere manier van aanpak, ik geloof dat we vaak dezelfde ideeën en gedachten hadden en zodoende hebben we het er maar mooi van afgebracht. Als je de naam Wija noemt, dan noem je automatisch ook de naam Lisette. Dr. Ir. De Groot, ik bewonder de manier waarop jij je leven en werk hebt georganiseerd. Ik waardeer je rustige maar waardevolle inbreng zeer. Op welk vlak dan ook, je hebt altijd kritische vragen klaar die niet altijd even gemakkelijk te beantwoorden zijn. Als goede gidsen lieten jullie oogluikend toe dat ik een enkele keer van mijn pad afweek zodat ook ándere interessante dingen werden gezien, naast de paden die al uitgestippeld waren, maar zonder dat ik te ver van dit pad geraakte.

Professor Kok, Frans, aan het begin van mijn onderzoek hebben we enkele constructieve besprekingen gehad. Daarna is onze samenwerking helaas wat verwaterd, maar het was je wel duidelijk dat ik bij Wija en Lisette in goede handen was. Jij leidt deze vakgroep op een bewonderenswaardige manier.

Professor Gert Jan Hiddink, diverse AIO's van Wija heb jij namens (voorheen de Stichting Zuivel, Voeding en Gezondheid) de Nederlandse Zuivel Organisatie wetenschappelijk en financieel ondersteund. Een telefoontje op onverwachte momenten maakte mij telkens weer duidelijk dat ik er niet alleen voor stond en dat je met ons meedacht. Veel dank hiervoor.

Halverwege mijn AIO-onderzoek zochten we contact met Professor Paul Lips. Deze samenwerking is zeer vruchtbaar gebleken. Paul, ik heb zelden iemand gezien die zo goed de tijd voor me nam zonder te laten merken hoeveel werk er nog lag te wachten op hem. Jouw enthousiaste inbreng werkte zeer aanstekelijk en maakte dat ik graag naar

Amsterdam kwam. Je bracht mij in contact met Nathalie Bravenboer en Huib van Essen. Al was ik niet echt een labpersoon, bedankt voor jullie hulp en samenwerking op het lab. Het Endocrinologie-lab zorgde voor snelle bepalingen van vitamine B_{12} in het bloed. Saskia Pluijm zorgde ervoor dat ik snel wegwijs raakte in de gegevens van de grote, uitstekend opgezette LASA-studie. Het was een genot om met deze data te kunnen werken; zo goed en duidelijk zat alles in elkaar. Ook kon ik altijd bij je terecht voor advies.

Binnen de VU is er nog een groep die ik wil bedanken: het Metabool Laboratorium onder leiding van Professor Karel Jacobs. Altijd kon ik bij jullie vele monsters meten op MMA en Hcy in het bloed. Iedereen in het lab wilde dat meisje uit Wageningen wel helpen, en daar maakte ik natuurlijk goed gebruik van!

Martine Lips, Diane ter Doest en Moniek van Zutphen hebben op verschillende momenten tijdens mijn AIO-project een afstudeervak onder mijn begeleiding uitgevoerd. Alle drie zijn jullie erg verschillend en was het een uitdaging voor mij om het beste in jullie naar boven te halen, ik hoop dat dat gelukt is. In ieder geval heb ik veel van jullie geleerd en genoten. Annemarie Wagemans, al deed je dan niet bij mij een afstudeervak, we hebben samen een flink aantal leuke, gezellige uurtjes beleefd in de verzorgingshuizen.

Een aantal co-auteurs, die een grote bijdrage hebben geleverd tot publicatie van onze manuscripten, wil ik graag bij naam noemen: Nynke de Jong, Marijke Chin A Paw, Marijke van Dusseldorp, Jörn Schneede, Joyce van Meurs, André Uitterlinden en Henk Blom. Ik was altijd blij met jullie commentaar. Joyce, jij nog even apart, jij hebt echt bergen werk verzet voor het NEJM-artikel. Geweldig dat het gelukt is.

Op de valreep mocht ik nog een interventiestudie uitvoeren. Veel mensen zijn hierbij betrokken geweest. Annemarie Braber en Anton van den Hoven van het NIZO zorgden ervoor dat de met vitamine B_{12} verrijkte melk op de juiste manier bereid werd. Gelukkig kwamen we er samen uiteindelijk goed uit. Alle deelnemers, die uit alle hoeken van het land trouw meededen aan de interventie, wil ik hartelijk bedanken. Lucy Okma bereidde de bloedafnames voor en regelde de analisten (Wilma Staring, Isabelle, Diana, Janneke en Miny). De samenwerking met Simone Eussen was ideaal om de verzamelde data uit onze studies te combineren. Simone, samen hebben we je Brain12-studie goed opgestart en vooral in het begin hebben we veel lol gehad. Bedankt voor alles.

Op de vakgroep lopen veel mensen rond die mij op allerhande manieren geholpen hebben, dit waren onder andere: Karen, Lidwien, Eric, Marie, Eva, Clive, Lous, Els, Saskia, Jan B, Paul, Tineke, Dione, Ben en Dirk. Dirk, ik mis nog altijd je goeie, soms onverstaanbare, humor. Karen, bij jou kon ik altijd binnenlopen en over van-alles-en-nog-wat heerlijk kletsen. Dank voor je gezelligheid!

Ik heb het altijd naar mijn zin gehad op de afdeling Human Voeding. Alle collega's en met name de (oud-)AIO's wil ik daarom bedanken voor hun gezelligheid en steun. Tijdens het kritisch bespreken van onze manuscripten in de oldsmobiles-meetingen hebben we veel van elkaar geleerd. Dit werkte zeer motiverend. Er waren ook een paar collega's die vriendinnen werden: Anouk, Astrid, Linette en Monica. Eerst hebben we samen de Study Tour georganiseerd en dat is een goede basis geweest voor onze vriendschap. Koffie, thee, of rode wijn het was altijd erg gezellig in ons hoekje. Of ging het jullie om het snoep dat ik altijd wel op mijn kamer had liggen? Petra, eigenwijze meid die je bent, ook jij bent een bijzondere collega.

Brenda, dat jij nu in Amerika bent, maakt onze vriendschap er niet minder om. Ik heb genoten van jouw perikelen, onze discussies, maar ook gezellige gesprekken. Dank voor je advies op allerlei gebied. Ik vind het erg leuk dat ik straks je paranimf mag zijn.

Marleen en Marieke, eventjes samen, leuk, heel leuk dat jullie mijn paranimfen willen zijn. Jullie tweeën weten samen heel veel over mij. Marleen, dat je meer dan alleen een collega van me bent, dat is eenieder allang duidelijk. We hebben lief en leed met elkaar gedeeld. Je hebt mij op veel zichtbare en onzichtbare momenten gesteund, meer dan je waarschijnlijk zelf beseft. Ondanks je eigen drukke onderzoek stond je altijd voor mij klaar: lief, oprecht en met goed advies. Geweldig bedankt daarvoor! Ik hoop dat ik ook iets voor jou heb kunnen betekenen. Eén ding heb ik echter in al die tijd nog steeds niet goed van je geleerd: je netheid!

Marieke, nu jij even apart, jouw interesse, aanstekelijk goede humeur en hartelijkheid hebben mij doen besluiten om jou als paranimf te vragen. Met jou aan mijn zijde op het podium, gaan we ervoor zorgen dat het een leuke dag wordt. Naast dat hockey een goede uitlaatklep was voor het werk, ben ik blij dat jullie, Marieke, Mariska, Wies, Franka, Fréderique, Sanne H, Sanne vd B, Janine, Sarah en Saskia meer dan alleen hockeymaatjes van me zijn. Bruiloften, babies, promoties, wie is er deze keer aan de beurt voor een leuk feestje of nieuwtje?

The Indian Curry group, with Shital and Basav as the main initiators and organisors, always took care of nice dinners and special gatherings during Indian celebrations. Shital and Basav, dhanyavaad for translating the Summary into a Hindi summary. Ajay, I appreciate your friendship and humor a lot. Alina, Carla, Odette en Ondine, elk uitje met jullie was heerlijk om de werkstress even van me af te laten gaan. Batian en Ondine, we hebben een bijzondere vriendschap met z'n ups en downs. Jullie aanwezigheid bij onze Indiase trouwceremonie was heel bijzonder. Annuska en Frank, jullie zijn op vele manieren getuige geweest van ons leven de afgelopen vijf jaar. Ik ben blij dat wij nu getuige mogen zijn van jullie geluk dat bij jullie rondkruipt.

Het is een rijk gevoel om zulke lieve broers en schoonzusjes te hebben die altijd geïnteresseerd zijn, maar soms dachten dat zo'n AIO-baan een luizenleventje inhield. Misschien dat jullie er nu anders tegenaan kijken? Merci à tous pour votre attention! Sjoerd & Stéphanie, we koesteren onze reisjes naar jullie in Frankrijk. Joris & Gilian, geweldig dat jullie meegingen naar India, dat was een speciale ervaring. Chère Carla, tu nous fais toujours rire, surtout continue!

I would like to thank my parents-in-law for their trust in us. Even though you live far away from us, it feels that you are close by as you are always in our heart. Sukryia. Vrishali, it was nice to have you close to us in Wageningen. I wish you all the best in your future life and with the choices you make. Mahesh, your wahini will always want the best for you, still you must work hard for your (still to be defined) goals but I am sure you will get there.

Lieve papa en mama, wat hebben jullie ons ongelooflijk veel hulp geboden op allerlei gebied. Ik ben heel blij dat ik zulke stimulerende, behulpzame en begripvolle ouders heb. Geweldig ook dat jullie meegingen met ons naar India. Waar ook ter wereld voel ik jullie steun en liefde. Ik vind het heel fijn dat wij in ons gezin zo'n sterke hechte band met elkaar hebben.

Pankaj, my love, far away from your family and home country, you always encouraged me and you found the strength and perseverance in me to continue. I appreciate and admire your efforts of building up your scientific career. I am confident that you will fulfill to the 'veni vidi vici' saying. I am happy we still have a long life in front of us because this *journey* with you cannot last long enough for me. Now it is about time for our next project.

List of publications

Peer-reviewed papers

Dhonukshe-Rutten RAM, Pluijm SMF, De Groot CPGM, Lips P, Smit JH, Van Staveren WA. Homocysteine and vitamin B_{12} status relate to bone turnover markers, broadband ultrasound attenuation and fractures in healthy elderly people. *Provisionally accepted for publication*

Dhonukshe-Rutten RAM, Van Dusseldorp Marijke, Schneede Jörn, de Groot CPGM, van Staveren WA. Low bone mineral density and bone mineral content are associated with low cobalamin status in adolescents. *European Journal of Nutrition.* 2004 30 Aug; Epub ahead of print

van Meurs JBJ, **Dhonukshe-Rutten RAM**, Pluijm SMF, van der Klift M, de Jonge R, Lindemans J, de Groot CPGM, Hofman A, Witteman JCM, van Leeuwen JPTM, Breteler MMB, Lips P, Pols HAP, Uitterlinden AG. Homocysteine levels and the risk of osteoporotic fractures. *The New England Journal of Medicine*. 2004 May 13;350(20):2033-41

Dhonukshe-Rutten RAM, Lips M, de Jong N, Chin A Paw MJ, Hiddink GJ, van Dusseldorp M, de Groot LC, van Staveren WA. Vitamin B-12 status is associated with bone mineral content and bone mineral density in frail elderly women but not in men. *Journal of Nutrition*. 2003 Mar; 133(3): 801-7

Dhonukshe-Rutten RAM, Van Zutphen M, de Groot CPGM, Eussen SJPM, Blom HJ, Van Staveren WA. Effect of supplementation with cobalamin carried either by a milk product or a capsule in mildly cobalamin deficient Dutch elderly people. *Submitted for publication*

Dhonukshe-Rutten RAM, Vossenaar M, West CE, Schümann K, Bulux J, Solomons NW. Day-to-day variation in the concentration of iron, zinc and copper in breast milk of Guatemalan mothers. *Accepted for publication in Journal of Pediatric Gastroenterology and Nutrition*

de Jong N, Chin A Paw MJ, de Groot LC, **Rutten RAM**, Swinkels DW, Kok FJ, van Staveren WA. Nutrient-dense foods and exercise in frail elderly: effects on B vitamins, homocysteine, methylmalonic acid, and neuropsychological functioning. *American Journal of Clinical Nutrition*. 2001 Feb; 73(2): 338-46

Abstracts in published conference proceedings

Dhonukshe-Rutten RAM, Lips P, Pluijm SMF, de Groot LCP, van Staveren WA. Homocysteine, bone density and fractures in healthy elderly people: The LASA study. *Journal of Bone and Mineral Research*. 2003; 18(S2): S53 [abstract]

Dhonukshe-Rutten RAM, de Groot LCPGM, Lips P, van Staveren WA. Vitamin B12 and homocysteine status in relation to fractures in healthy dutch elderly people – the LASA study. *The Journal of Nutrition, Health* & *Aging*. 2003; 7(4): 208 [abstract]

Rutten RAM, de Jong N, de Groot CPGM, Hiddink GJ, van Staveren WA. Vitamin B12 association with bone mineral density, bone mass and bone calcium in elderly men and women. *Revista Espanola de Geriatria y Gerontologia*. 2000; 35(6): 375-6 [abstract]

Other publication

Bakker-Zierikzee A, Geelen A, Mars M, Pellis L, **Rutten R**, Wark P. Diversiteit binnen voedingsonderzoek in Europa. *Voeding Nu*. 2002; 9: 26-7

Overview educational program

Discipline specific activities

- Diet and successful aging, Wageningen, NZO, Oct 1999
- De voeding van Nederland in de twintigste eeuw, Ede, NVVL, Oct 1999
- Meeting NWO Voeding, NWO, Oct 1999, 2001, 2002, 2003
- Geriatriedagen, Feb 2000
- Third European Congress on Nutrition and Health in the Elderly People, Madrid, Nov 2000
- The use of the dietary pattern approach in nutritional research, Wageningen, WUR, Dec 2001
- Symposium on Hcy, folate and vitamin B_{12} in cardiovascular and neurological diseases, Ravenstein, UMC Nijmegen, Dec 2001
- Epidemiologic Data Analysis, Kenneth J Rothman, RIVM, May 2002
- Symposium 'CHD and the 7-countries Study', RIVM, May 2002
- WEON, June 2002
- Nutrition and Lifestyle Epidemiology, VLAG, June 2003
- 2nd Annual International Academy on Nutrition and Aging, Albuquerque, IANA, July 2003
- 25th American Society of Bone and Mineral Research Annual Meeting, Minneapolis, ASBMR, Sep 2003
- Meeting Calcium & Bone, Zeist, NVCB, Oct 2003
- Masterclass Geriatric Nutrition, VLAG, April 2004

General courses

- Systematic reviews: theory and practice, VLAG, March 2000
- Scientific writing in English, Language centre WUR, Spring 2001
- Organizing & supervising MSc thesis work, WUR, April 2002

PhD student week

- VLAG AIO week Bilthoven, VLAG, Oct 1999
- Succesvol functioneren in organisaties, VLAG, Nov 2002

Optional courses and activities

- Preparation PhD research proposal, WUR, 1999
- PhD Study Tour, WUR, Sept 2001
- Journal Club, WUR, 1999-2003
- Methodology Club, WUR, 2000
- Literature Lunch, WUR, 2000-2001
- Hcy Club, WUR, 2000-2004
- Oldsmobiles Club, WUR, 1999-2004

The studies described in this thesis were mainly funded by the Dutch Dairy Association (NZO).

Cover design: Rosalie and Pankaj Dhonukshe, Wageningen

Printing: Grafisch bedrijf Ponsen & Looijen B.V., Wageningen