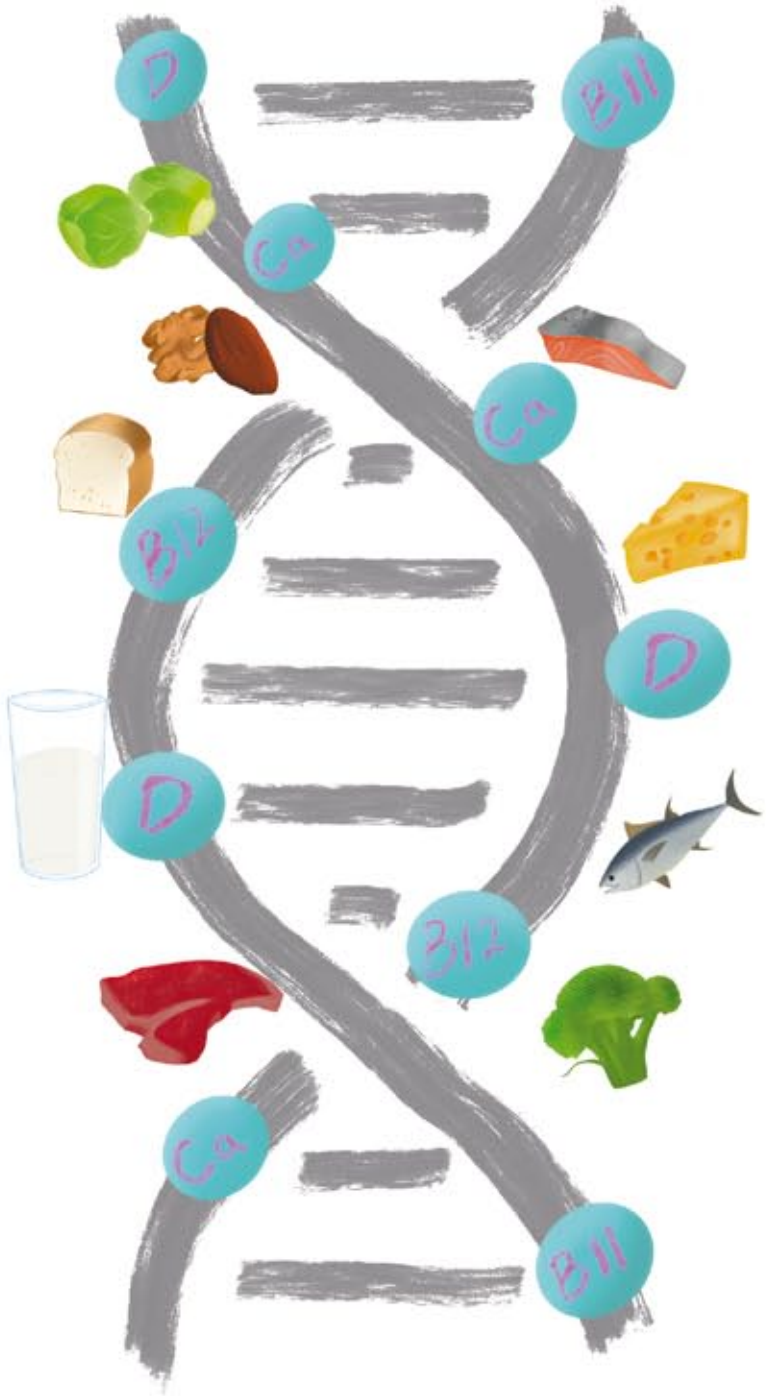
Micronutrients and Health in Older Population: Friend or Foe?



Sadaf Oliai Araghi

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Micronutrients and Health in Older Population: Friend or Foe?

Micronutriënten en gezondheid bij oudere populatie: vriend of vijand?

Thesis

to obtain the degree of Doctor from the
Erasmus University Rotterdam
by command of the
rector magnificus
Prof. dr. F.A. van der Duijn Schouten
and in accordance with the decision of the Doctorate Board.
The public defence shall be held on
Wednesday 17 March 2021 at 10.30 hrs

by

Sadaf Oliai Araghi
born in Arak, Iran.

Erasmus University Rotterdam

To my daughter

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Chapter 3. Long-term effect of micronutrients on health

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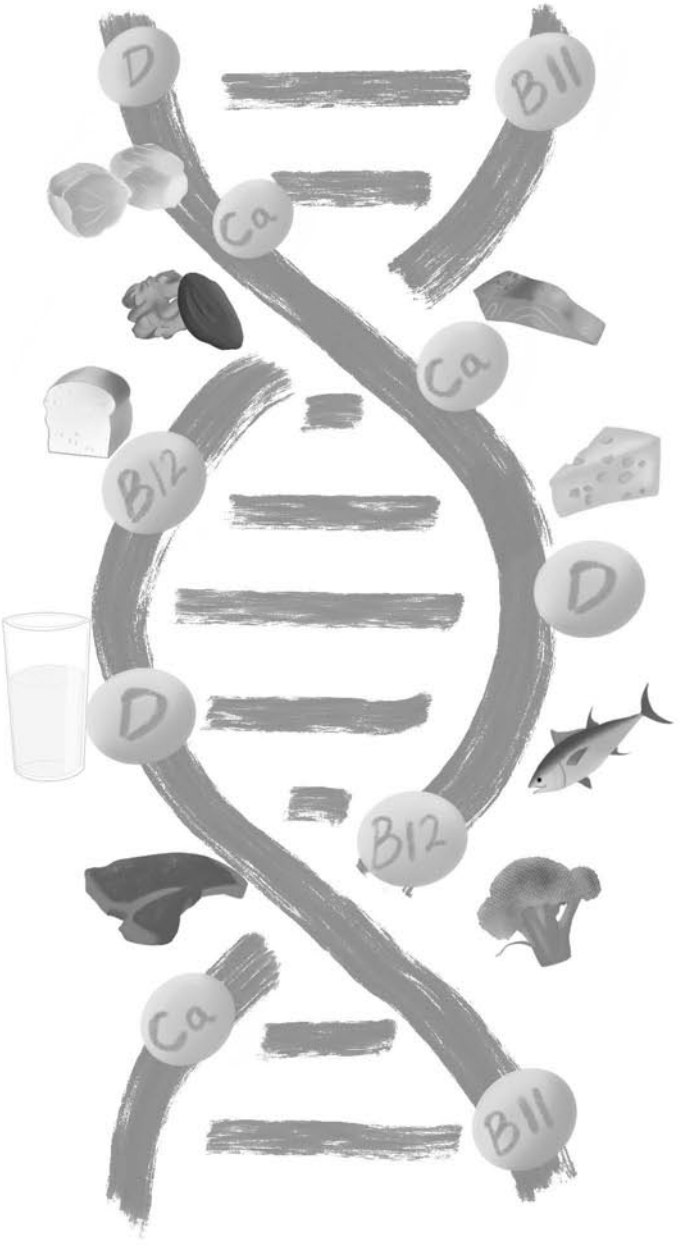
*Denotes equal contribution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GENERAL INTRODUCTION

GENERAL INTRODUCTION

Nutrition and Ageing

We live in an ageing society and thus the number of older people is increasing rapidly around the world (1). The ageing process makes people more prone to certain health conditions and many older people experience multiple health problems. Furthermore, the percentage of people with chronic diseases increases with age. What is more, due to an increase in life expectancy, the increasing prevalence of chronic health problems result in an equally increasing loss of healthy life years (2). Moreover, with aging accumulation of DNA damage and alterations in epi-genetic and cellular mechanisms occur that affect the trajectory of healthy ageing (3). Nutrients and nutritional status could also influence these epi-genetic and cellular mechanisms (4). Hence, cumulative effects of ageing and inadequate nutritional status result in a further increased risk of negative health outcomes.

Accordingly, nutritional status and body composition changes as a result of ageing, and there are many factors that influence nutritional status during ageing. These include gender, genetic variation in individuals, physical functional status (such as physical activity, strength and endurance), socio- economic status, cognition, nutrition and medical health status, such as chronic or acute health conditions and the use of medication (**figure 1**) (5).

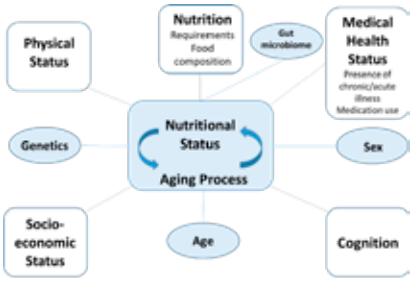

Figure 1. Factors that influence nutritional status as people age (5)

Furthermore, an age-related set of clinical syndromes characterized as “frailty”, results in an increased risk of worse health outcomes, such as falls, disability, hospitalization and mortality (6). It is possible that improving nutritional status in older people could help to inhibit the ageing process and reverse frailty (7). Poor nutritional status of the ageing population is caused partly by low intake of macro- and micronutrients (**figure 2**), in particular dietary protein intake, which is besides

associated with frailty (8). Community-dwelling older adults face health problems, and micronutrient deficiencies (deficiencies in vitamins and minerals) are similarly more common among this group due to inadequate diet, and age-related changes in the absorption, distribution, metabolism and/or excretion of nutrients (9). A common approach to measure vitamin deficiencies is measuring dietary intake or biomarkers in blood, urine or tissue (**figure 2**).

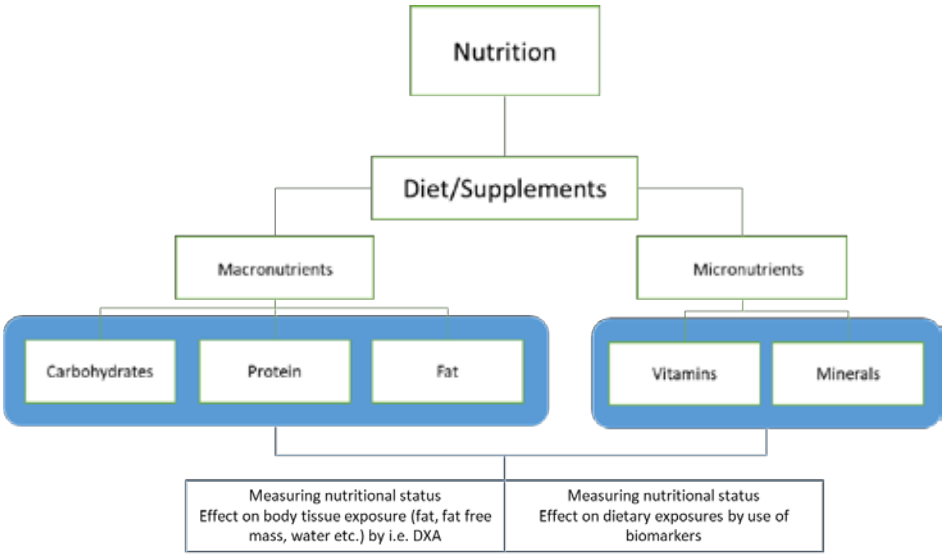

Figure 2. Nutrition and measuring nutritional status, effect on body tissue and dietary exposures

In the Netherlands older adults regularly use dietary supplements to prevent vitamin deficiencies (10). The dietary supplements that are most frequently used by older adults are multi-vitamins including B-vitamins, vitamin D and calcium (11). The global use of dietary supplements, such as vitamins and minerals has become a routine part of many people's lives, including many older people. For example, in the US the national Health and Nutrition Examination Survey showed that in 2011-2014, 70% of the older population used dietary supplements, and in the Netherlands around 45% of this part of the population used dietary supplements in 2010-2012 (12, 13). It has been shown that the use of supplements in the Netherlands is increasing with age (11). Yet, the role of dietary supplements on health, especially in the elderly, is not completely understood. Dietary supplements could play a role in optimizing health, however it could further be harmful and have negative effects on health (14).

Role of Genetic Factors in nutritional status

Genetic variation in individuals may play a role in nutrition and could result in differences in nutritional processes such as absorption, food metabolism, micronutrient metabolism and gut microbiome composition. Moreover, the genetic factors affect food preference(15, 16). An approach used in genetic research to associate specific genetic variations with particular diseases is called genome-wide association studies (GWAS) (17). GWAS helps us to increase our understanding of the genetic variation and diseases. With GWAS we are able to improve our knowledge of subtle differences between individuals, including behavioural characteristics and health (18). For example, different Loci for serum calcium concentrations were identified with GWAS (19). These Loci then could be used as a combined genetic score, like polygenetic risk score (PRS). Additionally, PRS can be applied as effect-modifier in association analysis or in Mendelian Randomisation studies to understand causality in the association between micronutrients (e.g. calcium) and disease (e.g. cancer). By using genetic variations as natural experiments, MR studies provides evidence about assumed causal relationships between a modifiable risk factor and the outcome of interest (20).

MICRONUTRIENTS AND HEALTH

Due to inadequate intake of nutrients and changes in absorption in our digestive system, micronutrient deficiencies, like vitamin D deficiency, are common in older persons (9).

Concomitant with age-related changes in body composition, the incidence of obesity increases too among older populations (21). These changes in body composition are further associated with lower micronutrient status, such as in the case of vitamin D, vitamin B12 and folic acid, for example (22, 23). So, in the older populations, changes in body composition as well as micronutrient deficiencies are more common. However, the association between intake and circulating levels of these micronutrients and body composition in older adults is unclear.

The commonly used measure of body composition, BMI (as weight in kg divided by square height in meters) may underestimate the prevalence of body composition among older adults. Ageing is associated with height loss due to changes in the bones, joints and muscles loss (24), therefore, in studies involving older people measuring fat mass and fat-free mass provides a better insight into the actual body composition and the prevalence of obesity. However, for a better insight into body composition,

especially in an ageing population, other measures than BMI are needed. For example, the DXA scan (or other alternatives such as Bod Pod) that measure the body fat and fat free mass, is considered a better alternative (**figure 2**) (25).

Vitamin deficiencies appear to play a role in several chronic and common diseases in older persons. Community-dwelling older persons are at risk of vitamin B12 and vitamin D deficiency However, the role of vitamin B12 and folic acid in common diseases in older adults, such as osteoporosis, cancer and cardiovascular disorders remains unclear. It is possible that hyperhomocysteinemia is an important factor in this relationship.

Homocysteine concentration is negatively associated with vitamin B12 and folate concentrations, as depicted below, and an effective method to reduce homocysteine concentration is the provision of B-vitamins (26, 27). The association between hyperhomocysteinemia and the risk of cardiovascular disease and fractures has been observed consistently (28, 29) and showed that elevated homocysteine level appears to be a strong predictor of ischemic heart disease, stroke and fractures. Consequently, observational studies have shown an association between B-vitamin intake (dietary and supplements) and lower risks of fractures and cardiovascular disease, however with conflicting results that may be explained by bias such as confounding. Thus, to assess the effect of B-vitamin intake (dietary and supplements) on the risk of fractures and cardiovascular disease, randomized controlled trials are needed. Several intervention trials have been performed to investigate the effect of treating these common conditions with B-vitamins, but again with conflicting results (30, 31). Due to few RCT's, in 2008 the B-PROOF (B-Vitamins for the PRevention Of Osteoporotic Fractures) trial was designed to investigate the effect of B-vitamins on different outcomes in an older population using a multicentre RCT design (more details below). After 2-3 years of supplementation with vitamin B12 and folic acid, a reduced risk of osteoporotic fractures was observed in only a subgroup of compliant persons aged 80 years and over (32). In addition, the B-vitamin intervention was observed to have no effect on the overall incidence of coronary heart disease, with the exception of a significantly reduced risk of cerebrovascular events among females (33). Unexpectedly, we found as well a higher self-reported incidence of cancer in the intervention group compared to the control group (32). In view of the relative short duration of the B-PROOF intervention, it was additionally speculated if longer follow up would reveal more consistent effects.

Thus, additional research questions arose following the B-PROOF trial: what is the long-term effect of the B-PROOF intervention on cancer risk? What is the long-term ef-

fect of the intervention on osteoporotic fractures and cardiovascular diseases? Other more general questions included: what is the effect of micronutrients supplementation (i.e. B-vitamins, vitamin D and calcium) on age related disease in the general population? Who benefits from taking supplements? What are the risks of excessive and over the counter micronutrient- and dietary supplements? In this thesis, we aimed to address these questions. In order to answer them, some pathophysiological background is needed first, which we will further explain in the next section, by starting with vitamin B12, folate, calcium and vitamin D and their potential influence on ageing and related-diseases.

B-VITAMINS IN ONE-CARBON METABOLISM

Vitamin B12 & Folate

Vitamin B12 (cobalamin) is water-soluble and naturally present in products derived from animals, such as meat, milk, cheese, fish, and eggs. Together with folic acid, vitamin B12 plays a role as an important co-factor in the one-carbon metabolism. Cells require one-carbon units for DNA synthesis and methylation (**figure 3**). In the methionine cycle, vitamin B12 is involved in the synthesis of methionine from homocysteine (both amino-acids) by two intermediates, namely S-adenosylmethionine (SAM) and S-adenosylhomocystine (SAH). Homocysteine is an amino acid that is not available from food but is made after the demethylation of methionine, which is an essential amino-acid available from food (34). So, methionine and homocysteine are necessary for DNA synthesis by the methyl group released when SAM converts to SAH (35).

Vitamin B12 in food is attached to animal protein (haptocorin) and is released from this protein by gastric acid and pepsin in the stomach to free vitamin B12. Vitamin B12 is absorbed in the intestine and in the small intestine vitamin B12 binds with the intrinsic factor (IF) (which is produced in the stomach) and absorption is facilitated by mucosa in the small intestine. It is transported to other tissues via the blood. Vitamin B12 that is not used immediately is stored in the liver (36). The daily recommended intake of vitamin B12 for adults is 2.8 micrograms and currently no limited upper intake level has been defined for vitamin B12 (37).

Folate is a water-soluble B-vitamin too, which is naturally present in grains and green leafy vegetables. The bioavailability of natural folate is lower than in its synthetic form, folic acid, which is used in supplements (38). The metabolism of folate starts in

the intestine in its bounded form. It is absorbed in the jejunum, and the hydrolysed folate attached to methyl groups is delivered to the liver and other body cells via the blood. The methyl group is in the one-carbon metabolism removed from 5-methyl-THF in the synthase reaction. The enzyme 5-methyl-THF together with vitamin B12 as co-factor is needed before homocysteine can be converted into methionine, which serves as a methyl group donor through transformation to SAM, and can be used to methylate DNA, for example (**figure 3**) (38). In the Netherlands, the daily recommended intake of folate for adults is 300 micrograms and 400 micrograms for pregnant women in order to prevent major birth defects in the baby's brain and spine, like neural tube defects. The European Food Safety Authority (EFSA) specifies an upper limit on the intake of folic acid from supplements of 1,000 micrograms per day, because vitamin B12 deficiency is not detected if the intake of folic acid is too high (39).

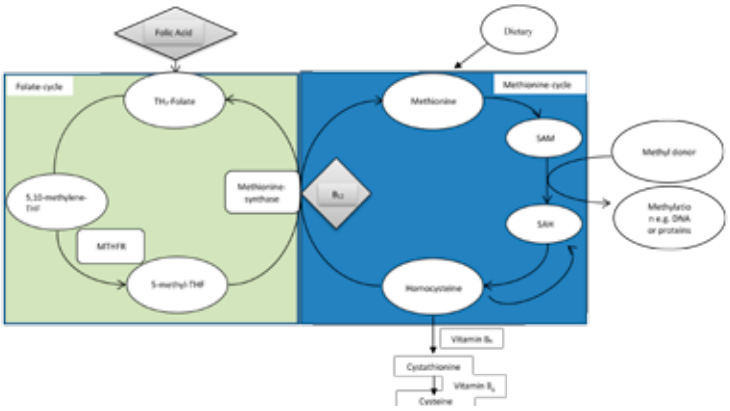

Figure 3. One-carbon metabolism

Calcium & Vitamin D

Calcium is a mineral needed in cellular metabolism and is, together with phosphate, incorporated in hydroxyapatite which is crucial for the structure and maintenance of the bones and teeth (40). Calcium plays a key role in the optimal functioning of bone, tooth formation, muscles and nerves, blood clotting, the transport of other minerals and hormone excretion (41). Furthermore, it plays a role in cell signalling and fluid balance (42).

As a dietary component, calcium is naturally present in milk, dairy products, vegetables, nuts and legumes. It is absorbed in the gut with the aid of a controlling hormone, 1.25-Dihydroxyvitmin D3 (1.25(OH2)D3), the active form of vitamin D (43).

Most calcium in the body is deposited in the skeleton and less than 1% is found in the blood, soft tissues and extracellular fluid (41). Circulating calcium in serum exists in 3 forms, whereby 5-15% is complexed calcium bound to anions, 30-50% is bound to albumin, and the remaining circulates as free ionized calcium (Ca2+) (44).

The Health Council of the Netherlands recommends 950 milligrams of calcium daily for men aged between 25-69 years and women aged 25-50 years. The daily recommended intake for persons older than 70 years is 1,200 milligrams daily; for women aged 51-69 years it is 1,100 milligrams. Pregnant women and lactating women, need 1,000 milligrams of calcium daily (45). The upper limit on intake from food and supplements is 2,500 milligrams daily, due to the elevated risk of kidney stones, renal insufficiency, vascular and soft issue calcification and hypercalciuria (45).

Vitamin D is a fat-soluble vitamin that is naturally present in fat fish, and in lower amounts in meat and eggs. Additionally, it is fortified in margarines. Sunlight is the most important source of vitamin D (3), which enables the synthesis of 1.25(OH2) D3 in the skin, produced by 7-hydrocholesterol through exposure to ultraviolet B. In the liver, 25-hydroxylation of vitamin D results in the formation of 25-hydroxyvitamin D (25(OH)D), which is considered a more longer term reliable measure of vitamin D status, reflecting vitamin D reservoirs in persons with normal kidney function (45).

As mentioned, vitamin D is important for calcium homeostasis (46). The daily recommended intake of vitamin D is 10 micrograms for adults and 20 micrograms a day for everyone aged above 70 years. The upper daily limit for vitamin D is 100 micrograms a day. High intake of vitamin D is not that common in healthy people, however, it can result in symptoms of malaise, drowsiness, loss of appetite and obstipation (45).

Diet and medication: Diuretics as an example

In addition to the increased use of dietary supplementation, effects of use of medications and polypharmacy on nutritional status are as well a growing concern among older population (47). A frequently prescribed medication group to treat heart failure and hypertension, especially in older people is diuretics (48, 49). A study in 2015 showed that between 33 and 47% of older people aged 50-90 years or older used diuretics. The potential adverse effects of diuretics may have serious impact on older persons. Recently, recognition of the relevance of food-drug interactions in clinical practice has further been growing (40). Interestingly, it has been widely documented that diuretics can have various effects on bone health (50-52). Thiazide diuretics have been shown to have a protective effect in protecting bone mass and in decreasing the

risk of fractures (51). In contrast, loop diuretics may have a negative impact on bone turnover by increasing urinary calcium excretion (53). Calcium and vitamin D have been hypothesized to play a role in the association between the use of diuretics and bone health (54). Thus, knowledge of the food-drug interaction regarding diuretics and the use of vitamin D and calcium supplementation in relation to the effects on bone health may be therefore relevant.

STUDY POPULATIONS AND METHOLOGY

For this thesis, the data from the B-PROOF trial and the Rotterdam study was used. For the initial B-PROOF study, the inclusion criteria were, age >65 years, a homocysteine level >12 µmol/L and <50 µmol/L and creatinine level <150 µmol/L. Initially 2,919 older persons participated. For the second follow-up study after 5-7 years, 1,298 participants responded to the extended studies follow-up questionnaire. Details on the design of the B-PROOF can be found elsewhere (55) and details of the follow-up study are provided in **chapter 3.2**. Exposures from B-PROOF trial studied in this thesis were serum vitamin B12 level, holotranscobalamin (HoloTC), methylmalonic acid (MMA), folic acid, vitamin D and the intervention (folic acid and vitamin B12 supplements). Furthermore, the outcomes assessed in this thesis were body composition (fat mass and fat free mass measured by DXA-scan), cancer (from national cancer registry), fractures (self-reported and verified by the GP's), cardiovascular and cerebrovascular diseases (self-reported).

The Rotterdam Study (RS) is a population-based prospective cohort that has been going on since 1990 and includes participants aged 40 years and over. The study focuses on the most common diseases in this age category, such as cardiovascular, locomotor, endocrine, neurological and respiratory diseases. There are various examination cycles in the RS. For this thesis, we included the participants from RS I, RS II and RS III. The rationale, design and the diagram of the cycles of the study is described elsewhere (56). Exposures from RS studied in this thesis were diuretics use (thiazide and loop diuretics prescriptions) and calcium (intake by FFQ, levels and supplements). Outcomes assessed in this thesis were bone mineral density (BMD) and the trabecular bone score (TBS, measured by DXA-scan) and cancer (from national cancer registry).

AIMS AND OUTLINE OF THIS THESIS

An important societal development of concern is the increasing use of the dietary supplementation due to growing interest in the role of micronutrients in optimising health and preventing certain diseases (14). Supplements of B-vitamins, calcium and vitamin D are now commonly used among older adults (11). However, there are a number of questions with regard to the effects of these vitamins on age-related changes in body composition, fracture risk, and the prevalence of cardiovascular disorders and cancer. Thus, the overall aim of this thesis is to study the role of micronutrients and body composition and the long-term effect of micronutrients on common negative health outcomes such as fractures, cancer and cardiovascular events in the older population **(Figure 4)**. Further objectives of this thesis are to study the role of drug-micronutrients interaction on bone health, specifically diuretics, and to use genetic variation involved in regulating calcium metabolism as a tool to understand causality in the association between micronutrients (e.g. calcium) and cancer (colorectal).

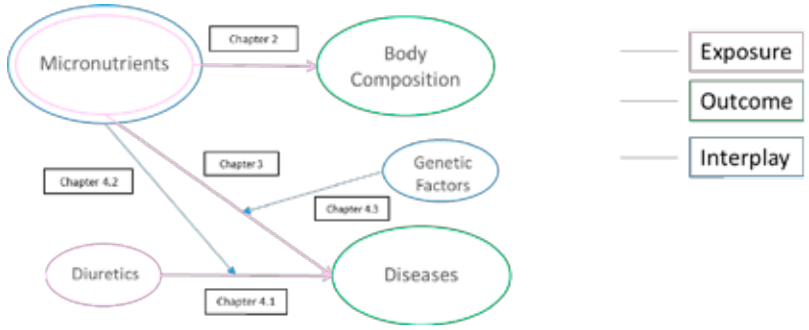

Figure 4. Overview of the topics of the several chapters in this thesis and their relationships

Chapter 2 focuses on the associations between vitamin D, B-vitamins and body composition using data from the B-PROOF study. In **Chapter 2.1**, the associations between vitamin D and BMI and fat mass are described. **Chapter 2.2** presents the observational and experimental evidence from the B-PROOF study on folic acid, vitamin B-12 and body compositions.

Chapter 3 focuses on the long-term effect of micronutrients on disease outcomes. **Chapter 3.1** shows the long-term effect of the folic acid and vitamin B12 intervention on the risk of cancer. The results concerning the long-term effect of folic acid and vitamin B12 intervention on the B-PROOF's primary and secondary outcome- osteoporotic fractures and cardiovascular disease- are presented in **Chapter 3.2**.

Chapter 4 focuses on the interplay between micronutrient intake and levels, genetic variation and drug use in relation to colorectal cancer and bone health, respectively. **Chapter 4.1** presents the impact of thiazide diuretics on bone mineral density and trabecular bone score in the Rotterdam study. **Chapter 4.2** examines whether vitamin D level and dietary calcium intake modified the association between loop diuretics and bone health. **Chapter 4.3** describes the associations between dietary calcium intake, calcium level and calcium supplementation and colorectal cancer and the interaction with genetic variation. In **Chapter 5**, the overall findings of this thesis are discussed regarding their implications, including some recommendations for future studies.

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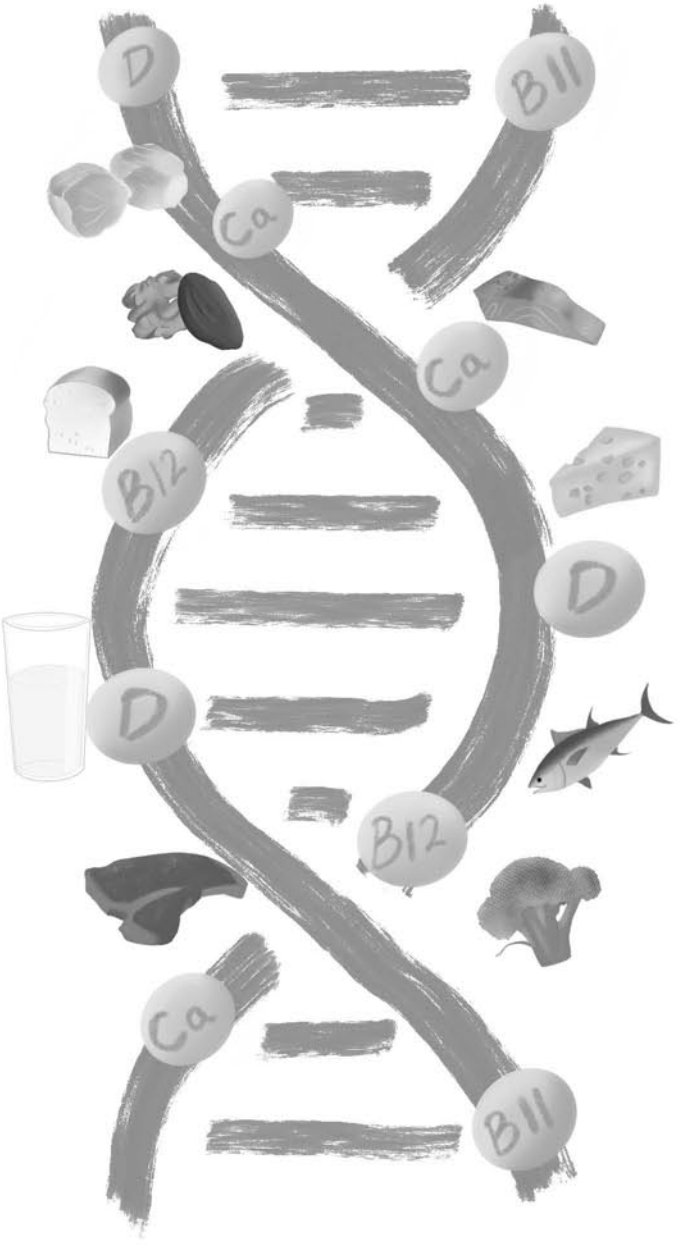
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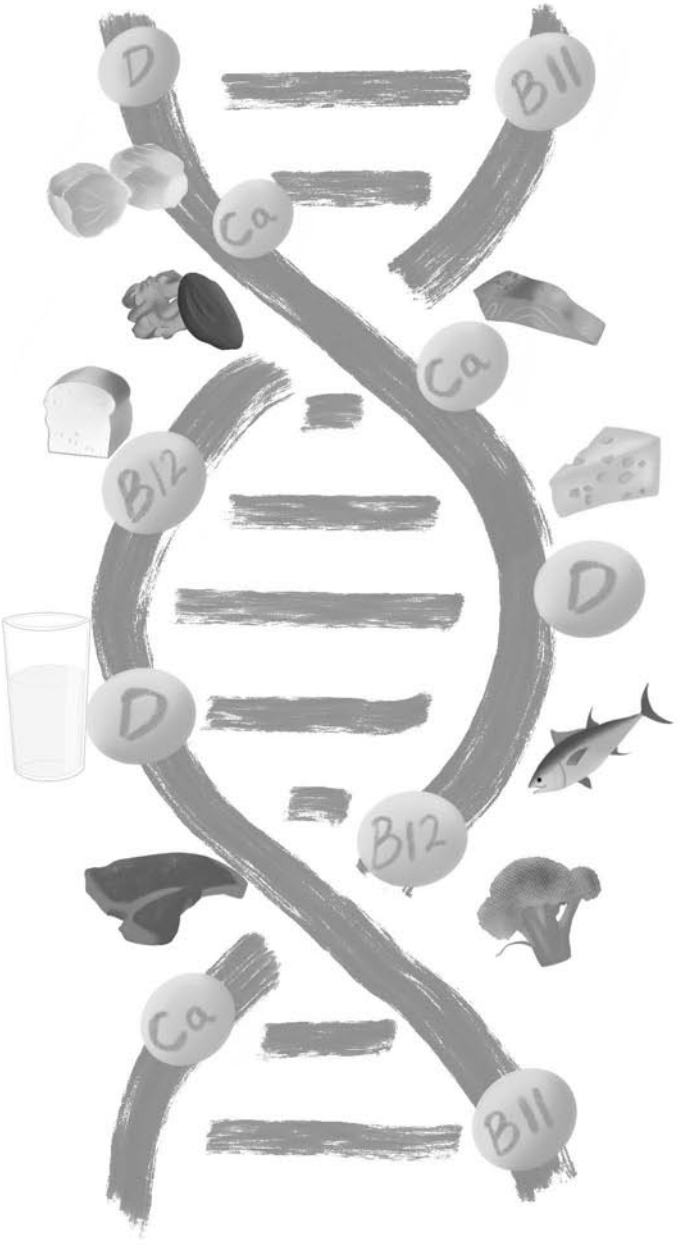
MICRONUTRIENTS & BODY COMPOSITION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Chapter 2.1

BMI and body fat mass is inversely associated with vitamin D levels in older individuals

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ABSTRACT

Objective

To assess the association between obesity (measured by Body Mass Index (BMI) and fat percentage) and serum 25(OH)D levels in older persons.

Design

Cross-sectional analysis of data from 'the B-PROOF study' (B-vitamins for the Prevention Of Osteoporotic Fractures).

Participants

2842 participants aged 65 years and older.

Measurements

BMI and fat percentage, measured by Dual Energy X-ray, and serum 25(OH)D levels.

Results

Mean age was 74 years (SD 6.5), with 50% women. Mean serum 25(OH)D levels were 55.8 nmol/L (SD 25). BMI and total body fat percentage were significant inversely associated with serum 25(OH)D levels after adjustment for confounders (ß-0.93; 95%CI [-1.15; -0.71], p<0.001 and ß-0.84; 95%CI [-1.04; -0.64], p<0.001). This association was most prominent in individuals with a BMI in the 'overweight' and 'obesity' range (ß -1.25 and -0.96 respectively) and fat percentage in the last two upper quartiles (ß-1.86 and -1.37 respectively).

Conclusion

In this study, higher BMI and higher body fat percentage were significantly associated with lower serum 25(OH)D levels in older persons. This association was particularly present in individuals with overweight, and higher fat percentages, suggesting that these persons are at increased risk of vitamin D insufficiency.

Keywords: BMI, Fat percentage, vitamin D, elderly people

INTRODUCTION

The percentage of individuals with overweight is growing in all age categories (1). This is an alarming issue (2), as overweight and obesity have been associated with a range of serious health consequences, including increased risk of metabolic syndrome, coronary heart disease, hypertension, type 2 diabetes, stroke and certain types of cancers (3,4). Furthermore, being overweight and obesity have been shown to alter the absorption, distribution, metabolism and/or excretion of micronutrients, which can cause several vitamin deficiencies (5-10). In particular in elderly, were vitamin deficiencies are more common (11,12). In this context, vitamin D deficiency has been associated with obesity (5-8). Because an accurate vitamin D level is important for calcium homeostasis5-8, and osteoporosis is a serious health problem in the older population (13), it is important to investigate the role of obesity in vitamin D deficiency.

A recent meta-analysis showed a significant inverse weak association between Body Mass Index (BMI) and serum 25-hydroxy vitamin D (25(OH)D) levels (14). However, this study did not analyze the relationship between body fat mass (fat percentage) and serum 25(OH)D levels. it needs to be emphasized that the way to measure overweight by BMI amongst the population of elderly is also under debate (15). Aging is associated with changes in body composition (16-18), this leads to loss in muscle mass and muscle strength (19). Therefore, BMI could underestimate the prevalence of obesity in this population, and fat percentage could be a better predictor for obesity than BMI in elderly individuals (20). So, dependent on the above mentioned arguments, body fat may be a better indicator of overweight than BMI. Consequently, we will investigated the association between BMI and fat percentage, and serum 25(OH)D levels, in a large population of older persons.

MATERIALS AND METHODS

Study participants

For the present cross-sectional analyses, baseline data of the 'B-PROOF study' (B-vitamins for the Prevention Of Osteoporotic Fractures) were used. B-PROOF is a multi-center, randomized, placebo controlled, double-blind, intervention study, investigating the effect of a 2-year daily oral vitamin B12 (500 µg) and folic acid (400 µg) supplementation on fracture incidence. The study was conducted in three research centers in the Netherlands: Vu University Medical Center (Amsterdam),

Wageningen University (Wageningen), and Erasmus Medical Center (Rotterdam). This study included 2919 individuals, aged 65 years and older with an elevated homocysteine levels (12 - 50 µmol/l). Participants were excluded if they had a renal insufficiency (creatinine level > 150 µmol/l) or presence of a malignancy in the past 5 years. A detailed description of the trial has been reported elsewhere (21).

All participants gave written informed consent before the start of the study. The B-PROOF study has been registered in the Netherlands Trial Register (NTRNTR1333) and with ClinicalTrials.gov (NCT00696514). The WU Medical Ethics Committee approved the study protocol, and the Medical Ethics committees of Erasmus MC and VUmc gave approval for local feasibility (21).

Clinical and anthropometrics measurements

Clinical and anthropometric measurements include height, weight and blood pressure. Height was measured in duplicate to the nearest 0.1 cm with the participant standing erect and without wearing shoes, using a stadiometer (21). Weight was measured to the nearest 0.5 kg using a calibrated weighing device (SECA 761) with the participant wearing light garments, empty pockets and without wearing shoes (21). BMI was calculated as weight in kilograms divided by square of height in meters and expressed as kg/m2. Participants were categorized in underweight (BMI < 20), normal weight (BMI 20 - 25.0), overweight (BMI 25.0 - 30) and obesity (BMI > 30) (22). Blood pressure measurements were performed two times on the left arm using an Omron M1 plus blood pressure device (Omron Healthcare Europe). The measurement with the lowest diastolic blood pressure was used for further analyses. Hypertension was defined as systolic blood pressure >140 mmHg and/or diastolic blood pressure >90 mmHg.

Demographic characteristics and health status variables, which included age, sex, self-reported medical history (cardiovascular disease and diabetes), alcohol intake, smoking habits, and vitamin supplement use, were determined using a structured questionnaire. Alcohol intake was categorised into ‘never’, ‘light’, ‘moderate’ and ‘(very) excessive’ drinkers, based on the number of days per week alcohol was consumed and the number of glasses per time, following the Dutch method of Garretsen et al. (23,24). Smoking habits were defined as never smoked, former smoker or current smoker and vitamin D supplement use was defined as users or non-users.

Physical activity

At baseline, participants were asked to complete a questionnaire about their daily physical activity during the past two weeks, including walking, biking, light and heavy household work, gardening and sports using a validated questionnaire (LAPAQ) (25) and was calculated in kilocalories (Kcal) per day.

Body composition

A subsample of participants underwent Dual Energy X-ray assessment (DXA) using the GE Lunar Prodigy device (GE Healthcare, USA, CV = 0.08%), (Erasmus MC) and the Hologic QDR 4500 Delphi device (Hologic Inc., USA, CV – 0.45%), (VuMC under standard protocols at baseline. The two devices were cross-calibrated by measuring a European spine phantom (ESP) five times on both devices and all results were adjusted accordingly. Total body composition was calculated by summing the amount of fat-free soft tissue (i.e. lean mass minus bone mineral content) and fat mass. Fat percentage was also calculated from the DXA scan (21). For analyses, fat percentage was divided in quartiles.

Biological sample collection and analysis

Venous blood samples were obtained in the morning, when the participants were in a fasted state, or had taken a restricted breakfast. Serum 25(OH)D was released from the protein through a denaturized internal standard (IS: 25(OH)D3-d6). Samples were extracted and analyzed by XLC-MS/MS (a Symbiosis online SPE system (Spark Holland, Emmen, the Netherlands) coupled to a Quattro Premier XE tandem mass spectrometer (Waters Corp., Milford, MA). The inter-assay coefficient of variation was 9% at the level of 10 ng/mL and 6% at the level of 25 ng/mL. All analyses were performed in the Endocrine Laboratory of the VU University Medical Center. The cut-off value for vitamin D deficiency was defined as a serum 25(OH)D levels < 50 nmol/L7,26 which was based on the current recommendations by the Institute of Medicine (27) and the recommendations for the older adults aged >70 years by the Dutch Health Council. Season of blood collection was dichotomized into summer (April – September) and winter (October – March) for the analyses.

Statistical analyses

The total B-PROOF population was included to investigate the association between BMI and 25(OH)D levels (n=2842). To study the association between body fat percentage

and serum 25(OH)D levels, a subsample of participants that underwent a DXA scans (n=1197) was used. Differences between subsamples were tested using the student t-test or Mann-Whitney U test, based on normally distributed or skewed data. Normal distribution for all variables was tested by visual inspection of histograms.

Second, linear regression analysis was used to determine associations between BMI, body fat mass and 25(OH)D levels (model 1, crude). Subsequently, age and gender were added as fixed confounders (model 2a). Thereafter, other potential confounders were added using the forward selection method (in model 2b). Potential confounders were smoking, alcohol intake, hypertension (yes or no), self-reported cardiovascular disease, total physical activity (in kcal/day), and season of blood collection. To address the potential mediating effect of vitamin D supplement use, this factor was added to the model. When the point estimate of interest changed >10%, vitamin D supplement use was regarded as a potential mediator and included in the final analysis. In addition, the interaction of age and total activity was tested in the crude model, and a P value < 0.1 for the interaction was considered statistically significant. If the interaction term was statistically significant, stratified analyses were performed. Stratification was performed as follow: age was dichotomized as younger or older than 80 years; and for total activity we created quartiles; both only when the interaction term was significant.

Further, we tested the associations between body fat percentage and serum 25(OH) D levels in different BMI categories (underweight, normal weight, overweight and obesity) and also per quartile of body fat percentage.

Statistical analysis was performed using the statistical software package of SPSS 21.0 (SPSS Inc., Chicago, Illinois, USA). P-values of < 0.05 were considered statistically significant for all the analyses other than the interaction analyses (<0.1).

RESULTS

Population characteristics

Population characteristics are presented in **Table 1**. Mean age was 74.0 years (6.5 SD) for the total population (n=2842) and 72.8 years (5.7 SD) for the DXA population (n=1197). Mean BMI for the total population was 27.2 (4.0 SD), and 27.0 (3.8 SD) for participants who underwent a DXA measurement. The participants who underwent a DXA scan were significantly younger, more active, largely included during summer; more likely to have hypertension and different alcohol consumption patterns (more moderate and excessive alcohol intake and less very excessive drinkers) when compared to the total B-PROOF population.

BMI and serum 25(OH)D levels

Results of the linear regression analyses of BMI and serum 25(OH)D levels are showed in **Table 2**. BMI was inversely associated with serum 25(OH)D levels after adjustments for covariates, indicating that for each unit increase in BMI there was a decrease in 25(OH)D level of 0.93 nmol/L(β -0.93, $p < 0.001$). Age was a significant interaction-term ($p = 0.02$) in this association and total physical activity was not. Stratification for age showed that the association between BMI and serum 25(OH)D levels was most pronounced in participants younger than 80 years (β -0.97 $p<0.001$) compared to the participants older than 80 years (β -0.72 $p=0.006$), **Table 3**.

When considering the categories of BMI, we observed that in overweight and obese individuals, BMI was significantly associated with serum 25(OH)D levels (β -1.25, $p=0.004$ and β -0.96, $p=0.004$ respectively, as showed in **Table 4**).

Fat percentage and serum 25(OH)D levels

The association between fat percentage and serum 25(OH)D levels is showed in Table 2. Fat percentage was inversely associated with serum 25(OH)D levels, after adjustments for covariates, (β -0.84, $p < 0.001$). No significant interaction effects were observed. We did observe a stronger association for the 3th and 4th quartile of body fat percentage (β -1.86, $p=0.01$ and β-1.37, $p<0.001$ respectively) and no association in the 1th and 2th quartiles (β-0.02, $p= 0.92$ and β-1.24, $p=0.16$ respectively, as showed in **Table 5**).

Table 1. Population characteristics

	B-PROOF Participants (N = 2842)	DXA-test Participants (N = 1197)	Comparison B-PROOF participants and DXA scan participants p-value
Age (years)[a]	74 (6.5)	73 (5.7)	<0.001*
Gender			
Female (%)	50	48	0.16
Body Mass Index (kg/m^2)	27.2 (4.0)	27.0 (3.8)	0.03
Underweight (%)	1	2	
Normal weight (%)	28	29	
Overweight (%)	51	50	
Obesity (%)	20	19	
Fat			
Total Fat Mass (Kg)	NA	25.6 (8.4)	NA
Total Fat Percentage (%)		32.5 (8.2)	
Smoking (%)			0.56
Current	10	9	
Former	56	57	
Never	34	34	
Alcohol intake (%)			0.001*
Light	67	64	
Moderate	29	32	
Excessive	3	4	
Very excessive	1	0	
Self-reported medical history of			
Cardiac disease (% yes)	25	25	0.97
Diabetes (% yes)	10	11	0.40
Measured hypertension (% yes)*	52	59	0.89
25(OH)D (nmol/L)[a]	55.8 (25)	55.1 (24)	0.26
Vitamin D <25 nmol/L (%)	10	8	0.01*
Vitamin D <50 nmol/L (%)	47	48	0.21
Vitamin D supplement use (% yes)	20	21	0.80
Total activity (Kcal/day)$_a$	649 (477)	714 (529)	<0.001*
Region (%)			<0.001*
Amsterdam	26	34	
Rotterdam	44	66	
Wageningen	30	0	
Season of blood collection(%)			<0.001*
Summer (April-September)	51	43	
Winter (October-March)	49	57	

[a]Presented as mean (SD) *significantly differences between total population and DXA-test participants

Table 2. Linear regression results of obesity parameters (BMI and fat-percentage) and serum 25(OH)D levels

Variable	Model 1			Model 2[a]			Model 2[b]		
	ß	[95% CI]	p	ß	[95% CI]	p	ß	[95% CI]	P
Body Mass Index (kg/m^2)	-0.78	[-1.01 ; -0.55]*	<0.001	-0.84	[-1.07 ; -0.62]*	<0.001	-0.93	[-1.15 ; -0.71]*	<0.001
Total Body Fat Percentage (%)	-0.52	[-0.68 ; -0.35]*	<0.001	-0.84	[-1.05 ; -0.64]*	<0.001	-0.84	[-1.04 ; -0.64]*	<0.001

Model 1: crude model. Model 2[a]: adjusted for age and sex. Model 2[b]: adjusted for total activities (sport and non-sport) in Kcal per day, smoking, alcohol and season of blood collection. *P-value <0.05.

Table 3. Linear regression results of BMI and serum 25(OH)D levels, stratified for age

BMI	Model 1			Model 2[a]			Model 2[b]		
	ß	[95% CI]	p	ß	[95% CI]	p	ß	[95% CI]	p
<80 year N = 2302	-0.91	[-1.16; -0.66]	<0.001	-0.91	[-1.16; -0.65]	<0.001	-0.97	[-1.22; -0.73]*	<0.001
≥ 80 year N = 540	-0.48	[-0.99; 0.03]	0.06	-0.61	[-1.12; -0.10]	0.02	-0.72	[-1.22; -0.21]*	0.006

Model 1: crude model Model 2[a]: adjusted for sex. Model 2[b]: adjusted for alcohol, total activities (sport and non-sport) in Kcal per day, smoking, alcohol and season of blood collection. *P-value <0.05.

Table 4. Linear regression results of BMI and serum 25(OH)D levels, BMI in categories

BMI	Model 1			Model 2[a]			Model 2[b]		
	ß	[95% CI]	p	ß	[95% CI]	p	ß	[95% CI]	p
Underweight N = 45	1.20	[-5.00; 7.35]	0.70	1.27	[-4.90; 7.44]	0.68	-	-	-
Normal weight N = 788	-1.02	[-2.50; 0.47]	0.18	-1.14	[-2.60; 0.32]	0.13	-1.16	[-2.59; 0.28]	0.11
Overweight N = 1444	-0.99	[-1.87; -0.10]*	0.03	-1.10	[-1.97; -0.22]*	0.01	-1.25	[-2.10; -0.40]*	0.004
Obesity N = 565	-0.82	[-1.43; -0.22]*	0.001	-0.89	[-1.49; -0.28]*	0.004	-0.96	[-1.54; -0.38]*	0.001

Model 1: crude model. Model 2[a]: adjusted for age and sex. Model 2[b]: adjusted for alcohol, total activities (sport and non-sport) in Kcal per day, smoking, alcohol and season. *P-value <0.05.

Table 5. Linear regression results of Fat% and serum 25(OH)D levels, fat% in quartiles

Fat%	Model 1			Model 2[a]			Model 2[b]		
	ß	[95% CI]	p	ß	[95% CI]	p	ß	[95% CI]	p
Quartile 1 N = 298	-0.06	[-0.87; 0.75]	0.88	-0.04	[-0.86; 0.78]	0.92	-0.02	[-0.80; 0.77]	0.97
Quartile 2 N = 299	-0.69	[-2.45; 1.07]	0.44	-1.29	[-3.03; 0.46]	0.15	-1.24	[-2.95; 0.48]	0.16
Quartile 3 N = 299	-1.12	[-2.66; 0.42]	0.15	-1.96	[-3.50; -0.42]*	0.01	-1.86	[-3.29; -0.43]*	0.01
Quartile 4 N = 298	-1.21	[-1.87; -0.55]*	<0.001	-1.33	[-2.00; -0.66]*	<0.001	-1.37	[-2.02; -0.71]*	<0.001

Model 1: crude model. Model 2[a]: adjusted for age and sex. Model 2[b]: adjusted for alcohol, total activities (sport and non-sport) in Kcal per day, smoking, alcohol and season. *P-value <0.05.

DISCUSSION

In our study we observed that BMI was significantly associated with serum 25(OH)D levels in older adults. After stratification for age, the association between BMI and serum 25(OH)D was only modest in the oldest group (>80 year), but stronger in the individuals younger than 80 years of age. This finding is consistent with the results of a recent meta-analysis, which showed that BMI inversely associated with 25(OH)D levels in a younger population14. Furthermore, we also observed that fat percentage was significantly associated with serum 25(OH)D levels and the association was more pronounced in the third and fourth quartiles of fat percentage. This finding supports the hypothesis that compared to BMI, body fat percentage is possibly a more accurate marker of obesity in our study group, to analyze the association between 'overweight' and serum 25(OH)D levels and could be used instead or next to BMI for measuring obesity in an older population.

A recent bi-directional genetic study, a design known to reduce the possibility of confounding, suggested that higher BMI leads to lower 25(OH)D levels in a younger population (mean age 53.4 years). Additionally, this study suggested an only modest relationship between lower 25(OH)D levels and BMI (28). The mechanism underlying the association between obesity and serum 25(OH)D levels is not yet completely understood. Several factors may be responsible for the observed association in this population of older adults, including limited sun exposure due to impaired mobility or clothing habits (29). In addition, it may be speculated that older obese persons have a lower vitamin D dietary intake, which may also lead to decrease in serum 25(OH)D levels (30,31). Moreover, laboratory findings indicated that adipose tissue is a storage site for 25(OH)D (32,33), and therefore it has been proposed that the

obesity-associated vitamin D deficiency may be due to the decreased bioavailability of vitamin D owing to its deposition in body fat compartments (34). Studies have also shown that weight loss and reduced body fat mass in obese persons is often accompanied with improvements in serum 25(OH)D levels (35).

The association between body fat and serum 25(OH)D levels may also be explained by metabolic pathways related to glucose intolerance. Particularly, it has been shown that a higher 25(OH)D levels may result in a higher insulin sensitivity, decrease in appetite and food intake, and thus a lower body fat percentage (36). Conversely, a higher body fat percentage may also result in lower serum 25(OH)D levels (36). As a result, vitamin D deficiency is more common in obese people (37), and this was also observed in our study. Based on the studies described above we can hypothesized that obese persons would require higher dosage of vitamin D supplementation to achieve accurate25(OH) vitamin D levels (38).

Limitations and future research

The main strengths of our study are its large population, and the use of both BMI and fat percentage measured by DXA. Limitations include, the cross-sectional approach, which prevents us from drawing conclusions regarding causality and the direction of the association. Secondly, there were some differences between the sub-samples, which may be explained by the fact that the younger and fitter persons were the ones that were able to visit the hospital to undergo the DXA scan, and this may have biased our results.

CONCLUSION

We observed an inverse association between BMI, body fat percentage, and serum 25(OH)D levels in elderly people. Thus, a higher BMI and a higher body fat percentage were associated with lower serum 25(OH)D levels. Although it is well known that elderly people overall are at risk for vitamin D deficiency, the results of the current study indicate that vitamin D deficiency is particularly important for obese older adults. This study suggests furthermore that, other anthropometric measurements, including fat mass percentage, may be more reliable measures of obesity than BMI, particularly if study outcomes are fat-mass related. Thus, it can be concluded that further research is needed to assess the direction and potential causality of this association in older persons and the effect of fat percentage on the dose-response effect of vitamin D supplementation in older persons.

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ETHICAL STANDARDS

We have worked according to the current laws in the Netherlands

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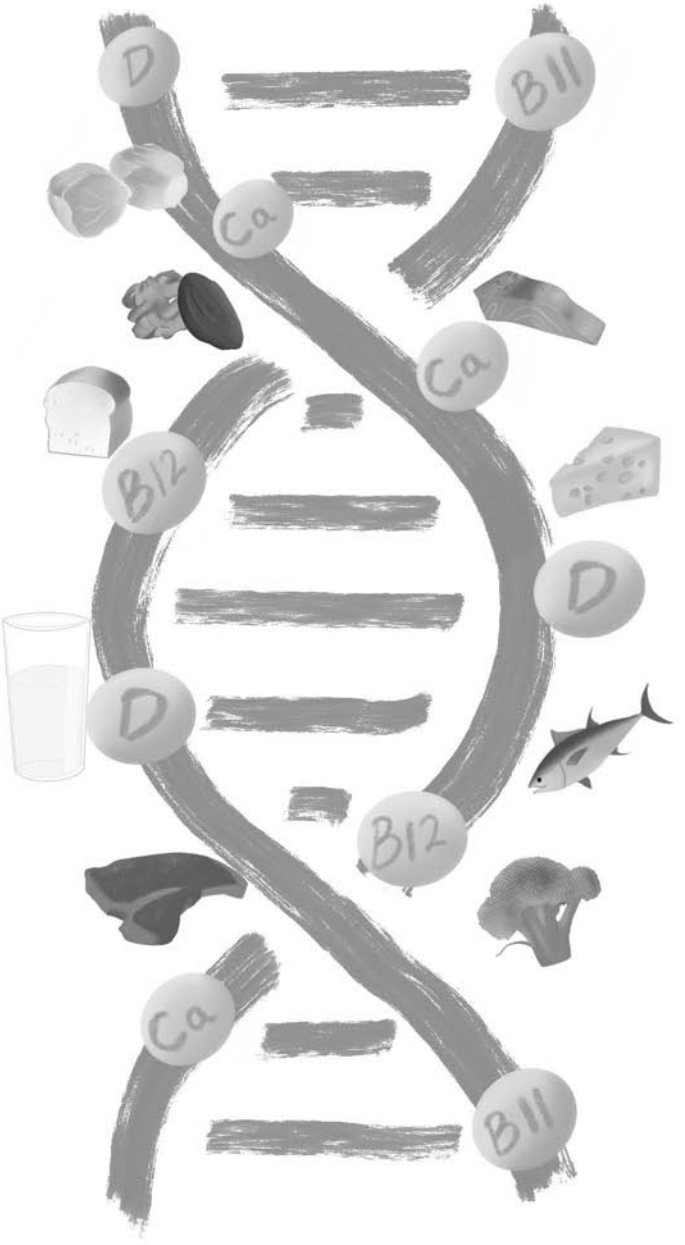
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Chapter 2.2

B-vitamins and body composition: integrating observational and experimental evidence from The B-PROOF study

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ABSTRACT

Purpose

Higher folate and vitamin-B12 have been linked to lower risk of overweight. However, whether this is a causal effect of these B-vitamins on obesity risk remains unclear and evidence in older individuals is scarce. This study aimed to assess the role of B-vitamin supplementation and levels on body composition in older individuals.

Methods

A double-blind, randomized controlled-trial in 2919 participants aged ≥ 65 years with elevated homocysteine levels. The intervention comprised a 2-year supplementation with a combination of folic acid (400 µg)and vitamin B12 (500 µg), or with placebo. Serum folate, vitamin-B12, active vitamin-B12 (HoloTC), methylmalonic acid (MMA), and anthropometrics were measured at baseline and after 2 years of follow-up. Dietary intake of folate and vitamin-B12 was measured at baseline in a subsample (n=603) using a validated food-frequency questionnaire. Fat mass index (FMI) and fat-free mass index (FFMI) were assessed with Dual Energy X-ray absorptiometry (DXA).

Results

Cross-sectional analyses showed that a 1 nmol/L higher serum folate was associated with a 0.021 kg/m^2 lower BMI (95%CI -0.039; -0.004). Higher HoloTC (per pmol/L log-transformed) was associated with a 0.955 kg/m^2 higher FMI (95%CI 0.262; 1.647), and higher MMA (per µgmol/L) was associated with a 1.108 kg/m^2 lower FMI (95%CI -1.899; -0.316). However, random allocation of B-vitamins did not have a significant effect on changes in BMI, FMI or FFMI during 2 years of intervention.

Conclusions

Although observational data suggested that folate and vitamin B12 status are associated with body composition, random allocation of a supplement with both B-vitamins combined versus placebo did not confirm an effect on BMI or body composition.

Keywords

Vitamin B12 and folic acid, Body composition, BMI, Fat (Free) mass, effect of vitamin B12 and folic acid on obesity.

INTRODUCTION

The prevalence of overweight is growing in all age categories (1). Among people older than 65 year in the European Union the prevalence of overweight, defined as a BMI of ≥ 25, has been estimated at between 58 and 66% (2). Overweight increases the risk of several chronic diseases, including cardiovascular diseases, metabolic syndrome, and type 2 diabetes (3). For adequate prevention of these diseases, it is therefore important to identify factors that influence the development of overweight across age categories.

Several studies showed that there is an association of overweight and obesity with lower serum vitamin-B12 and folate levels (4-8). However, it is currently not clear whether deficiencies of these B vitamins are a cause or a consequence of obesity (9). It is suggested that being overweight or obese can alter the absorption, distribution, metabolism and/or excretion of micronutrients, which may cause vitamin deficiencies, including those of folate and vitamin-B12 (10-12). This may be particularly of importance in older persons, as this group is at higher risk for deficiencies (13), including vitamin-B12 deficiency (14), due to inadequate dietary intake as well as malabsorption and higher depletion (15). Another explanation of the observed associations could be that B-vitamin deficiencies may contribute to adiposity, for example by the role of folate and vitamin-B12 in epigenetics. Vitamin-B12 and folate act as co-factors in one-carbon metabolism, which is important for the production of methyl donors for DNA methylation (16, 17). Deficiency of these nutrients could lead to dysregulation of DNA methylation and might generate metabolic disturbances, including disturbed energy and lipid metabolism, contributing to adiposity (18, 19).

To date, studies exploring the association between folate and vitamin-B12 with body composition in older individuals are scarce. Furthermore, BMI as a measure of overweight in elderly population is still under debate (20). As aging is associated with loss of muscle mass (21), BMI could underestimate the prevalence of obesity in this population. Hence, it is important to study more comprehensive measures of body composition in older people, e.g. by making a distinction between fat mass and fat free mass.

We aimed to 1) assess the cross-sectional associations of serum folate and vitamin-B12 levels as well as dietary intake of folic acid and vitamin-B12 from both food and supplements with body composition in older individuals; and 2) to study the causal effect of these vitamins by assessing the effect of supplementation with folic acid and vitamin-B12 combined on body composition in a randomized controlled trial.

METHODS

Study design and participants

For the present study, baseline data (2008-2011) and two years follow-up (2010-2013) data from the B-PROOF study (B-vitamins for the Prevention Of Osteoporotic Fractures) were used. The B-PROOF study is a multi-center, randomized, placebo-controlled, double-blind intervention study, investigating the effect of a 2-year daily oral vitamin-B12 (500 µg) and folic acid (400 µg) supplementation on fracture incidence. The study was conducted by three research centers in the Netherlands: VU University Medical Center (Amsterdam), Wageningen University (Wageningen), and Erasmus University Medical Center (Rotterdam). This study included 2919 individuals, aged 65 years and older with elevated homocysteine levels (12 - 50 µmol/L). Participants were excluded if they had renal insufficiency (creatinine level > 150 µmol/L) or diagnosed with malignant cancer in the past 5 years. A detailed description of the trial has been reported elsewhere (22). All participants gave written informed consent before the start of the study. The B-PROOF study has been registered in the Netherlands Trial Register (NTRNTR1333) and with ClinicalTrials.gov (NCT00696514). The Wageningen University Medical Ethics Committee approved the study protocol, and the Medical Ethics committees of Erasmus MC and VUmc gave approval for local feasibility (22).

Anthropometrics measurements

At baseline and follow-up, height was measured in duplicate to the nearest 0.1 cm with the participant standing erect and without wearing shoes, using a stadiometer (22). Weight was measured to the nearest 0.5 kg using a calibrated weighing device (SECA 761) with the participant wearing light garments, empty pockets and without wearing shoes [22]. BMI was calculated as weight in kilograms divided by square of height in meters and expressed as kg/m^2. Participants were categorized in underweight (BMI < 20 kg/m^2), normal weight (BMI 20 to <25 kg/m^2), overweight (BMI 25 to <30 kg/m^2), or obese (BMI ≥ 30 kg/m^2) (23).

Body composition measurements

At baseline and follow-up, a subsample of participants from the Amsterdam and Rotterdam research centers (n=424 and =803 at baseline; n=380 and n=732 at follow-up, respectively) underwent Dual Energy X-ray assessment (DXA) using the GE Lunar Prodigy device (GE Healthcare, USA, CV = 0.08%), (Erasmus MC) or the Hologic QDR 4500 Delphi device (Hologic Inc., USA, CV = 0.45%), (VuMC). The two devices were

cross-calibrated by measuring a European spine phantom (ESP) five times on both devices and all results were adjusted accordingly. Total fat mass and total fat free mass were estimated from the DXA scan (22). Fat Mass Index (FMI) and Fat Free Mass Index (FFMI) were calculated as total fat mass or total fat free mass in kilograms divided by square of height in meters and expressed as kg/m^2. Android/gynoid fat ratio was calculated as fat mass android region (g)/ fat mass gynoid region (g) and was only available for the participants from Rotterdam (n=800).

Laboratory measurements

Venous blood samples were obtained in the morning at baseline, when the participants were in a fasted state, or had taken a restricted breakfast. Plasma homocysteine was determined at baseline, using the Architect i2000 RS analyser (VUmc, intra assay CV – 2%, inter assay CV = 4%), HPLC method [22] (WU, intra assay CV = 3.1%, inter assay CV = 5.9%) and LCMS/MS (EMC, CV = 3.1%). According to a cross-calibration, different methods of determined plasma homocysteine of the three centers did not differ significantly. Serum folate was determined by imunoelectrochemiluminescence on a Roche Modular E170 (Roche, Almere, The Netherlands) (CV = 5.9% at 5.7 nmol/L and 2.8% at 23.4 nmol/L). Serum methylmalonic acid (MMA) was measured by LC-MS/MS (CV < 9%) and holo-transcobalamin (HoloTC), and was determined by the AxSYM analyser (Abbott Diagnostics, Hoofddorp, the Netherlands) (CV < 8%) (22, 25). HoloTC was used as measure of vitamin-B12 status, because it has been shown to better reflect vitamin-B12 status than serum total vitamin-B12 (26). To isolate DNA, buffy coats were used. The MTHFR genotypes, 677CC, 677CT or 677 TT, were determined using the Illumina Omni-express array (Illumina Inc., San Diego, CA, USA).

Food intake measurements

Dietary intake was estimated at baseline in a subsample, i.e. all participants of the Wageningen region (n=603), by a 190 item Food Frequency Questionnaire (FFQ), originally designed to assess intake of energy, total fat, fatty acids and cholesterol. This FFQ was updated using the Dutch National Food Consumption Survey of 1998 and extended with questions to estimate other macronutrients, vitamin-B12, folate, vitamin D, and calcium intake by the dietetics group of Wageningen University (27, 28). When one item contributed to more than 0.1% of the intake of macronutrients, vitamin B12, folate, vitamin D or calcium in this national survey, this item was added to the FFQ. In the end, all items in the FFQ accounted for 90% of the total folate and vitamin-B12 intake according to Dutch National Food Consumption Survey data of

1998. The Dutch Food Composition database (NEVO) was used to calculate daily folate and vitamin-B12 intake (29).

All dietary nutrient intake were adjusted for total energy intake using the residual method (30). The participants were also asked to write down the brand names of each supplement they used in the questionnaire at the baseline. Current use of folic acid and/or vitamin-B12 supplement was defined as users or non-users (22). Total amount of folic acid and vitamin-B12 supplement was also measured from the FFQ.

Covariates

Demographic characteristics and health status, which included age, sex, self-reported medical history (cardiometabolic diseases i.e. cardiovascular disease, diabetes, hypertension and hypercholesterolemia), alcohol intake, smoking habits, physical activity (PA, from the LASA Physical Activity Questionnaire, total activity expressed in kcal/day), education, were determined using a structured questionnaire. Alcohol intake was categorized into 'never', 'light', 'moderate' and 'excessive' , based on the number of days per week alcohol was consumed and the number of glasses per time, following the Dutch method of Garretsen et al. (31, 32) smoking habits were defined as never smoked; former smoker, or current smoker.

Statistical analyses

Normal distribution for all variables was examined by visual inspection of histograms. When necessary, data were log-transformed. For the cross sectional analyses at baseline, linear regression analysis was used to determine associations of serum folate, vitamin-B12, HoloTC, MMA, folic acid intake, vitamin-B12 intake, folic acid supplement use, and vitamin-B12 supplement use, and total folic acid and vitamin B-12 (from food and supplement) with BMI, FMI and FFMI. All analyses were adjusted for age and sex, and for energy intake for the associations for folic acid and vitamin-B12 intake (from food and supplements) (model 1). Subsequently, based on literature, smoking, alcohol, PA, education, hypertension, and hypercholesterolemia were added as confounders (33, 34). In addition, the associations and correlations between energy-adjusted folate and vitamin-B12 intake and status were calculated using linear regression and Spearman's correlation coefficient. Furthermore, low serum vitamin-B12 and folate levels are characteristics of overweight and obese subjects, therefore we stratified the population into three different groups, normal weight (BMI <25 kg/m2), overweight (BMI 25-30 kg/m2) and obesity (BMI>30 kg/m2).

For the analyses on the effect of the intervention, the primary analyses were performed according to the intention-to-treat (ITT) principle. To study the effect of the treatment on BMI, FMI and FFMI, linear regression analyses were performed for the subjects with at least one observation of height and weight (baseline and/or follow-up). The independent variable as randomization group (intervention vs. placebo) and the dependent variables were measures of body composition at follow-up as well as the difference between baseline and follow-up measurement (delta BMI, delta FMI and delta FFMI).The effect of the treatment on delta body composition was adjusted for baseline measurement of body composition and HoloTC (because significant differences in HoloTC was present between the treatment and control group).

In addition, for the experimental analyses, we assessed whether our findings were different by age, gender, homocysteine concentrations, presence of cardiometabolic disease, and genetic vulnerability, by testing the interaction of these potential effect modifiers with the intervention. If the P value for interaction was < 0.1, stratified analyses were performed. Statistical analyses were performed using the statistical software package of SPSS 24.0 (SPSS Inc., Chicago, Illinois, USA). P-values of < 0.05 were considered statistically significant for all the analyses other than the interaction analyses (<0.1).

RESULTS

Population characteristics

Population baseline characteristics are presented in **Table 1**. Mean age was 74.0 years (SD 6.5) for the total population (n=2919), 72.9 years (SD 5.7) for the DXA population (n=1227) and 72.8 years (5.7 SD) for the population with dietary intake data (n=603). In the total population, mean BMI at baseline was 27.1 kg/m^2 for the intervention group and 27.2 kg/m^2 and control group. In the DXA population, mean FMI and FFMI were 8.9 and 18.0 kg/m^2 respectively, for the intervention group and 8.9 and 18.1 kg/m^2 respectively, for the control group at baseline. Mean baseline folic acid level of total population was 21.0 nmol/L (11.62 SD) and mean baseline vitamin B12 level was 285.5 pmol/L (116.0 *SD).* Follow-up measurements were conducted 2 years after in 2636 participants. The characteristics of these participants are shown in **Supplemental Table 1.** Mean folic acid level of total population raised to 40.5 nmol/L and for vitamin B12 level the mean raised to 472.6 pmol/L (378.2 SD) after the intervention.

Table 1. Population baseline characteristics

	B-PROOF Participants (N =2919)	DXA-test Participants (N = 1227)	FFQ Participants (N=603)
Age (years)[a]	74 (6.5)	72.9 (5.7)	72.8 (5.7)
Sex			
Female (%)	50	48.3	42.1
Body Mass Index (kg/m^2)[a]	27.1 (4.0)	27.0 (3.8)	26.9 (3.6)
Underweight (%)	0.4	0.2	0.3
Normal weight (%)	28.6	30.4	28.4
Overweight (%)	50.9	50.1	54.7
Obesity (%)	20.1	19.2	16.6
Fat	NA		NA
Total Fat Mass (kg)		25.5 (8.4)	
Total Fat Percentage (%)		32.4	
FMI (kg/m^2)		8.9 (3.2)	
FFMI (kg/m^2)		18.0 (2.2)	
Smoking (%)			
Current	56.5	57.6	58.8
Former	9.6	9.0	10.4
Never	33.9	33.3	31.0
Alcohol intake (%)			
Light	67.4	64.1	64.2
Moderate	28.8	31.4	32.8
Excessive	3.4	4.0	2.5
Very excessive	0.4	0.6	0.5
Self-reported medical history of			
Cardiac disease (% yes)	25.1	25	25.5
Diabetes (% yes)	10.3	10.8	7.1
Hypercholesterolemia (%yes)	24.7	28.5	21.2
Measured hypertension (%yes)	51.5	58.8	38.6
Homocysteine (mmol/L)[b]	14.4 [3.4]	14.3 [3.2]	14.0 [3.2]
Serum Folate (nmol/L)[a]	21.0 (11.62)	21.3 (9.3)	20.1 (17.2)
Serum Vitamin-B12 (pmol/L)[a]	285.5 (116.0)	287.5 (115.2)	281.2 (107.9)
Holotranscobalamin (pmol/L)[b]	64.0 [251.0]	67.0 [41.0]	60.0 [34.0]
MMA (µgmol/L)[b]	0.2 [0.1]	0.2 [0.1]	0.2 [0.1]
MTHFR (%)			
CC	44.9	46.4	44.3
CT	42.1	40.8	42.6
TT	13.0	12.8	13.1
Folic Acid supplement use (%)	16.0	16.3	11.1
Vitamin-B12 supplement use (%)	16.2	16.8	11.1
Folate intake from food (mcg/day)[a]	NA	NA	191.5 (53.9)
Vitamin-B12 intake from food (mcg/day)[a]	NA	NA	4.1 (2.0)
Total activity (Kcal/day)[b]	560 [489]	595 [504]	593 [506]

Table 1. Population baseline characteristics (continued)

	B-PROOF Participants (N =2919)	DXA-test Participants (N = 1227)	FFQ Participants (N=603)
Total energy intake (Kcal/day)[a]	NA	NA	2006 (473)
Education (%)			
Low	32.1	32.5	25.9
Middle	42.0	41.1	40.8
High	26.0	26.4	33.3
Region (%)			
Amsterdam	26.6	34.6	0
Rotterdam	29.4	65.4	0
Wageningen	44.0	0	100

[a]Presented as mean (SD)b median [IQR]

Baseline associations of vitamin B12 and folic acid with body composition

Results of the cross-sectional linear regression analyses of serum folate, serum vitamin-B12, HoloTC, MMA, folic acid supplement use, and vitamin-B12 supplement use with BMI for the total population, are shown in **Table 2**. Higher serum folate (per nmol/L) was associated with a 0.021 kg/m^2 lower BMI (95% CI -0.039; -0.004) after adjustments for covariates. Serum vitamin-B12, HoloTC, MMA, folic acid supplements and vitamin-B12 supplements were not associated with BMI after adjustment for covariates (**Table 2**). We found also no significant associations between intake of folic acid and vitamin-B12 (total and from food only) and BMI (**Table 2**).

Results in a subgroup (n=1227) with DXA measurements showed that there was an association between HoloTC and FMI (β 0.955 kg/m^2 per pmol/L; 95% CI 0.262; 1.647) in the adjusted model, indicating that for each pmol/L higher log-transformed HoloTC, there was a 0.955 kg/m^2 higher FMI. MMA was significantly inversely associated with FMI, indicating that for each μgmol/L higher MMA, there was a 1.108 kg/m^2 lower FMI (95% CI -1.899; -0.316). None of the examined exposures were associated with FFMI (**Table 3**) or with android/gynoid fat ratio (data not shown).

Stratified analysis of different groups of BMI showed that higher serum folate level was associated with a lower BMI in the population with overweight and obesity, however the associations were not statistically significant (**Supplemental Table 2**).

Table 2. Baseline associations of serum folate, vitamin-B12, HoloTC, MMA, folic acid supplements and vitamin-B12 supplements with BMI

	BMI	
	Model 1 *B 95% CI*	*Model 2* *B 95% CI*
Serum Folate (nmol/L) (n=2919)	-0.018 [-0.030; -0.005]*	-0.021 [-0.039; -0.004]*
Serum Vitamin-B12 (pmol/L) (n=2919)	-0.001 [-0.003; -0.00001]*	-0.001 [-0.002; 0.001]
HoloTC_log[a] (pmol/L) (n=2919)	0.533 [-0.085; 1.151]	0.612 [-0.099; 1.323]
MMA (µgmol/L) (n=2919)	-0.258 [-0.742; 0.225]	-0.675 [-1.470; 0.120]
Folic acid supplement use (n=2919)	-0.271 [-0.666; 0.124]	-0.104 [-0.543; 0.335]
Vitamin-B12 supplement use (n=2919)	-0.366 [-0.758; 0.026]	-0.268 [-0.705; 0.169]
Folic acid total intake (FFQ) (n=1227)	-0.0002 [-0.002; 0.001]	0.0005 [-0.003; 0.002]
Vitamin-B12 total intake (FFQ)[a] (n=1227)	-0.795 [-2.086; 0.496]	-1.045 [-2.735; 0.646]
Folic acid intake from food (FFQ) (n=1227)	0.005 [-0.002; 0.011]	0.007 [-0.001; 0.015]
Vitamin-B12 total intake from food (FFQ) (n=1227)	0.033 [-0.013; 0.080]	0.069 [-0.006; 0.143]

Values are regression coefficients and 95% CIs based on linear regression models and reflect differences in BMI per 1 unit increase of serum folate, serum vitamin-B12, HoloTC, MMA, folic acid supplements, vitamin-B12 supplements; Model 1 is adjusted for age and sex. Model 2 is additionally adjusted for smoking, alcohol consumption, physical activity, education, hypertension and hypercholesterolemia. alog transformed *P-value <0.05.

The effect of the intervention

After 2 years follow-up, mean BMI was 27.2 kg/m^2 for both groups, mean FMI and FFMI were 9.1 and 18.0 kg/m^2 respectively, for the intervention group, and 9.0 and 18.0 kg/m^2 respectively, for the control group. Linear regression analyses showed that the combined vitamin B12 and folic acid intervention did not affect changes in BMI (ß= -0.051; 95%CI: -0.368; 0.265 for the effect on FU BMI and ß= -0.031; 95%CI: -0.156; 0.093 for the effect on delta BMI in the total population) (**Table 4**). Also, no significant effect of the intervention on changes in FMI or FFMI were observed (**Table 4**).

Additional analyses on the intervention

For the experimental analyses, we observed a significant interaction between treatment and gender with BMI (p for interaction 0.001), cardiometabolic diseases with FMI and FFMI at the end of follow-up (p for interaction 0.06 and 0.02 resp.), and MTHFR with change in FFMI (p for interaction 0.05). Stratified analyses showed only a significant effect of the intervention for people with MTHFR genotype TT, compared to

Table 3. Baseline associations of serum folate, vitamin-B12, HoloTC, MMA, folic acid supplements and vitamin-B12 supplements with FMI and FFMI in the population with DXA data (n=1227)

	FMI		FFMI	
	Model 1	*Model 2*	*Model 1*	*Model 2*
	B 95% CI	*B 95% CI*	*B 95% CI*	*B 95% CI*
Serum Folate (nmol/L)	-0.002 [-0.019; 0.016]	-0.002 [-0.019; 0.016]	-0.009 [-0.020; 0.002]	-0.010 [-0.021; 0.0005]
Serum Vitamin-B12 (pmol/L)	0.0002 [-0.002; 0.001]	-0.000003 [-0.001; 0.001]	0.0001 [-0.001; 0.001]	0.0003 [-0.001; 0.001]
HoloTC_log[a] (pmol/L)	0.748 [0.073; 1.423]*	0.955 [0.262; 1.647]*	0.232 [-0.191; 0.654]	0.403 [-0.032; 0.839]
MMA (µgmol/L)	-0.746 [-1.530; 0.039]	-1.108 [-1.899; -0.316]*	-0.138 [-0.629; 0.353]	-0.170 [-0.669; 0.328]
Folic acid supplements	-0.031 [-0.462; 0.400]	0.174 [-0.272; 0.621]	-0.175 [-0.444; 0.094]	-0.105 [-0.384; 0.174]
Vitamin-B12 supplements	-0.184 [-0.610; 0.242]	-0.054 [-0.494; 0.386]	-0.191 [-0.457; 0.075]	-0.112 [-0.388;0.163]

Values are regression coefficients and 95% CIs based on linear regression models and reflect differences in BMI per 1 unit increase of serum foliate, serum vitamin-B12, HoloTC, MMA, folic acid supplements, vitamin-B12 supplements. Model 1 is adjusted for age and sex. Model 2 is additionally adjusted for smoking, alcohol consumption, physical activity, education, hypertension and hypercholesterolemia. alog transformed *P-value <0.05.

Table 4. The effect of the intervention on follow-up and changes of body composition

	Intervention group			Placebo group				
	Baseline estimated mean (SD)	*FU estimated mean (SD)*	*2-year change*	*Baseline estimated mean (SD)*	*FU estimated mean (SD)*	*2-year change*	Treatment effect on FU *B 95% CI*	Treatment effect on Δ BC *B 95% CI*
BMI (n=2919)	27.116 (3.975)	27.158 (4.229)	0.042	27.171 (3.959)	27.207 (4.008)	0.049	-0.051 [-0.368; 0.265]	-0.031 [-0.156; 0.093]
FMI (n=1227)	8.951 (3.201)	9.120 (3.244)	0.169	8.929 (3.227)	8.975 (3.110)	0.046	0.105 [-0.270; 0.479]	0.015 [-0.101; 0.130]
FFMI (n=1227)	17.979 (2.150)	17.976 (2.182)	-0.003	18.057 (2.273)	17.951 (2.172)	-0.106	0.039 [-0.220; 0.298]	0.052 [-0.073; 0.177]

Values are regression coefficients and 95% CIs based on linear regression models and reflect differences in BMI, FMI, and FFMI for intervention compared to the placebo group. Adjusted for HoloTC at baseline FU= follow-up Δ BC= difference between body composition at baseline and follow-up.

CT and CC (ß 0.46, 95%CI 0.13; 0.79 and ß-0.05, 95%CI -0.26; 0.16 and ß-0.04, 95%CI -0.25; 0.18 resp.) (**Supplemental Table 3**).

DISCUSSION

In the current study, we integrated observational and experimental data on B-vitamins and body composition in a large elderly population. Although we found that higher serum folate was associated with a lower BMI and, that indices of a higher vitamin B12 status were associated with a higher FMI, *we did not observe* any effect of random allocation with both B-vitamins combined on BMI or body composition *after 2 years of intervention*.

Hypotheses of the observed associations between B-vitamins and body composition have been proposed for both directions: folate and vitamin-B12 deficiency may be a consequence of obesity due to inadequate dietary intake, altered absorption and (urinary) excretion of folate and vitamin-B12 (35), but may also cause obesity via epigenetic mechanisms, such as DNA methylation and miRNA expression involved in lipid homeostasis and inflammatory pathways (18, 19, 36). In our observational data, we confirmed results from previous studies showing associations between B-vitamin status and body composition. However, we did not find any differences in body composition after supplementation with folic acid and vitamin B12 after 2 years of follow-up, suggesting that B-vitamins may not have a role in the etiology of obesity or changes in body composition in the older individuals. In addition, to evaluate whether insufficient dietary intake explained the results, we examined the associations between dietary intake of folate and vitamin-B12 with body composition which also did not show that (dietary intake) vitamin-B12 and folate were related to differences in body composition. Thus, on the basis of our findings, it may be argued that obesity or increased fat mass may lower B-vitamins status (37), but not the other way around. This is in line with a previous study in which the causality of the relation between vitamin-B12 and BMI was studied with use of a Mendelian randomization approach. The authors reported that vitamin-B12 levels were associated with BMI, however, there was no evidence that lower vitamin-B12 levels caused a higher BMI (38). In our observational data, we found a non-significant association of a lower BMI with higher intake of vitamin-B12. In contrast to our hypothesis, we observed that several biomarkers of higher vitamin-B12 status, including higher levels of HoloTC and lower levels of MMA, were associated with a higher FMI. In contrast, in another study among adults from a primary care-based setting vitamin-B12 level was negatively correlated with BMI [6]. With regard to folate, Kimmons et al. showed that, compared

with normal-weight adults, overweight and obese adults were more likely to have low folate levels [4]. Similarly, a study by Mahabir et al. showed that adiposity was associated with lower serum folate levels in postmenopausal women [5], suggesting that obese individuals are at increased risk of folate deficiency.

We found an indication that the B-vitamin intervention had an effect on changes in FFMI in participants with the MTHFR TT variant, and not in the those with the CT and CC variant. It is known that the TT variant is associated with higher risk of folate deficiency and increased homocysteine concentrations, which may suggest that a potential effect of B-vitamins on change in fat free mass may be restricted to elderly population at risk for folate deficiency or disturbances in one-carbon metabolism, but this needs further replication in larger intervention studies.

Strengths and limitations

The current study has several strengths and limitations. An important strength is the size of the population included and the combination of both observational and experimental data. Another strength of this study was that detailed body composition measurements were available using DXA. Although BMI may be practical to measure, it is limited regarding prediction of several health outcomes, especially in older individuals, because it does not take into account body composition (39, 40). We had also several methods to measure vitamin-B12 levels which provided better insight into potential deficiencies. For instance, HoloTC has been shown to be a more accurate to detect vitamin-B12 deficiency compared to other methods (41). Also serum concentrations of MMA are considered to be metabolic indicators of vitamin-B12 status through the L-Methylmalonyl-CoA mutase (42).

To appreciate our findings also some limitations should be taken into account. We used the intervention data to show that folic and vitamin B12 supplementation did not affect body composition. Thus, our findings suggest that obesity or increased fat mass may lower B-vitamin status, but not vice versa. However, due to potential residual confounding we cannot conclude that the cross-sectional association between biomarkers of vitamin-B12 with FMI is explained by an effect of adiposity on B12 levels. Associations may also be due to e.g. other dietary and lifestyle factors, which unfortunately we could not explore in this population. Furthermore, due to the low variation of B-vitamin status in our study population, we may not have been able to detect any possible effect of the intervention on body composition and the results are less generalizable in other populations. The combination of vitamin B12 and folic acid in the intervention may have limited our ability to detect an individual effect of these

vitamins on body composition. In cross sectional analyses, we observed associations in different directions for folate levels and biomarkers of vitamin B12. Therefore, a potential effect of either of these vitamins on body composition could not be detected in our experimental data, as these may have canceled each other. Replication of findings forthcoming this study are thus required to examine whether results are true associations and should be examined for vitamin B12 and folic acid separately.

CONCLUSION

In this large population of older individuals, although observational evidence suggested that folate and vitamin B12 are associated with body composition, random allocation of a supplement with both B-vitamins versus placebo did not confirm an effect on body composition. Future studies should further investigate mechanisms underlying potential effects of body composition on B-vitamin status and how these effects may differ for certain subgroups.

Author contributions

The authors' responsibilities were as follows— SOA, KVEB, TV and JCKdJ: designed the research; SAO and KVEB: analyzed the data; SOA, KVEB, TV and JCKdJ: wrote the manuscript; TV and JCKdJ: had primary responsibility for the final content of the manuscript; NvdV, SCvD, NMvS, CMZ, LCPGMdG, AGU, and BS: critically revised the manuscript for important intellectual content; and all authors: read and approved the final manuscript.

Ethical standard

All participants gave written informed consent before the start of the study. The B-PROOF study has been registered in the Netherlands Trial Register (NTRNTR1333) and with ClinicalTrials.gov (NCT00696514). The Wageningen University Medical Ethics Committee approved the study protocol, and the Medical Ethics committees of Erasmus MC and VUmc gave approval for local feasibility [24] and have, therefore, been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments.

Conflicts of Interest: The authors declare no potential conflicts of interest.

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SUPPLEMENTARY DATA

Supplemental Table 1. Population follow up characteristics

	B-PROOF Participants (N =2636)
Age (years)[a]	75.6 (6.2)
Sex	
Female (%)	50
Body Mass Index (kg/m^2)[a]	27.2 (4.1)
Underweight (%)	0.4
Normal weight (%)	29.5
Overweight (%)	48.8
Obesity (%)	21.2
Fat	NA
Total Fat Mass (kg)	
Total Fat Percentage (%)	
FMI (kg/m^2)	
FFMI (kg/m^2)	
Smoking (%)	
Smoking not changed	96.1
Stopped smoking	1.2
Started smoking	0.2
Alcohol intake (%)	
Light	69.9
Moderate	27.2
Excessive	2.6
Very excessive	0.3
Self-reported medical history of	
Cardiac disease (% yes)	16.2
Diabetes (% yes)	11.1
Hypercholesterolemia (%yes)	25.1
Measured hypertension (%yes)	56.5
Homocysteine (mmol/L)[b]	12.2 [5.0]
Serum Folate (nmol/L)[a]	40.5 (22.6)
Serum Vitamin-B12 (pmol/L)[a]	472.6 (378.2)
Holotranscobalamin (pmol/L)[b]	87.0 [70.0]
MMA (µmol/L)[b]	0.2 [0.1]
MTHFR (%)	
CC	44.9
CT	42.1
TT	13.0
Folic Acid supplement use (%)	12.0
Vitamin-B12 supplement use (%)	12.1
Folate intake from food (mcg/day)[a]	NA
Vitamin-B12 intake from food (mcg/day)[a]	NA

Supplemental Table 1. Population follow up characteristics (continued)

	B-PROOF Participants (N =2636)
Education (%)	
Low	32.1
Middle	42.0
High	26.0
Region (%)	
Amsterdam	26.6
Rotterdam	29.4
Wageningen	44.0

[a]Presented as mean (SD)b median [IQR]

Supplemental Table 2. Associations between vitamin B12 and folate intake and serum and BMI - stratified for BMI (normal weight, overweight and obesity).

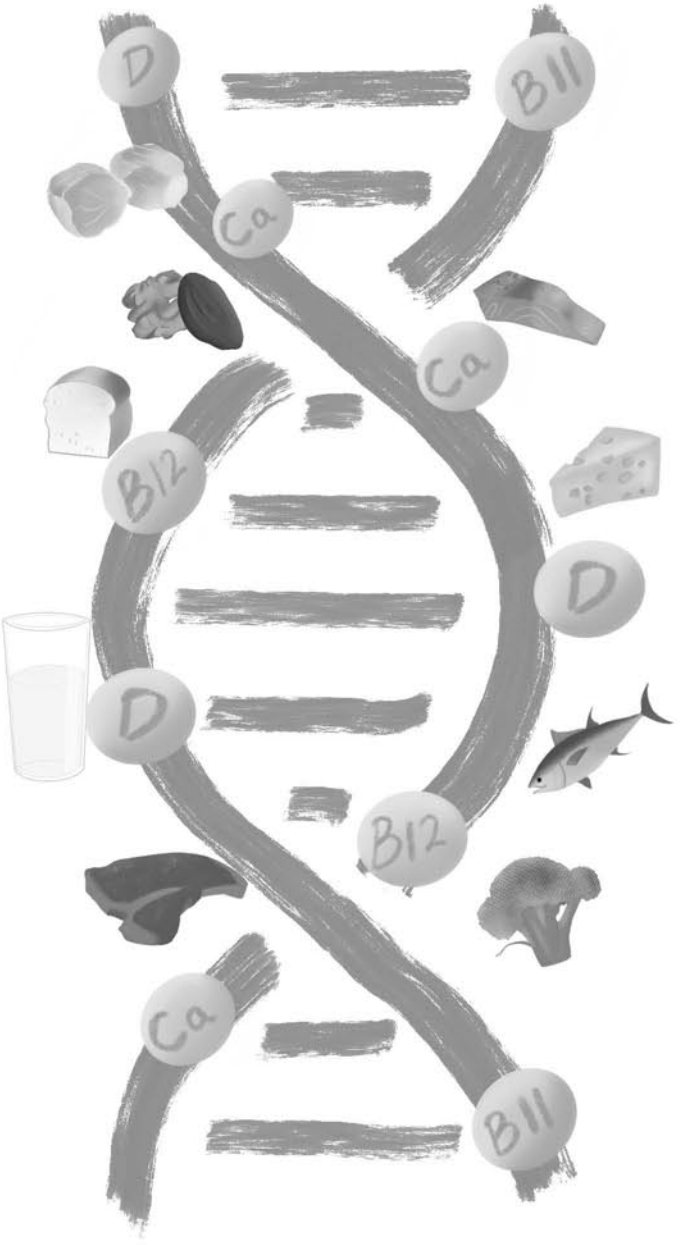
	BMI					
	Model 1 β	95% CI		Model 2 β	95% CI	
Normal weight (BMI <25)						
Serum folate	0.002	-0.008	0.013	0.003	-0.010	0.015
Serum vitB12	-0.0002	-0.001	0.001	-0.0003	-0.001	0.001
Overweight (BMI 25-30)						
Serum folate	-0.002	-0.007	0.003	-0.0003	-0.009	0.008
Serum vitB12	0.0001	-0.001	0.001	0.0002	-0.001	0.001
Obesity (BMI >30)						
Serum folate	-0.024	-0.057	0.009	-0.013	-0.053	0.026
Serum vitB12	-0.0005	-0.003	0.002	-0.001	-0.004	0.002

Model 1 is adjusted for age and sex; Model 2 is additionally adjusted for smoking, alcohol consumption, physical activity, education, hypertension and hypercholesterolemia.

Supplemental Table 3: The effect of the intervention on follow-up and changes of body composition stratified for effect modifiers (p-interaction < 0.10)

	Treatment effect on FU *B 95% CI*	Treatment effect on Δ BC *B 95% CI*	Treatment effect on FU *B 95% CI*	Treatment effect on Δ BC *B 95% CI*	Treatment effect on FU *B 95% CI*	Treatment effect on Δ BC *B 95% CI*
	Without cardiometabolic diseases		**With cardiometabolic diseases**			
FMI	-0.118 [-0.545; 0.309]	-	0.661 [-0.092; 1.431]	-	-	-
FFMI	0.227 [-0.069; 0.522]	-	-0.432 [-0.935; 0.070]	-	-	-
	Male		**Female**			
BMI	-0.113 [-0.480; 0.254]	-	0.011 (-0.508; 0.530)	-	-	-
	MTHFR TT		**MTHFR CT**		**MTHFR CC**	
FFMI	-	**0.489 [0.157; 0.821]***	-	-0.057 [-0.261; 0.147]	-	-0.032 [-0.237; 0.173]

Values are regression coefficients and 95% CIs based on linear regression models and reflect differences in BMI, FMI, and FFMI for intervention compared to the placebo group. FU= follow-up Δ BC= difference between body composition at baseline and follow-up. * p-value <0.05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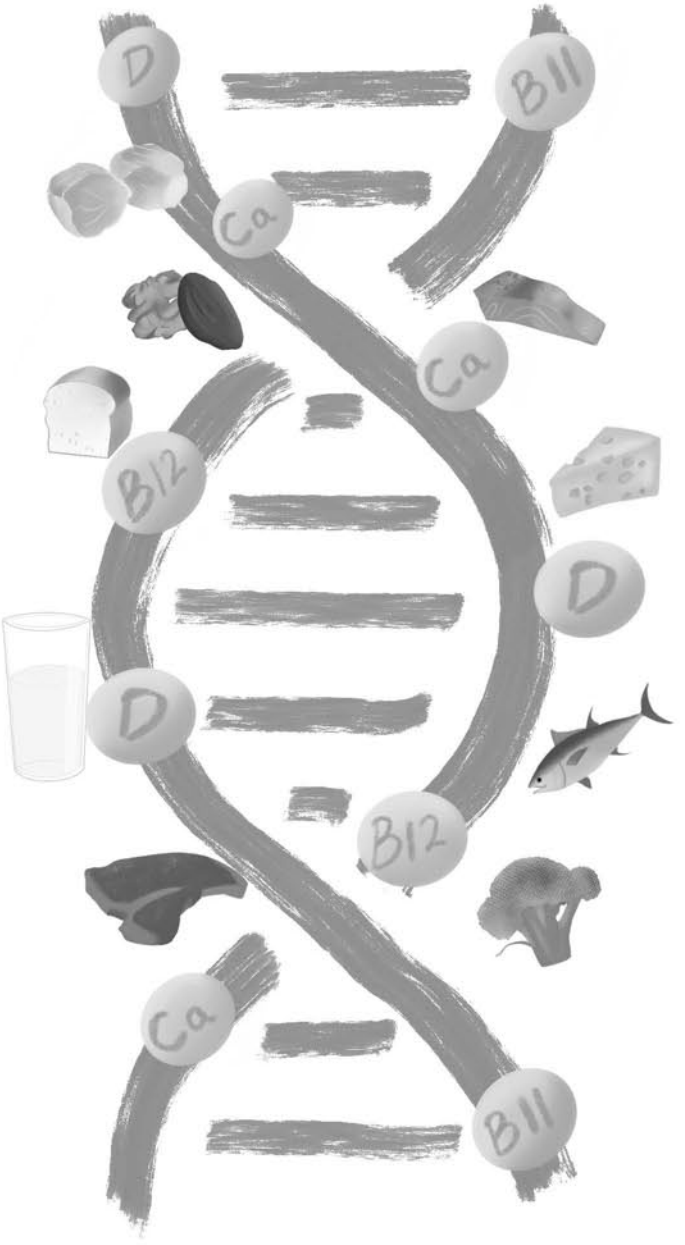
LONG-TERM EFFECT OF MICRONUTRIENTS ON HEALTH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Chapter 3.1

Folic acid and vitamin-B12 supplementation and the risk of cancer: long-term follow-up of the B-vitamins for the Prevention Of Osteoporotic Fractures (B-PROOF) trial

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ABSTRACT

Background

Folic acid and vitamin-B12 play key roles in one-carbon metabolism. Disruption of one-carbon metabolism may be involved in the risk of cancer. Our aim was to assess the long-term effect of supplementation with both folic acid and vitamin-B12 on the incidence of overall cancer and on colorectal cancer in the B-PROOF trial.

Methods

Long-term follow-up of B-PROOF trial participants (N=2,524), a multi-center, double-blind randomized placebo-controlled trial designed to assess the effect of 2-3 years daily supplementation with folic acid (400 µg) and vitamin-B12 (500 µg) versus placebo on fracture incidence. Information on cancer incidence was obtained from the Netherlands cancer registry (Integraal Kankercentrum Nederland), using the International Statistical Classification of Disease (ICD-10) codes C00-C97 for all cancers (except C44 for skin cancer), and C18-C20 for CRC.

Results

Allocation to B-vitamins was associated with a higher risk of overall cancer (171 [13.6%] vs. 143 [11.3%]), HR 1.25; 95%CI 1.00-1.53, p=0.05). B-vitamins were significantly associated with a higher risk of colorectal cancer (43[3.4%] vs. 25[2.0%]), HR 1.77; 95%CI 1.08-2.90, p=0.02).

Conclusion

Folic acid and vitamin-B12 supplementation was associated with an increased risk of colorectal cancer.

Impact

Our findings suggest that folic acid and vitamin-B12 supplementation may increase the risk of colorectal cancer. Further confirmation in larger studies and in meta-analyses combining both folic acid and vitamin-B12 are needed to evaluate whether folic acid and vitamin B12 supplementation should be limited to patients with a known indication such as a proven deficiency.

INTRODUCTION

A large proportion of the population globally, especially older people, use dietary supplements to promote good health (1). Studies of National and International Food Consumption Surveys, reported for example in the USA, UK and The Netherlands that 56%, 39% and 27% of older adults respectively use dietary supplements (2-5). However, supplements may not always be favorable, and in certain cases or doses they may even have adverse health effects (6). In addition, potential effects need to be put into perspective according to different fortification policies in countries (e.g. mandated vs. voluntary folic acid fortification). Together with vitamin-B12, folic acid plays a key role in one-carbon metabolism being involved in DNA methylation and DNA synthesis (7, 8). Several studies have suggested that altered DNA methylation is associated with a higher risk of certain cancers, including breast, prostate and colorectal cancer (CRC). Until recently, it was believed that folate and folic acid supplementation may have a protective effect on the risk of malignancies (9, 10). However, over the past decade, there have been some concerns that folic acid supplementation may actually increase the risk of cancer (11-15), possibly by promoting the progression of pre-neoplastic and undiagnosed neoplastic lesions (8, 16). Although some countries, including The United States, South Africa and Australia, have introduced population-wide folic acid fortification to prevent neural tube defects in the fetus (17-21), mandatory folic acid fortification has not been implemented in New Zealand or in several Western European countries partly because of these concerns about potential adverse effects on cancer incidence or progression (21, 22).

A recent meta-analysis of 10 studies (n=19,106; age range 26-69 years), reported no significant excess risk of folic acid (0.4 to 1 mg) supplementation on overall cancer incidence (23). However, previous results from the B-PROOF study, a randomized controlled trial on vitamin-B12 and folic acid supplementation on fracture risk in older persons, reported a higher incidence of self-reported cancer in the intervention group relative to the control group after a follow-up of 2-3 years (HR 1.56; 95% CI 1.04-2.31). Additional subgroup analysis revealed that the excess risk was predominantly explained by a higher CRC incidence, and that the effect appeared to be strongest in people aged older than 80 years (24).

Since the adverse effect of folic acid and vitamin-B12 supplementation on self-reported (colorectal) cancer was previously observed within 2-3 years in the B-PROOF study (24), the objective of this study was to validate these findings with data on confirmed cancer diagnosis and assess the long-term effects of folic acid and vitamin-B12 co-supplementation on the risk of overall cancer incidence and on CRC using prolonged

follow-up of trial participants. As such this secondary analysis of the B-proof study will contribute to current understanding of the biological plausibility of the effect of folic acid and vitamin- B12 co-supplementation on cancer (CRC) risk which will contribute to the ongoing fortification debate ongoing in several countries.

MATERIALS AND METHODS

The B-PROOF study (B-vitamins for the Prevention Of Osteoporotic Fractures) is a large multi-center (Erasmus MC Rotterdam, VU University Medical Center Amsterdam (VUmc) and Wageningen University (WUR), the Netherlands), randomized, placebo-controlled, double blind study, investigating the effect of daily oral vitamin-B12 and folic acid supplementation over a period of 2 to 3 years on fracture incidence.

Recruitment of participants took place between September 2008 and March 2011. A detailed description and study protocol of the trial has been reported elsewhere (25). Participants (n=2919) aged 65 years and over with an elevated homocysteine level (Hcy 12-50 µmol/l) were included. Participants were excluded if they had a renal insufficiency (creatinine level > 150 µmol/l) or history of a malignancy (excluding non-melanoma skin cancer) in the past 5 years or if they used high dosages of B-vitamins (intramuscular injections of vitamin-B12 and/or folic acid intake >300 µg/day, this was reported at the time of recruitment and was asked again by the questionnaire at the baseline).

Written informed consent was obtained before allocated treatment for all participants. For the present analysis, we used only the information of participants who gave permission to contact health institutes and medical doctors for their health details and medical history (n=2,524).

The B-PROOF study was registered in the Netherlands Trial Register (NTRNTR1333) and ClinicalTrials.gov (NCT00696514). The Ethics Committee approval for the study protocol was obtained from the Medical Ethics committees of Erasmus MC, VUmc and WU universities, according to declaration of Helsinki (25).

The intervention group received a daily tablet with 500 µg vitamin-B12 and 400 µg folic acid. In addition, both the control and intervention groups received 15 µg (600 IU) of vitamin D3 daily to ensure a normal vitamin D status. The intervention and placebo tablets, produced by Orthica, Almere, the Netherlands, are indistinguishable in taste, smell and appearance. The duration of intervention was 2 years, and to

increase power, individuals who finished their participation extended their participation for 1 more year (n=339 had 3 years intervention)(25).

The primary outcome of this study was the incidence of any cancer defined based on the International Statistical Classification of Disease (ICD-10) codes C00-C97. Individual data were obtained by linkage to the Netherlands cancer registry (Integraal Kankercentrum Nederland IKNL) from baseline until May, 2017. The Netherlands Cancer Registry is linked to the International Agency for Research on Cancer (IARC) and delivers pseudonymous data to the European database of the European Network of Cancer Registries (ENCR). Hence, employees of the cancer registry were unaware of treatment allocation of the participants. We used C00-C97 ICD codes for overall cancer (except C44 for skin cancer), and C18-C20 for CRC (26).

At baseline, height was measured using a stadiometer in duplicate to the nearest 0.1 cm and weight by using a calibrated weighing device (SECA 761) to the nearest 0.5 kg, both without wearing shoes (25). Body mass index (BMI) was calculated as weight in kg/height in m^2. A structured questionnaire was used to assess self-reported medical history (cardiovascular disease and diabetes mellitus), current use of medication and supplements, alcohol intake and smoking habits. Blood was collected, plasma homocysteine (Hcy), serum folate, vitamin-B12, holotranscobalamin (HoloTC), 25(OH) D and methylenetetrahydrofolate reductase (MTHFR)-genotype were determined; details of the methods used have been described previously (25).

Statistical analyses

We extended the follow-up of the original B-proof study to study the incidence of pathology-proven solid cancers. For all variables, mean with standard deviations (SD) or percentages were reported for each group. Differences between groups were tested with the t-test for continuous variables, Mann-Whitney U for normally skewed variables, and Chi-squared test for categorical variables. The cumulative event-free survival for cancer was analyzed by a Kaplan-Meier event curve. We calculated follow-up time as the number of months from the baseline measurement until the first diagnosis of incident cancer, death, loss-to-follow-up, or end of the study period, whichever occurred first. The incidence rate ratio was calculated on the incidence-rate of cancer for both treatment groups, which is defined as the number of cancer cases divided by the total sum of the follow-up in each group (cases/persons years). To avoid bias, primary analysis was based on the intention to treat (ITT) principle, where participants were analyzed based on the initial treatment allocation. Unadjusted Cox proportional hazard analyses were conducted with treatment (intervention vs.

control group) as the independent variable and the cancer diagnosis as the dependent outcome variable. Multivariable Cox proportional hazard regression analyses were applied adjusted for serum HoloTC, because this variable differed significantly between the intervention and the control group despite randomization. All other potential confounders were equally distributed between both groups. Additionally, subgroup analyses were performed to assess whether the treatment effect was different in strata of sex, age, plasma Hcy and MTHFR polymorphism, interaction with these variables were evaluated in the multivariable model for both overall cancer and CRC. When *p* for interaction was <0.1, subgroup analyses were performed. Second, per protocol (PP) analyses were performed that included data only from subjects who were compliant (>80% of pills consumed) to the study protocol, details have been described previously (25) and also a sensitivity analysis was performed in participants who were not using folic acid and vitamin- B12 supplements. Exploratory analysis has been done by duration of treatment (2 years vs. 3 years). Furthermore, for comparison purposes, we also calculated the risk ratio (RR) and log-rank observed – expected statistic. p-values <0.05 were considered to be statistically significant. Analyses were performed using IBM SPSS 21.

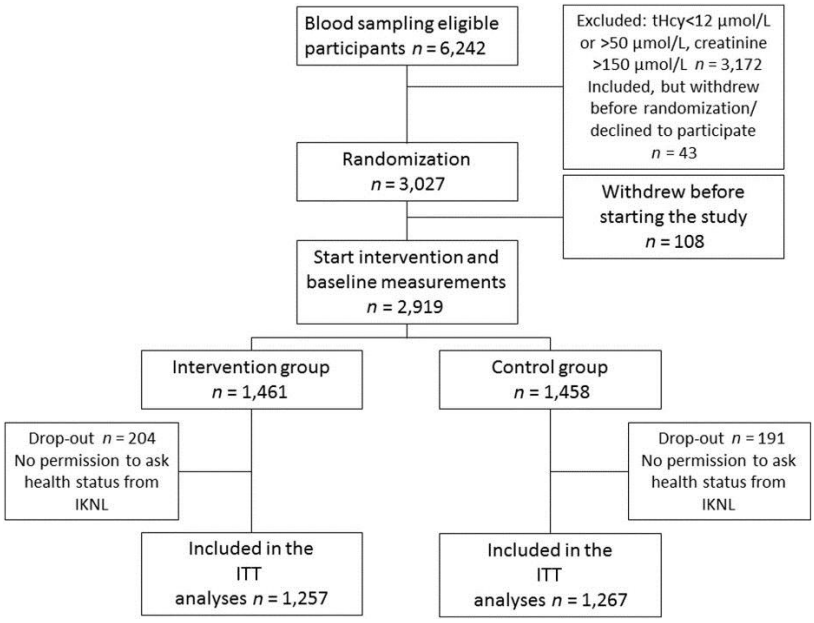

Figure 1 Flow-chart of the B-PROOF Trial based on the CONSORT 2010 Statement. A total of 2,524 participants were included in the intention to treat analyses.

Table 1. Selected characteristics of the trial population (n=2,524)

	Control: Vitamin D (n=1,267)	Missing (n)	Intervention: Folic acid, vitamin-B12 and vitamin D (n=1,257)	Missing (n)	p-value
Age(years)[a]	74.0 (6.2)	0	73.9 (6.6)	0	0.63
Sex (% Women)	48.6	0	50.4	0	0.36
Education years (%)		2		0	0.30
5-10	67.3		65.3		
11-18	32.7		32.7		
Alcohol consumption(%)		1		0	0.57
Light	66.7		66.9		
Moderate	28.8		29.6		
Excessive	4.0		3.0		
Very excessive	0.4		0.5		
Smoking status (%)		0		0	0.80
Never	33.9		33.5		
Current	9.4		10.2		
Former	56.7		56.3		
BMI[a]	27.2 (4.0)	8	27.1 (4.0)	10	0.67
Homocysteine (micromol/l)[b]	14.5 (13.0-16.7)	0	14.3 (13.0-16.5)	0	0.40
MTHFR (%)		161		145	0.27
CC	42.8		44.2		
CT	41.2		39.3		
TT	12.6		12.3		
Folic acid use supplements (%)		0		0	0.94
Yes	14.8		14.5		
No	83.3		83.8		
When necessary	1.9		1.8		
Vitamin-B12 use supplements (%)		0		0	0.96
Yes	15.1		14.5		
No	83.2		79.3		
When necessary	1.9		1.8		
Vitamin D use supplements (%)		0		0	0.85
Yes	19.7		18.8		
No	78.5		79.3		
When necessary	1.7		1.8		
Serum 25(OH)D (nmol/L)[a]	55.8 (23.9)	30	56.0 (25.9)	26	0.82
Serum Folate (nmol/L)[a]	20.1 (7.3)	44	20.3 (7.4)	45	0.47
Serum Vitamin-B12 (pmol/L)[a]	283.6 (115.0)	17	289.7 (116.2)	11	0.19
Serum Holotranscobalamin (pmol/L)[a]	70.9 (42.5)	11	74.3 (44.5)	7	0.05*

[a]mean (SD)[b]median (IQR) *p<0.05

RESULTS

A flow chart of 2,524 participants (86.5% of the initial 2,919 participants) is shown in **Figure 1**. Baseline characteristics were similar for the participants with and without informed consent for medical follow-up (n=2,524 vs. n=395).

Table 1 presents the selected baseline characteristics of the B-PROOF population, by allocated treatment. Mean (SD) age was 74 years (6.2) in both treatment and control groups and mean values for all other baseline characteristics were similar for treatment (n=1,257) and control groups (n=1,267), except for serum HoloTC concentration which was slightly higher in the treatment group (mean 74.3 (44.5 SD) vs. 70.9 (42.5 SD); p<0.05).

Intention To Treat (ITT) analyses showed that 314 persons were diagnosed with any cancer (171 cases [13.6%] in the intervention group vs 143 cases [11.3%] in the control group) and 68 persons were diagnosed with CRC (43 cases [3.4%] in the intervention group vs 25 cases [2.0%] in the control group) during a median follow up of 78 months; IQR: 74-83. Crude Cox proportional hazards models showed that persons in the intervention group did not have a significantly higher risk of any cancer than persons in the control group (HR 1.23; 95%CI 0.98-1.53; **Table 2**). However, the risk of CRC was significantly higher in the intervention group than persons in the control group (HR 1.76; 95% CI 1.07-2.88; **Table 2**) (**Figures 2 and 3**). After additional adjustment for baseline HoloTC, the risk of any cancer tended to be higher in the intervention group than in the control group (HR 1.25; 95%CI 1.00-1.57) and a significant increased risk remained for CRC (HR 1.77; 95%CI 1.08-2.90; **Table 2**).

Table 2. Effect of folic acid and vitamin-B12 on overall cancer, and on CRC incidence. Cox proportional hazard analysis of risk of cancers (ITT) in total group

Type of cancer (cases treatment vs. control group)	Cases/100 PY treatment vs. control group	HR [95%CI][a]	p-value	HR [95% CI][b]	p-value
Any cancers (171 vs. 143)	2.3 vs. 1.9	1.23 [0.98; 1.53]	0.07	1.25 [1.00; 1.57]	0.05
CRC (43 vs. 25)	0.6 vs. 0.3	**1.76 [1.07; 2.88]***	0.03	**1.77 [1.08; 2.90]***	0.02

[a] Crude model [b] adjusted for HoloTC (significant difference between intervention and control group) *p<0.05 CRC=colorectal cancer; HR: Hazard Ratio; ITT: Intention To Treat; PY= person years.

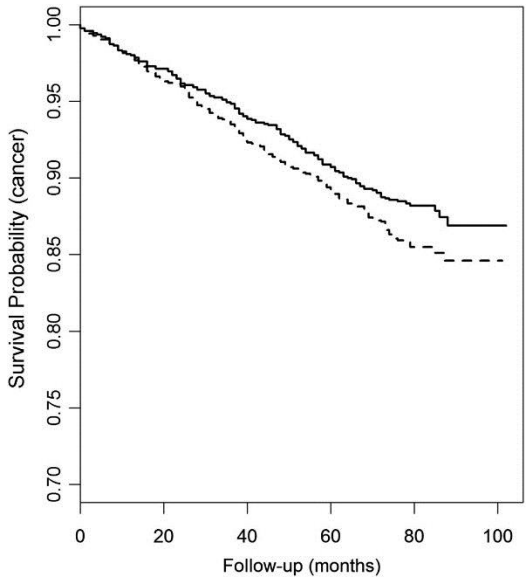

Figure 2. Kaplan-Meier curve of survival analysis of any cancers for the intervention (dashed line) and the control group (continuous line) and the follow-up time in months.

Interaction analyses revealed that the effect of the intervention did not significantly differ by age (<80 vs. >80 years), sex, plasma Hcy and MTHFR polymorphism (p-interaction > 0.10*)* for overall cancer and CRC.

Table 3. Effect of folic acid and vitamin-B12 on overall cancer, and on CRC incidence. Cox proportional hazard analysis of risk of cancers (PP) in compliance participants >80%

Type of cancer (cases treatment vs. control group)	Cases/PY treatment vs. control group	HR [95%CI][a]	p-value	HR [95% CI][b]	p-value
Any cancers (160 vs. 124)	2.3 vs. 1.7	**1.32 [1.05; 1.67]***	0.02	1.00 [0.99; 1.00]	0.10
CRC (40 vs. 19)	0.6 vs. 0.3	**2.15 [1.25; 3.72]***	0.01	**2.17 [1.26; 3.75]***	0.01

[a] Crude model [b] adjusted for HoloTC (significant difference between intervention and control group) *p<0.05 CRC=colorectal cancer; HR: Hazard Ratio; ITT: Intention To Treat; PY= person years.

Per Protocol (PP) analysis was conducted in compliant participants (n=2,330). After PP analyses, the HR on any cancer weakened but the HR on CRC became stronger relative to the ITT analyses (HR 1.00; 95%CI 0.99-1.00 and HR 2.17; 95%CI 1.26-3.75 respectively in the adjusted model; **Table 3**). Sensitivity analysis in participants who were not using folic acid and/or vitamin-B12 supplements, showed that the HR for

any cancer and CRC became stronger relative to the ITT analyses (HR 1.30; 95%CI 1.01-1.66 and HR 2.10; 95%CI 1.21-3.63 respectively in the adjusted model).

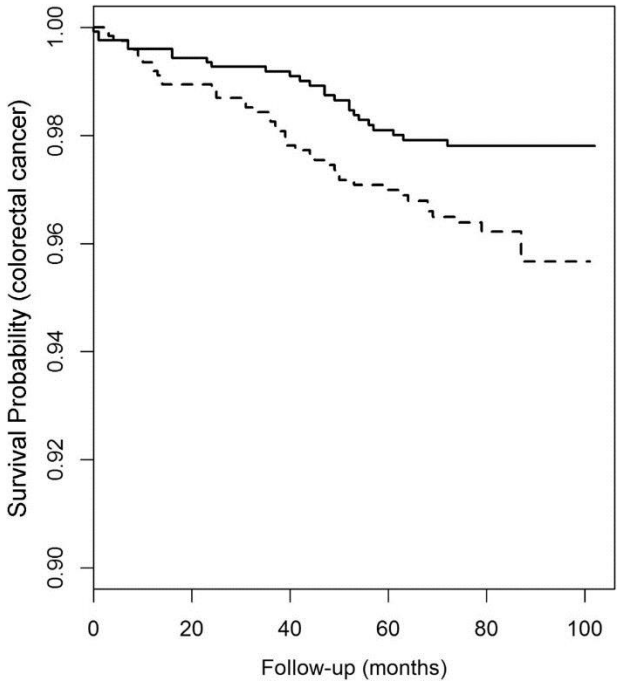

Figure 3. Kaplan-Meier curve of survival analysis of colorectal cancer (CRC) for the intervention (dashed line) and the control group (continuous line) and the follow-up time in months.

Exploratory analysis stratified by duration of the treatment (2 years vs. 3 years) showed that the HR on CRC were slightly weaker for participants with 2 years of intervention relative to the ITT analyses, but was still significant (HR= 1.72; 95%CI: 1.03-2.88 in the adjusted model).

We compared the results from the Cox proportional hazard model with the risk ratio (RR) and log-rank statistics and found similar results.

DISCUSSION

The findings of this study showed that allocating older persons with mildly elevated homocysteine levels to receive combined folic acid and vitamin-B12 supplementation was associated with a slight excess risk of overall cancer but a statistically significant increased risk for CRC when compared to placebo. The effect on CRC risk was even

more extreme in compliant participants (>80%). As difference in cancer risk was already apparent within the first years of follow-up, these findings are consistent with evidence that folic acid (combined with vitamin-B12) may promote the growth of early precursor mucosal lesions (8, 16). However, on the basis of the previous observations and the results of the B-PROOF study, we cannot yet ascertain whether this is due to an individual effect of folic acid or vitamin-B12, or an interactive effect of both folic acid and vitamin-B12 combined. The current findings confirm previous observations from the primary analyses of the B-PROOF trial (using self-reported cancer data), which showed an increased cancer risk in participants using folic acid and vitamin-B12 supplementation(24). A major strength of the current study is the extended follow-up of the B-PROOF study combined with the use of pathology-proven malignancies as an outcome measure. This is particularly important because of the long latency period between dietary risk-factors and cancer as well as the timeframe from premalignant lesions to cancer diagnosis in elderly. However, the findings differ from three recent meta-analyses, with mostly overlapping trials, which studied the effects of folic acid on cancer risk. Qin et al. found no significant overall effect of folic acid supplementation (mean dosage 1.64 mg) on cancer and CRC during a mean follow-up time of 5.3 years (mean age: 62.5 yrs.) (27). Vollset et al demonstrated no significant effect on cancer incidence including colon cancer in a time-frame of 1.8 to 7.4 years with a mean dosage of 4.7 mg (mean age: 64 yrs.) (28). In contrast, Baggott et al. (2012) reported a higher cancer risk in participants receiving folic acid supplementation during 3-8 years (mean dosage 1.3 mg), in a meta-analysis of a subset of these trials (mean age: 62.0)(29). The results of the present study differ from the three meta-analyses, probably because these studies addressed a younger population, as well as different dosages of supplementation and different outcome measures. It should also be noted that most studies included individual folic acid supplementation without vitamin-B12. Besides B-PROOF, only two other RCT's studied the effect of both folic acid and vitamin-B12. Although these trials included a selected population of people with ischemic heart disease, they also observed a significantly higher overall cancer risk (HR 1.21; 95% CI 1.03-1.41; p=0.02)(11), which is consistent with our findings. Most of the previous trials had a shorter follow-up, albeit they had a longer duration of treatment than B-PROOF. Whether folic acid, vitamin B-12, or both explain the results, cannot yet be confirmed. Especially because the studies of the effect of dietary, supplements, and plasma levels of folate and vitamin-B12 on cancer risk showed opposing results and none of these were randomized controlled trials (30-33). For example, Matejcic et al. (2017) found that overall, folate and vitamin-B12 status was not clearly associated with breast cancer risk in their prospective cohort study. They did, however, find potential interactions between vitamin-B12 and folate on the risk of breast cancer and suggested that low plasma folate concentrations

(mainly 5-methyl THF), as a consequence of high vitamin-B12 status, may impair DNA methylation (32). Price et al. (2016) reported a small increased risk of prostate cancer with higher folate and vitamin-B12 concentrations, in data from six cohorts (33). Another recent study, reported a 30-40% increase in lung cancer risk in men using vitamin-B12 supplements (not from multivitamins). They found no association of use of folic acid supplements in men and women in risk of lung cancer (31).

The previous analysis of the B-PROOF study showed that curves for cancer incidence separated shortly after the start of the intervention, which may imply that the effect of the treatment was on cancer progression rather that cancer induction. Since our previous analysis of the B-PROOF study showed a higher risk in persons aged >80y (24), and given that the results of the current study showed that 18 of the 28 excess cancers were CRC, it may be argued that the older age group has a higher prevalence of latent colorectal neoplastic cells (8) since the risk of CRC increases with advanced age (34) and older individuals may therefore be more prone to the effects of folic acid and vitamin-B12 supplementation. However, we did not have data on the presence of early neoplastic lesions in the colorectal mucosa to confirm this hypothesis.

The intervention dosage was 500µg vitamin-B12 and 400 µg folic acid per day. Although the dosage of folic acid was close to the recommended daily intake and well below the Tolerable Upper Intake Level for folic acid of 1 mg/d in Europe (35), the dosage of vitamin-B12 was almost 200 times higher than the recommended intake. For vitamin-B12, no systematic toxicological effects have been reported so far, (35), but we cannot rule out that the high dosage of vitamin-B12 supplementation influenced the risk of CRC in our study.

There may be several plausible mechanisms by which folic acid and vitamin-B12 supplementation increase the risk of CRC in particular. First, the epithelial cells of the colorectal mucosa have the most rapid turnover rate of any tissue in the body. Hence, it may be speculated that this tissue may be particularly sensitive to nutrients involved in cell growth such as B-vitamins. Folic acid and vitamin-B12 play a key role in one-carbon metabolism and cells require one-carbon units for DNA synthesis and methylation (36). Thus, these nutrients may influence pathways enhancing proliferation of cancer cells and modulate DNA and therefore the chance of developing a neoplastic cell (36). Folate has been demonstrated to affect neoplastic cells by enhancing growth in both animal and in-vitro models in DNA synthesis (36). Both folic acid and vitamin-B12 are essential for the synthesis of methionine and S-adenosyl methionine (SAM), which are required as the common methyl donor for the regulation of DNA methylation patterns in DNA influencing gene expression (36-38). DNA

methylation occurs mainly in CpG dinucleotides, concentrated in short CpG-rich DNA fragments so-called ÇpG islands' (39, 40). In normal cells, CpG island in active promoters can be methylated, which lead to long-term silencing of transcription. However, gene expression may be inactivated in genes that are hypermethylated at their CpG island-containing promotors, through which a neoplastic cell can develop (41). Currently little is known about the possible relation between vitamin-B12 and cancer risk. However, since vitamin-B12 has a key role in one-carbon metabolism and cells require one-carbon units for DNA synthesis, methylation as well as redox and reductive metabolism, vitamin-B12 may influence pathways enhancing the proliferation of cancer cells (42).

A second potentially relevant mechanism for CRC specifically may be via the gut microbiome. Several studies have shown that microbial imbalance of *Fusobacterium spp., Streptococcus gallolyticus susp. gallolyticus* may play a role in CRC etiology (43-45). Vitamin-B12 and folate can be synthesized by human gut microbes as a valuable resource in the gut (46). It has been suggested by others that competition and exchange of vitamin-B12 and cofactors from both dietary intake and gut microbes affect the gut microbial community (46). Thus, there may be an interaction between the gut microbiome and B-vitamins but further exploration of this hypothesis is needed.

The present study has several strengths as well as potential limitations. The main strengths of the present study were the randomized controlled study design, the pathology-proven cancer, the large sample size of elderly subjects, and the prolonged follow-up relative to other trials. A limitation of the present study is that it presents secondary analyses of a randomized controlled trial primarily designed to study the effect on fracture risk. As a result, a significant results of such analysis have to be interpreted in the context of other evidence in the literature. However, with a sample size of 2,524, an alpha of 5% and a power of 80% our study was able to detect a HR of 0.85/1.18 on overall cancer (47). In addition, the decision to study CRC in the B-PROOF study and other (non-site specific) was made on the basis of prior results on self-reported cancer as adverse event of the trial. We did not include other GI cancers, due to the limited power. It can be argued that this approach may increase the probability of type I errors because we did not adjust for multiple comparisons. For possible type I errors, stringent interpretation of p-values, especially for the results on all (non-site specific) cancers (p=0.05) should be made with caution. The initial B-PROOF study included 2,919 participants, but for the current extended follow-up analyses we collected data from a subgroup of 2,524 participants which could introduce a source of bias. However, there was no difference between the intervention and

control group in baseline characteristics between the participants with and without informed consent for medical follow-up.

Another source of potential bias was that the allocation to the intervention and control group was no longer blinded to the researchers. However, since the data collection of cancer was derived from the independent national cancer registry, and the physicians involved in the cancer diagnosis were blinded to the allocation of the intervention, observer bias is unlikely. Another possible limitation is that we only included Caucasian participants aged 65 years and over with elevated homocysteine levels in a country where no mandated folic acid fortification has been implemented. Therefore, the results may not be generalizable to other populations. Nonetheless, this trial is one of the few that were done in a population without mandated folic acid fortification and relatively low supplement use. As a result we were able to clearly discern the effect of supplementation in a population with limited intake of folic acid above the tolerable upper intake level.

CONCLUSION

The present study reported a higher risk of CRC among those allocated to folic acid and vitamin-B12 compared with placebo, which persisted over time (6-9 years). This was observed in older ambulant persons with mildly elevated homocysteine concentrations. The primary analyses of the B-PROOF trial did not show any protective effect of folic acid and vitamin-B12 supplementation on fracture, falls, and cardiovascular disease (with the exception of CVA). However, since secondary analyses of this trial, showed potential adverse effects on cancer, careful monitoring of long-term hazards of B-vitamins is required before making any recommendations for public health related to the implementation of fortification policies. To clarify the role of combined supplementation with B-vitamins on CRC, further confirmation for example by individual meta-analyses of existing, large RCT of folic acid and vitamin-B12, with additional information on the presence of early neoplastic lesions in the colorectal mucosa is needed.

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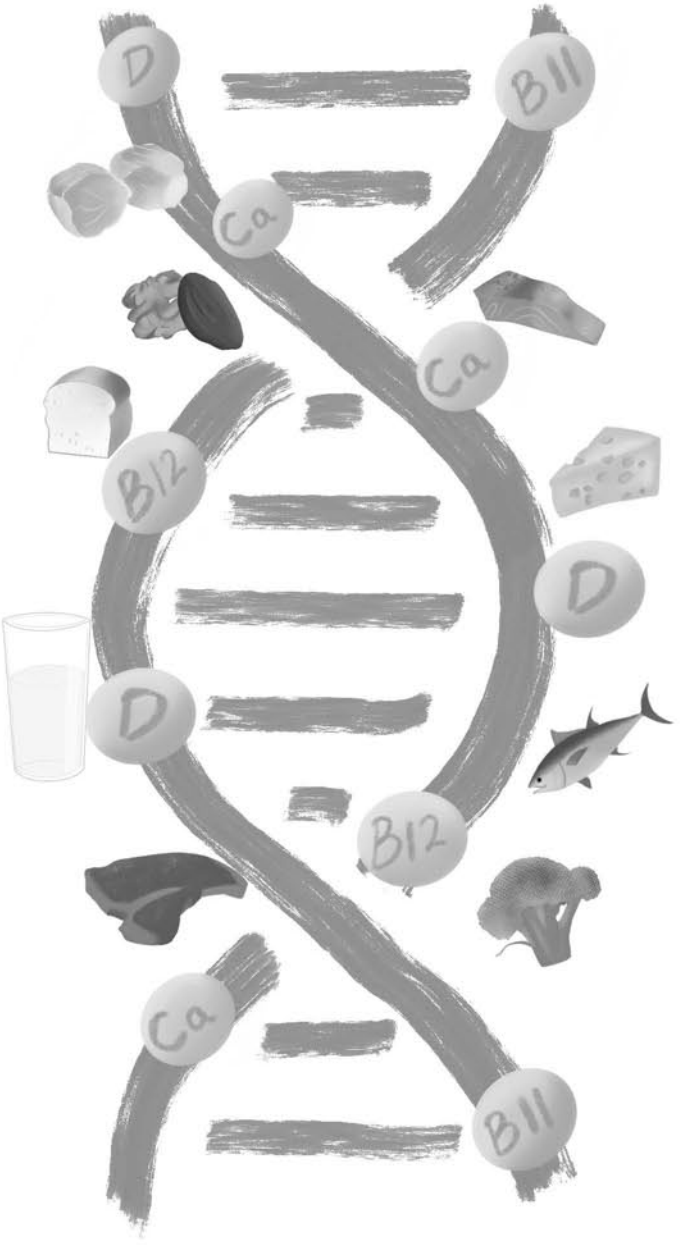
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Chapter 3.2

Long-term effects of folic acid and vitamin-B12 supplementation on fracture risk and cardiovascular disease: extended follow-up of the B-PROOF Trial

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ABSTRACT

Background & aims

In the initial B-proof we found inconsistent results of B vitamin supplementation. However the debate regarding the effects of B vitamins on age-related diseases continues. Therefore, our aim was to investigate the long-term effects (5-7 years follow-up) of an intervention with folic acid and vitamin-B12 supplementation on fracture and cardiovascular disease risk.

Methods

Extended follow-up of the B-PROOF trial, a multi-center, double-blind randomized placebo-controlled trial designed to assess the effect of 2-3 years daily supplementation with folic acid (400 µg) and vitamin-B12 (500 µg) versus placebo (n=2,919). Primary outcome was verified self-reported fracture incidence and secondary outcomes were self-reported cardiovascular endpoints, which were collected through a follow-up questionnaires Proportional hazard analyses was used for the effect of the intervention on risk of fracture(s) and logistic regression for the effect of the intervention on risk of cardiovascular disease.

Results

A total of 1,298 individuals (44.5%) participated in the second follow-up round with median of 54 months [51-58], (n=662 and n=636, treatment versus placebo group). Median age at baseline was 71.0 years [68.0-76.0] for both groups. No effect was observed of the intervention on osteoporotic fracture or any fracture risk after a follow-up (HR: 0.99, 95% CI: 0.62-1.59 and HR: 0.77; 95% CI: 0.50-1.19, respectively), nor on cardiovascular or cerebrovascular disease risk (OR: 1.05; 95%CI: 0.80-1.44 and OR: 0.85; 95%CI: 0.50-1.45, respectively). Potential interaction by baseline homocysteine concentration was observed for osteoporotic- and any fracture (p= 0.10 and 0.06 respectively), which indicated a significantly lower risk of any fracture in the treatment group with higher total homocysteine concentrations (>15.1 µmol/l). No age-dependent effects were present.

Conclusions

This study supports and extends previous null-findings of the B-PROOF trial and shows that supplementation of folic acid and vitamin-B12 has no effect on fracture risk, nor

on cardiovascular disease in older individuals over a longer follow-up period. However, B-vitamin supplementation may be beneficial in reducing fractures in individuals with high total homocysteine concentrations, a finding which needs to be replicated.

Keywords

B-vitamins, fracture, cardiovascular disease, long-term follow-up.

INTRODUCTION

Previously, homocysteine-lowering therapy has been suggested as a potential treatment option for common diseases such as osteoporosis and cardiovascular disease (1). An effective method of normalizing homocysteine concentration is treatment with B-vitamins, which play a central role in homocysteine metabolism (2). Over the years, several intervention trials have been performed, but conflicting effects of treatment with B-vitamins and homocysteine were observed for both fracture and cardiovascular outcomes (3, 4).

An association between increased homocysteine concentration and risk of cardiovascular disease and fracture has been observed in particular in older individuals (5). Recently within the older B-PROOF population, we observed no effect of B-vitamin intervention on the overall incidence of coronary heart disease, but a significantly but slightly lower risk of cerebrovascular events was observed among females. This was further confirmed by a recent meta-analysis that also showed a reduced risk of stroke with folic acid alone and B-complex supplementation (5). Yet, this meta-analysis included, beside our initial B-PROOF study, studies with mostly younger individuals which makes it difficult to extrapolate these findings to older populations. With regard to fractures, the B-PROOF trial did show lower osteoporotic fracture incidence but only in a subgroup of compliant persons aged 80 years and over (6).

The mechanism of B-vitamins in bone health is not yet completely understood, but B-vitamins appear to influence the development of collagen and alter the metabolism of osteoblasts in a dose-dependent manner (7, 8). Moreover, low levels of B-vitamins have been associated with low bone mineral density (BMD) and increased fracture risk (9). The recent meta-analysis by Gracia Lopez et al., however, did not show a significant reduction of fractures after B-vitamin supplementation (10). From all included RCT's in this meta-analysis with different dosages of B-vitamins, only our initial B-PROOF trial had fractures as main outcome and included older participants (6). Other trials which included selected patients with cardiovascular disease or colorectal adenomas, found no significant differences in fracture risk (as secondary outcome) between the groups (4, 11-13).

Another potential explanation for previous inconsistent and null-findings may be the relatively short follow-up time of the trials. Besides increasing power, prolonged follow-up could account for the potential latency period between exposure (B-vitamins) till event. As known, the latency period of coronary heart disease (from exposure to mortality) may be 10 years or more (14). Thus, additional post-trial follow-up on risk

of fractures and cardiovascular diseases could provide valuable scientific information on potential long-term effects of supplementation (15). For that reason, this study aimed to examine the primary and secondary endpoints of the B-PROOF study (fracture and cardiovascular disease) with a longer follow-up time to validate our previous findings (16).

MATERIALS AND METHODS

The initial B-vitamins for the Prevention Of Osteoporotic Fractures (B-PROOF) trial was a multi-center (Erasmus MC Rotterdam, VU University Medical Center Amsterdam (VUmc) and Wageningen University (WUR), the Netherlands), double blinded, randomized placebo-controlled study. This trial was designed to investigate the effect of daily oral folic acid (400 µg) and vitamin-B12 (500 µg) supplementation (treatment group) on fracture incidence as a primary outcome. Secondary outcomes included amongst others cardiovascular events. Placebo- and treatment group received daily 15 µg (600 IU) vitamin D3 to ensure a normal vitamin D status. The duration of the intervention was 2 years, and this was extended in a subgroup for 1 more year with the aim to increase power (n=339 out of 2,919 participants had 3 years intervention) (17).

The recruitment period was between September 2008 and March 2011. A total of 2,919 participants aged 65 years and over with an elevated homocysteine concentration (12-50 µmol/l) were included in the study. Exclusion criteria were renal insufficiency (creatinine level > 150 µmol/l), history of malignancy (except non-melanoma skin cancer) in the past 5 years before the recruitment and use of high dosage of B-vitamins (folic acid intake >300 µg/day and/or intramuscular injections of vitamin-B12 at recruitment and baseline). The study protocol of the trial and a detailed description of the study have been reported elsewhere (17). The B-PROOF study was registered in the Netherlands Trial Register (NTRNTR1333) and ClinicalTrials.gov (NCT00696514). The study protocol was approved by The Medical Ethics committees of Erasmus MC, VUmc and WU universities (17). In 2015, we extended the follow-up of the original B-PROOF study by sending the participants who gave permission to contact them additional questionnaires to investigate the long-term effect of the intervention on risk of cancer, fracture and cardiovascular diseases (n=1,298). The long-term effect on cancer has been described in a separate paper (18). The current paper describes the outcomes of the extended follow-up on fracture and cardiovascular diseases. End of follow-up for this study was December of 2017.

Covariates and outcomes

A wide set of measurements (e.g. BMI, medical history, plasma homocysteine (Hcy), serum folate, vitamin-B12, holotranscobalamin (HoloTC), methylmalonacid (MMA) and 25(OH)D) was performed at baseline and at 2-y follow-up (17). The extended (5-7y) follow-up structured questionnaire was used to assess self-reported medical history (fractures and cardiovascular disease (CVD)), alcohol intake and smoking habits. All reported fractures were verified with the general practitioner of the participants and were categorized as osteoporotic (all fractures excluding head, hand, finger, foot or toe fractures, fractures caused by traffic accidents or by cancer) or any fracture. CVD was assessed, in concordance with the original B-PROOF trial outcomes, as self-reported and without date of event. Self-reported CVD events were also verified by the GP in order to obtain information on the validity of these events. Cohen's kappa coefficient was calculated for the agreement between the self-reported and verified CVD events. For CVD the coefficient was 0.89 (excellent agreement between self-reported and verified events) and for Cerebrovascular accident (CVA) 0.72 (fair to good agreement between self-reported and verified events). CVD was classified as any type of CVD, and subgroups of myocardial infarction (MI), angina pectoris (AP), hearth failure and cardiac valve disease were assessed. CVA and Transient Ischemic Attack (TIA) were included as cerebrovascular disease.

Statistical analysis

Mean with standard deviations (SD), or median with interquartile range (IQR) or percentages were reported. Differences between groups at baseline were tested with t-test for continuous variables and Mann-Whitney U for not normally distributed data. Chi-squared tests were used for categorical variables. Kaplan-Meier event curve was used for the cumulative event-free survival on the basis of fracture incidence. The follow-up time was calculated as the number of months from the baseline measurement until the first diagnosis of incident fracture, death (derived from the national institute 'Centrum voor familiegeschiedenis', CBG), loss-to-follow-up, or end of the study period, whichever occurred first for the participants with complete follow-up. The incidence rate ratio was calculated on the incidence-rate of fracture for both treatment groups, which is defined as the number of events divided by the total sum of the follow-up in each group (cases/persons years). Participants were analysed based on the initial treatment allocation (intention to treat principle (ITT)). Unadjusted Cox proportional hazard analyses were conducted with treatment (treatment vs. placebo group) as the independent variable and the fracture diagnosis as the dependent outcome variable. Subsequently, per protocol (PP) analyses were performed

that included data only from participants who were compliant during the intervention (>80% of pills consumed) (17). Then, cluster proportional hazard model was used to analyse the effect of the intervention on the multiple osteoporotic fracture risk (19). Multiple osteoporotic fracture was defined as total osteoporotic fractures during the intervention in the total population.

For the analyses with cardiovascular and cerebrovascular disease as outcome, binary logistic regression analysis was used for the population with complete follow-up, due to the absence of a precise time of onset, a Cox regression analysis was not possible. Self-reported events were used as the dependent factor and the treatment group as the independent factor. Multivariable Cox proportional hazard regression analyses and binary logistic regression analysis were adjusted for serum HoloTC since this variable differed significantly between the treatment and the placebo group regardless of randomization. All other potential confounders were equally distributed between both groups. Furthermore, as in the original B-PROOF trial, multivariable analyses were performed to test the interaction between sex, age (continuous) study center, homocysteine concentration, MTHFR polymorphism, vitamin-B12 and folate level and baseline CVD. Appropriate subgroup analyses were performed when *p* for interaction was <0.10.

P-values <0.05 were considered to be statistically significant (except the interaction analysis). Analyses were performed using IBM SPSS 24, the library survival R statistical packages(20).

RESULTS

The baseline characteristics of the total population (initial B-PROOF trial with 2-3 years follow-up, n=2,919) and population with (n= 1,298) and without 5-7 years follow-up (n=1,621) are shown in **table 1**. The median or mean values for all variables were similar for treatment (n=1,485) and placebo (n=1,461) group at baseline, except for serum HoloTC levels, with higher levels in the treatment group compared to placebo group (65.0 [48.0-86.0] vs. 63.0 [45.0-84]). A total of 1,298 participants (n=662 in the treatment group and n=636 in the placebo group) sent the second follow-up questionnaire back, with the median age at baseline of 71.0 years [68.0-76.0] for both groups. The median follow-up time was 54 months [IQR 51-58]. There were some differences in the baseline characteristics of the participants who did not return the second questionnaire compared to the participants with 5-7 y follow-up. For example, the non-responders were older, with a higher percentage of women with median age

Table 1. Baseline characteristics of total study population (n=2,919) and the participants with (n=1,298) and without (1,621) follow-up

	Population with 2-3Y FU (n=2,919)		Population with 5-7Y FU (n=1,298)		Population without 5-7Y FU (n=1,621)	
	Placebo (n=1,458)	Treatment group (n=1,461)	Placebo (n=636)	Treatment group (n=662)	Placebo (n=822)	Treatment group (n=799)
Age (years)[b]	73.0 [69.0; 78.0]	73.0 [69.0; 78.0]	71.0 [68.0-76.0]	71.0 [68.0-76.0]	75.0 [70.0-80.0]	74.0 [70.0-80.0]
Sex (%women)	49.7	50.4	43.9	46.1	54.1	53.9
Study center (%)						
WU	29.6	29.2	23.7	20.7	34.1	36.2
VUmc	26.8	26.4	23.4	24.3	29.4	28.2
EMC	43.6	44.4	52.8	55.0	36.5	35.7
Education years (%)						
Low	53.6	52.4	47.8	48.6	57.9	55.6
Intermediate	21.1	21.1	22.5	21.2	20.0	21.0
High	25.4	26.5	29.7	30.2	22.1	23.4
Height (cm)[a]	169.2 (9.3)	169.4 (9.4)	170.6 (9.1)	170.6 (9.0)	168.1 (9.3)	168.3 (9.6)
Weight (kg)[a]	77.8 (13.3)	77.9 (13.3)	78.7 (12.0)	77.0 (13.6)	77.2 (14.2)	77.0 (13.6)
BMI (kg/m^2)[a]	27.2 (4.0)	27.1 (4.0)	27.0 (3.5)	27.1 (3.8)	27.3 (4.3)	27.2 (4.1)
Alcohol consumption (%)						
Light	66.8	68.0	74.1	66.4	69.0	70.2
Moderate	29.0	28.5	22.9	28.9	26.9	26.4
Excessive	4.2	3.5	2.9	4.7	4.2	3.4
Smoking status (%)						
Current (cigarette)	9.7	9.5	8.0	9.2	11.1	9.8
Homocysteine (mmol/l)[b]	14.4 [13.0-16.7]	14.3 [13.0-16.5]	14.1 [12.9-16.0]	14.0 [12.8-15.9]	14.8 [13.2-17.2]	14.6 [13.2-17.2]
HoloTC (pmpl/l)[b]	63.0 [45.0-84.0]	65.0 [48.0-86.0]	65.0 [46.5-87.0]	66.0 [51.0-87.5]	60.0 [44.0-82.25]	62.0 [45.0-84.75]
25 (OH)D (nmol/l)[a]	55.8 (23.9)	55.5 (25.8)	57.5 (23.9)	55.9 (24.9)	54.5 (23.9)	55.2 (26.5)
Vitamin-B12 (pmol/l)[a]	282.3 (114.0)	288.6 (117.9)	287.8 (119.9)	283.9 (102.3)	278.1 (109.0)	292.4 (129.2)

Table 1. Baseline characteristics of total study population (n=2,919) and the participants with (n=1,298) and without (1,621) follow-up (*continued*)

	Population with 2-3Y FU (n=2,919)		Population with 5-7Y FU (n=1,298)		Population without 5-7Y FU (n=1,621)	
	Placebo (n=1,458)	Treatment group (n=1,461)	Placebo (n=636)	Treatment group (n=662)	Placebo (n=822)	Treatment group (n=799)
MMA (mcgmol/l)[a]	0.3 (0.4)	0.3 (0.2)	0.3 (0.2)	0.3 (0.2)	0.3 (0.5)	0.3 (0.2)
Folate (nmol/l)[a]	20.7 (8.7)	21.4 (13.9)	20.9 (8.1)	21.4 (10.0)	20.5 (9.1)	21.4 (16.5)
Folic acid supplement use (% yes)	14.8	14.0	14.7	13.9	14.9	14.1
Vitamin-B12 supplement use (% yes)	15.2	16.4	15.7	15.0	15.6	15.5
Medication use (% yes)	84.3	83.9	81.3	82.0	86.6	85.4
Falls frequency (12 months before baseline)	32.5	32.6	30.1	31.2	34.3	33.6
History of fracture (% yes)	42.9	41.3	40.3	60.3	45.0	57.3
Cardiovascular diseases (%)	19.1	18.6	21.5	21.0	17.3	16.6

[a]mean (SD)[b]median (IQR). FU= Follow-up, MMA= methylmalonacid.

at baseline of 75.0 years [70.0-80.0] for the treatment group (n=799) and 74.0 years [70.0-80.0] for the placebo group (n=822) (**Table 1**).Furthermore, the baseline characteristic of the participants with 5-7 y follow-up was different for vitamin B12 level between placebo and the treatment group (15.7% vs. 15.0%, respectively).

Fracture incidence

Table 2 shows the incidence of osteoporotic fractures, any fractures, and the effect of the intervention on these outcomes. The incidence of osteoporotic fractures was not different between the treatment and placebo group for participants with complete follow-up (n=1,298, 35 vs. 35). However, the incidence of any fractures tended to be lower in the treatment group compared to the placebo group (37 vs. 47, p=0.19). The results of ITT analyses of the effect of folic acid and vitamin B-12 on verified first osteoporotic fractures in participants with complete follow-up showed no effect of treatment of folic acid and vitamin-B12 on first osteoporotic fracture and any fracture risk after a follow-up of 5-7 years in the adjusted model (HR: 0.99, 95% CI: 0.62; 1.59 and HR: 0.77, 95% CI: 0.50; 1.19 respectively, **table 2 and figure 2**). PP analyses showed also no effect of the treatment of folic acid and vitamin-B12 on both osteoporotic fractures and any fractures in the final model (HR: 1.09, 95% CI: 0.66; 1.79 and HR: 0.81, 95% CI: 0.52; 1.28 respectively, **table 2**).

Total fractures and first osteoporotic fractures from baseline to the end of follow-up periods for the total population (participants with only FU1) and participants with complete follow-up (participants with FU1 and FU2), are shown in **supplemental table S1**. **Supplemental table S3** shows the number of osteoporotic fractures in the treatment and placebo group at FU1 and FU2 in detail. In **table 2**, the effect of the intervention on the multiple osteoporotic fracture risk is shown. In the total population, the number of osteoporotic fractures was lower in the treatment group than in the placebo group (81 vs. 86). The intervention had no effect on multiple osteoporotic fracture incidence in the total population (HR=0.93; 95% CI: 0.65; 1.33, **table 2**).

In the interaction analysis, we found only a significant interaction term for homocysteine concentration for the effect of the intervention on first verified osteoporotic and any fracture (p=0.10 and p=0.06 respectively). After stratification for homocysteine in tertiles, the risk of osteoporotic fracture did not significantly differ between the groups. However, we found a lower risk of any fractures for those with homocysteine concentration above 15.1 mmol/l for the treatment group compared to the placebo group (HR: 0.42, 95%CI: 0.19; 0.92, **figure 1**). Baseline characteristics of population with homocysteine concentration < and ≥ 15.1 mmol/l are shown in **supplemental**

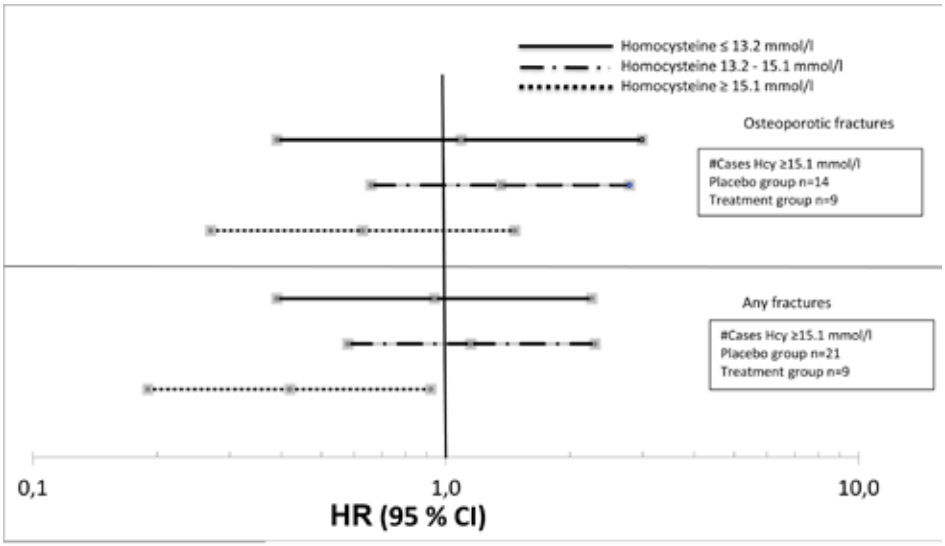
Table 2. Effect of folic acid and vitamin-B12 intervention on verified first osteoporotic fracture in participants with complete follow-up (n=1,298)

Outcome	Placebo group		Treatment group		5-7 years FU	
	n	Cases/100 PY	n	Cases/100 PY	HR [95%CI][a]	HR [95%CI][b]
ITT analysis (n=1,298: 636 in placebo and 662 in control group)[c]						
Osteoporotic Fractures	35	1.3	35	1.2	0.96 [0.60; 1.53]	0.99 [0.62; 1.59]
Any Fractures	47	1.7	37	1.3	0.75 [0.49; 1.16]	0.77 [0.50; 1.19]
PP analysis (n=1,245 1,325 in placebo and 1,336 in control group)[c]						
Osteoporotic Fractures	30	1.1	33	1.2	1.04 [0.64; 1.71]	1.09 [0.66; 1.79]
Any Fractures	42	1.6	35	1.2	0.79 [0.51; 1.24]	0.81 [0.52; 1.28]
Multiple fracture incidence analysis (n=2,919 1,458 in placebo and 1,461 in control group)[d]						
Osteoporotic fractures	86	NA	81	NA	0.92 [0.65; 1.31]	0.93 [0.65; 1.33]

[a] unadjusted model; [b] adjusted model for HoloTC (significant difference between treatment and placebo group); [c] Values were derived from Cox proportional hazards, ITT, and PP analyses; [d] Values were derived from Cluster proportional hazards *p<0.05. HR= Hazard Ratio; ITT= Intention To Treat; PP= Per Protocol; PY= person years, FU= Follow-up.

table S2. Besides, we found differences in baseline characteristics between placebo and intervention group within each category by homocysteine level. For the participants with homocysteine concentration <15.1 mmol/l (lower two tertiles), weight (p=0.04), vitamin B12 level (p=0.01), smoking (p=0.03) and kidney problems (p=0.04) were different between placebo and intervention group. For the participants with homocysteine ≥ 15.1 (higher tertile), there were no differences between placebo and intervention group in baseline characteristics.

Median homocysteine concentration for the participants with the extended follow-up changed more after the intervention in the treatment group in the highest tertile compared to the first two tertiles (-3.1 for the first tertile and -4.1 mmol/l for the second tertile, -6.0 for the last tertile, data not shown).



Hcy= Homocysteine, HR= Hazard Ratio.

Figure 1. the effect of folic acid and vitamin-B12 on verified first osteoporotic fracture and any fracture in participants with complete follow-up (n=1,298) stratified by homocysteine tertiles (≤ 13.2, 13.2-15.1 and ≥15.1 mmol/l) in the adjusted model (p- for interaction=0.10 and p=0.06 respectively).

Hcy= Homocysteine, HR= Hazard Ratio.

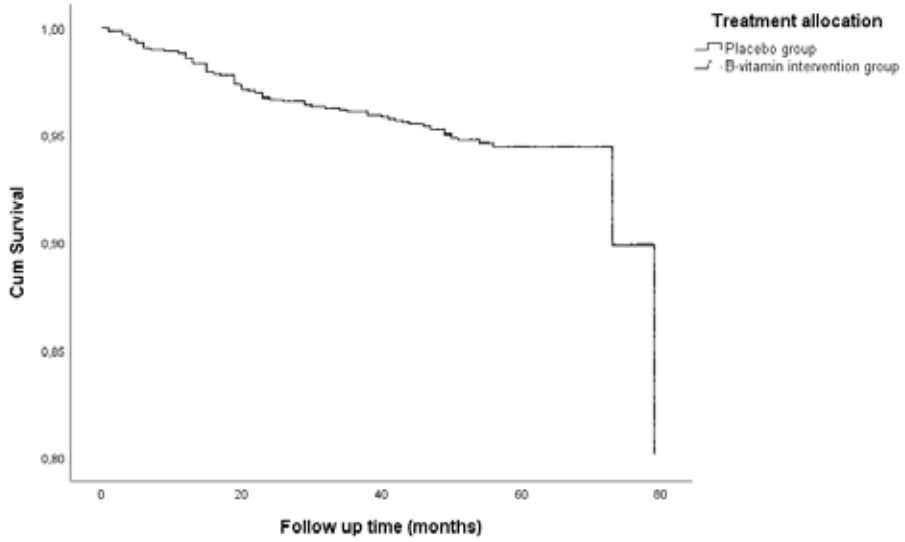


Figure 2. Kaplan-Meier curve of survival analysis of any type of fractures for the treatment (continue line) and the placebo (dotted line) group.

The interaction analysis of the effect of the intervention on multiple fracture incidence showed no significant interaction terms for age, gender, study center and total homocysteine and MTHFR (data not shown).

Cardiovascular disease and cerebrovascular disease

The incidence of any type CVD, MI, AP, heart failure and/or cardiac valve disease during the follow-up period for the participants with complete follow-up was higher in the treatment group compared to the placebo group (130 vs. 120, 20 vs. 18, 43 vs. 42 and 41 vs. 35 respectively), except for cerebrovascular disease (27 vs. 30). However, the differences were not statistically significant (logistic regression analysis, **table 3**). Sex and study center were significant modifiers in the intervention effect on any type of CVD (P-for interaction=0.03 and 0.09, respectively). In addition, sex had a potential interaction with the intervention effect on heart failure/cardiac valve disease (P-for interaction=0.10). After stratification for sex, the intervention showed a higher risk of any type of CVD, heart failure and/or cardiac valve disease for women compared to men, but these differences were non-significant (for women: OR=1.53; 95% CI: 0.99-2.35 for any type of CVD and OR=1.52; 95% CI: 0.78-2.97 for heart failure and/or cardiac valve disease, for men: OR=0.81; 95% CI: 0.56-1.17 for any type of CVD and OR=0.71; 95% CI: 0.39-1.31 for heart failure and/or cardiac valve disease). After stratification for study center, the intervention showed a higher risk (not significant) of any type of CVD for the participants from Rotterdam than participants from Amsterdam or Wageningen (OR=1.36; 95%CI: 0.93-2.00, OR=0.80; 95%CI: 0.45-1.42 and OR=0.78; 95%CI: 0.44-1.40 respectively).

DISCUSSION

The extended follow-up of 5-7 years of a 2-year supplementation with folic acid and vitamin-B12 within a multi-center, randomized, double-blind, placebo-controlled trial in older adults, showed no effect on incidence of first osteoporotic or any type of fractures and multiple osteoporotic fractures. However, after stratification, the intervention led to a significantly lower incidence of any fractures in the participants with the highest total baseline homocysteine. Furthermore, the intervention had no effect on the incidence of CVD or cerebrovascular disease.

No overall long-term effect of 2 years of supplementation of folic acid and vitamin-B12 on fracture risk was found in our extended follow-up study. This is in line with the results of recent meta-analysis where no association between homocysteine-lowering

Table 3. Effect of folic acid and vitamin-B12 intervention on self-reported cardiovascular outcomes investigated with logistic regression analysis according to the intention-to-treat principle in participants with complete follow-up (n=1,298)

			Effect of the intervention treatment group compared to placebo group			
			Model 1		Model 2	
Outcome	No. of cases in placebo group (cumulative incidence)	No. of cases in treatment group (cumulative incidence)	OR [95%CI]	p-value	OR [95% CI]	p-value
Any type CVD	120 (0.88)	130 (0.94)	1.06 [0.80; 1.39]	0.71	1.05 [0.80; 1.39]	0.72
MI	18 (0.33)	20 (0.44)	1.07 [0.56; 2.05]	0.83	1.08 [0.56; 2.05]	0.84
Heart Failure and/or heart Valve	42 (2.00)	43 (2.15)	0.99 [0.64; 1.54]	0.95	1.02 [0.66; 1.59]	0.98
Angina Pectoris	35 (1.94)	41 (2.92)	1.14 [0.72; 1.81]	0.59	1.13 [0.71; 1.80]	0.61
Cerebrovascular disease	30 (0.65)	27 (0.69)	0.86 [0.51; 1.47]	0.59	0.85 [0.50; 1.45]	0.55

Model 1: crude model; Model 2: adjusted for HoloTC (significant difference between treatment and placebo group); *p<0.05. OR= Odds Ratio; MI= Myocardial Infarction.
Reference group =placebo group

treatment with these B-vitamins and the risk of fracture was shown including long term follow-up studies (10). However, only 2 of the 6 included trials had a longer (extended) follow-up (7 years and 11.1 years) (3, 21). Although the initial B-PROOF trial was also included in this meta-analysis, differences in study design and population with B-PROOF should be noted. The study population of other included trials was younger compared to B-PROOF (62.5-68.8 years). Nevertheless, there was no indication of an age-dependent effect in our current extended follow-up analyses. The baseline total homocysteine concentration of the included participants was also different compared to our study (9.8-13.4 mmol/l versus 14.4 mmol/l). Interestingly, we found a lower fracture incidence for the group with higher total homocysteine concentration at baseline (>15.1 µmol/l). The findings were supported by the tendency toward fracture reduction in the total group, but not by the findings on osteoporotic fractures. Yet the numbers of cases were low in the stratified analysis and for this reason, these explorative findings should be interpreted with caution. Also, the participants in the treatment group with higher baseline homocysteine concentration had a steeper decline of total homocysteine concentration after the supplementation of folic acid and vitamin-B12 than the participants with lower baseline homocysteine concentration suggesting that the effect of the intervention was more pronounced in participants with higher total homocysteine concentration. This is in line with treatment of vitamin D deficiency, where the effect on serum parathyroid hormone concentration is greater when the baseline serum 25-hydroxyvitamin D is lower (22). In general, vitamin supplementation may show a threshold effect, working only in deficient people (23). In a similar way of reasoning, the effects may soon disappear after discontinuation of supplementation. This follow-up study reports outcomes after a follow-up of 5 to 7 years, including treatment for 2 to 3 years only, thus, the effect of supplementation may be disappeared. Besides, from our previous findings of an increased risk of colorectal cancer with B-vitamins supplementation, we do not recommend supplementation of these vitamins in not-deficient general population (18).

It may be speculated that the latter indicates a (intracellular) B-vitamin deficiency (24). As known, B-vitamins lower total homocysteine concentration and play an important role in the homocysteine metabolism (1). However, the studies of the relation between high homocysteine concentration and bone show conflicting results (25). From the previous studies which reported an association between elevated total homocysteine concentration and fracture risk, it remains unclear whether this could be explained by disrupted one-carbon metabolism or whether residual confounding by other physiological and lifestyle factors that associate with hyperhomocysteinemia may play a role (25). The one-carbon metabolism can be disrupted by vitamin-B12 and

folate deficiencies. However, other causes of hyperhomocysteinemia are high intake of methionine, certain diseases (chronic renal failure, hypothyroidism and malignant tumors in the breast, ovary or pancreas) and ingestion of certain drugs (26-29). However, in our study, vitamin-B12 and folate level was not an effect modifier in the effect of the intervention on fracture risk, suggesting that different levels of vitamin-B12 and folate would not make a difference in the risk of fracture. Our population was also not deficient in B-vitamin measured by different methods (active vitamin-B12, HoloTC and MMA). Since the methods to detect vitamin-B12 and folate deficiency are under debate, (30) due to its low biased value of B-vitamin level, the effectiveness of the intervention in the high homocysteine group might be explained by a subclinical deficiency of B-vitamins, that warrants further study.

With regard to the cardiovascular diseases, we found no effect of the intervention with B-vitamins and risk of cardiovascular diseases in this extended follow-up. However, like the initial B-PROOF study we found a lower incidence of cerebrovascular events in the treatment group, but this was not significant. In line with our results, an update of a Cochrane review showed no effect of homocysteine-lowering B-vitamins supplementation compared to placebo on MI, but they did show a small reduced risk of stroke with B-vitamin interventions (vitamin B6, B9 or B12 given alone or in combination compared to placebo RR=0.90; 95% CI: 0.82-0.99) (5). Also, the recent meta-analysis of Jenkins et. al. showed reduced risk of stoke with folic acid alone and B-vitamins with folic acid, B6 and B12 (RR= 0.83; 95%CI: 0.69-0.93 for folic acid treatment and RR=0.90; 95%CI: 0.81-1.00 for B-complex treatment) (31). However, these latter results were driven by one large Chinese trial of 20,000 participants.

For the effect of the intervention on CVD and heart failure/cardiac valve disease, the risks were higher in women compared to men, however, the differences were not significant. A possible greater vulnerability of women to folic acid and vitamin B12 supplementation could be explained by the influences of sex hormones in one-carbon metabolism and the differences between men and women in the expression level of enzymes in this metabolism (32).There is a gap in the knowledge of CVD in different sex and age groups, due to under-representation of women and the older population (because of higher comorbidities) (33). It has been suggested that there is more variability of the increased risk factors by ageing, related to sex differences that could change between middle-aged and elderly adults (33). In addition, heart failure, occurs mostly in (postmenopausal) women (34). Thus, probably, the differences in incidence of CVD are due to sex-differences in baseline risk, regardless of the intervention.

Strengths and limitations

A strength of our study is that the B-PROOF trial was initially designed to study fracture risk as primary outcome in an older population. The extended follow-up period allowed us to study long-term effects as well as increase power, and it allowed us to analyse the risk of multiple fractures.

A limitation of our study was that some of the baseline characteristics of the responders were different compared to the total populations and non-responders of the second follow-up questionnaire. The participants who did not return the second questionnaire, were older, and the high mortality and morbidity rate within this age group, may have influenced our results through competing risk bias. Also, the differed in HoloTC and vitamin-B12 levels. So, the results are less powered and need to be interpreted with caution. However, the variables were not different between the treatment and placebo group in responders and non-responders, respectively, which indicate that the randomisation and internal validity was still intact.

A second limitation is the self-reported cardiovascular events used both in the initial and the extended B-PROOF study. However, the agreement between self-reported and verified events for CVD and cerebrovascular events were more than adequate, respectively 0.89-0.72 (excellent and fair to good agreement). Due to missing of completely verified data, we were not able to do time to event analysis for these outcomes. Additionally, events in the subgroups of CVD (MI, AP, heart failure and cardiac valve disease) as well as cerebrovascular disease group were too small to conduct in depth analysis.

Moreover, there were more participants recruited from Rotterdam than other regions (1,285 participants from Rotterdam and, 857 from Wageningen and 777 from Amsterdam). Other differences in characteristics were found between study centers: participants from Rotterdam had lower 25(OH)D level, lower SES, higher use of vitamin D, folic acid and vitamin-B12 supplements, compared to other regions. Also the incidence of CVD was already higher at the baseline for the participants from Rotterdam with an extended follow-up. Nonetheless, after stratification by study center, the intervention showed a higher risk of any type of CVD for participants who were recruited from Rotterdam, however not significant.

Finally, the population with extended follow-up reported a lower use of over the counter vitamin B supplements at FU1 and FU2 compared to the total population at FU1, but the treatment group reported a higher intake of folic acid and vitamin-

B12 supplements compared to the placebo group at FU2. Other differences between groups might have arisen after the baseline visit and FU1 (for example drug use and other diseases), which we were unfortunately not able to measure. Furthermore, we have no information about the B-vitamin level and total homocysteine concentration at FU2 to evaluate the effect of the intervention on actual blood biomarkers.

In conclusion, in the extended follow-up of B-PROOF, an overall effect of supplementation of folic acid and vitamin-B12 on fracture risk, CVD and cerebrovascular risk in older individuals with elevated homocysteine concentration was not observed. However, the results of the stratified analyses suggest a reduced fracture risk in individuals with higher total homocysteine concentration. This needs further replication. Currently, we do not recommend supplementation of these B-vitamins in healthy (non-deficient) general population for fracture prevention.

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SUPPLEMENTARY DATA

Table S1 Characteristics of total population (n=2,919) and population with complete follow-up (n=1,298)

	Total population (n=2,919)		Population with complete follow-up (n=1,298)	
	Placebo (n=1,458)	Treatment group (n=1,461)	Placebo (n=636)	Treatment group (n=662)
Age at follow-up (years)[a]	75.0 [71.0-80.0]	75.0 [71.0-80.0]	76.0 [73.0-81.0]	76.0 [72.0-81.0]
Sex (%women)	49.7	50.4	43.9	46.1
Study center (%)				
WU	29.6	29.2	23.7	20.7
VUmc	26.8	26.4	23.4	24.3
EMC	43.6	44.4	52.8	55.0
Education years (%)				
Low	53.6	52.4	47.8	48.6
Intermediate	21.1	21.1	22.5	21.2
High	25.4	26.5	29.7	30.2
Height (cm)	169.1 (9.3)	169.2 (9.3)	NA	NA
Weight (kg)	77.9 (13.3)	77.9 (13.8)	77.4 (14.0)	77.3 (14.5)
BMI at FU (kg/m^2)	27.2 (4.0)	27.2 (4.2)	NA	NA
Alcohol consumption (%)				
Light	69.4	70.3	74.1	66.4
Moderate	27.6	26.8	22.9	28.9
Excessive	3.0	2.9	2.9	4.7
Smoking status (%)				
Status not changed at FU	95.4	96.8	93.8	90.7
Homocysteine (mmol/l)[b]	14.3 [12.4-17.0]	10.3 [8.9-12.0]	NA	NA
HoloTC (pmpl/l)[b]	60.7 [42.0-84.0]	121.0 [92.0-172.0]	NA	NA

Table S1 Characteristics of total population (n=2,919) and population with complete follow-up (n=1,298) (*continued*)

	Total population (n=2,919)		Population with complete follow-up (n=1,298)	
	Placebo (n=1,458)	Treatment group (n=1,461)	Placebo (n=636)	Treatment group (n=662)
25 (OH)D (nmol/l)	NA	NA	NA	NA
Vitamin-B12 (pmol/l)	295.1 [225.4-391.8]	606.3 (215.1)	NA	NA
MMA (mcgmol/l)	0.3 [0.2-0.3]	0.2 (0.1)	NA	NA
Folate (nmol/l)	24.3 [19.7-31.3]	53.8 (21.9)	NA	NA
Total Fractures during FU	74	62	48	42
Number of participants with at least one osteoporotic fracture during FU	61	48	35	35
Mortality (%)	11.7	12.3	4.1	2.4
Folic acid supplement use (%)	16.7	16.0	7.9	9.4
Vitamin-B12 supplement use (%)	16.8	16.4	12.3	14.7
Medication use (%)	NA	NA	85.5	87.4

[a]mean (SD)[b]median (IQR) *p<0.05. MMA= methylmalonacid.

Table S2. Baseline characteristics of population with homocysteine concentration < and ≥ 15.1 mmol/l)

	Population with Hcy < 15.1 (n=876, lower two tertiles)		Population with Hcy ≥ 15.1 (n=422, highest tertile)		p-value
	Placebo group (n=421)	Treatment group (n=455)	Placebo group (n=215)	Treatment group (n=207)	
Age at baseline (years)[a]	71.0 [68.0-75.0]	71.0 [67.0-75.0]	72.0 [69.0-77.0]	73.0 [68.0-77.0]	**<0.001***
Sex (%women)	47.0	53.0	40.8	39.0	**0.013**
Study center (%)					0.075
WU	24.9	25.3	22.6	16.0	
VUmc	24.3	24.7	22.6	23.9	
EMC	50.8	50.0	54.9	60.1	
Education years (%)					0.598
Low	47.3	50.2	48.3	47.0	
Intermediate	22.7	20.9	22.2	21.4	
High	30.0	28.9	29.5	31.6	
Height (cm)	170.2 (8.9)	170.1 (8.7)	171.1 (9.2)	171.2 (9.2)	0.301
Weight (kg)	78.1 (11.7)	78.2 (12.7)	79.7 (12.2)	79.7 (12.9)	0.869
BMI at baseline (kg/m^2)	27.0 (3.5)	27.0 (3.9)	27.1 (3.5)	27.2 (3.6)	0.303
Alcohol consumption (%)					0.137
Light	65.2	67.9	62.6	62.9	
Moderate	31.3	29.8	32.1	32.5	
Excessive	3.5	2.4	5.3	4.6	
Smoking status (%)					0.115
Current (cigarette)	6.9	10.1	9.1	8.3	
HoloTC (pmpl/l)[b]	68.0 [52.0-91.0]	70.0 [54.0-91.0]	56.0 [40.0-77.0]	58.0 [42.0-80.3]	**0.038***
25 (OH)D (nmol/l)	56.5 (23.3)	58.1 (24.9)	58.5 (24.4)	53.7 (24.7)	0.125
Vitamin-B12 (pmol/l)	310.5 (133.9)	302.0 (99.8)	265.4 (99.6)	265.6 (101.7)	0.066
MMA (mcgmol/l)	0.2 [0.2-0.3]	0.2 [0.2-0.3]	0.1 [0.2-0.3]	0.2 [0.2-0.3]	**<0.001***

Table S2. Baseline characteristics of population with homocysteine concentration < and ≥ 15.1 mmol/l) (*continued*)

	Population with Hcy < 15.1 (n=876, lower two tertiles)		Population with Hcy ≥ 15.1 (n=422, highest tertile)		p-value
	Placebo group (n=421)	Treatment group (n=455)	Placebo group (n=215)	Treatment group (n=207)	
Folate (nmol/l)	22.7 (8.4)	23.3 (10.8)	19.2 (7.4)	19.5 (8.8)	**0.016***
Vitamin-B12 supplement use (%)	60.6	57.8	42.5	43.3	**0.021***
Folic acid supplement use (%)	56.7	55	40.2	38.5	**0.014***
Vitamin D supplement use (%)	62.5	69.7	67.8	51.7	0.987
Living status (%)					0.372
Independent	96.8	97.9	98.8	97.4	
Assistant living	2.1	0	1.2	2.6	
Service flat	0.5	2.1	0	0	
Home for elderly	0.5	0	0	0	
Marital status (%)					1.00
Unmarried	4.5	4.6	4.2	4.8	
Living together	4.8	2.6	2.3	5.3	
Married	68.9	65.1	66.0	68.6	
Widow	16.2	19.6	20.0	15.0	
Divorced	5.7	8.1	7.4	6.3	
Total activity (kcal/d)	712.6 (514.7)	656.8 (446.1)	691.1 (481.6)	701.6 (546.5)	0.137
Fall frequency 12m before baseline (%)	31.2	32.5	30.2	30.1	0.808
Elevated blood pressure (%yes)	37.2	37.0	37.6	43.1	0.286
Kidney problems (%yes)	0.9	3.1	3.0	5.3	0.056
Diabetes (%yes)	6.9	9.0	9.7	11.2	0.163
Hypercholesterolemia (%yes)	24.0	25.2	26.3	22.9	0.994
CVD (%yes)	21.8	21.9	27.7	25.0	0.132
Leg problems/peripheral arterial disease) (%yes)	6.9	7.1	10.3	9.1	0.108

Table S2. Baseline characteristics of population with homocysteine concentration < and ≥ 15.1 mmol/l) (*continued*)

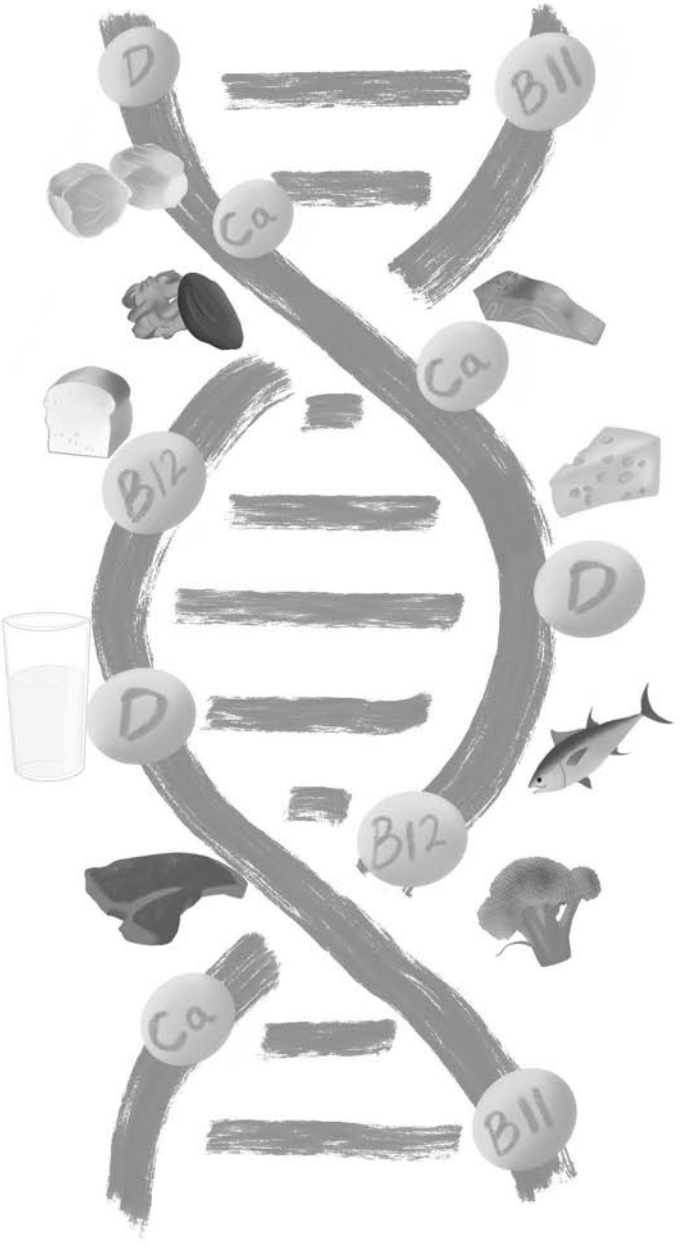
	Population with Hcy < 15.1 (n=876, lower two tertiles)		Population with Hcy ≥ 15.1 (n=422, highest tertile)		p-value
	Placebo group (n=421)	Treatment group (n=455)	Placebo group (n=215)	Treatment group (n=207)	
TIA or Stroke (%yes)	7.5	5.2	8.7	9.0	0.103
Thrombosis or embolism	4.1	4.7	4.6	7.4	0.240
Medication use (%)	79.1	79.7	86.9	83.4	**0.011***

[a]mean (SD)[b]median (IQR) *p<0.05. Hcy= Homocysteine, CVD= cardiovascular disease.

Table S3: Number of osteoporotic fractures in the placebo and treatment group at FU1 and FU2

N=1,298	Number of any fractures	Placebo group	Treatment group	Number of osteoporotic fractures	Placebo group	Treatment group
FU1	**0**	596	646	**0**	611	647
	1	32	12	**1**	22	12
	2	5	3	**2**	2	2
	3	2	1	**3**	1	21
	4	1	0	**4**	0	0
	Missing	0	0	**Missing**	0	0
FU2	**0**	608	627	**0**	625	642
	1	14	20	**1**	11	17
	2	1	2	**2**	0	3
	3	1	1	**3**	0	0
	4	0	0	**4**	0	0
	Missing	12	12	**Missing**	0	0

FU1=Follow-up 1; FU2=Follow-up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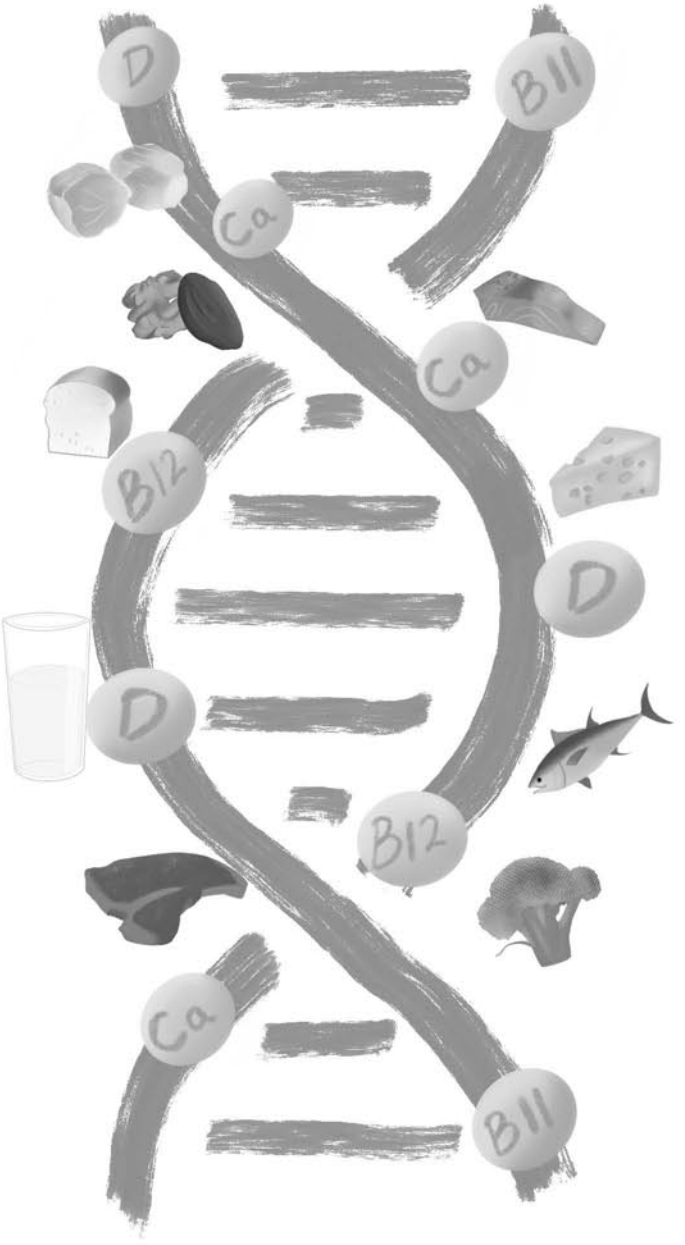
4

INTERPLAY BETWEEN MICRONUTRIENTS & BONE HEALTH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Chapter 4.1

The Impact of Thiazide Diuretics on Bone Mineral Density and the Trabecular Bone Score: The Rotterdam Study

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ABSTRACT

The decreased risk of osteoporotic fractures in thiazide diuretics (TD) users is possibly not only caused by an increase in bone mineral density (BMD), but by an increase in other determinants of bone strength as well, such as the trabecular bone score (TBS). To test this hypothesis, we studied the association between TD use and both lumbar spine BMD (LS-BMD) and lumbar spine TBS (LS-TBS) cross-sectionally in 6,096 participants from the Rotterdam Study, as well as the association between TD use and bone turnover estimated by serum osteocalcin levels. We found that past and current use of TD were associated with an increase of LS-BMD (ß = 0.021 g/cm^2 (95% CI: 0.006;0.036) and ß = 0.016 g/cm^2 (95% CI: 0.002;0.031), respectively). Use of ≥1 defined daily dose (DDD) (ß = 0.028, 95% CI: 0.010;0.046; p for trend within DDD of use <0.001) and use of >365 days (ß = 0.033, 95% CI: 0.014;0.052; p for trend within duration of use < 0.001) were positively associated with LS-BMD. No significant association between TD use and LS-TBS was observed. Mean serum osteocalcin levels were significantly different between users and non-users of TD (20.2 ng/ml (SD 8.3) and 22.5 ng/ml (SD 17.0), respectively, p<0.001). Furthermore, linear regression analysis showed that the use of TD was associated with a 3.2 ng/l (95% CI: -4.4.;-2.0) lower serum osteocalcin level compared to non-use of TD, when adjusted for Rotterdam Study cohort, age and sex. Our results suggest that the decreased fracture risk in TD users is explained by increased bone mass rather than by improved bone microarchitecture. Alternatively, changes in bone microarchitecture might not be detected through TBS and more sophisticated techniques are possibly needed to study a potential effect of TD on bone microarchitecture.

INTRODUCTION

Thiazide diuretics are known to have a small but positive effect on bone mineral density (BMD) [1-8]. Furthermore, our research group demonstrated in 2003 that the use of thiazide diuretics was associated with a significantly reduced risk of hip fracture, which disappeared after four months of discontinuation of use [9]. Similarly, several other studies have shown a reduced risk of hip fractures as well as of other osteoporotic fractures when using thiazide diuretics [10-13].

Thiazide diuretics can affect bone through different mechanisms. These drugs were shown to directly stimulate osteoblast differentiation and bone formation [14]. This could result in an increase in serum osteocalcin, which is considered as a marker of osteoblast activity, bone formation, and bone turnover in general [15-17]. However, bone histomorphometric studies have presented evidence for a reduced bone resorption, and markers of bone resorption such as N-telopeptide and of bone formation such as osteocalcin have been shown to be reduced especially during the first six months of therapy with thiazide diuretics [6,18]. Furthermore, use of thiazide diuretics directly stimulates calcium uptake by the bones [19] and indirectly increases the calcium concentrations in the human body via calcium retention through the kidneys [20-22]. In addition, thiazide diuretics use has been associated with lower parathyroid hormone (PTH) levels, independently of serumcalcium levels [23]. PTH plays an important role in skeletal home-ostasis and lower levels of this hormone can lead to a decrease in bone remodeling [24].

BMD is an important determinant of bone strength [25] and fracture risk [26]. However, previous studies have shown that use of thiazide diuretics is associated with only a small increase in BMD and a much larger decrease in the risk of osteoporotic fractures, suggesting that this decrease is not only caused by an increase in BMD, but by an increase in other determinants of bone strength as well. This highlights the importance of measuring and studying determinants of bone strength other than BMD. Recently, the trabecular bone score (TBS), estimated from dual-energy X-ray absorptiometry (DXA) scan images, has been approved by the Food and Drug Administration (FDA) as a non-invasive technique for producing a metric that correlates with the trabecular microarchitecture of bones [27]. BMD and TBS are independent measures of bone strength [25]. In addition, TBS has been shown to be a predictor of fracture risk independently of both BMD and the Fracture Risk Assessment Tool (FRAX) and adjusting the FRAX score for TBS could also improve the assessment of fracture risk [28-30]. Thus, investigating the effect of thiazide diuretics on both BMD and TBS could provide new and important

insights into the mechanism by which the decreased risk of osteoporotic fractures in thiazide diuretics users can be explained.

To the best of our knowledge, the effect of thiazide diuretics on TBS has not been studied before. In view of the frequent use of thiazide diuretics and the high prevalence of osteoporosis in the ageing population [31,32], it is important to evaluate the association between thiazide diuretics and several aspects of bone strength. Therefore, our objective was to investigate the association between thiazide diuretics use and both BMD and TBS as well as the association between thiazide diuretics use and bone formation estimated by serum osteocalcin levels, in a large, population-based cohort study.

MATERIALS AND METHODS

Study design and population

This cross-sectional analysis was conducted in individuals who participated in the Rotterdam Study, an ongoing prospective population-based cohort study. The design and rationale of the Rotterdam Study have been described elsewhere in detail [33]. In brief, the Rotterdam Study originated in 1990 and was designed to investigate chronic diseases in the elderly. The study started with 7,983 participants aged 55 years and older, living in Ommoord, a suburb of Rotterdam, The Netherlands. This original cohort (RS-I) was extended with a second cohort (RS-II) in 2000 and a third cohort (RS-III) in 2006, adding 3,011 (aged ≥ 55 years) and 3,932 (aged ≥ 45 years) participants, respectively. This resulted in a total study population of 14,926 participants aged 45 years and older. All participants were examined at baseline and asked to participate in follow-up examinations every 3-4 years. For this analysis, we studied participants from the fourth visit of RS-I (RS-I-4, 2002-2004), the second visit of RS-II (RS-II-2, 2004-2005), and the first visit of RS-III (RS-III-1, 2006-2008) for whom there was both a LS-BMD and a LS-TBS measurement available. In total, 6,601 participants gave written informed consent to participate in the study. Ever users of bisphosphonates and current users of loop diuretics were excluded from the study. Ever users of bisphosphonates were excluded because previous literature suggests that the effect of bisphosphonates might persist for years after discontinuation of use [29]. Loop diuretics have been suggested to influence BMD, however, to the best of our knowledge, literature about the persistence of the effect after discontinuation of use is lacking. Therefore, we only excluded current users of loop diuretics. The Rotterdam Study has been approved by the Medical Ethics Committee of the Erasmus Medical

Center and by The Dutch Ministry of Health, Welfare and Sports. The approval has been renewed every five years.

Assessment of thiazide diuretics use

Information about thiazide diuretics exposure was obtained through linkage with the pharmacies in Ommoord which use one shared computer network. The drug exposure period was calculated by dividing the total number of units per dispensed prescription by the prescribed daily number of units. The daily dose was expressed in 'defined daily dose' (DDD), as defined by the World Health Organization (WHO) [35]. When a renewed prescription was filled within seven days after ending the previous one, it was considered as one continuous episode of drug use. The number of days between the last episode of drug use and the date of the DXA scan was used to determine the time a participant was unexposed to the drug prior to undergoing the DXA scan. We defined three different groups of users: current users, past users and never users. If the date of the performed DXA scan was within the drug exposure period or when the participant had a drug exposure period within 120 days prior to the performed DXA scan, the participant was classified as being a current user. This cutoff was set because previous literature has shown that the protective effect of thiazide diuretics on hip fracture, one of the most important types of osteoporotic fractures, disappears after 4 months of discontinuation of thiazide diuretics use [9]. If the date of the performed DXA scan was not within the drug exposure period but when the participant had a drug exposure period in the past not within 120 days prior to the performed DXA scan, the participant was classified as being a past user. Participants for whom the date of the performed DXA scan was not within the drug exposure period and who did not have a drug exposure period in the past were considered as never users. We used the following WHO's Anatomical Therapeutic Chemical codes for thiazide and thiazide-like diuretics: C03AA and C03BA; for the combination of thiazide diuretics and potassium-sparing agents: C03EA; for the combination of thiazide diuretics and beta blocking agents: C07BB; and for the combination of thiazide diuretics and ACE inhibitors: C09BA.

Measurement of BMD and TBS

As TBS is measured at the lumbar spine and in order to study BMD and TBS measurements of the same skeletal site, measurements of lumbar spine BMD (LS-BMD) and lumbar spine TBS (LS-TBS) were used in the current study. These measurements were carried out with DXA using a GE Lunar Prodigy densitometer (Lunar Radiation Corp., Wadison, WI). DXA scans were analyzed with GE lunar software for LS-BMD and with

iNsight software version 4.0 for LS-TBS. LS-BMD values are expressed in g/cm^2. LS-TBS values are expressed as a score. LS-TBS was calculated as the slope of the log-log representation of a two-dimensional variogram, which is derived from gray-level differences on the DXA image. The higher the LS-TBS value, the higher the microstructural quality of the bones. The method of TBS measurement has been described elsewhere in detail [25]. As measurements of LS-TBS were not reliable when having a body mass index (BMI) above 37, participants with a BMI above 37 were excluded.

Osteocalcin measurements

As measurements of osteocalcin were not available for RS-I-4 and RS-II-2, osteocalcin measurements from the third visit of RS-I (RS-I-3, 1997-1999) and the first visit of RS-II (RS-II-1, 2000-2001) were used in the current analysis. In addition, measurements of osteocalcin from RS-III-1 were available and added to the analysis as well. In total, 8,707 participants were included in the osteocalcin analysis. Osteocalcin levels were measured in blood samples by the Department of Clinical Chemistry of the Erasmus Medical Center using the Roche/Hitachi cobas e411/e601/e602, Elecsys 2010 and MODULAR ANALYTICS E170 analyzers (Roche Diagnostics, Indianapolis, IN, USA). Information about thiazide diuretics, bisphosphonates and loop diuretics use was obtained in the same way as described above. For the osteocalcin analysis, the participant was classified as being a user if the date of the blood sampling was within the drug exposure period. This definition of users is different from the definition of current users in the other analyses, because a change in osteocalcin by thiazide diuretics is expected to occur rapidly whereas it takes time to increase the bone mineralization and microarchitecture. Every participant who was not classified as being user, was classified as being a non-user. Current users of bisphosphonates and loop diuretics were excluded from the analysis.

Assessment of covariables

Information on bisphosphonate, loop diuretic and oral glucocorticoid use was obtained in the same way as information about thiazide diuretics was obtained. Ever use of bisphosphonates was defined as having at least one current or past drug exposure period. Current use of loop diuretics and oral glucocorticoids was defined in the same way as current use of thiazide diuretics, in order to be consistent in the definitions of use. Information on alcohol intake, smoking and physical activity was acquired using home interviews. Alcohol intake was measured continuously in g/day and categorized into three categories, based on quantiles of use: none, low, medium, and high. Smoking was expressed categorically as never, past, and current smoker. BMI

was calculated by dividing the weight in kilograms by the height in meters squared. Physical activity was expressed in total metabolic equivalent (MET) hours per week. Ascertainment methods for diabetes mellitus (DM), stroke and coronary heart disease (CHD) have been previously described in detail [31-33]. In short, history of DM, stroke and CHD were assessed during the baseline home interviews and verified by reviewing medical records. Subsequently, DM, stroke and CHD were assessed during follow-up in the Rotterdam Study using information from general practitioners' records, hospital records, and lab measurements of serum glucose for DM. Serum vitamin D, serum calcium, serum sodium, serum potassium, serum magnesium, and serum phosphate were measured in blood samples by the Department of Clinical Chemistry of the Erasmus Medical Center using standard methods. Season of blood sample collection was divided in two categories based on possible sunlight exposure: 1) autumn and winter and 2) spring and summer.

Statistical analyses

Continuous variables were expressed as means and standard deviations (SD), while categorical variables were expressed as frequency and percentage or valid percentage. Univariable and multivariable linear regression were used to examine the relationship of the use of thiazide diuretics with LS-BMD and LS-TBS. Furthermore, categories were created based on the mean defined daily dose (DDD) of thiazide diuretics to study the effect of dosage of thiazide diuretics on LS-BMD and LS-TBS. This resulted in two categories: <1 DDD and ≥ 1 DDD. To study the effect of the duration of thiazide diuretics use, three different categories of use were created: 1-120 days, 120-365 days and more than 365 days. The higher cut-off of 365 days was chosen because of its clinical relevance: it has been shown that the use of thiazide diuretics for more than 365 days was associated with a reduced risk of osteoporotic fractures [9,13] and that the protective effect of thiazide diuretics on femur fractures is largest among those using the medication for > 365 days [39]. In addition, it is shown that the protective effect of thiazide diuretics on hip fracture disappears after 4 months of discontinuation of use [9], which implies that bone needs 4 months to remodel after a change in thiazide diuretics use. Subsequently, we hypothesized that the effect of thiazide diuretics on bone will only appear after 4 months of thiazide diuretics use and, therefore, the lower cut-off of 120 days was chosen. In both the DDD and the duration analyses, never use of thiazide diuretics was used as the reference category. Subsequently, both univariable and multivariable linear regression was used to examine the relationship of the DDD and duration of medication use with LS-BMD and LS-TBS. For all analyses, we used four models. The first model was adjusted for Rotterdam Study cohort, age and sex, whereas the second model was additionally adjusted for BMI, vitamin D

level, serum calcium, serum sodium, serum potassium, serum magnesium, serum phosphate, alcohol intake, smoking and, diabetes mellitus. The third model was additionally adjusted for LS-TBS in the models where LS-BMD was the outcome, and *vice versa*. In addition, in a fourth model, we investigated if additional adjustment for season of vitamin D measurement, corticosteroids use, physical activity, stroke and coronary heart disease significantly changed the results. A p for trend was calculated across both the 3 categories of duration of use and the two categories of DDD of use, and the reference category.

In case of a significant association between thiazide diuretics use and LS-BMD or LS-TBS, we tested interactions of thiazide diuretics use with age, sex and BMI. Interaction terms were considered to be significant when the p for interaction was below 0.10.

Serum osteocalcin levels for current users and non-users of thiazide diuretics were expressed as means and SD. Linear regression analysis was used to examine the relationship of the use of thiazide diuretics with serum osteocalcin levels. For this analysis, we used two models: the first model was unadjusted and the second model was adjusted for Rotterdam Study cohort, age and sex.

Multiple imputation was performed to impute missing values in the covariables, using the 'Multivariate Imputation by Chained Equations' package in R [40]. The number of imputed datasets was based on the average percentage of missing values per variable [41]. The average percentage of missing values in the total population was 3.2%. Rounding to a value which is a multiplication of 5, generated a total number of 5 imputed datasets. The number of iterations was increased with 5 at a time until convergence was achieved. With the exception of the characteristics of the study population, results are reported for imputed data.

A two-sided p-value below 0.05 was considered as statistically significant. Data were analyzed using R version 3.4.4 (R Foundation for Statistical Computing, Vienna, Austria) and SPSS Statistics for Windows, Version 24.0 (IBM, Armonk, NY, USA).

RESULTS

Population characteristics

The flowchart of the study population is shown in **Figure 1.** A total number of 6,640 participants were eligible for the current study as there was both a LS-BMD and a

LS-TBS measurement available. Of the total study population, 41 participants were excluded because they did not provide written informed consent, resulting in an eligible study population of 6,599 participants. Subsequently, 503 participants were excluded because of ever use of bisphosphonates and/or current use of loop diuretics, resulting in a final study population of 6,096. This population could be further divided into 4,883 never users, 551 past users and 662 current users of thiazide diuretics.

Characteristics of the final study population as well as the characteristics of never, past and current users of thiazide diuretics, before imputation, are shown in **Table 1.** Mean age of the total study population at the time of the performed DXA scan was 65.5 years and 56.2% were female. A total of 662 participants (10.9%) were current users of thiazide diuretics.

Association between thiazide diuretics use and LS-BMD

Table 2. shows the association between the use, dosage and duration of use of thiazide diuretics and LS-BMD. The use of thiazide diuretics was found to be positively associated with LS-BMD in the fully adjusted model (model 3). Past use of thiazide diuretics was associated with a 0.021 g/cm^2 (95% CI: 0.006;0.036) higher LS-BMD value, while current use of thiazide diuretics was associated with a 0.016 g/cm2 (95% CI: 0.002;0.031) higher LS-BMD value, both compared to never use of thiazide diuretics.

When taking dosage into account, the use of <1 DDD of thiazide diuretics was associated with a decrease in LS-BMD of 0.0002 g/cm^2 (95% CI: -0.021;0.021) compared to never users in the fully adjusted model (model 3), although this difference was not statistically significant. Conversely, the use of ≥1 DDD of thiazide diuretics was positively and significantly associated with LS-BMD (β = 0.028, 95% CI: 0.010;0.046, model 3). A significant trend within the DDD of use was found (p for trend = 0.004). Analysis of the duration of thiazide diuretics use showed a positive association between thiazide diuretics use and LS-BMD when using the medication for more than 365 days (β = 0.033, 95% CI: 0.014;0.052). In addition, a significant trend within the duration of use was found (p for trend < 0.001).

Additionally adjusting the analyses for season of vitamin D measurement, corticosteroids use, physical activity, stroke and coronary heart disease did not change the results (data not shown).

Table 1. Characteristics of the study population

	Total population (n = 6,096)	Never users of thiazide diuretics (n = 4,883)	Past users of thiazide diuretics (n = 551[a])	Current users of thiazide diuretics (n = 662[a])
General characteristics				
Female sex, n(%) (n= 6,096)	3,423 (56.2)	2,639 (54.0)	353 (64.1)	431 (65.1)
Age at DXA scan, years (n = 6,096)	65.5 ± 10.3	64.6 ± 10.2	69.9 ± 10.0	68.5 ± 10.0
Alcohol use, n(%) (n = 5,276)				
No alcohol use	572 (10.8)	419 (10.0)	87 (17.5)	66 (11.5)
Light drinking	774 (14.7)	621 (14.8)	66 (13.3)	87 (15.2)
Moderate drinking	2,687 (50.9)	2,137 (50.8)	246 (49.5)	304 (53.0)
Heavy drinking	1,243 (23.6)	1,028 (24.4)	98 (19.7)	117 (20.4)
Smoking, n(%) (n = 6,021)				
Never smoker	1,806 (30.0)	1,400 (29.0)	179 (33.0)	227 (34.8)
Former, non-smoker	3,036 (50.4)	2,204 (49.8)	297 (54.8)	335 (51.4)
Current smoker	1,179 (19.6)	1,023 (21.2)	66 (12.2)	90 (13.8)
Physical activity, hours/week (n = 5,215)	78.0 ± 52.6	77.1 ± 53.3	84.6 ± 49.0	79.5 ± 50.0
Comorbidities and medication use				
Diabetes mellitus, n(%) (n = 5,632)	544 (9.7)	386 (8.5)	64 (12.9)	94 (15.4)
Stroke, n(%) (n = 5,788)	68 (1.2)	45 (1.0)	15 (2.9)	8 (1.3)
CHD, n(%) (n = 5,702)	232 (4.1)	174 (3.8)	28 (5.5)	30 (4.8)
Oral corticosteroid use, n(%) (n = 6,096)	153 (2.5)	119 (2.4)	20 (3.6)	14 (2.1)
Measurements				
BMI, kg/m2 (n = 6,036)	27.1 ± 3.6	26.8 ± 3.5	28.2 ± 3.7	28.6 ± 3.7
LS-BMD, g/cm2 (n = 6,096)	1.144 ± 0.205	1.140 ± 0.205	1.156 ± 0.204	1.162 ± 0.205
LS-TBS (n = 6,096)	1.319 ± 0.103	1.324 ± 0.103	1.299 ± 0.103	1.305 ± 0.101
Systolic blood pressure, mmHg (n = 6,075)	142 ± 22	140 ± 21	153 ± 24	152 ± 23
Diastolic blood pressure, mmHg (n = 6,075)	81 ± 11	81 ± 11	83 ± 12	84 ± 12
Blood measurements				
Season of blood collection, summer and spring, n(%) (n = 5,874)	2,733 (46.5)	2,197 (46.6)	256 (48.8)	280 (44.4)
Serum vitamin D, ng/ml (n = 5,086)	58.97 ± 27.42	60.15± 27.56	54.78 ± 26.87	53.41 ± 25.78
Serum calcium, mg/dl (n = 5,772)	2.4 ± 0.1	2.4 ± 0.1	2.4 ± 0.1	2.5 ± 0.1
Serum sodium, mmol/L (n = 5,669)	142 ± 2	142 ± 2	142 ± 3	142 ± 3
Serum potassium, mmol/L (n = 5,666)	4.4 ± 0.3	4.4 ± 0.3	4.3 ± 0.4	4.2 ± 0.4
Serum magnesium, mmol/L (n = 5,657)	0.85 ± 0.06	0.85 ± 0.06	0.84 ± 0.06	0.83 ± 0.06
Serum phosphate, mmol/L (n = 5,655)	1.11 ± 0.16	1.11 ± 0.16	1.12 ± 0.17	1.11 ± 0.16

Data are presented as number (%), number (valid %), or mean ± standard deviation. Values are shown for non-imputed data. For variables with missing data, valid % is given.

[a] Percentage of past users = 9.0%, percentage of current users = 10.9%

Abbreviations: n = number; DXA = dual-energy X-ray absorptiometry; CHD = coronary heart disease; BMI = body mass index; LS-BMD = lumbar spine bone mineral density; LS-TBS = lumbar spine trabecular bone score.

Table 2. Univariable and multivariable linear regression of the use of thiazide diuretics, dosage and duration of thiazide diuretics use, and LS-BMD (in g/cm^2) (n = 6,096)

	Model 1 Beta (95% CI)	Model 2 Beta (95% CI)	Model 3 Beta (95% CI)
Thiazide diuretics use			
Never users (n = 4,883)	Reference	Reference	Reference
Past users (n = 551)	0.044 (0.026;0.061)[a]	0.025 (0.008;0.042)[a]	0.021 (0.006;0.036)[a]
Current users (n = 662)	0.047 (0.031;0.062)[a]	0.020 (0.004;0.036)[a]	0.016 (0.002;0.031)[a]
Dosage of thiazide diuretics			
Never users (n = 4,883)	*Reference*	*Reference*	*Reference*
Current users (n = 662)			
< 1 DDD (n = 274)	0.029 (0.005;0.052) [a]	0.004 (-0.019;0.028)	-0.0002 (-0.021;0.021)
≥ 1 DDD (n = 388)	0.059 (0.039;0.079)[a]	0.031 (0.011;0.051)[a]	0.028 (0.010;0.046)[a]
P for trend	<0.001[a]	0.003[a]	0.004[a]
Duration of thiazide diuretics use			
Never users (n = 4,883)	*Reference*	*Reference*	*Reference*
Current users (n = 662)			
1-120 days (n = 256)	0.014 (-0.010;0.039)	-0.012 (-0.037;0.012)	-0.008 (-0.030;0.014)
120-365 days (n = 53)	0.059 (0.007;0.111)[a]	0.037 (-0.013;0.088)	0.025 (-0.021;0.070)
>365 days (n = 353)	0.069 (0.047;0.091)[a]	0.042 (0.020;0.063)[a]	0.033 (0.014;0.052)[a]
P for trend	<0.001[a]	<0.001[a]	<0.001[a]

Model 1: adjusted for Rotterdam Study cohort, age and sex; model 2: additionally adjusted for BMI, vitamin D level, serum calcium, serum sodium, serum potassium, serum magnesium, serum phosphate, alcohol intake, smoking, systolic blood pressure, diastolic blood pressure and, diabetes mellitus; model 3: additionally adjusted for LS-TBS. [a] p<0.05.
Abbreviations: LS-BMD = lumbar spine bone mineral density; CI = confidence interval; n = number; DDD = defined daily dose; BMI = body mass index; LS-TBS = lumbar spine trabecular bone score.

Association between thiazide diuretics use and LS-TBS

Results of the linear regression analyses of the use, dosage and duration of use of thiazide diuretics and LS-TBS are shown in **Table 3.** No statistically significant result was found, neither in the model adjusted for Rotterdam Study cohort, age and sex only, nor in the fully adjusted models. Additionally adjusting for season of vitamin D measurement, corticosteroids use, physical activity, stroke and coronary heart disease did not change the results (data not shown).

Interaction terms

As a significant association between the use of thiazide diuretics and LS-BMD was found, interaction terms of age, sex and BMI with thiazide diuretics were tested. None of these interaction analyses showed a statistically significant interaction with thiazide diuretics (data not shown).

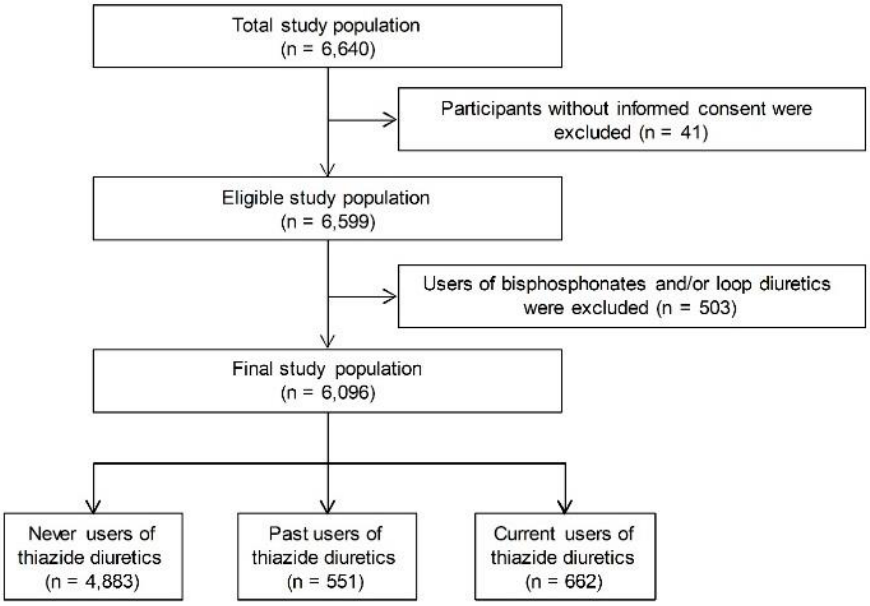

Figure 1. Flowchart of the study population. Of the total population of 6,640 eligible participants, 41 were excluded because of no informed consent, resulting in an eligible study population of 6,599 participants. Subsequently, 503 participants were excluded because of ever use of bisphosphonates and/or current use of loop diuretics. This resulted in a final study population of 6,096 participants. This population could be further divided into 4,883 never users, 551 past users and 662 current users of thiazide diuretics.
Abbreviations: n = number; LS-TBS = lumbar spine trabecular bone score; LS-BMD = lumbar spine bone mineral density.

Serum osteocalcin levels in users and non-users of thiazide diuretics

Of the 8,707 eligible participants, 64 were excluded because they did not provide written informed consent and another 325 of current bisphosphonate and/or loop diuretics use, resulting in a total population of 8,318 participants. The mean level of serum osteocalcin was 20.2 ng/ml (SD 8.3) in users of thiazide diuretics and 22.5 ng/ml (SD 17.0) in non-users of thiazide diuretics ($p<0.001$). Linear regression analyses

Table 3. Univariable and multivariable linear regression of the use of thiazide diuretics, dosage and duration of thiazide diuretics use, and LS-TBS (n = 6,096)

	Model 1 Beta (95% CI)	Model 2 Beta (95% CI)	Model 3 Beta (95% CI)
Thiazide diuretics use			
Never users (n = 4,883)	*Reference*	*Reference*	*Reference*
Past users (n = 551)	0.005 (-0.003;0.013)	0.005 (-0.003;0.013)	-0.0007 (-0.008;0.006)
Current users (n = 662)	0.006 (-0.002;0.013)	0.004 (-0.003;0.012)	-0.0002 (-0.007;0.007)
Dosage of thiazide diuretics			
Never users (n = 4,883)	*Reference*	*Reference*	*Reference*
Current users (n = 662)			
< 1 DDD (n = 274)	0.006 (-0.005;0.013)	0.005 (-0.006;0.016)	0.004 (-0.006;0.014)
≥ 1 DDD (n = 388)	0.005 (-0.004;0.017)	0.004 (-0.006;0.013)	-0.003 (-0.011;0.005)
P for trend	0.17	0.33	0.66
Duration of thiazide diuretics use			
Never users (n = 4,883)	*Reference*	*Reference*	*Reference*
Current users (n = 662)			
1-120 days (n = 256)	-0.003 (-0.015;0.008)	-0.005 (-0.017;0.007)	-0.002 (-0.013;0.009)
120-365 days (n = 53)	0.014 (-0.010;0.038)	0.013 (-0.011;0.037)	0.005 (-0.016;0.026)
>365 days (n = 353)	0.010 (0.0005;0.020) [a]	0.009 (-0.001;0.019)	-0.00001 (-0.009;0.009)
P for trend	0.04[a]	0.08	0.95

Model 1: adjusted for Rotterdam Study cohort, age and sex; model 2: additionally adjusted for BMI, vitamin D level, serum calcium, serum sodium, serum potassium, serum magnesium, serum phosphate, alcohol intake, smoking, systolic blood pressure, diastolic blood pressure and, diabetes mellitus; model 3: additionally adjusted for LS-BMD. [a] $p<0.05$.
Abbreviations: LS-BMD = lumbar spine bone mineral density; CI = confidence interval; n = number; DDD = defined daily dose; BMI = body mass index; LS-TBS = lumbar spine trabecular bone score.

showed that the use of thiazide diuretics was associated with a 2.3 ng/l (95% CI: -3.5;-1.1) lower serum osteocalcin level in the unadjusted model and with a 3.2 ng/l (95% CI: -4.4.;-2.0) lower serum osteocalcin level when adjusted for Rotterdam Study cohort, age and sex, both compared to non-use of thiazide diuretics (**table 4., figure 2.**). Excluding the outliers did not significantly change the results.

DISCUSSION

In this cross-sectional analysis of 6,096 participants from the Rotterdam Study, past and current use of thiazide diuretics were significantly associated with an increase in LS-BMD. Use of ≥1 DDD and use of thiazide diuretics for more than 365 days were posi-

Table 4. Linear regression analysis of the use of thiazide diuretics and serum osteocalcin levels (in ng/ml) (n = 8,318)

	Model 1	Model 2
	Beta (95% CI)	**Beta (95% CI)**
Use of thiazide diuretics	-2.3 (-3.5;-1.1)	-3.2 (-4.4.;-2.0)

Model 1: unadjusted; model 2: adjusted for Rotterdam Study cohort, age and sex

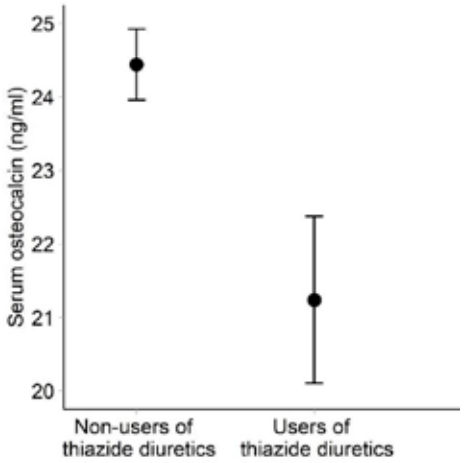

Figure 2. Serum osteocalcin levels in ng/ml in users and non-users of thiazide diuretics, adjusted for Rotterdam Study cohort, age and sex (estimate with standard error).

tively and significantly associated with LS-BMD. In addition, a significant trend within the DDD and duration of use was found as well. On the other hand, no significant association between the use, the DDD and the duration of use of thiazide diuretics and LS-TBS was found. Furthermore, analyses regarding the association between thiazide diuretics use and serum osteocalcin levels showed that the use of thiazide diuretics was significantly associated with lower serum osteocalcin levels.

The findings of our study regarding the association between current use of thiazide diuretics and LS-BMD are consistent with several previous studies which have shown a positive association between the use of thiazide diuretics and LS-BMD as well [1,5-7,42]. However, we also found an unexpected positive association between past use of thiazide diuretics and LS-BMD. We hypothesized that the effect of thiazide diuretics on LS-BMD would disappear after four months of discontinuation of use and our definition of current use of thiazide diuretics derived from this hypothesis. Our hypothesis was based on an important finding of a previous study of our research group, namely that

the protective effect of thiazide diuretics on the incidence of hip fractures disappeared after four months of discontinuation of use [9]. As known, the risk of hip fracture is predominantly related to femoral neck BMD and not to LS-BMD. Nevertheless, we expected similar effects of thiazide diuretics on the BMD of different skeletal sites. Our finding could imply that the effect of thiazide diuretics on BMD lasts longer than their effect on the risk of osteoporotic fractures, but it is also possible that the effect of thiazide diuretics on BMD differs per skeletal site. Another explanation for this unexpected association could be the presence of residual confounding. In addition, as we found a significant association between thiazide diuretics use and LS-BMD, interaction terms of age, sex and BMI with thiazide diuretics were tested. None of the tested interaction terms were statistically significant. This implies that the effect of thiazide diuretics on LS-BMD does not differ by age, sex and BMI.

Furthermore, we found that the use of ≥1 DDD was positively and significantly associated with LS-BMD, while the use of <1 DDD was not. Similarly, when studying the effect of the duration of thiazide diuretics use on LS-BMD, a positive effect of thiazide diuretics on LS-BMD is only seen when using the medication for more than 365 days. Furthermore, we showed a significant trend within the DDD and duration of use as well, suggesting that the positive effect of thiazide diuretics on LS-BMD increases with dosage and time of thiazide diuretics use. Thiazide diuretics stimulate osteoblast differentiation, bone formation and calcium uptake [19] by the bones. Higher dose and longer duration of thiazide diuretics use could stimulate those three processes to a greater extent, causing a larger increase in the LS-BMD value. To the best of our knowledge, we are the first showing this positive trend and further studies are needed to confirm this finding before implications for clinical practice can be made.

In order to study determinants of bone strength other than BMD, we investigated the association between thiazide diuretics use and bone microarchitecture, estimated by LS-TBS and measured using DXA. No statistically significant association between the use of thiazide diuretics and LS-TBS was seen, neither overall nor when categorized according to DDD or duration of thiazide diuretics use. This suggests that the use of thiazide diuretics is not associated with changes in LS-TBS. However, another explanation for our findings could be that the measurement of LS-TBS by using DXA is not able to detect the small changes in bone microarchitecture caused by thiazide diuretics use and that more sophisticated techniques for measuring bone microarchitecture are needed. High-resolution peripheral quantitative computed tomography (HR-pQCT) is one of those techniques which has the potential to measure different aspects of bone quality, including bone microarchitecture.

Bone is a dynamic tissue that is continuously remodeled in order to preserve its strength and integrity [43-45]. In the normal bone remodeling process, bone resorption is coupled to bone formation, ensuring that the resorbed bone is completely replaced by new bone [46]. Currently, several biochemical markers are available for the assessment of bone turnover [16]. In osteoporosis, the bone turnover rate is increased [16]. However, bone resorption and bone formation will be partly uncoupled, causing bone resorption to exceed bone formation [47]. Bone resorption will thus still be followed by bone formation in osteoporosis patients, only to a lesser extent, which results in an increase in both bone resorption and bone formation markers in osteoporosis. One of the important bone formation markers is osteocalcin [16] and the serum levels of this marker will be increased in osteoporosis patients. In the current study, we investigated the association between the use of thiazide diuretics and serum osteocalcin levels. Previous literature investigating the effect of thiazide diuretics use on serum osteocalcin levels, has shown conflicting results. In 1993, it was reported that users of thiazide diuretics had lower levels of serum osteocalcin compared to non-users of thiazide diuretics [48]. In addition, a randomized controlled trial investigating the effect of hydrochlorothiazide on rates of bone loss in 320 men and women, showed a decrease in serum osteocalcin levels in subjects treated with thiazide diuretics [6]. In contrast, no significant change in serum osteocalcin levels was seen in a study of 50 postmenopausal women treated for 7 days with bendroflumethiazide compared to postmenopausal women treated with placebo [49]. Two years later, a study of the same research group was published, showing a dose-dependent increase in serum osteocalcin levels in users of bendroflumethiazide [50]. In our study, we found that users of thiazide diuretics had a significantly lower mean serum osteocalcin level compared to non-users of thiazide diuretics. Furthermore, linear regression analysis showed that the use of thiazide diuretics was significantly associated with lower serum osteocalcin levels after adjustment Rotterdam Study cohort, age and sex. These findings suggests that the higher BMD in users of thiazide diuretics may be explained by a decrease in bone turnover.

In this study, there was an unexpected finding, namely a significantly lower vitamin D level in users of thiazide diuretics compared to non-users. A possible explanation for this result may be found in the indication for prescribing thiazide diuretics. Thiazide diuretics are one of the cornerstones in the treatment of hypertension, but are also used in the treatment of conditions related to volume-overload such as chronic kidney disease and heart failure [51]. Both chronic kidney disease and heart failure can lead to impaired mobility [52,53], which can cause a possible decrease in vitamin D level due to a shortage of sunlight exposure in these patients.

Our study has a number of strengths and limitations. The main strength of our study is that we are the first to investigate the effect of thiazide diuretics, including dosage and duration of use, on multiple aspects of bone strength, namely BMD as well as TBS and bone turnover. Another strength is that we used a large prospective population-based cohort study to investigate our research question, which limits the chance of selection and information bias and creates a high level of generalizability of our results to the general population. The Rotterdam Study is conducted in the elderly population, which is an important population of interest when performing studies of bone strength and osteoporosis. The main limitation of our study is the fact that our study is cross-sectional and therefore, we could not study changes in LS-BMD and LS-TBS over time. As a consequence, we were not able to establish a causal relationship between thiazide diuretics use and markers of bone strength. Second, potential misclassification of the exposure could have occurred in our study. Users of thiazide diuretics were identified using filling data at the pharmacies, which does not disclose whether the patient took the medication as prescribed and does not give information about treatment adherence. However, this potential misclassification of the exposure would probably be non-differential, as the probability of the exposure being misclassified is independent of the LS-BMD and LS-TBS values of the participants. Third, confounding by indication could be a possibility in our study, as thiazide diuretics are prescribed for hypertension and hypertension is associated with a lower LS-BMD. However, we tried to address this confounding by indication by adjusting for both systolic and diastolic blood pressure. Fourth, the Rotterdam Study consists of a predominantly Caucasian population aged above 45 years of age, which could limit the generalizability of our results to other populations.

CONCLUSION

The results from our study suggest that thiazide diuretics exert positive effects on LS-BMD, but not on LS-TBS in the general population. This could imply that the reduced risk of osteoporotic fractures in thiazide diuretics users is explained by an increased BMD without improving bone microarchitecture. However, it is also possible that the measurement of LS-TBS is not able to detect small changes in bone microarchitecture and that more sophisticated techniques such as high-resolution peripheral quantitative computed tomography (HR-pQCT) are needed to investigate the association between thiazide diuretics use and bone microarchitecture. Furthermore, our study indicates that only a high dose and longer duration of thiazide diuretics use exert positive effects on LS-BMD. These results could be relevant for clinical practice when treating elderly individuals with both hypertension and osteoporosis.

Disclosures

All authors state that they have no conflicts of interest.

Acknowledgments

We gratefully acknowledge the dedication, commitment and contribution of the study participants, the staff of the Rotterdam Study, and the participating general practitioners and pharmacists.

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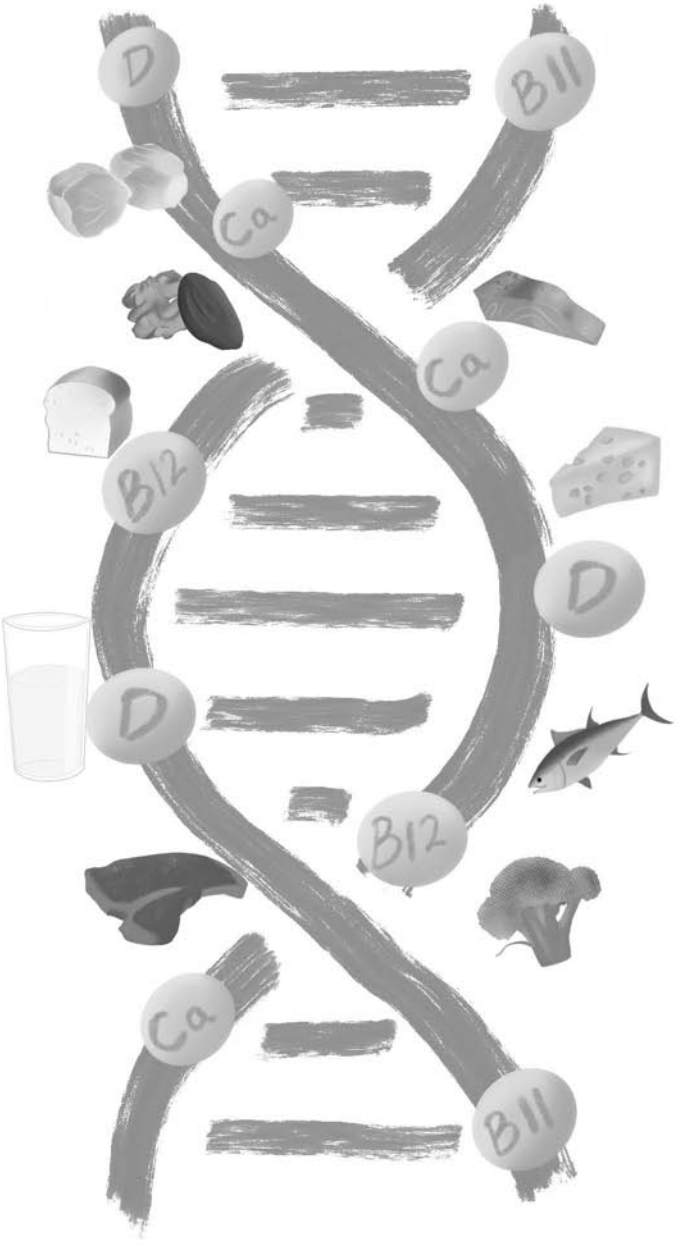
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Chapter 4.2

Do vitamin D level and dietary calcium intake modify the association between loop diuretics and bone health?

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Calcif Tissue Int. 2020 Feb;106(2):104-114.

ABSTRACT

Loop diuretics (LD) may affect bone health by inhibiting renal calcium reuptake. However, whether vitamin D status and dietary calcium intake modify the association between LD and bone outcome is unclear. Therefore, this study aimed to evaluate whether vitamin D level or calcium intake modify the association between LD and various indices of bone health including bone mineral density (BMD), and Trabecular Bone Score (TBS). From The Rotterdam Study, a prospective population-based cohort study, we used data from 6,990 participants aged >45yr with a DXA scan (2002-2008); 6,908 participants with femoral neck (FN)-BMD, 6,677 participants with lumbar spine (LS)-BMD and 6,476 participants with LS-TBS measurements. Use of LD was available from pharmacy dispensing records. Vitamin D (25(OH)D) level was measured in serum, and dietary calcium intake was measured with a validated food frequency questionnaire. Almost eight percent of the participants used LD. The association between LD (past-users compared to never-users) and LS-TBS was significantly different by 25(OH)D concentrations (P for interaction=0.04). A significantly lower LS-TBS among LD past-users was observed for 25(OH)D ≥50 nmol/l compared to ≤20 and 20-50 nmol/l (ß=-0.036, 95% CI -0.060; -0.013 versus ß=-0.012, 95% CI -0.036; 0.013 and ß=-0.031, 95% CI -0.096; 0.034 respectively). However, no other significant effect-modification by 25(OH)D and dietary calcium intake was found in the associations between LD-use and bone health outcomes (P-interaction > 0.13). This study suggests that the association between LD-use and indices of bone health is not consistently modified by vitamin D or dietary calcium intake.

Keywords

Loop Diuretics; Bone Mineral Density; bone geometry; Trabecular Bone Score.

INTRODUCTION

In the past decade, recognition of the importance of food and drug interactions has been growing in clinical practice (1). Especially in older people, however, more knowledge is needed because of the frequent use of medications and polypharmacy and higher risk of poor nutritional status (2). Food-drug interactions may be relevant in older people using loop diuretics (LD). Diuretics are frequently prescribed in the treatment of heart failure and hypertension (3, 4), and they have been shown to influence calcium homeostasis and bone metabolism.

Thiazide diuretics have shown to have a protective effect in preserving bone mass and in decreasing the risk of fractures (5). Yet, these effects have been shown to vary in relation to dosage, duration of treatment and do not last long after discontinuation of treatment (6-8). In contrast, few studies have been carried out assessing the effect of loop diuretics (LD) on skeletal health. Some studies suggest that LD can have a negative impact on bone turnover by increasing urinary calcium excretion (9, 11-13) whereas other show no association between LD-use and bone health on long-term use (6-13).

These conflicting findings may be the result of differences in calcium intake and vitamin D levels across the studied population, considering that bone mineral deposition/formation and bone resorption can be relatively normal as long as serum calcium and phosphate and calciotropic hormone levels like vitamin D and parathyroid hormone (PTH) are normal (14). Vitamin D has a major role in calcium homeostasis through three mechanisms: I) increased intestinal absorption of calcium, II) reduced renal excretion of calcium by stimulating resorption of the distal tubules and III) deposition and mobilization of calcium from bones (15). An inverse association between LD-use and serum 25(OH)D level has been reported before (16). LD-users have a lower concentration of 25(OH)D than non-users (17). As a result vitamin D deficiency may amplify any potential adverse effects of LD on bone health due to increased urinary calcium losses. Also, the use of LD may be harmful for bone health through increased plasma PTH and $1.25(OH)_2D$ levels as a result of increased renal calcium losses (13). Higher calcium loss may increase bone turnover resulting in a negative calcium balance. Furthermore, in case of very low external calcium supply from diet or supplementation, high levels of $1.25(OH)_2D_3$ mobilise the bone calcium reservoir for serum calcium homeostasis at the (temporary) expense of bone mass and strength (18). Accordingly, in people with a low calcium intake, the effect of LD on bone loss and increased fracture risk may be enhanced (10).

Therefore, in view of the frequent use of LD and a high prevalence of osteopenia and osteoporosis in the ageing population, in combination with a higher risk of mal-

nutrition and vitamin D deficiency in older individuals, it is important to evaluate whether vitamin D level or calcium intake may modify the association between loop diuretics and various indices of bone health including bone mineral density (BMD) and Trabecular Bone Score (TBS) in elderly population.

MATERIALS AND METHODS

Study Population

This study was embedded in the first three cohorts of The Rotterdam Study (RS-I, RS-II and RS-III), an ongoing, population-based cohort study in Ommoord, a suburb of Rotterdam, the Netherlands (19). Since January 1990 participants of 55 years and over were recruited for RS-I (N=7,983). In 2000, the study was extended to 3,011 participants (RS-II). Later in 2006, the study was extended with a third cohort of participants of 45 y and older (RS-III). Overall response for all three cycles at baseline was 72% (14,926 of 20,744) (19). Participants were interviewed at home by a trained research assistant, after which they were invited for a physical examination and dietary assessment at the research center. The study has been approved by the institutional review board (Medical Ethics Committee) of the Erasmus Medical Center and by the review board of the Netherlands Ministry of Health, Welfare and Sports, and approval has been renewed every 5 years. From all subjects written informed consent was obtained. More details on the main objectives, design and diagram of examination cycles of the Rotterdam Study (RS) have been published elsewhere (19). Because of a possible persisting effect on bone, users of bisphosphonates were excluded from the study. For the current analysis, 6,908 participants with data available for femoral neck (FN)-BMD, 6,677 participants with lumbar spine (LS)-BMD and 6,476 participants with LS-TBS were included from the fourth examination of the first cohort (RS-I-4, 2002-2004), the second examination of the second cohort (RS-II-2, 2004-2005) and the first examination of the third cohort (RS-III-1, 2006-2008) (**Figure 1**).

Dietary intake and serum 25(OHD) level

Dietary data were collected at baseline (between 1989 and 1993 in RS-I-1, between 2000 and 2001 in RS-II-1 and between 2006 and 2008 in RS-III-1) using a validated semi-quantitative Food Frequency Questionnaire (FFQ) managed by a trained dietician, at the study center (20, 21). For RS-I-1 and RS-II-1, a two-stage 170-items FFQ was used (during first stage, participants mentioned on 170 food item, which foods they consumed at least twice a month in the preceding year, and in the second stage,

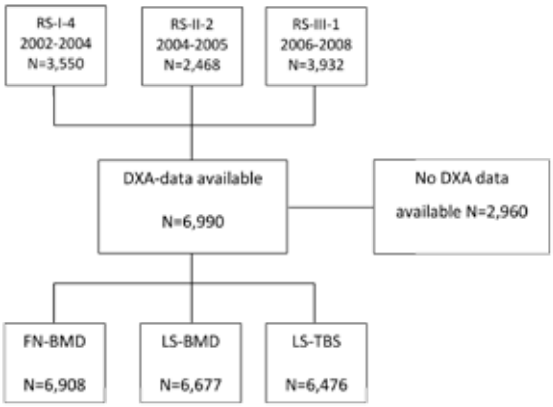

Figure 1. Flow-chart of sub-cohorts included in the study

dietician identified how often and in which amounts the foods were consumed). For RS-III-1, a one-stage 389-items FFQ was used. Dietary intake of nutrients (incl. total energy and dietary calcium intake) was determined using the Dutch Food Composition Tables (NEVO) from 1993, 2001 and 2006, using standardized portion sizes (20, 22). Intake of calcium was adjusted for energy corresponding to the residual method (23). Serum 25(OH)D was measured in the blood collected at the same time as dietary data, between 1990 and 2008 using electrochemiluminescence immunoassay (COBAS, Roche Diagnostics GmbH). The sensitivity of the test was 10 nmol/L, the range of serum 25(OH)D concentrations was from 7.5 nmol/L to 175 nmol/L, the within-run accuracy was less than 7.8%, and the intermediate precision accuracy was less than 13.1% (24, 25). Vitamin D deficiency was defined as a serum 25(OH)D level ≤ 50 nmol/L according to the current recommendations for older adults aged >70 years by the Institute of Medicine and the Dutch Health Council (26). In addition, we used also a vitamin D deficiency threshold <20 nmol/L in our analysis and showed the stratification analysis in categories of <20 nmol/L, between 20-50 nmol/L and >50 nmol/L.

Loop diuretics

As of 1st January 1991, pharmacy records of prescriptions were collected via all pharmacies in the Ommoord region with details on product name, ATC-code, number of tablets/capsules in each prescription, and prescribed daily number (27). LD-use (ATC-code C03C) was determined from baseline to the date of the DXA scan [19] and defined in three different categories: current-users, past-users and never-users. Current-users were defined when the participant had a drug exposure period between the date of the DXA scan and 120 days prior to the performed DXA scan. Past-users were defined when the participant had a drug exposure period more than 120 days

prior to the performed DXA scan. In addition, if the participants had no drug exposure from baseline till the DXA scan, the participants were considered as never-users. The duration of LD-use among current-users was categorized into 1-120 days, 120-365 days, and more than 365 days. Never-use of LD was used as the reference category.

Assessment of co-variables

Co-variables related to lifestyle, body composition and socioeconomic status (SES) were included. Weight (kg) and height (cm) were measured at study entry. BMI was calculated as weight divided by height squared (kg/m^2). During home interviews physical activity (PA) was assessed by means of the Zutphen Physical Activity Questionnaire (28). Metabolic equivalent of tasks were calculated (MET hours/week) according to time spent in categories of light, moderate and vigorous activity (28). Socioeconomic status variables (i.e., educational level and income level), smoking (yes/no), pack years, use of alcohol, prevalence of Coronary Heart Diseases (CHD), stroke and Diabetes Mellitus (DM) were assessed by home interview. Also the use of bisphosphonates was determined using pharmacy dispensing records in the same way as the use of LD. Serum calcium, magnesium and sodium were measured in blood samples by the Department of Clinical Chemistry of the Erasmus Medical Center using the Roche/ Hitachi cobas c501 analyzer (Roche Diagnostics, Indianapolis, IN, USA).

Outcome assessment

Femoral neck and lumbar spine BMD was measured at RS-I-4, RS-II-2 and RS-III-1 (between 2002 and 2008) by dual energy X-ray absorptiometry (DXA) using a ProdigyTM fan-beam densitometer (GE Lunar Corp, Madison, WI, USA for all participants (19). The DXA-derived Trabecular Bone Score (TBS), which is measured in the Lumbar Spine (LS-TBS) is a grey-level texture measurement that correlates with 3D parameters of bone micro-architecture, connectivity density, trabecular separation and trabecular number (29). LS-TBS predicts future fractures (all type) independent of areal BMD [30]. Moreover, recent studies have shown that LS-TBS may be an applicable measure of trabecular bone integrity to study in regard to lifestyle factors that are adaptable, such as a dietary intake (31, 32). LS-TBS was derived from the same lumbar DXA scans that BMD was obtained from and it was analyzed using TBS iNsight software (version 1.9; Medimaps, Geneva, Switzerland) at the Bone Disease Unit of the University of Lausanne (Lausanne, Switzerland). LS-TBS was calculated for a subgroup of RS-I-4, RS-II-2 and RS-III-1 and represents the mean value of the individual vertebral measurements from L1 to L4. Subjects with a BMI higher than 37 kg/m2 were excluded from the study since LS-TBS measurements in morbidly obese persons are not ac-

curate. Furthermore, the LS-TBS was standardized according to sex using the residual method, due to technical differences between LS-TBS across sexes. The method of LS-TBS calculation has been described in detail elsewhere (32).

Statistical analyses

First, for all variables normal distribution was examined by visual check of histograms. When necessary, data were log transformed. Linear regression analysis (cross-sectional) was used to assess the association between LD-use (past-, current-use and the duration of 1-120 days, 120-365 days and >365 days) and FN-BMD, LS-BMD and LS-TBS. All the analyses were adjusted for age and sex and cohort (model 1). Thereafter, the co-variables were added additionally to model 2, based on literature relevance as well as principles of causal inference combined with the change-in-effect-criterion of ≥10% (33, 34). Potential confounders were BMI, smoking (pack-years), alcohol intake (g/day), SES (education and job), total physical activity (METhours/wk) and comorbidities (prevalence of CHD, stroke and DM). For the analysis of the duration (continuous), the model was additionally adjusted for the past-users. An earlier study showed that in subjects with osteopenia/normal BMD levels, TBS is significantly associated with vertebral fractures (35). Even though BMD and TBS are correlated, they present different aspects of bone health (35). For that reason, as sensitivity analyses, we wanted to assess how the association between LD and BMD may depend on measures of bone architecture (i.e. TBS) and how a potential association between LD and TBS is dependent on BMD. So, the association between LD and FN-BMD and LS-BMD were additionally adjusted for LS-TBS and vice versa (model 3). In addition, in all analyses, sensitivity analyses was performed with additional adjustment for serum vitamin D, season of blood collection of vitamin D: (winter (September till end of February) and summer (March till end of August)) serum calcium, magnesium and sodium concentrations.

To assess whether effect-modification by serum 25(OHD) level or dietary calcium intake was present in the association of LD (ever-use) on FN-BMD, LS-BMD and LS-TBS, we evaluated the effect-modification by serum 25(OH)D level and energy adjusted dietary calcium intake on FN-BMD, LS-BMD and LS-TBS in model 2 (P for interaction<0.10), since the beta did not changed after additional adjustment in model 3. Thereafter, we stratified the analyses of LD-use (LD never-use, current-use and past-use with never use as reference category) and bone outcomes according to serum 25(OH)D level, in subsequent categories of ≤20, between 20-50 and ≥50 nmol/L) and energy adjusted dietary intake of calcium in following categories: ≤950 mg/day, 950-1200 mg/day and ≥1200 mg/day. The association between LD and LS-TBS, LS-BMD and FN-BMD was also evaluated by sex through effect-modification.

To account for missing data in co-variables (varied from 1.6% to 66.8%), we used a multiple imputation approach (n=10 imputations, 10 iterations). Briefly, as described by Sterne et al, multiple imputation is created on the correlation between each variable and missing values with other participant characteristics [36]. Linear regression analyses were then separately accomplished in each of the 10 datasets [36]. Beta's were pooled by taking the average of the effect sizes of the 10 imputed datasets. The pooled standard errors and respective 95% Confidence Intervals (CI) were then calculated by using Rubin's rules (37). For details of the multiple imputation, see supplement **supplemental tables S1 and S2**. The statistical software package of SPSS 24.0 was used for the statistical analyses (SPSS Inc., Chicago, Illinois, USA). For all the analyses except the effect-modification analysis, p-values of < 0.05 were considered statistically significant.

RESULTS

Population characteristics

Baseline characteristics are presented for the total population, LD-users and non-users (**Table 1**) and baseline characteristics before and after multiple imputation are shown in **supplemental table S2**. The median age, on the DXA scan date, was 65.0 years [57.0-99.0 IQR] for the total population (n=6,990). Of the total population, 7.8% (n=543) ever used LD from baseline till the DXA scan with a mean duration of 51.2 days (325.1 SD). The median age of the 543 LD-users was 77.0 years [52.0-99.0 IQR] and 64.0 years [51.0-97.0 IQR] for the LD non-users (**Table 1**).

Table 1. Characteristics total population, users and non-users of LD

	Total population (n=6,990)	LD ever users (n=543)	LD never users (n=6,447)	p-value LD ever- vs. never users
Age (years)[b]	65.0 [57.0-99.0]	77.0 [52.0-99.0]	64.0 [51.0-97.0]	**<0.001***
Sex (%women)	3,985 (57.0)	309 (56.9)	3,675 (57.0)	0.92
BMI (kg/cm2)[a]	27.6 (4.1)	28.9 (4.7)	27.5 (4.7)	**0.002***
Cohort (%)				**<0.001***
RS-I	2,799 (40.0)	437 (80.5)	2,360 (36.6)	
RS-II	980 (14.0)	62 (11.4)	918 (14.2)	
RS-III	3211 (46.0)	44 (8.1)	3,167 (49.1)	

Table 1. Characteristics total population, users and non-users of LD (*continued*)

	Total population (n=6,990)	LD ever users (n=543)	LD never users (n=6,447)	p-value LD ever- vs. never users
Loop diuretics use (%ever use)	543 (7.8)	543 (100)	0	NA
Duration (days)[a]	NA	194 [40-893]	0	
25(OH)D (nmol/l)[b]	54.4 [36.5-76.0]	42.3 [28.8-62.3]	55.4 [37.2-76.7]	**<0.001***
Cutoff (%):				
<50 nmol/L	2,580 (44.2)	247 (45.5)	2,333 (36.2)	**<0.001***
>50 nmol/L	3,257 (55.8)	156 (28.7)	3,101 (48.1)	
Season measurement (%):				0.41
Spring	1,928 (27.6)	147 (27.0)	1,854 (28.8)	
Summer	1,143 (16.4)	82 (15.1)	1,107 (17.2)	
Autumn	2,219 (31.7)	177 (32.5)	2,126 (33.0)	
Winter	1,432 (20.5)	138 (25.4)	1,360 (21.0)	
Dietary calcium intake (mg/day)[a]	1126.5 (388.9)	1189.2 (394.1)	1120.8 (394.1)	0.81
Tertiles (%):				
<950.0 mg/day	1,691 (24.2)	103 (19.0)	1,588 (24.6)	
950-1200 mg/day	1,504 (21.5)	120 (22.1)	1,384 (21.5)	
>1200 mg/day	1,795 (25.7)	186 (34.3)	2,609 (25.0)	
Lumbar Spine TBS[a]	1.24 (0.13)	1.17 (0.12)	1.25 (0.12)	0.32
Femoral Neck Bone Mass Density (g/cm2)[a]	0.91 (0.15)	0.86 (0.15)	0.91 (0.15)	0.98
Lumbar Spine Bone Mass Density (g/cm2)[a]	1.14 (0.21)	1.15 (0.22)	1.14 (0.20)	0.40
Smoking (%)				**0.003***
Never smoker	2,080 (29.8)	157 (28.9)	1,921 (29.8)	
Current smoker	1,358 (19.4)	58 (10.6)	1,302 (20.2)	
Former, non-smoker	3,506 (50.2)	319 (58.8)	3,185 (49.4)	
Pack years[a]	9.4 (18.6)	20.3 (28.0)	8.5 (28.0)	**<0.001***
Education category (%)				**<0.001***
Low education	3,575 (51.6)	319 (58.7)	3,256 (50.9)	
Higher education	3.353 (48.4)	219 (40.3)	3,134 (49.1)	
Alcohol Intake (g/day)[b]	7.3 [0.8-20.0]	3.1 [0.1-15.7]	7.9 [0.9-20.0]	**0.11**
PA (MET hours/week)[b]	70.5 [39.4-103.9]	70.8 [47.6-100.1]	70.4 [38.5-104.4]	**0.84**
Energy intake (Kcal/day)[a]	2176.6 (710.2)	1956.9 (533.8)	2195.2 (533.8)	**<0.001***
Comorbidities (%yes)	1,112 (15.9)	150 (27.6)	963 (14.9)	**<0.001***
CHD	315 (4.5)	69 (12.7)	246 (3.8)	
Stroke	129 (1.8)	13 (2.4)	116 (1.8)	
DM	790 (11.3)	98 (18.0)	691 (10.7)	

[a]mean (SD); [b]median (IQR); %=percentage; * p-value<0.05.

Table 2. Linear regression of the use of LD and FN-BMD and LS-TBS in RS-I-4, RS-II-2 & RS-III-1

	Model 1 β (95%CI)	Model 2 β (95%CI)	Model 3 β (95%CI)
LS-TBS[a] (6,476)			
LD never-use (5,972)	*Reference*	*Reference*	*Reference*
LD past-use (n=310)	**-0.031 [-0.044; -0.017]***	**-0.019 [-0.033; -0.006]***	**-0.025 [-0.038; -0.012]***
LD current-use (n=194)	**-0.018 [-0.034; -0.001]***	-0.011 [-0.028; 0.006]	-0.015 [-0.032; 0.002]
Duration of LD-use categories[b]			
Never users (n=5,972)	*Reference*	*Reference*	*Reference*
Current users 1-120 days (n=37)	-0.008 [-0.027; 0.011]	-0.0005 [-0.043; 0.042]	-0.003 [-0.044; 0.039]
Current users 121-365 days (n=27)	-0.026 [-0.048; 0.003]	-0.026 [-0.070; 0.018]	-0.041 [-0.084; 0.002]
Current users >365 days (n=310)	-0.019 [-0.039; 0.001]	-0.010 [-0.031; 0.010]	-0.013 [-0.032; 0.007]
LS-BMD (n=6,677)			
LD never-use (6,146)	*Reference*	*Reference*	*Reference*
LD past-use (n=321)	**0.048 [0.026; 0.071]***	**0.029 [0.006; 0.052]***	**0.038 [0.016; 0.060]***
LD current-use (n=210)	**0.037 [0.010; 0.065]***	0.021 [-0.008; 0.050]	0.025 [-0.003; 0.052]
Duration of LD-use categories[b]			
Never users (n=6,146)	*Reference*	*Reference*	*Reference*
Current users 1-120 days (n=41)	0.020 [-0.040; 0.081]	0.012 [-0.059; 0.083]	0.014 [-0.054; 0.082]
Current users 121-365 days (n=32)	**0.072 [0.004; 0.140]***	0.068 [-0.003; 0.139]	**0.075 [0.007; 0.143]***
Current users >365 days (n=137)	**0.034 [0.0004; 0.068]***	0.012 [-0.022; 0.047]	0.016 [-0.017; 0.049]
FN-BMD (n=6,908)			
LD never-use (6,376)	*Reference*	*Reference*	*Reference*
LD past-use (n=319)	**0.016 [0.001; 0.031]***	0.006 [-0.009; 0.021]	0.010 [-0.005; 0.025]
LD current-use (n=213)	0.006 [-0.012; 0.024]	-0.006 [-0.025; 0.013]	-0.006 [-0.024; 0.013]

Table 2. Linear regression of the use of LD and FN-BMD and LS-TBS in RS-I-4, RS-II-2 & RS-III-1 (*continued*)

	Model 1 β (95%CI)	Model 2 β (95%CI)	Model 3 β (95%CI)
Duration of LD-use categories[b]			
Never users (n=6,376)	*Reference*	*Reference*	*Reference*
Current users 1-120 days (n=44)	0.020 [-0.019; 0.058]	0.024 [-0.021; 0.069]	0.021 [-0.025; 0.067]
Current users 121-365 days (n=32)	0.035 [-0.011; 0.080]	0.019 [-0.028; 0.066]	0.023 [-0.023; 0.069]
Current users >365 days (n=137)	-0.005 [-0.027; 0.018]	-0.019 [-0.042; 0.003]	-0.018 [-0.040; 0.004]

Model 1: sex, age, cohort. Model 2: additional adjusted for BMI, Alcohol, smoking, SES, PA and comorbidities (for duration of LD-use additional adjusted for past users). Model 3: + LS-TBS (for the analysis of FN-BMD)/FN-BMD and LS-BMD (for the analysis of LS-TBS); *p<0.05; [a]standardized according to sex by residual method; [b]Past users depicted in the rows above.

The median serum 25(OH)D level of the LD-users was 42.3 nmol/L [28.8-62.3 IQR] and for LD non-users, 55.4 nmol/l [37.2-76.7 IQR]. LD-users showed a significantly lower serum 25(OH)D level than non-users (p<0.001). The mean dietary calcium intake of the LD-users was 1189 mg/day (394 SD) and 1121 mg/day (394 SD) for LD non-users. Results of the linear regression analysis of LD-use and indices of bone health are shown in **table 2** and discussed below.

Loop diuretics and LS-TBS

Compared to LD never-use, current-use of LD was only associated with LS-TBS in the crude model (ß=-0.018, 95% CI: -0.034; -0.001) and past-use of LD was associated with lower LS-TBS, in model 1 (ß=-0.031, 95% CI: -0.044; -0.017). Adjustment for co-variates in model 2 attenuated the association somewhat whereby the effect size was 30% lower. The analysis of categories of the duration of LD-use among users and LS-TBS, showed no significant associations (**Table 2**).

Loop diuretics and LS-BMD

Current-use of LD in 210 participants was associated with significantly higher LS-BMD compared to never-use of LD in the crude model (ß=0.037, 95% CI: 0.010; 0.065). Past-use of LD was associated with higher LS-BMD compared to never-use of LD in the crude model, model 2 and fully adjusted model (model3) (ß=0.048, 95% CI: 0.026; 0.071, ß=0.029, 95% CI: 0.006; 0.052 and ß=0.038, 95% CI: 0.016; 0.060). In the analyses of the duration of LD-use, current-use of LD between 121-365 days showed a significantly higher LS-BMD in fully adjusted model (ß=0.075, 95% CI: 0.007; 0.143 **table 2**).

Loop diuretics and FN-BMD

LD current-users showed no significant association with FN-BMD compared to never-use of LD. However, past-use of LD showed a significantly higher FN-BMD only in the crude model (ß=0.016, 95% CI: 0.001; 0.031, **table 2**).

Additional adjustment for serum vitamin D, season of blood collection of vitamin D, serum calcium, magnesium and sodium did not change the results (data not shown).

Furthermore, we evaluated the association between LD and LS-TBS, LS-BMD and FN-BMD by sex and found no evidence that the association between LD and LS-TBS was significantly different according to sex (P-interaction=0.83). However, the association

Table 3. Linear regression of LD-use (yes/no) on FN-BMD and LS-TBS for 25(OH)D level in categories (≤20, 20-50 and ≥50 nmol/l) in model 2

	25(OH)D ≤20 nmol/l (n=302)	25(OH)D 20-50 nmol/l (n=2,240)	25(OH)D ≥50 nmol/l (n=3,225)	p-value interaction term
	Β (95%CI)	Β (95%CI)	Β (95%CI)	
LS-TBS[b] (6,476)				
LD never-use	*Reference*	*Reference*	*Reference*	**0.04***
LD past-use (n=310)	-0.012 [-0.036; 0.013]	-0.031 [-0.096; 0.034]	**-0.036 [-0.060; -0.013]***	
LD current-use (n=194)	0.001 [-0.036; 0.039]	-0.029 [-0.099; 0.041]	-0.014 [-0.046; 0.017]	
	25(OH)D ≤20 nmol/l (n=287)	**25(OH)D 20-50 nmol/l (n=2,146)**	**25(OH)D ≥50 nmol/l (n=3,108)**	**p-value interaction term**
	Β (95%CI)	**Β (95%CI)**	**Β (95%CI)**	
LS-BMD (n=6,677)				
LD never-use	*Reference*	*Reference*	*Reference*	0.30
LD past-use (n=321)	0.079 [-0.054; 0.148]	0.003 [-0.034; 0.041]	0.038 [-0.003; 0.080]	
LD current-use (n=210)	-0.052 [-0.157; 0.053]	**0.044 [0.002; 0.087]***	0.039 [-0.016; 0.095]	
	25(OH)D ≤20 nmol/l (n=267)	**25(OH)D 20-50 nmol/l (n=2,054)**	**25(OH)D ≥50 nmol/l (n=3,056)**	**p-value interaction term**
	Β (95%CI)	**Β (95%CI)**	**Β (95%CI)**	
FN-BMD (n=6,908)				
LD never-use	*Reference*	*Reference*	*Reference*	0.13
LD past-use (n=319)	0.027 [-0.037; 0.092]	-0.006 [-0.031; 0.019]	0.008 [-0.020; 0.036]	
LD current-use (n=213)	-0.022 [-0.089; 0.045]	-0.004 [-0.031; 0.023]	0.015 [-0.021; 0.051]	

*p<0.05; **p<0.10; [b]standardized according to sex by residual method

Table 4. Linear regression of LD-use (yes/no) on FN-BMD and LS-TBS for dietary calcium intake categories (≤950, 950-1200 and ≥1200 mg/day) in model 2

	Dietary calcium intake ≤950 mg/day (n=1,822)	Dietary calcium intake 950-1200 mg/day (n=1,600)	Dietary calcium intake ≥1200 mg/day (n=1,875)	p-value interaction term
	ß (95%CI)	**ß (95%CI)**	**ß (95%CI)**	
LS-TBS[b] (6,476)				
LD never-use	*Reference*	*Reference*	*Reference*	0.58
LD past-use (n=310) (yes/no)	-0.018 [-0.047; 0.010]	-0.022 [-0.050; 0.005]	-0.015 [-0.037; 0.007]	
LD current-use (n=194) (yes/no)	-0.009 [-0.044; 0.025]	0.007 [-0.028; 0.043]	-0.015 [-0.043; 0.013]	
	Dietary calcium intake ≤950 mg/day (n=1,732)	**Dietary calcium intake 950-1200 mg/day (n=1,548)**	**Dietary calcium intake ≥1200 mg/day (n=1,838)**	**p-value interaction term**
	ß (95%CI)	**ß (95%CI)**	**ß (95%CI)**	
LS-BMD (n=6,677)				
LD never-use	*Reference*	*Reference*	*Reference*	0.87
LD past-use (n=321) (yes/no)	0.017 [-0.034; 0.068]	0.039 [-0.008; 0.086]	0.026 [-0.012; 0.063]	
LD current-use (n=210) (yes/no)	0.038 [-0.023; 0.100]	0.045 [-0.016; 0.106]	0.016 [-0.031; 0.064]	
	Dietary calcium intake ≤950 mg/day (n=1,695)	**Dietary calcium intake 950-1200 mg/day (n=1,509)**	**Dietary calcium intake ≥1200 mg/day (n=1,771)**	**p-value interaction term**
	ß (95%CI)	**ß (95%CI)**	**ß (95%CI)**	
FN-BMD (n=6,908)				
LD never-use	*Reference*	*Reference*	*Reference*	0.99
LD past-use (n=319) (yes/no)	0.001 [-0.032; 0.034]	0.087 [-0.023; 0.039]	0.008 [-0.017; 0.034]	
LD current-use (n=213) (yes/no)	-0.003 [-0.043; 0.037]	0.024 [-0.015; 0.064]	-0.025 [-0.057; 0.007]	

*p<0.05; **p<0.10; [b]standardized according to sex by residual method

between LD and LS-BMD and FN-BMD was significantly different according to sex (P-interaction for both=0.04) **(Supplemental table S3)**.

Serum 25(OH)D level and dietary calcium intake in the association of the use of loop diuretics and FN-BMD, LS-BMD and LS-TBS

Effect-modification analysis of serum 25(OH)D level and dietary calcium intake in the association between LD-use on FN-BMD and LS-TBS are shown in **tables 3 and 4**. P-value for interaction terms varied from 0.04 to 0.99. Serum 25(OH)D level was an effect modifier in the association between LD use and LS-TBS (P for interaction=0.04). After stratification in model 2, the group of serum 25(OH)D level ≥50 nmol/L showed a significantly lower LS-TBS compared to serum 25(OH)D ≤20 nmol/L and between 20-50 nmol/L for LD past-use (ß=-0.036, 95% CI: -0.060; -0.013 vs. ß=-0.012, 95% CI: -0.036; 0.013 and ß=-0.031, 95% CI: -0.096; 0.034, respectively, **table 3**).

There was no effect modification by serum 25(OH)D level on the association between LD-use and FN-BMD and LS-BMD (**Table 3**). After stratification, for the analysis of the association between LD and LS-BMD, participants with serum 25(OH)D level between 20-50 nmol/L had a significantly higher LS-BMD compared to participants with serum 25(OH)D level ≤20 and ≥50 nmol/l for LD current use, however there was no significant interaction (P for interaction=0.30) (**Table 3**).

No significant effect modification by dietary calcium intake was observed on the association between use of LD and FN-BMD, LS-BMD and LS-TBS (**Table 4**). After stratification of dietary calcium intake in categories of intake ≤950, 950-1200 and ≥1200 mg/day**,** no significant associations were found with FN-BMD, LS-BMD and LS-TBS (**Table 4**).

DISCUSSION

In this study, we observed a modest increase in BMD of the lumbar spine and a modest decrease in TBS in LD past-users, therefore our study conclude that if any, LD use does not have a strong association with bone health, in a population with a high calcium intake. Furthermore, this study found effect-modification by serum 25(OH)D level in the association between LD and LS-TBS. However, after stratification on serum 25(OH)D, no consistent findings were found in the association between LD-use and bone outcomes, suggesting no strong modifying effect of serum 25(OH)D on these associations. Additionally, no effect-modification was found by dietary calcium intake.

In our study, current LD-use showed a negative association with LS-TBS, however not significant.

In contrast to the findings of the association between LD and LS-BMD, we found that a history of LD was associated with a decreased LS-TBS if LD was used in the past. To our knowledge there have been no studies on LD-use and LS-TBS. TBS is a measurement related to bone microarchitecture provides skeletal information that is not captured from the standard BMD measurements (31). Also, TBS might be an appropriate measure to study in regard to lifestyle factors that are adaptable, such as dietary intake (31, 32). Other studies have reported lower TBS among individuals with primary hyperparathyroidism (38). And as mentioned, LD's increase the plasma PTH and 1.25(OH)2D levels as a result of increased renal calcium losses (11). Thus a possible explanation for our finding is that LS-TBS may be decreased by secondary hyperparathyroidism caused by long-time LD-use (13, 39). However, we could not confirm this possible pathway in our current study, since we do not have the availability of serum PTH. Other explanation for the opposite direction between BMD and TBS due to potential residual confounding (i.e. body composition and health status) in the association between LD and TBS. Further studies are needed to explore the association between LD and overall bone health, especially TBS.

Additionally, LD-use was not associated with a lower BMD. In contrast, it has been shown that LD increases renal calcium excretion (8-11), with a potential negative effect on BMD (10, 13, 38). We expected that LD-use would result in a lower FN-BMD and LS-BMD, because treatment with LD may induce secondary hyperparathyroidism with raised bone resorption resulting in a lower BMD (39, 40). This was, however, not confirmed by our findings. Current-use of LD showed a non-significant negative association with FN-BMD and a non-significant positive association with LS-BMD. Furthermore, the study of Rejnmark et. al. in 2005 showed also a non-significantly higher LS-BMD for the group with LD (who had been treated with a LD for at least 2 years prior to inclusion in the study), compared with non-users (38). Another study of Rejnmark et. al. in 2006 showed a decrease in BMD for the LD-users compared to placebo treated for 1 year. The effect was however weakened after 6 months end of treatment. Unfortunately, we cannot confirm this result in our study. In our analysis of the association between the duration of LD and FN-BMD, we found a positive association for LD-use between 1-365 days and a negative association with FN-BMD for longer use of LD (>365 days) compared to never-users of LD, nevertheless this was not significant. Also we found an increase in LS-BMD when LD was used for 121-365 days. This suggests that LD-use might be weakly associated with higher BMD but only for a short time period.

Some studies have shown differences in vitamin D level according to LD-use. In line with our results, there is evidence that LD-users have a lower serum 25(OH)D level than non-users (34) and the inverse association between LD-use and serum 25(OH)D level has been reported before (16). To the best of our knowledge, information about the effect modification by serum 25(OH)D level on the association between LD-use and FN-BMD and LS-TBS as bone health parameters has not been reported earlier. In our study, stratified analyses did not show consistent patterns and the differences were not statistically significantly different between cut-offs of serum 25(OH)D level. However, we found evidence for potential effect modification (P-interaction<0.10) by serum 25(OH)D level for the association of LD past-use and LS-TBS. Stratified analyses showed a slightly stronger inverse association between past LD-use and LS-TBS in those with serum 25(OH)D level ≤20, however the association was not significant in strata of serum 25(OH)D level between 20-50 and above 50 nmol/L. This finding may imply that past-use of LD may have affected LS-TBS which can have a higher impact in those with extremely low 25(OH)D levels. However, because this effect-modification was not further confirmed by other categories of LD-use, further replication is needed.

Moreover, even though we adjusted our analysis for body fat mass, reduced vitamin D levels may still be confounded other measures of body composition and health status.

Our initial hypotheses was that an adequate serum 25(OH)D level and dietary calcium intake would counteract the potential adverse effects of LD on bone health. In contrast, we found no consistent significant differences between categories of dietary calcium intake, in the association between LD and FN-BMD, LS-BMD and LS-TBS. Thus, given that LD increases the renal excretion of calcium (40), the effects of LD on bone health showed unexpected results. This is in contrast with earlier studies, showing that a higher dietary intake of calcium prevented the expected decrease of FN-BMD in LD-users (38, 41, 42). Potentially, the lack of calcium modifying the association between LD and bone health in our study may result from the fact that in our study calcium intake was higher than in other studies.

Strengths and limitations

A strength in our study is the availability of data on dietary intake of calcium, serum 25(OH)D level and LD-use, and the different indices of bone health (i.e. FN-BMD as well as LS-BMD and LS-TBS). Our study also adds data to the quite unexplored field of nutrient-drug interactions in a population of community-dwelling older persons, who are at risk of musculoskeletal diseases, malnutrition and consequences of polypharmacy. However, some limitations need to be taken into account when interpreting our

results. These include the observational study design and potential residual confounding (for example, underlying disease), which prevents us from drawing conclusions regarding causality and the direction of the effect size of the association. Furthermore, as all our analyses were hypothesis-based, we did not adjust for multiple comparisons. With regard to possible type I errors, stringent interpretation of p-values should be made with caution. Likewise, potential misclassification of LD-use using pharmacy records may occur because data regarding actual compliance was lacking. Also, dietary calcium intake was assessed using self-reported dietary assessment, which is subject to bias and measurement error. Also, dietary intake was measured only once (at baseline) and as a result we were unable to account for differences in dietary habits over time (e.g. due to disease or medication use). Moreover, the complete data on calcium or vitamin D supplement use was not available, which could lead to an underestimation of dietary intake. In addition, vitamin D and calcium prescriptions was not analysed as exposure variable because of confounding by indication. Also, we did not have a comprehensive assessment of calcium homeostasis. For example, the free calcium concentration in plasma is strongly controlled through a complicated physiological system including the interaction of calcitrophic hormones such as PTH and 1,25(OH)D and only in extreme situations, will the serum calcium concentration deflect from the normal range (14). Since we did not have the availability of serum PTH, we could not investigate potential pathways between the use of LD and calcium intake and vitamin D level. Finally, our result of inconsistent effect modification by serum25(OH)D and dietary calcium intake on the associations between LD and the parameters of bone health could be explained by managing the parathyroid hormones (PTH) and 1.25 (OH)D that maintain calcium homeostasis, especially when serum calcium is reduced (43).

In conclusion, this study does not support the hypothesis that the association between loop diuretic use and indices of bone health is modified by serum 25(OH)D level and calcium intake. However, because of polypharmacy effects and a higher risk of malnutrition in elderly, further research and replication is warranted on nutrient-drug interaction on bone health (considering the subjects with osteopenia and osteoporosis and people with malnutrition), using other biomarkers as PTH and bone turnover markers as well as long term loop diuretic use.

Supplementary Materials: Table S1: Details of the multiple imputation modelling, Table S2: Basic characteristics before and after multiple imputation.

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Author Contributions: Author's roles: S.O.A., J.C.K.d.J., A.G.U., B.H.S., and N.vd.V. discussed the hypothesis and interpreted the data. K.T., F.K., and F.R. provided the data. S.O.A., and J.K.d.J. analysed the data. A.G.U, B.H.S. and M.A.I. coordinated and directed the project. S.O.A., J.C.d.J., and N.vd.V wrote the article. S.O.A., J.C. K.d.J., K.T., F.K., F.R., M.C.Z., N.M.v.S., L.C.G.M.d.G. M.A.I., A.G.U., B.H.S. and N.vd.V. edited the article.

Informed consent: The study has been approved by the institutional review board (Medical Ethics Committee) of the Erasmus Medical Center and by the review board of the Netherlands Ministry of Health, Welfare and Sports, and approval has been renewed every 5 years. From all subjects written informed consent was obtained.

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SUPPLEMENTARY DATA

Table S1. Details of the multiple imputation modelling

	Multiple imputation procedure
Software used	SPSS 24.0 for Windows
Imputation method	Fully conditional specification (Markov chain Monte Carlo method)
Maximum iterations	10
Imputed datasets created	10
Exposures and outcomes (not imputed, used in model as predictor)	Calcium intake, vitamin D level, LD, LS-TBS, LS-BMD and FN-BMD
Covariates (imputed)	BMI, scan age, smoking, METhours and comorbidities
Additional predictors	Sex, cohort, packyears, alcohol, use of bisphosphonate
Treatment of non-normally distributed variables	Predictive mean matching
Treatment of binary/ categorical variables	Logistic regression models

Number of missing for the variables imputed can be found in supplementary table S2.

Table S2. Basic characteristics before and after multiple imputation

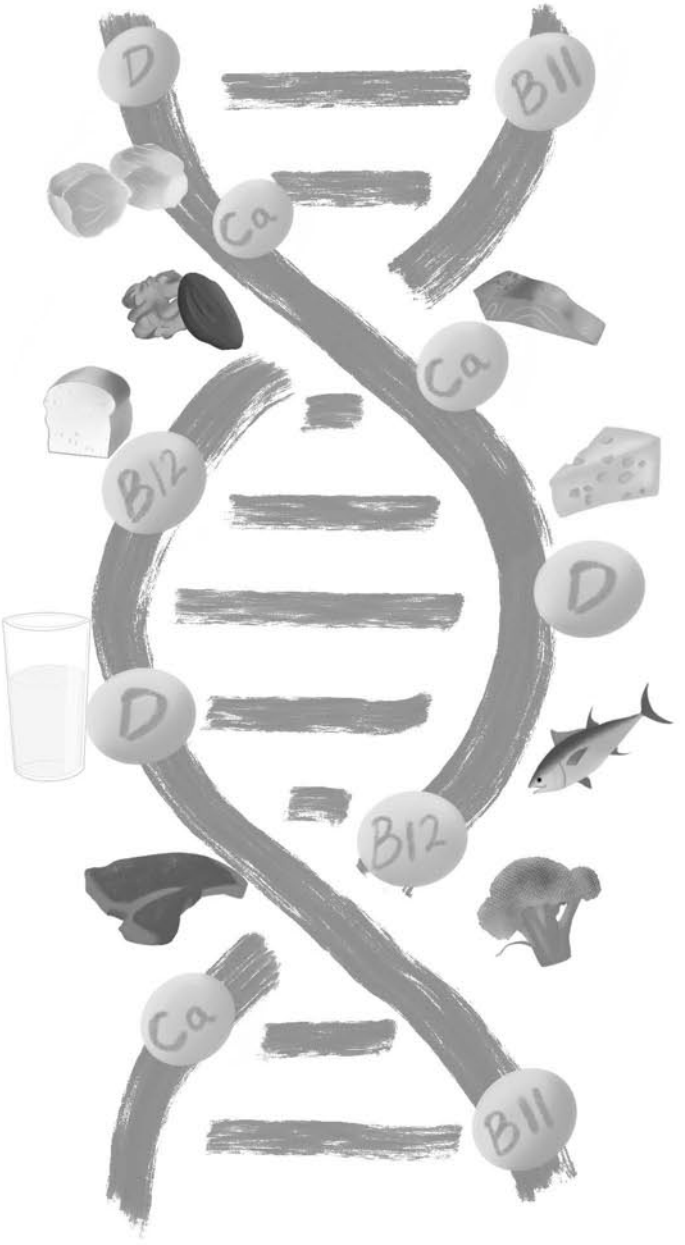
	N	Missing (n)	Original data	Imputed data
Age (years)[b]	6,990	0	65.0 [57.0-99.0]	*No missing*
Sex (%women)	6,990	0	3,985 (57.0)	*No missing*
BMI (kg/cm2)[a]	6,916	74	27.6 (4.1)	27.6 (4.1)
Loop diuretics use (%yes)	6,990	0	543 (7.8)	*No missing*
Duration (days)[a]			51.2 (325.1)	
25(OH)D (nmol/l)[b]	5,837	1,153	54.4 [36.5-76.0]	*Not imputed*
Cutoff (%):				
<50 nmol/L			2,580 (44.2)	
>50 nmol/L			3,257 (55.8)	
Season measurement (%):				
Spring			1,928 (27.6)	
Summer			1,143 (16.4)	
Autumn			2,219 (31.7)	
Winter			1,432 (20.5)	
Dietary calcium intake (mg/day)[a]	4,993	1,997	1126.5 (388.9)	*Not imputed*
Tertiles (%):				
<950.6 mg/day				
951-1234.7 mg/day				
>1235 mg/day				
Lumbar Spine TBS[a]	6,476	514	1.24 (0.13)	*Not imputed*
Femoral Neck Bone Mass Density (g/cm2)[a]	6,677	313	0.91 (0.15)	*Not imputed*
Lumbar Spine Bone Mass Density (g/cm2)[a]	6,908	82	1.14 (0.21)	*Not imputed*
Smoking (%)	6,909	81		
Never smoker			2,069 (29.6)	2,080 (29.8)
Current smoker			1,352 (19.3)	1,358 (19.4)
Former, non-smoker			3,484 (49.8)	3,506 (50.2)
Pack years[b]	6,990	0	9.4 (18.6)	*No missing*
Education category (%)	6,982	8		
Low education			3,570 (51.1)	3,575 (51.6)
Higher education			3351 (47.9)	3.353 (48.4)
Alcohol Intake (g/day)[b]	6,990	0	7.3 [0.8-20.0]	*No missing*
PA (MET hours/week)[b]	5,899	1,091	70.5 [39.4-103.9]	70.5 [39.4-103.9]
Energy intake (Kcal/day)[a]	5,405	1,585	2159.7 (680.4)	2176.6 (710.2)
Comorbidities (%yes)			13.9	1,112 (15.9)
CHD	6,645	345	4.6	315 (4.5)
Stroke	6,660	330	1.2	129 (1.8)
DM	6,467	523	10.7	790 (11.3)

[a]presented as mean (SD); [b]presented as median [interquartile range]

Table S3. The association between LD and LS-BMD and FN-BMD stratified by sex

	Male (n=2,772) ß (95%CI)	Female (n=3,637) ß (95%CI)	p-value interaction term
LS-TBS[b] (6,476)			0.83
LD never-use	Reference	Reference	
LD past-use (n=310)	**-0.030 [-0.050; -0.010]***	-0.011 [-0.029; 0.007]	
LD current-use (n=194)	-0.016 [-0.042; 0.011]	-0.007 [-0.029; 0.015]	
	Male (n=2,844) ß (95%CI)	**Female (n=3,833) ß (95%CI)**	**p-value interaction term**
LS-BMD (n=6,677)			**0.04***
LD never-use	Reference	Reference	
LD past-use (n=321)	-0.003 [-0.038; 0.033]	**0.057 [0.028; 0.086]***	
LD current-use (n=210)	0.027 [-0.020; 0.074]	0.018 [-0.017; 0.053]	
	Male (n=2,985) ß (95%CI)	**Female (n=3,923) ß (95%CI)**	**p-value interaction term**
FN-BMD (n=6,908)			**0.04***
LD never-use	Reference	Reference	
LD past-use (n=319)	-0.0002 [-0.024; 0.023]	0.013 [-0.006; 0.033]	
LD current-use (n=213)	-0.008 [-0.039; 0.023]	-0.005 [-0.028; 0.019]	

***p<0.05**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Chapter 4.3

Interaction between calcium and variations in the calcium concentrations SNP's and the risk of colorectal cancer risk: The Rotterdam study.

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ABSTRACT

Objectives

Previous studies showed that high calcium intake may be associated with reduced colorectal cancer (CRC) risk, but results were inconclusive. We evaluated whether calcium intake from diet and supplements, as well as the calcium levels itself were associated with CRC risk in middle-aged and older individuals. Also we evaluated whether these associations were modified by genetic variation of calcium homeostasis.

Design

This study was embedded in The Rotterdam Study, a prospective cohort study among adults aged 55 years and older without CRC at baseline, from the Ommoord district of Rotterdam, The Netherlands (N=10,941). Effect modification by a pre-defined genetic risk score (GRS) from seven loci known to be associated with calcium concentrations, was evaluated.

Results

Relative to the recommended dietary calcium intake, only higher than the recommended dietary calcium intake (≥1,485 mg/day) was associated with a reduced risk of CRC (HR: 0.66; 95% CI: 0.44 – 1.00). All seven loci for serum calcium concentrations, as well as the GRS, were not associated with CRC but showed effect modification by the GRS in the association between calcium intake and CRC (p for interaction=0.08). After stratification of GRS into low, intermediate and high, we found a lower CRC risk for low weighted GRS per increase in calcium intake.

Conclusion

The results from this study demonstrate that there is no consistent association between calcium indices on CRC. However, the association between calcium intake and CRC may be modified by genetic variation associated with serum calcium concentrations that deserves further replication in other studies with different population.

Keywords: dietary calcium, calcium supplements, colorectal cancer, calcium SNPs, prospective cohort

INTRODUCTION

Colorectal cancer (CRC) is a growing public health concern worldwide, with over 1.8 million new cases in 2018 globally (1). Several lifestyle factors such as physical inactivity, low dietary fiber intake, high red and processed meat intake and high alcohol intake are associated with an increased risk of developing CRC (2).

Previous studies have demonstrated that high dietary calcium intake (from dairy products) may be associated with reduced CRC risk, but the evidence for the association for non-dairy calcium and CRC is still inconclusive (2). These differential results may be explained by differences in bioavailability, for example some of the green vegetables have a low bioavailability of calcium due to the presence of oxalate (3, 4).

Several biological mechanisms may explain a potential protective role of calcium in the development of CRC. For example, experimental studies in animals and humans showed that calcium may protect against CRC development by binding to bile acids and fatty acids in the gastrointestinal tract, and subsequently protecting the colon mucosa from these potentially toxic products (5, 6). Also, calcium may influence differentiation and apoptosis of colonic epithelial cells and might reduce inflammation and oxidative damage in these cells (7).

Moreover, a potential role of the calcium-sensing receptor (CASR) in influencing carcinogenesis of colon epithelium and mediating antineoplastic effect of calcium was suggested (8). In this study, it was found that higher calcium intake was associated with a lower risk of CASR-positive tumours but not with CASR-negative tumours. Besides CASR, seven other Single Nucleotide Polymorphisms (SNPs) associated with serum calcium have been identified (i.e. rs1801725, rs10491003, rs1550532, rs1570669, rs7336933, rs7481584, rs780094); these may be relevant in CRC etiology as well (9). However, inconsistent results on (colorectal) cancer have been reported for calcium-related SNPs so far (e.g. related to CASR or Vitamin D Receptor (VDR) genes) (8, 10, 11). Also, the association of these SNPs and CRC, and the effect modification of these SNPs on the association of calcium indices and CRC have not been investigated yet. Our working hypothesis is that higher intake of calcium could decrease the risk of CRC but that this depends on the genetic variability related to calcium homeostasis. Therefore, the aim of the present study was to determine whether calcium intake from diet and supplements as well as total calcium levels was associated with CRC risk in middle-aged and older individuals. Furthermore, we aimed to assess whether 7 loci for serum calcium concentration are associated with CRC and whether the association

between calcium indices and colorectal cancer risk differs according to pre-specified SNPs involved in calcium homeostasis.

Subjects and methods

The Rotterdam Study

This study was embedded in The Rotterdam Study, an ongoing prospective population-based cohort study originally designed to investigate the occurrence and determinants of common age-related diseases (12). Briefly, The Rotterdam Study is composed of four cohorts. We used the existing data from the first and second cohort of The Rotterdam Study (RS-1 and RS-II). Individuals aged 55 years and older, who were living in the Ommoord district of Rotterdam, the Netherlands were recruited for RS-I, between 1990 and1993 (n=7,983). The study was extended in 2001 with 3,011 participants in RS-II. Baseline data, were obtained by a home interview and two subsequent visits to the research center in Ommoord. Follow-up examinations were repeated approximately every three to four years, with a response rate of 78%, which is in line. Clinical outcomes such as morbidity and mortality were continuously monitored throughout the study period. All participants provided written informed consent, and ethical approval was obtained from the Medical Ethical Committee of the Erasmus Medical Center (12, 13).

Calcium

Dietary calcium intake data were obtained by using semi-quantitative food frequency questionnaires (FFQ) at baseline (between 1989 and 1993 in RS-I-1 and between 2000 and 2001 in RS-II-1), managed by a trained dietician at the study center (12, 14). Portion size of each food item was specified in standardized units, household measures or grams, and the frequency of each item was collected in times per day, week or month. Food items were coded using the corresponding NEVO-code (Dutch Food Composition Table) (15). Dietary intake of nutrients (incl. total energy and calcium) was calculated using the Dutch Food Composition database (NEVO) (15). Dietary calcium intake was adjusted for total energy intake using the residual method to adjust for measurement error and residual confounding (16, 17). In a validation study (*n*=80) of The Rotterdam Study nutrient intake assessed with the FFQ was validated against multiple food records (18). The validation study showed a good correlation for calcium intake (Pearson's correlation after adjustment for age, sex, energy intake, and within-person variation: 0.72) (18).

In addition, drug *use of participants of The Rotterdam Study was continuously monitored since January 1, 1991, through computerized records from the pharmacies in the Ommoord district. The pharmacy data included the Anatomical Therapeutical Chemical (ATC)-code, the dispensing date, the total number of drug units per prescription, the prescribed daily number of units, and product name of the drugs. On the basis of this information, the number of calcium dispensings was extracted (A12AA and A12AX) (19).*

Serum calcium levels were determined at baseline using a cresolphthalein complexone method (Merck Diagnostica, Amsterdam, the Netherlands) with a Kone auto-analyser (Kone Diagnostics, Espoo, Finland) (12).

Single Nucleotide Polymorphisms (SNPs) selection

The Rotterdam Study RS-I and II consist of 8.448 DNA samples at baseline, and from all Rotterdam Study samples the genotypes of SNPs are being estimated using the basis Illumina 500 K SNP dataset configurations in each subject (12). The selection of SNPs in this study, was pre-specified using the seven loci (six new regions) known to be associated with serum calcium based on literature and based on our hypothesis only for the included participants of RS-I and RS-II (9). The selected SNPs were rs1801725, rs10491003, rs1550532, rs1570669, rs7336933, rs7481584, rs780094.

Colorectal Cancer (CRC)

Diagnosis of incident cancer was based on medical records of general practitioners (including hospital discharge letters) and furthermore through linkage with Dutch Hospital Data (Landelijke Basisregistratie Ziekenhuiszorg), histology and cytopathology registries in the region (PALGA), and the Netherlands Cancer Registry. Cancer diagnoses were coded independently by two physicians and classified according to the International Classification of Diseases, 10th revision (ICD-10) (20). In case of discrepancy, consensus was sought through consultation with a physician specialised in internal medicine. In these analyses, only pathology proven CRC were used from baseline of the cohort until the end of follow up on December 31, 2014. Date of diagnosis was based on date of biopsy or—if unavailable—date of hospital admission or hospital discharge letter. Codes C18-C20 about malignant neoplasms of the colon, recto-sigmoid junction and rectum, were used to classify CRC diagnosis.

Covariates

The following characteristics of the study population and other information were assessed during the home interviews and the visit to the research center at baseline of the cohort between 1990 and 2001: gender, age, education level, income, smoking status, other dietary variables (including intake of alcohol, dietary fiber and processed red meat), height, weight, waist circumference, history of diabetes mellitus type II, serum total cholesterol levels and serum total calcium levels. Level of education and net monthly household income were used as indicators of socioeconomic status. Highest attained educational level was classified according to the International Standard Classification of Education using the following categories: primary education, lower/intermediate general and lower vocational education, higher general and intermediate vocational education, and higher vocational education and university (21). For the present study, education level was categorized into two categories: low education (primary education solely) and intermediate to high education (secondary education and higher). Income of the participants was expressed in net monthly household income. For the present study, income was categorized into low to intermediate income (<2,400 gulden/per month) and intermediate to high income (≥2,400 gulden/per month) based on net modal household income of the study population. Smoking status was categorized into two categories: never or ever smokers and current smokers.

Intake of energy (kcal/day), alcohol (g/day), dietary fiber (g/day) and processed red meat (g/day) were assessed with the FFQ as described previously. All dietary nutrient intake were adjusted for total energy intake using the residual method (17).

Height and weight were measured at the research center, and Body Mass Index (BMI) was calculated by weight in kilograms divided by the square of height in meters (kg/m^2) (22). Waist circumference was measured midway between the lowest rib and the iliac crest using a measuring tape (12). Diabetes mellitus type II was defined as fasting plasma glucose concentrations of ≥7 mmol/l or the use of glucose lowering drugs or insulin using the World Health Organization (WHO) and American Diabetes Federation (ADA) guideline (23, 24). Serum total cholesterol levels were determined with blood samples using an automated enzymatic procedure (25).

Physical activity and vitamin D level were obtained during the visit to the research center at the third follow-up visit of the cohort between 1997 and 2001. Physical activity was determined by means of an adapted version of the Zutphen Physical Activity Questionnaire (ZPAQ) (26). The ZPAQ was previously validated with a test-retest reliability of 0.93. The correlation of ZPAQ with doubly labeled water which

is the golden standard measurement of physical activity was 0.60 (27). The adapted questionnaire consisted of questions about walking, cycling, gardening, diverse sports, hobbies and housekeeping. The Metabolic Equivalent of Task (MET) was used to express the intensity of physical activity of each activity. MET-values were based on the metabolic rate for that specific activity compared to the resting metabolic rate using the 2011 Compendium of Physical Activities (28). Vitamin D status was assessed with plasma concentrations of 25-hydroxyvitamin D (25OHD) (nmol/l) from non-fasting blood samples using electrochemiluminescence immunoassay (COBAS, Roche Diagnostics GmbH, Germany (12).

Statistical Analyses

Continuous variables with a normal distribution were expressed as mean with its standard deviation (SD), and continuous variables with a skewed distribution were expressed as median with its interquartile range (IQR). Categorical variables were presented in frequencies and relative percentages.

Cox regression analyses were performed to determine the associations between calcium diet, supplements, calcium level andCRC risk separately. Follow-up time (in years) was used as underlying timescale in the analyses. The proportional hazard assumption was explored by performing an interaction test of exposure with time in the Cox proportional hazard models. The proportional hazard assumption is assumed to hold when the *P*-value of the interaction between exposure and time is >0.05 (29). The association between prescribed calcium supplement intake and CRC was analyzed using Cox regression analysis with prescribed calcium supplement intake as a time-dependent covariate. In these analyses the prescriptions of calcium supplement was compared with non-prescriptions of calcium supplements at the same time point. Time since first calcium dispensing was used as underlying timescale (30).

Dietary calcium intake was first analyzed continuously (per 200 mg). Subsequently, dietary calcium intake was analyzed as a categorical variable after stratification into four categories on the basis of the Recommended Dietary Allowance (RDA) for dietary calcium intake in the Netherlands (1,100 mg/day) (31) ± standard deviation (SD) of the study population. The category that included the RDA was used as reference group for further analyses. To assess linear trends between dietary calcium intake and CRC risk, tests for trend were calculated using the categorical variable as a continuous variable in the Cox proportional hazard models. In addition, prescribed calcium supplement intake were analyzed continuously (per each prescription of calcium supplements) and dichotomously (yes/no) for the analyses. The category that included

no prescribed supplement intake was used as reference group for the dichotomous analyses. Because calcium homeostasis is affected by albumin concentrations, serum total calcium level was adjusted for serum albumin level in a subgroup (available only in RS-I) with the use of the following formula: 0.8 (4.0 – serum albumin level) + serum calcium level (32). Serum total calcium level was analyzed continuously (per each mmol/L) and in quartiles (first quartile as reference).

Potential confounders were added to the sex- and age-adjusted model (Model 2). We also included another model in which we additionally adjusted for BMI and waist circumference (Model 3).

The weighted Genetic Risk Score (GRS) was calculated from seven SNPs for calcium concentration (80% of variance is explained by these SNPs) by multiplying with the effect estimate of each SNP from GWAS on calcium concentration (9). After that, we summed all the scores of all seven SNPs (33). The association between seven SNPs separately as well as the GRS was analyzed by a Cox proportional hazard model. Furthermore, we tested the effect modification by weighted GRS on the association between calcium intake and CRC (*P*-value for statistically significantly interaction <0.10). Additionally, the associations of calcium level and calcium intake were tested (in Model 3) for effect modification by serum 25(OH)D level. The associations were stratified by 25(OH)D level (< 50 and ≥ 50 nmol/l) if the interaction term was below 0.10.

To reduce bias associated with missing data, the multiple imputation procedure according to the fully conditional specification method was used (*n*=10 imputations) (**Supplemental tables S1 and S2**) (34). The percentage of missing data of variables varied from 5.1% for smoking status to 42.8 % for history of diabetes type II (**Supplemental table S2**).

Results are presented as hazard ratios (HRs) and 95% confidence intervals (Cis). The pooled results from the multiple imputation procedure are given for all analyses. Statistical significance was set at $P < 0.05$. All analyses were performed with IBM SPSS Statistics version 25 for Windows.

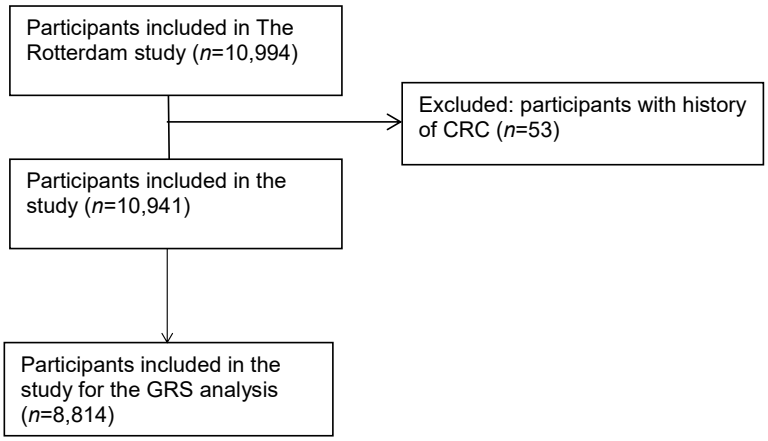

Figure 1. Flowchart of the included study participants

RESULTS

Population characteristics

Baseline characteristics of the study population are presented in **table 1**. Of the 10,941 subjects included in the study (**figure 1**), 427 subjects (3.9 %) were diagnosed with CRC. The incidence rate was 2.9 per 1,000 person-years. The median age of the study population was 67.4 [IQR: 61.0-76.0 years]. The mean intake of dietary calcium intake was 1,116.7 (±390.0) mg/day (unadjusted for energy). Dispensed calcium supplement intake was reported by 17.3% of the study population. The mean of serum total calcium level was 2.4 (±0.1) mmol/L. As shown in **table 1**, the median (IQR) serum 25(OH)D level of the study population was 45.8 [IQR: 29.2-67.7].

Dietary calcium intake and CRC risk

Associations between dietary calcium intake and CRC risk are shown in **table 2**. Dietary calcium was only significantly associated with higher CRC risk in the crude model, when analyzed continuously (HR: 0.93; 95% CI: 0.87-0.99). When analyzed the dietary calcium intake in categories with using the RDA as reference, lower risk was found for high dietary calcium intake (≥1,485 mg/day), compared to the RDA (≥1,100-1,485 mg/day; HR: 0.66; 95% CI: 0.44-1.00 in fully adjusted model).

Table 1. Baseline characteristics of the study population (*n*=10,941)

Characteristics	
CRC cases, n (%)	427 (3.9)
Follow-up, years	13.6 (7.5)
Age, years[c]	67.4 [61.0-76.0]
Women, n (%)	6,543 (59.8)
Education level, n (%)	
Primary solely	6,094 (55.7)
Secondary and higher	4,847 (44.3)
Income, n (%)	
Low to intermediate (<2,400)	5,049 (46.1)
Intermediate to high (≥2,400)	5,892 (53.9)
Total energy intake, kcal/d	1,954.5 (552.4)
Total dietary calcium intake[b], mg/d	1,116.7 (393.0)
Total dietary fiber intake[b], g/d	26.3 (79.4)
Total processed red meat[b], g/d	101.1 (79.4)
Total alcohol intake g/d	9.7 (14.9)
Smoking status, n (%)	
Never/ever	8,679 (79.3)
Current	2,262 (20.7)
History of diabetes mellitus type II, n (%)	1,326 (12.1)
Physical activity[a], MET hours per week	80.8 (44.4)
Body mass index, kg/m2	26.5 (3.9)
Waist circumference, cm	91.4 (11.5)
25(OH)D status[a], nmol/l, median[c]	45.8 [29.2-67.7]
Serum total cholesterol levels, mmol/l	6.4 (1.2)
Serum total calcium level, mmol/l	2.4 (0.1)
Prescribed calcium supplement intake, n (%)	
Once or more	17.3
No	82.7

Values presented as means (SD), unless noted otherwise
[a] Measured during the third follow-up, not at baseline
[b] Adjusted for energy intake
[c] Median [IQR]

Prescribed calcium supplementation and CRC risk

Associations between dispensed calcium supplement intake and CRC risk are shown in **table 3**. No associations were found between dispensed calcium supplement and CRC risk when analyzed continuously or dichotomously (**Table 3**).

Table 2. The association between dietary calcium intake and CRC risk

	CRC cases	Model 1[a] HR (95% CI)	Model 2[b] HR (95% CI)	Model 3[c] HR (95% CI)
Dietary calcium intake - Continuous	322	**0.93 (0.87-0.99)***	0.95 (0.89-1.02)	0.95 (0.89-1.02)
Dietary calcium intake (mg/day) in categories				
Category 1 (≤ 715)	59	**1.47 (1.06-2.04)***	1.32 (0.93-1.89)	1.33 (0.93-1.89)
Category 2 (715-1,100)	120	0.85 (0.65-1.10)	0.83 (0.63-1.09)	0.82 (0.62-1.09)
Category 3 (1,100-1,485)	111	Reference	Reference	Reference
Category 4 (≥ 1,485)	31	**0.65 (0.44-0.97)***	**0.66 (0.44-0.99)***	**0.66 (0.44-1.00)***
***P*-trend[d]**		**0.01***	0.06	0.07

Continuous: per each 200 mg
Categories: on the basis of the Recommended Dietary Allowance and standard deviation
[a]Model 1 was adjusted for cohort, age (years) and sex
[b]Model 2 was adjusted for age (years), sex, education (primary solely, secondary or higher), income (low to intermediate, intermediate to high), history of diabetes type II (no/yes), smoking status (never/ever, current), alcohol intake (g/day), dietary fiber intake (g/day), red meat intake (g/day), serum total cholesterol levels (mmol/l) and physical activity (hours/day)
[c]Model 3 was additionally adjusted for BMI (kg/m^2) and waist circumference (cm)
[d]Test for trend were carried out by entering the categorical variables as continuous variables in Model 3 of the Cox's proportional hazard models
*p-value of < 0.05

Table 3. The association between prescribed calcium supplement and CRC risk

	CRC cases	Model 1[a] HR (95% CI)	Model 2[b] HR (95% CI)	Model 3[c] HR (95% CI)
Prescribed calcium supplement	453			
- Continuous		0.99 (0.98-1.01)	0.99 (0.96-1.01)	0.99 (0.97-1.01)
- Dichotomous (y/n)		0.91 (0.70-1.17)	0.91 (0.58-1.43)	0.94 (0.59-1.49)

Continuous (prescribed calcium intake): per each prescription of calcium supplements
Dichotomous: yes/no
[a]Model 1 was adjusted for cohort, age (years) and sex
[b]Model 2 was adjusted for age (years), sex, education (primary solely, secondary or higher), income (low to intermediate, intermediate to high), history of diabetes type II (no/yes), smoking status (never/ever, current), alcohol intake (g/day), dietary fiber intake (g/day), red meat intake (g/day), serum total cholesterol levels (mmol/l) and physical activity (hours/day)
[c]Model 3 was additionally adjusted for BMI (kg/m^2) and waist circumference (cm)
*p-value of < 0.05

Calcium concentration and CRC risk

The association between total serum calcium level and CRC in RS-I and RS-II is depicted in **table 4**. Total serum calcium level were not associated with CRC risk (**Table 4**), and no linear trend was found (P-trend value >0.19). However, in the sensitivity analysis in RS-I population, where serum total calcium level was adjusted for serum albumin level in a subgroup (RS-I), we found a statistically significantly reduced CRC risk for total serum calcium level and a significantly increased CRC risk for higher albumin-adjusted calcium level **(Supplemental table S3)**.

Table 4. The association between serum total calcium level and CRC risk

	CRC cases	Model 1[a] HR (95% CI)	Model 2[b] HR (95% CI)	Model 3[c] HR (95% CI)
Serum total calcium level - Continuous	257	0.49 (0.19-1.25)	0.47 (0.14-1.51)	0.48 (0.15-1.57)
Serum total calcium level - Categorical				
Quartile 1 (≤ 2.31)	63	Reference	Reference	Reference
Quartile 2 (2.31-2.39)	56	0.82 (0.59-1.15)	0.81 (0.57-1.15)	0.82 (0.58-1.16)
Quartile 3 (2.39-2.46)	40	0.85 (0.60-1.21)	0.80 (0.55-1.16)	0.81 (0.55-1.14)
Quartile 4 (>2.46)	48	0.84 (0.60-1.18)	0.77 (0.53-1.13)	0.78 (0.54-1.14)
P-trend[d]	50	0.35	0.19	0.21

Continuous: per each mmol/l
Categorical: quartiles (mmol/l)
[a]Model 1 was adjusted for cohort, age (years) and sex
[b]Model 2 was adjusted for age (years), sex, education (primary solely, secondary or higher), income (low to intermediate, intermediate to high), history of diabetes type II (no/yes), smoking status (never/ever, current), alcohol intake (g/day), dietary fiber intake (g/day), red meat intake (g/day), serum total cholesterol levels (mmol/l) and physical activity (hours/day)
[c]Model 3 was additionally adjusted for BMI (kg/m^2) and waist circumference (cm)
[d]Test for trend were carried out by entering the categorical variables as continuous variables in Model 3 of the Cox's proportional hazard models
*p-value of < 0.05

Effect modification by calcium concentrations SNP's

The association between 7 SNPs separately as well as GRS is shown in **table 5**: we found no statistically significantly associations.

After evaluating the effect modification by weighted GRS from the calcium concentrations SNPs, we found effect modification with dietary calcium intake by the GRS on CRC risk (p=0.08). No statistically significant effect modification by SNP with serum

Table 5. The association between GRS, 7 SNPs for calcium concentrations and CRC risk (Cox analysis in Model 3)

SNPs of calcium concentration	HR (95% CI)
rs1801725	1.06 (0.78; 1.32)
rs1550532	1.06 (0.87; 1.30)
rs780094	0.96 (0.80; 1.16)
rs10491003	0.93 (0.68; 1.28)
rs7336933	1.14 (0.90; 1.45)
rs1570669	1.13 (0.93; 1.38)
rs7481584	0.99 (0.81; 1.20)
GRS	0.42 (0.02; 7.68)

calcium level and calcium supplementation for CRC risk was found (*P* for interaction 0.56 and 0.98, respectively). After stratification of GRS in low, intermediate and high weighted GRS in the association between dietary calcium intake and CRC risk, we found a significant lower CRC risk for the participants with lower GRS (HR= 0.78 per increase in calcium intake; 95%CI: 0.67-0.92, **table 6**).

Table 6. The association between dietary calcium intake and CRC risk, stratified by GRS score (low, intermediate and high GRS) (n=8,814)

Dietary calcium intake	CRC cases	HR (95% CI)
Low GRS	108	**0.78 (0.67-0.92)***
Intermediate GRS	124	0.94 (0.82-1.08)
High GRS	109	1.05 (0.94-1.18)

Sensitivity analyses on effect-modification by 25(OH)D status

Serum 25(OH)D level was a significant effect-modifier in the association between calcium intake and CRC (p=0.001) and in the association between calcium level and CRC (p=0.04). After stratification for serum 25(OH)D level, dietary calcium intake was associated with lower risk of CRC in subgroup of 25(OH)D level <50 nmol/l, and calcium level was associated with lower risk of CRC in subgroup of 25(OH)D level ≥ 50 nmol/l. (**Supplemental table S4**).

The association of calcium concentration and calcium supplementation with CRC risk was not modified by serum 25(OH)D level (*P* for interaction 0.13 and 0.72, respectively). Finally, a list of results from this study and comparison with literature has been added on **supplemental table S5**.

DISCUSSION

Main findings

In this prospective population-based cohort study, we did not find a consistent association between calcium intake from diet or supplements or total serum calcium level and CRC risk. However, our findings suggest that the association between dietary calcium intake and CRC risk may be modified by the weighted GRS for SNPs for calcium concentrations, with calcium intake associated with a lower CRC risk for those with a low GRS.

Comparison with literature

Our results regarding dietary calcium intake and CRC risk are not fully in line with previous studies. Some prospective cohort studies found inverse associations of dietary calcium intake on CRC risk (8, 35). Moreover, results of combined prospective cohort studies showed a linear association; each 300 mg/day intake of total calcium was inversely associated with approximately 8% reduced CRC risk (36). In our study, we found an inverse association between dietary calcium intake and risk of CRC (Table 2). The discrepancy between our results and those from previous studies may be explained by differences in average dietary calcium intake. The average intake of total calcium intake was below 800 mg/day for the previously studies (8, 16, 36), whereas our study population had a relatively high dietary calcium intake (1,116.7) mg/day).

In contrast to previous findings of studies of the association between dietary intake of calcium and CRC, a meta-analysis of randomized trials found no association between calcium supplement intake and CRC risk over a period of four years (3). It may be argued that the duration of the included trials was too short and probably lacked power to detect effects on CRC risk. As we know, calcium from diet mainly contains calcium phosphate, whereas calcium from supplements generally contains other compounds such as calcium citrate malate. Calcium from supplements has a higher bioavailability than calcium from diet (10).The duration in our study was longer, however, the percentage of calcium prescriptions was around 17%, which is also low powered to discover any association. Moreover, we had no reliable data of the dosage, frequencies of the prescribed supplementation and over-the-counter calcium supplementation.

In our study, serum total calcium level was also not associated with CRC risk. Most studies on serum calcium levels and CRC have been conducted in selected patient group, where hypercalcemia is a well-known characteristic of various malignancies

(19). One previous study showed that serum total calcium levels were associated with a slightly higher risk of CRC risk in women (37). Another study showed that lower serum calcium levels may be a prognostic factor for CRC development (38). Most of the previous studies were conducted using serum calcium levels uncorrected for albumin. Sensitivity analysis in this study showed interestingly a higher risk for CRC for albumin-adjusted calcium levels, which is an important result and were also found in another study (37). This finding suggests that the true association between calcium and colorectal cancer may depend on other factors regulating calcium homeostasis. Differences may also be explained by suggested potential role of SNPs of calcium concentrations in influencing carcinogenesis of colon epithelium and mediating antineoplastic effect of calcium (8). CASR could be associated with CRC survival (39); however, others showed no statistically significant effect modification investigating genome-wide SNPs, associated with calcium level and risk of CRC (40). In the present study, we evaluated whether the seven loci known to be associated with serum calcium concentration, discovered from genome-wide study (9), were associated with CRC. In our study, we found no association between seven SNPs as well as weighted GRS score with CRC risk. Furthermore, only effect modification by the weighted GRS score in the association between total dietary calcium intake and CRC risk was found, suggesting that the protective effect may differ according to different genetic variability for altered calcium levels .

Additionally, the association of dietary calcium intake or calcium level with CRC risk was modified by 25(OH)D status. It is well known from previous evidence that vitamin D can modify the association between calcium level and CRC (41, 42). Previous study showed that 25-hydroxyvitamin D levels were associated with reduced CRC risk for concentrations of >80 nmol/l (43). Stratification by 25(OH)D status, showed that the association between dietary calcium intake and CRC risk appeared to be lower in subjects with a serum 25(OH)D level below 50 nmol/l suggesting that high calcium intake may inhibit the adverse impact of vitamin D deficiency. Besides, the association between calcium level and CRC risk was lower in subjects with a serum 25(OH)D level above 50 nmo/l. Vitamin D and calcium are interrelated. As described previously, vitamin D is important for the absorption of calcium in the gut (41). Like calcium, vitamin D plays an important role in growth restraining, controlling differentiation and apoptosis in cells of the intestines (41). Based on this, the association between calcium intake and CRC risk is hypothesized to become weaker for higher levels of 25(OH)D status (5).

Potential mechanisms

We hypothesized that calcium may be associated with CRC risk through several mechanisms. First, calcium may influence cell growth. Calcium has growth inhibiting properties on normal and tumor intestinal cells and may thereby influence CRC development (5). Also, in *vivo* and in *vitro* studies on human colonic epithelial cells showed that calcium suppresses proliferation and induces apoptosis in the lining of the colon, and thereby protects against CRC development (5, 42). Furthermore, experimental studies in animals and humans showed that calcium may bind to bile acids and fatty acids in the gastrointestinal tract, forming insoluble complexes, such as calcium soaps that protect the lining of the colon, and thereby reduces the risk of CRC (5, 6).

Moreover, we hypothesized that individual common genetic variants of calcium concentrations do modify the association between dietary calcium intake and CRC risk, and indeed we observed such an effect modification by the GRS on the association of calcium intake with CRC. To investigate effect modification by other genetic variants, larger studies with sequence data and genome-wide studies of calcium and CRC risk are needed.

Strengths and limitations

Our study has several strengths and limitations. One of the strengths is the prospective study design, which minimizes recall bias associated with CRC diagnosis. Also, this study had a long follow-up period, which is important because of the long latency period of CRC (44) and it may reduce the influence of reverse causation. Another strength of this study is the large study sample from a population-based setting, which increases the generalizability of the results.

Several potential limitations of our study need to be considered. First, information on dietary intake was obtained by self-report and at baseline of the study. Although diet in middle-aged and older individuals remains fairly consistent over time (45) and we adjusted our analyses for total energy intake to reduce potential measurement error (18), misclassification in calcium intake may still have occurred. Also, measurement error may have occurred since not all dietary supplement intakes were specified in dosages and frequency of usage. Furthermore, calcium homeostasis is affected by i.e. albumin concentrations. Unfortunately, we were only able to perform albumin-adjusted calcium level analysis in a subgroup. Finally, the association between calcium intake and CRC risk may differ in parts of the colon or rectum (46), we could not evaluate potential differences because of a limited number of CRC cases.

CONCLUSION

In this prospective population-based cohort study, we did not find a consistent association between calcium intake from diet , supplements or total serum calcium levels and CRC risk. However, on the basis of SNPs related to calcium concentrations, we observed effect modification of the weighted GRS on the association between dietary calcium intake and CRC risk, with lower risk of CRC by increasing calcium intake in subjects with low weighted GRS score. Considering the increasing incidence of CRC, it is important to further investigate other factors regulating calcium homeostasis and its role on CRC etiology.

Acknowledgments

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Abbreviations used

BMI: body mass index, CRC: colorectal cancer, FFQ: food frequency questionnaire, HR: hazard ratio, IQR: interquartile range, RDA: Recommended Dietary Allowance, SD: standard deviation

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SUPPLEMENTARY DATA

Table S1. Details of the multiple imputation modelling

	Multiple imputation procedure
Software used	SPSS 25.0 for Windows
Imputation method	Fully conditional specification (Markov chain Monte Carlo method)
Maximum iterations	10
Imputed datasets created	10
Exposures and outcomes (not imputed, used in model as predictor)	CRC, calcium level and dietary calcium intake
Covariates (imputed)	
Additional predictors	Sex, cohort, packyears, alcohol, smoking
Treatment of non-normally distributed variables	Predictive mean matching
Treatment of binary/ categorical variables	Logistic regression models

Number of missing for the variables imputed can be found in supplementary table S2.

Table S2: Basic characteristics before and after multiple imputation

Characteristics	N	Missing (n)	Original data	Imputed data
CRC cases, n (%)	10,941	0	427 (3.9)	*No missing*
Follow-up, years	10,941	0	13.6 (7.5)	*No missing*
Age, years[c]			67.4 [61.0-76.0]	
Women, n (%)	10,941	0	6,543 (59.8)	*No missing*
Education level, n (%)	8,263	2,687		
Primary solely			4,348 (39.7)	6,094 (55.7)
Secondary and higher			3,915 (35.8)	4,847 (44.3)
Income, n (%)	8,912	2,029		
Low to intermediate (<2,400)			3,808 (34.8)	5,049 (46.1)
Intermediate to high (≥2,400)			5,104 (46.7)	5,892 (53.9)
Total energy intake, kcal/d	6,638	4,303	1,968.7 (549.7)	1,954.5 (552.4)
Total dietary calcium intake[b], mg/d	6,188	4,753	1,116.7 (393.0)	*Not Imputed*
Total dietary fiber intake[b], g/d	6,207	4,734	26.3 (75.9)	26.3 (79.4)
Total processed red meat[b], g/d	6,207	4,734	101.1 (78.0)	101.1 (79.4)
Total alcohol intake[b], g/d	6,404	4,537	9.9 (15.0)	9.7 (14.9)
Smoking status, n (%)	10,379	562		
Never/ever			8,217 (75.1)	8,679 (79.3)
Current			2,162 (19.8)	2,262 (20.7)
History of diabetes mellitus type II, n (%)	6,263	4,678	827 (7,6)	1,326 (12.1)
Physical activity[a], MET hours per week	7,273	3,668	80.8 (44.4)	80.8 (44.4)
Body mass index, kg/m2	9,545	1,396	26.6 (3.9)	26.5 (3.9)
Waist circumference, cm	8.954	1,987	91.4 (11.5)	91.4 (11.5)
25(OH)D status[a], nmol/l, median[c]	6,269	4,672	49.1 [32.4-71.1]	45.8 [29.2-67.7]
Serum total cholesterol levels, mmol/l	9,591	1,350	6.4 (1.2)	6.4 (1.2)
Serum total calcium level, mmol/l	6,636	4,308	2.4 (0.1)	*Not Imputed*

[a]presented as mean (SD); [b]presented as median [interquartile range]

Table S3. The association between serum total calcium level (unadjusted and adjusted for albumin level) and CRC risk in RS-I

	CRC cases	Model 1[a] HR (95% CI)	Model 2[b] HR (95% CI)	Model 3[c] HR (95% CI)
Albumin-adjusted calcium level	356	1.03 (0.95-1.11)	**1.11 (1.00-1.23)***	**1.11 (1.00-1.23)***
Total serum calcium level	356	0.44 (0.16-1.22)	**0.24 (0.06-0.94)***	**0.26 (0.06-1.03)* (p=0.054)**

Continuous: per each mmol/l
[a]Model 1 was adjusted for age (years) and sex
[b]Model 2 was adjusted for age (years), sex, education (primary solely, secondary or higher), income (low to intermediate, intermediate to high), history of diabetes type II (no/yes), smoking status (never/ever, current), alcohol intake (g/day), dietary fiber intake (g/day), red meat intake (g/day), serum total cholesterol levels (mmol/l) and physical activity (hours/day)
[c]Model 3 was additionally adjusted for BMI (kg/m^2) and waist circumference (cm)
*p-value of < 0.05

Table S4. The association between dietary calcium intake and CRC risk stratified by serum 25(OH)D (< and ≥ 50 nmol/l)

Dietary calcium intake	CRC cases	HR (95% CI)	P=0.001**
25(OH)D <50 nmol/l	299	**0.88 (0.79-0.97)***	
25(OH)D ≥50 nmol.l	181	1.01 (0.90-1.12)	
Calcium level			**P=0.04****
25(OH)D <50 nmol/l	299	0.94 (0.21-4.25)	
25(OH)D ≥50 nmol.l	181	**0.06 (0.01-0.65)***	

Continuous: per each 200 mg
*p-value of < 0.05 **p-value of <0.10

Table S5. List of Results from literature

Results from our study	In line with the following studies	In contrast to the following studies
Low CRC risk for high dietary calcium intake (≥1,485 mg/day), compared to the RDA (≥1,100-1,485 mg/day)	Garland et al., 1985 (1) Flood et al., 2005 (2) Abid et al., 2014 (3) Park & Kim, 2015 (4) Zhang et al., 2016 (5) Yang et al., 2018 (6) Meng et al., 2019 (7)	
No association between dispensed calcium supplement and CRC risk	Bristow et al., 2013 (8)	Flood et al., 2005 (2) Barry et al., 2019 (9)
No association between serum calcium level and CRC risk		Fuszek et al., 2004 (10)
A higher CRC risk for higher albumin-adjusted calcium level in a subgroup analysis	Proctor et al., 2010 (11) Wulaningsih et al., 2013 (12)	
No association between 7 SNPs separately as well as GRS and CRC risk	Mahmoudi et al., 2014 (1 of the SNPs) (13)	Jacobs et al., 2010 (some of the SNPs) (14) Zhu et al., 2017 (some of the SNPs) (15)
Effect modification by weighted GRS from calcium concentrations SNPs were found on the association between dietary calcium level and CRC risk. After stratification, a lower CRC risk was found for the participants with lower GRS	Park & Kim, 2015 suggest that gene-diet interactions may possibly alter the associations among dietary intake, genetic polymorphisms, and CRC risk (4)	Figueiredo et al., 2011 (16)
Serum 25(OH)D was also an effect-modifier in the association between calcium intake and calcium level and CRC. After stratification for serum 25(OH)D level, dietary calcium intake was associated with lower risk of CRC in subgroup of 25(OH)D level <50 nmol/l, and calcium level was associated with lower risk of CRC in subgroup of 25(OH)D level ≥ 50 nmol/l.	Ng et al., 2014 (17) Park et al., 2007 (18)	

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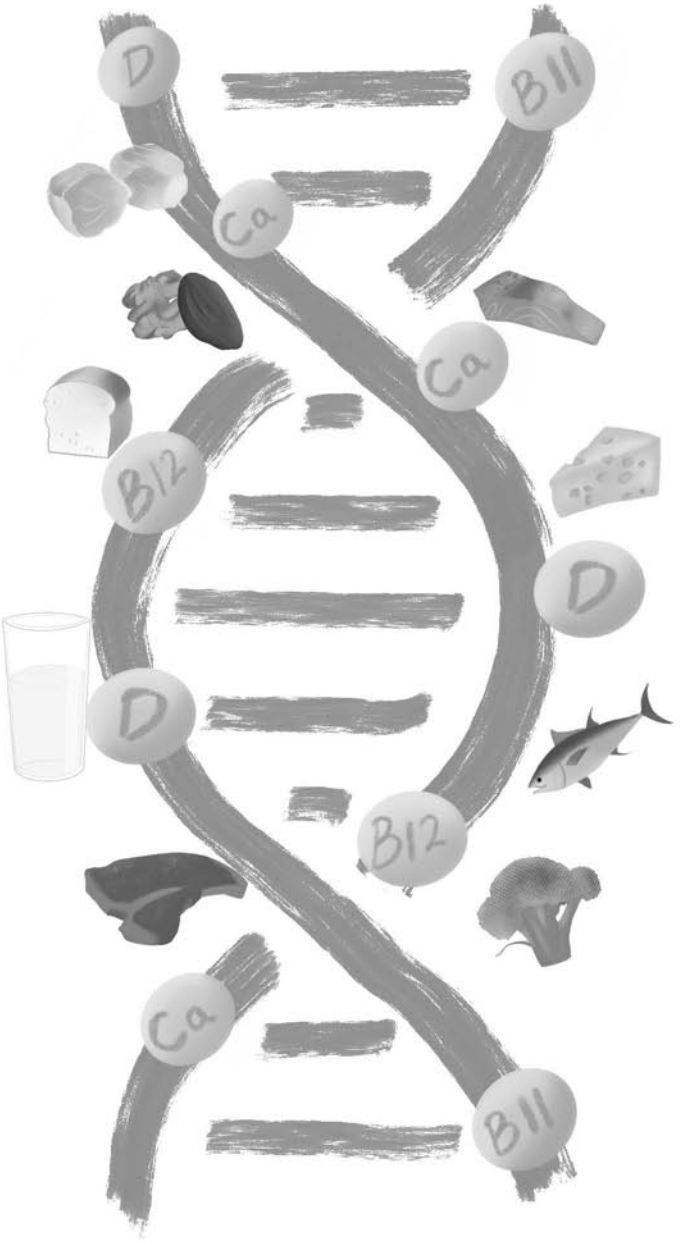
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GENERAL DISCUSSION & SUMMARY

GENERAL DISCUSSION

As the usage of dietary supplements grows worldwide, especially among older people, the objective of this thesis was to understand more about the role of micronutrients in health and diseases in the older population. The micronutrients that were the main topic of this thesis were folic acid, vitamin B12, vitamin D and calcium because they are widely used as dietary supplements, and have pleiotropic effects on several age-related diseases.

The association between vitamin D and body composition was studied, as well as the effect of folic acid and vitamin B12 on body composition (**Chapter 2**). The long-term effects of folic acid and vitamin B12 supplementation (the B-PROOF study) on common age-related diseases as such as cancer, fractures and cardiovascular diseases was assessed (**Chapter 3**). Lastly, the role of micronutrients on bone health and colorectal cancer in interactions with diuretics and genetic variation on bone health and colorectal cancer, respectively was assessed (**Chapter 4**). This chapter provides an overview and discussion of the main findings of this thesis, followed by an outline of the clinical and public health implications and suggestions for potential further research.

MAIN FINDINGS AND COMPARISONS WITH RECENT STUDIES

Micronutrients and body composition

The prevalence of micronutrient deficiencies is increased in people with obesity, which is expressed as having a BMI >30. However, obesity is a multifaceted physiological phenomenon leading to changes in body composition and is sometimes difficult to assess in an ageing population. Therefore, BMI as a measure may underestimate the prevalence of obesity among older adults. In studies involving this population group, measuring fat mass and fat-free mass as aspects of this changing body composition provides a better insight into the actual body composition and the prevalence of obesity. Therefore, the association between micronutrients and body composition (by measuring body fat percentage next to BMI) in an older population was assessed. We found that a higher BMI and higher body fat percentage were both significantly associated with lower serum 25(OH)D levels. In this study we confirmed that overweight older persons and those with a higher percentage of body fat were at risk of vitamin

D insufficiency (**Chapter 2.1**). This finding corresponds with other studies involving various populations (1, 2).

This association can be due to a decreased bioavailability of vitamin D due to its deposition in adipose tissue (3). But to assess the causality and the direction of the association between the level of serum 25(OH)D and the change in BMI and fat percentage, one can use vitamin D supplementation studies and Mendelian Randomization studies. Mendelian randomisation studies suggested that vitamin D is causally related to markers of obesity, e.g. adiponectin (4). In the B-PROOF intervention trial with B vitamin supplements, all participants were also given vitamin D supplementation, therefore whether vitamin D supplementation might change body composition could not be assessed. Neither did we have any information on serum 25(OH)D levels after the intervention. However, a recent RCT showed that vitamin D supplementation had no effect on body composition (5). Yet, a systematic review and meta-analysis of RCTs showed that obesity reduced the effect of vitamin D supplementation on vitamin D level (6, 7). In addition, evidence from RCTs has also shown that optimal vitamin D supplementation may benefit obesity-related disorders (3). Altogether these results suggest that increase in overweight and fat mass seems to reduce serum 25(OH)D level and that obese people may need a higher doses of vitamin D to achieve the normal range of 25(OH)D compared to non-obese people. Further RCTs on the role of vitamin D supplementation in obesity-related disorders are needed (see implications and future research).

Another aspect that was evaluated in this thesis, was the effect of folic acid and vitamin B12 supplementation on body composition. In **chapter 2.2** we observed that higher serum folate was associated with a lower BMI, and a higher vitamin B12 status was associated with a higher Fat Mass Index (FMI) from observational data, which was partly in line with previous studies (8). However, this association was not confirmed as causal, because the intervention with folic acid and vitamin B12 supplementation in the B-PROOF study had a null-effect on body composition, suggesting that B-vitamins may not have a role in the aetiology of obesity or changes in body composition in older individuals. Neither was the causal role of a lower vitamin B status in obesity supported in a Mendelian randomisation study of genetic determinants of vitamin B12 (9). However, a relatively strong association has been observed between the pleiotropic Fucosyltransferase 2 (*FUT2)* gene variant rs602662 and serum vitamin B12 (9). FUT2 is thought to be involved in interactions between host factors and gut microbiome composition, which is involved in production of vitamin B12. Therefore, it can be hypothesized that differences in the gut microbiome composition might lead to the observed associations with obesity and body composition, and the concomitant

vitamin B12 changes might be a by-stander effect (10). Other recent studies support the causal role of obesity on vitamin B12 status by finding an association between *FTO* gene variants (which are associated with obesity (11)), and serum vitamin B12 levels (12). On the basis of these findings it can be concluded that obesity may influence B-vitamin status, but not the other way around.

Long-term effect of micronutrients on cancer

In the B-PROOF trial, the combination of vitamin B12 and folic acid supplementation was observed to be associated with an elevated risk of cancer, and especially colorectal cancer (**Chapter 3.1**). Previous studies on the association between folic acid and vitamin B12 supplementation and cancer risk were inconclusive, with some studies showing a null-effect or even a reduced risk of cancer after supplementation (13). The proposed underlying mechanism is that the carbon group of folic acid supplementation contribute to cell proliferation through purine nucleotide synthesis that accelerate the process of cell growth or by the provision of methylgroup donors to influence the process of DNA methylation and expression of genes that may be involved in carcinogenesis. Indeed, in a follow-up study of the B-PROOF study, when assessing genome wide methylation patterns on 450.000 CpG sites in genomic DNA of circulating blood cells using the Illumina array, variations in DNA-methylation were observed in several genes involved in carcinogenesis (14) in the intervention group.

One explanation for the opposing outcomes of several trials compared to our findings is that most studies on the effects of B vitamins on cancer have examined the effect of folic acid supplementation only, rather than in combination with vitamin B12 supplementation. Since vitamin B12 also plays a role in the one-carbon mechanism, it might also play a role in the aetiology of cancer. The VITamins and Lifestyle cohort-study (VITAL), assessed the association of vitamin B12 supplementation and showed a higher risk of lung cancer in men between 50 and 76 years with a higher use of vitamin B12 supplements (15).

However, studies on the association between cancer on the one hand and dietary intake or plasma levels of these B vitamins on the other hand, have shown conflicting results. Results from observational studies have suggested that the intake of these micronutrients individually is associated chiefly with a decreased risk of several cancers (16-18). However, studies of plasma levels of folic acid and vitamin B12 did not find an association with an increased risk of several cancers (19, 20). For example, in a study of subjects in UK primary care, having higher plasma B12 levels was found to be associated with an increased one-year cancer risk compared to normal B12 levels (21).

In a nested case-control study including a Mendelian randomisation approach based on 8 genetic variants for circulating vitamin B12, an increased risk of lung cancer was found to be associated with an increase in circulating vitamin B12 concentrations suggesting a causal effect of B vitamins on cancer risk (22).

Taken the results of previous studies, the B-PROOF study and RCT together, it seems that folic acid and/or vitamin B12 supplements, due to higher dose and higher bio-availability, might potentially increase the risk of cancer in older persons (such as the participants in the B-PROOF study) (23). This could be due to individual effect of folic acid or vitamin-B12, or due to an interactive effect of both folic acid and vitamin-B12 combined, and that this effects could be due to changes in DNA methylation patterns.

Effects of B vitamins on fractures, CVD and stroke

With regard to the findings of the B-PROOF trial during a prolonged follow-up period on the main pre-specified outcome, no effect on fracture risk was observed, and neither an effect on cardiovascular disease was seen (**Chapter 3.2**). Similar to B-PROOF, another long-term RCT by Stone *et al.* found that vitamin B12 and folic acid supplementation had no effect on the risk of fractures in women (24). One of the limitations of B-PROOF could be the limited power to detect any effect of the B-vitamin supplementation due to relatively low incidence of events in the limited follow period. We therefore extended the follow-up period (of 2-3 years) of the initial B-PROOF trial to 6-9 years to capture more events, and evaluated the long-term effect of the intervention. A questionnaire was sent to the participants with informed consent for contacting in the future. In this extended study of the B-PROOF trial, however, only 90 new fractures were captured. We hypothesize that this may be an underestimation due to high loss to follow-up, mainly due to the high morbidity and mortality rates in these older adults (with a median age of 76 years when contacted in this extended follow-up study). During the second follow-up questionnaire, extra information was documented on the non-responders shown in the flow-chart below (**Figure 1**). In contrast with B-PROOF extended follow-up, a recent study showed that higher intake of vitamin B12 (≥ 30 vs < 5 µg, diet and supplements) in a longer follow-up period (of 20.9 years), was associated with increased fracture risk (25). This study documented 2304 cases with hip fracture of 75 864 included in women nurse health study with lower intake of vitamin B12 than B-PROOF study. Nevertheless as this study has a cohort design of the study, residual confounding may be present and thus randomized controlled trials with a larger follow-up are needed to establish adverse effects in the long run.

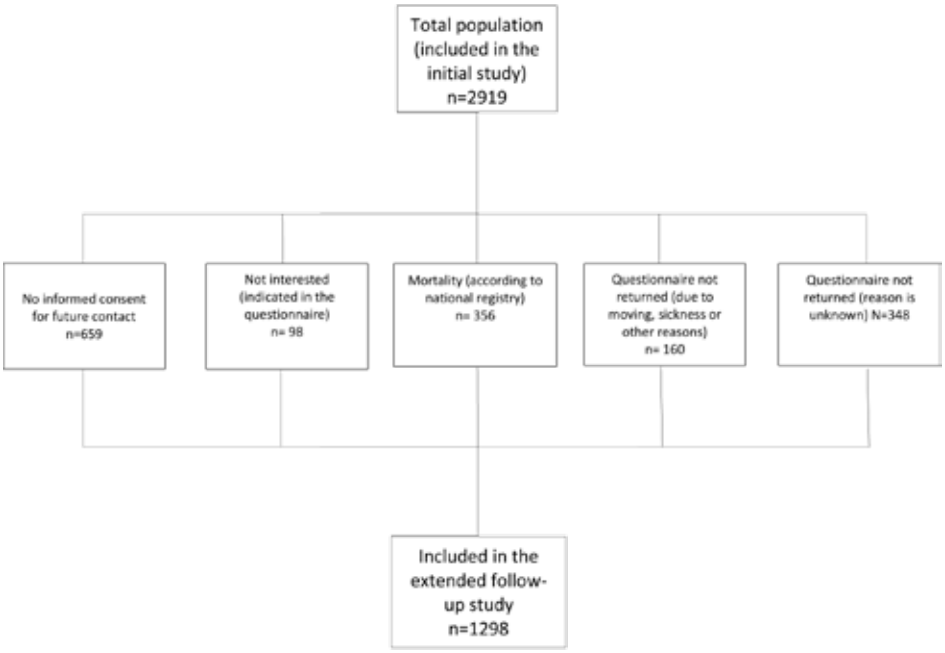

Figure 1. Flow-chart showing an overview of reasons for non-response of participants in the secondary analysis of the B-PROOF trial (Chapter 3.2).

In the B-PROOF trial, which selected and included participants with elevated homocysteine levels, B-vitamin supplementation was observed to may have been beneficial in reducing fractures, but only in individuals with high total homocysteine concentrations **(Chapter 3.2)**. This suggests that in subgroups of older persons or those with a higher risk of a deficiency of B-vitamins, supplementation may decrease the risk of fracture. However, it was a subgroup analysis and because of the low number of cases (n= respectively 16, 31, 23) in the stratified analysis of different levels of homocysteine levels (≤ 13.2, 13.2-15.1 and ≥15.1 mmol/l), these findings should be interpreted with caution. Similarly, in the study by Stone *et al.,* the number of participants was too low to study the effects of B-vitamin supplementation among women with deficiency in the Women's Antioxidant and Folic Acid Cardiovascular Study (WAFACS) (24). Thus, replication is needed to test the effect of B-vitamin supplementation especially in the deficient individuals. Potentially there is benefit of supplementation and the effect of B-vitamins on fracture risk in this deficient older persons.

With regard to the long-term follow-up effect of B-vitamin intervention in the B-PROOF trial on CVD and stroke, no effect was observed from analysis of the self-reported data. Systematic reviews on homocysteine-lowering with B-vitamins, showed only a

decrease in stroke risk and not in the risk of other cardiovascular diseases, identical as the outcomes of the initial B-PROOF study (26, 27).

Interplay between micronutrients, Single Nucleotide Polymorphisms (SNPs) and drugs on disease

Diuretics and bone health

In the Rotterdam Study (RS), a positive association was observed between thiazide use (past and current) and bone mineral density at the lumbar spine (LS-BMD) but not with lumbar spine trabecular bone score (LS-TBS), a DXA-derived measure of bone micro-architecture and quality. This relation was observed in the general population and in particular in participants older than 65 years, compared to participants younger than 65 years (**Chapter 4.1**). In line with this observation, other studies showed a lower fracture risk with the use of thiazide diuretics (TDs) (28). This is further supported by another study among women that showed that TD users had a slower annual change in femoral neck and spine BMD compared to non-users, which suggests that BMD loss was lower in TD users, confirming the results observed in RS (29). Thus, the possible protective effects in relation to fracture risk could be explained by an increase in BMD, which increased with the dosage and duration of thiazide use, without improving bone microarchitecture. The increased BMD in TD users may be explained by estimated decrease in osteocalcin level, a marker of osteoblast activity, bone formation, and bone turnover (30) the expression of which might be influenced by TD use (**Chapter 4.1**).

In contrast to thiazide diuretics, loop diuretics (LDs) may adversely affect bone health by inhibiting calcium reuptake. Indeed, in the Rotterdam Study, TBS was found to be only decreased if LDs were used in the past and LDs seem to be weakly associated with higher BMD when used for a period of 121-365 days, compared to never users, but the results were not consistent (**Chapter 4.2**). The potentially harmful effect of LDs on bone were more firmly confirmed in other studies (31-33). Because of the common use of LDs among older persons and the high prevalence of osteopenia and osteoporosis, we investigated the effect-modification of vitamin D and calcium on the association between LDs and bone health. Yet, in this study, no consistent associations were found between LD use and bone outcomes after stratification by serum 25(OH)D levels. Even though the analyses were adjusted for body fat mass, residual confounding may have biased the association between serum 25(OH)D levels and bone health caused by other co-morbidities, such as other measures of body composition, physical activity, lifestyle and health status.

Calcium and cancer

In **chapter 4.3**, we studied the association between the intake of calcium (dietary and supplements), serum calcium level, and colorectal cancer (CRC). In this study, an association was only found for a higher calcium intake (≥ 1,485 mg/day), compared to an average calcium intake (1,100-1,485 mg/day), which suggests that normal intake of dietary calcium is not associated with higher risk of CRC. This association was also not seen between calcium supplement use and CRC, and neither between calcium levels and CRC risk. In addition, we found no statistically significant linear trend between dietary calcium intake and CRC, perhaps suggesting a threshold effect. From previous studies, calcium intake from dairy products may be associated with reduced CRC risk while the association for non-dairy calcium intake and CRC risk is still inconclusive (34).

The different results from this and previous studies may be explained by differences in bioavailability of calcium (35). After adjusting serum calcium levels for albumin in a subgroup, circulating serum calcium levels were observed to be positively associated with an increased risk of CRC. This supports the observation of the relation between calcium dietary intake with increased CRC risk. However, these observations do not necessarily indicate a causal relationship. Calcium is partly (around 40%) bound to albumin (36) and it is known that calcium serum level is under close homeostatic control (under normal circumstances), and disturbed calcium homeostasis is often found in diseases including cancer (37). Studying genetic determinants of calcium concentrations, as a stable proxy, could give more insight into calcium homeostasis. This is possible because through GWAS several such genetic determinants have been found (38), which are combined into a so-called polygenic risk score, or polygenetic risk score (PRS), which can explain a certain percentage of the variance in calcium levels in the population. Therefore, we also studied the interaction between calcium intake, levels and supplementation and a PRS of calcium concentrations, in relation to colorectal cancer risk (**Chapter 4.3**). Indeed, the associations between dietary calcium intake or serum calcium levels and CRC, were modified by the calcium PRS, with a lower CRC risk for subjects with a lower PRS, compared to subjects with a higher PRS. Taken together, results of the calcium PRS analysis suggest a relationship between circulating calcium and risk of cancer, but further replication in other studies with different population are needed.

METHODOLOGICAL AND ETHICAL CONSIDERATIONS

Influence by a case of scientific misconduct?

For this thesis specifically the methodological and ethical considerations relevant to the B-PROOF trial concern two complexities: data integrity in the community of science, and secondary post-hoc analyses as an approach for analysis of the unexpected adverse events, which we will discuss in this section.

The design of the B-PROOF study was based on findings of earlier observational studies on the association between homocysteine levels and osteoporotic fractures, including those in the Dutch study populations of the Rotterdam Study and LASA (39-41). Interestingly, an intervention trial appeared, soon after these initial epidemiological association studies, to show that B-vitamin supplementation had a beneficial effect on fracture risk, which was the study by Sato *et. al.* (42). Many other trials, including the B-PROOF trial, have since been carried oud to investigate the effect of B-vitamin supplementation on bone health, but have not been able to replicate the results of the RCT by Sato *et al.* Therefore, around 10 years after publication of Sato's RCT, concern was expressed regarding the integrity and scientific validity of Sato's study (43). In the meantime, due to the null-findings of the primary results of the B-PROOF trial and concerns regarding the possible adverse effect of B-vitamin supplementation on cancer risk, the researchers in the B-PROOF trial were interested in conducting a meta-analysis including Sato's trial. However, the authors did not respond to this opportunity despite numerous attempts to get in touch with them. At the same time, a study to evaluate the validity of the results reported by the *Journal of the American Medical Association* had begun, and as a result, the article by Sato *et al.* was retracted along with 21 (of 33) of his other articles due to scientific misconduct and concerns about data integrity (44). Unfortunately, the results published by Sato *et al.* had -by that time- already resulted in several large-scale intervention trials being conducted, based in part on the spurious results of Sato's work.

The question therefore arises whether the B-proof study would have been conducted if the study by Sato *et al.* had not been published. Because of the positive results of other observational studies on the association between homocysteine level and osteoporotic fractures and the limited evidence from RCTs, the B-PROOF trial was designed to study the effect of homocysteine-lowering intervention through B-vitamin supplementation on osteoporotic fractures irrespective of Sato *et al.* Thus, most likely the B-PROOF study would still have been designed and conducted given the

order of events and available evidence at that time. Nevertheless, the power analysis of the B-PROOF trial was based on Sato's publications, and if the study by Sato *et al.* had not been published, the power analysis based on the observational studies would have resulted in recruitment of a larger number of study participants and/or a longer follow-up.

Importantly, the question also arises if unnecessary harm could have been prevented if the Sato *et al.* study was not published. The dosage of the B-vitamin intervention of the initial B-PROOF trial was chosen also based on safety considerations, since there were already concerns regarding the adverse effects of pharmacological doses of vitamin B supplementation. Furthermore, individuals with a five-year history of cancer were excluded from the study, and if any participant reported a cancer diagnosis during the study, they were advised to discontinue the supplementation, while follow-up of the other outcomes continued. Participants were advised to discontinue supplementation when reached high dosage of folic acid and vitamin B12 (beyond recommended daily intake) when combined with the intervention. Moreover, half way through the study, we compared the number of incident cancer cases with the incidence of cancer in the general population, without de-blinding the intervention, and observed these figures to be similar at that point in time. As mentioned, at the end of the B-PROOF trial and de-blinding, there were further concerns about the possible adverse effect of folic acid and or vitamin B-12 supplementation on cancer risk based in results from the B-PROOF trial. This was studied further in a secondary analysis, by linking the B-PROOF data to data of the national cancer registration. Subsequently, the concerns were confirmed by indeed showing an adverse effect for these B-vitamins on incidence of cancer overall, and colorectal cancers in particular. So, taken together it is unlikely that unnecessary harm was inflicted on B-PROOF subjects by including the Sato *et al.* study, and all required precautions were taken to prevent such harm from happening.

Thus, even without the RCT conducted by Sato *et al.*, the B-PROOF trial would have been designed and performed, however, probably with a different power calculation.

Lessons learned

Because we cannot turn back time, we can learn a great deal from this, one of science's major scandals, which by now has been widely publicized. This was however 7 years after B-PROOF was initiated.

Negative results are important

First of all, the number of publications is increasing, but the number of retracted papers is also increasing. Currently, the pressure to publish positive and novel results in science is high due to pressure on citation numbers in order to secure prizes and funding (45). Previous work has also shown that statistically significant results are more likely to be published than papers with null results. This could feed scientific misconduct, as was the case with Sato's RCT, and potential publication bias (*i.e.*, leading to distortion of the scientific literature and misleads health professionals, and policymakers in their decision-making (46)). It is therefore essential to be able to publish the negative results, too. A change in mind-set is needed when it comes to accepting negative papers, such as the follow-up B-PROOF manuscript with its null-findings on the risk of fractures and cardiovascular disease.

Secondary analyses are informative

A second methodological consideration in this thesis was the secondary *post hoc* analysis of the long-term effect of primary and secondary outcomes as well as the adverse events. Most of the studies focusing on the effect of B-vitamins on the relevant health outcomes (fracture or cancer) are secondary analyses alongside other outcomes (such as cardiovascular disease), which are the primary outcome. Selection bias and sample size calculations are some examples of issues that need to be taken into account with respect to interpreting these findings. Being aware of this, in future studies, issues of selection- and information bias and confounding can be dealt with in the design.

For the analysis of the long-term effect of the intervention on primary and secondary outcomes and the adverse events in the extension of the B-PROOF study, participants with informed consent for future studies from the B-PROOF trial were selected. Luckily as there were no differences between the intervention and the control group in the selected population, the randomisation was still intact. Another issue to address in secondary analyses is sample size. However, for studies on the unexpected adverse effects of supplementation such as our initial B-PROOF trial, or studies involving rare diseases as an outcome, it is maybe difficult to predict the correct sample size in advance, but assumptions are possible. It is important to report clearly that the study is a secondary analysis or an extended follow-up. Also, the methods of the secondary analysis and the number of participants which were included in the analysis derived from the primary trial should be reported. The recommendation of Hopewell *et al.* (2013) (47) is adapted from the 2010 CONSORT Statement, and states which information should be included in these reports in order to enable readers to evaluate the

validity and reliability of the results of the secondary analysis, and ultimately to use these to assess the scientific implications. Possible biases should also be mentioned, as well as their potential effects. In the secondary analysis of the B-PROOF in **chapter 3.1**, all these items are mentioned.

Longer follow up is beneficial

Moreover, a post-trial follow-up of a randomized controlled trial can provide valuable information about the long-term effect of the intervention on the primary outcomes of the trial, especially in this age category (48). Most of the RCTs are designed as short-term trials, but a longer follow-up time could help to detect persistent effects in the years following the intervention and identify the latency-effect of the intervention. We expected the primary outcomes of the B-PROOF trial to detect the persistent effects on fracture years after the intervention, and to detect the latency-effect of the intervention on CVD as in the baseline and at the end of the first follow-up, a follow-up questionnaire was used for the post-trial follow-up. Unfortunately, given the older age group the prolonged follow-up period resulted in a high loss to follow-up, resulting in limitations of the generalizability of the results. In light of this knowledge, prolonged follow-up is preferably already taken up in the initial study design. For example through patient consent forms a route could be to secure access to routine health records in order to access data on detailed health information relating to these participants using diagnostic codes (e.g. ICD-10), including mortality data and the cause of death in follow-up participants, where relevant. Of course, valid informed consent at the baseline would need to be in accordance with the new privacy guidelines. In the Netherlands, the electronic patient record (EPD) is useful but sometimes difficult to obtain information source and at the time of data collection the use of the EPD as researcher would have let to unanticipated regulatory issues. Additionally, from the mortality data and the cause of death, a cause-specific mortality analysis can provide more information on the effect of the intervention, since for example several studies have indicated that cancer patients may have a higher risk of CVD mortality (49).

Better documentation of supplement use helps interpretation

The examination of the supplement use (over the counter or additional supplementation on top of the intervention) of the participants in the B-PROOF trial and the Rotterdam study was based on self-reported data, which could result in misclassification of supplement use due to insufficient recall problems. The use of supplementation also varies over the years. Another method of monitoring dietary supplement use

more truthfully, was used in the study by White *et al.*. In this study, researchers collected the supplements data by repeated questionnaires and then calculated an high validity and reliability compared with questionnaire at baseline, three months after, supplement inventory and to the biomarkers of the nutrients (50). In the future, better monitoring of the supplement use will be necessary (see below, implications and future research).

Dosage counts matter

Another point of discussion that needs mentioning, is the dosage of the intervention. For the B-BROOF study, a dosage of 500 µg/day for vitamin B12 and 400 µg/day for folic acid was chosen. For vitamin B12, the dosage was higher than the recommended daily intake (51). However, this dosage was chosen based on a dose-finding study which showed that a dose of 647 µg/day for four months is sufficient in an older population with mild deficiency to normalize vitamin B12 status (52). The majority of dietary supplements contains a high level of micronutrients. For some micronutrients, such as folic acid, there is an upper intake limit, but this is not yet the case for vitamin B12 (51). As some colleagues showed previously, dietary intake of vitamin B12 is significantly associated with vitamin B12 biomarkers (53). Furthermore, the association between total vitamin B12 intake (from diet and supplements) showed a stronger association with vitamin B12 biomarkers, probably due to the higher bioavailability and higher absorption of the free vitamin B12 from supplements (54).

Other micronutrients are also important

As we mentioned previously, other nutrients are also involved in one-carbon mechanism, such as choline and methionine, and these are also relevant to the study of the effect of micronutrients on health. For example, choline metabolism disruption could affect DNA methylation (55). Additionally, high methionine is associated with methionine/transmethylation metabolism, which could increase DNA damage and carcinogenesis (56). However, more studies on the restriction of methionine and the prevention of cancer are needed. Unfortunately, there is no data available on these nutrients, but it is important to evaluate these mechanisms. However, given the fact that the B-PROOF trial was randomized and no major differences in baseline characteristics were observed between the intervention and control group, we assume that the effect of the B-vitamin intervention is not explained by differences in the intake of other methyl donors. On the other hand, for the cross-sectional analysis of the B-PROOF trial and in The Rotterdam Study, other nutrients involved in one-carbon mechanism could have played a role. Unfortunately, these were not available.

IMPLICATIONS AND FUTURE RESEARCH

What determines deficiency

The most common deficiencies in Western Europe are iron, vitamin D, folate and vitamin B12 (57). These nutrient deficiencies are fairly easy to detect, diagnose with laboratory tests and treat with the appropriate supplements. However, subclinical deficiency is difficult to recognize and can still result in pathological changes, which may subsequently lead to clinical diseases. It is therefore important to recognize the 'at-risk' population and to treat them appropriately (for example by a dietician) in order to prevent clinical diseases due to the depletion of micronutrients. Community-dwelling older persons are at risk of vitamin B12 deficiency induced anaemia that may be masked by the use of high dosages of folic acid (58). These potential underdiagnoses that can cause neurological complications needs further investigation. General Practioners should therefore be alert to the correct diagnosis of vitamin B12 deficiency, such as for example by assessing serum methylmalonic acid analysis (MMA) (59).

As well as the method of measuring deficiencies, the cut-off for some biomarkers is also under debate. For example, for vitamin D the Endocrine Society (ES) uses the cut-off of 75 nmol/L (60) and the Institute of Medicine (IOM) uses a cut-off of 50 nmol/L (61) for 25(OH)D concentrations. Yet, also based on the studies presented here it is highly recommended that a different cut-off for deficiency should be used for each age group due to different nutritional needs in different stages of life, particularly among older age persons, and due to physiological variations, the pathological ageing process, the role of medication and how it influences nutritional biomarkers (62).

Body composition

Since the studies in this thesis demonstrated that body composition, and in particular obesity, may have an effect on micronutrient status, future studies should focus on the effect of changes in body composition and the mechanisms that underlie their potential effects on micronutrient status. For example, in a meta-analysis of studies that measure the changes in the body composition and changes in B-vitamin levels along with the interaction of the genes associated with body composition and B-vitamins. Furthermore, due to the possible risks of unnecessary supplementation with high doses of B-vitamins (and possibly vitamin D) (63), a meta-analysis is needed to examine the differences in vitamin D and B-vitamin status in an obese population and whether vitamin D influences obesity-associated health risks over the short and long

term. The prevention of obesity (especially in early life) could be an important public health strategy for reducing metabolic syndrome and obesity-associated health risks.

Aspects of micronutrient research

Certain other important questions remain unanswered, including the following. Is it the structure of B-vitamins that makes the difference in the effect on cancer (cells), or the amount of intake regardless of the source of the folate/folic acid and vitamin B12? Is there a cut-off point for these micronutrients, after which they cease to be preventive and start to be harmful? What is the effect of these supplements in deficient people? Future research is required to understand the potential beneficial and adverse effects of micronutrient supplementation in more detail. Ideally, randomized controlled trials would provide more insight into the negative effects of dietary supplements but, due to potential ethical issues, this is not possible. Analysing the potential harm in the relevant subgroups within a meta-analysis of all the trials that have measured the effect of B-vitamins and vitamin D and accurately addressing adverse event reports, would therefore be a better solution. It is necessary to report adverse events in randomized controlled trials like the B-PROOF study, but trials in Good Clinical Practice (GCP)-based pharmaceutical settings have stricter rules for monitoring of adverse effects. In the future, there should be better monitoring of the use of dietary supplementation and adverse effect in the general population, including all age groups, similar to the clinical trials used to test the (new) drugs. A mandatory improved report on adverse effects, drafted in accordance with the International Conference on the Harmonisation of Good Clinical Practice (ICH GCP) guidelines (64), should cover all adverse effects and analyse the risks of dietary supplements in randomized controlled trial studies. However, clinical trials are not always feasible (due to earlier mentioned potential ethical issues), and so a population-based cohort such as the Rotterdam Study, with improved repeated measurement of the use of dietary supplements by the participants (including the use of over-the-counter supplements), could also provide valuable information and facilitate the analysis of the adverse effects of micronutrients (from food and dietary supplements). This type of study (population-based cohort) may also have better generalizability to the community-dwelling population as a whole (65).

Disentangling cause and effect

However, it is important to note that, in population-based studies, the reliability of the results derived from association analysis, whereby the direction of the association is not certain, i.e. potential ‘reverse causation’, is not always adequate.

Moreover, residual confounding is a problem in observational studies, and therefore Mendelian randomization (MR) could provide a solution, because this method is less likely to be affected by reverse causation or confounding. This method provides evidence about assumed causal relationships between a modifiable risk factor and the outcomes of interest, by using genetic variations as natural experiments. In our case, the genetic variants could be that for homocysteine to support causal inferences regarding the effect of cardiovascular disease (modifiable risk factors) (66). It should be noted that this method depends on assumptions for a valid instrumental variable: the relevance assumption (the instrumental variables should associate with the risk factor of interest); the independence assumption (the instrumental variables should share no common cause with the outcome); and the exclusion restriction assumption (the instrumental variables should do not affect the outcome except through the risk factor). Together this means that pleiotropy, i.e., one gene and/or variant of a gene can have multiple biological functions, can be a problem here, and will need to be addressed in future MR studies.

The challenge of drug-nutrient interactions

In our aging society prevalence of medications use is high and continues to rise, with accompanying polypharmacy. Furthermore, older age is an important risk factor for nutritional status, thus also prevalence of poor nutritional status is rising. As such, recognition of the importance of food and drug interaction has been growing in clinical practice (67). Diuretics are frequently prescribed in the treatment of heart failure and hypertension (68, 69), and they have been shown to influence calcium homeostasis and bone metabolism. As mentioned previously, the association between the use of diuretics and trabecular bone score has not been investigated previously, and the possible changes in bone microarchitecture need more detailed research using more refined techniques, such as high-resolution peripheral quantitative computed tomography (HR-pQCT). Further studies are needed to investigate the association between loop diuretics and general bone health, especially trabecular bone score. Moreover, research and replication is needed on nutrient-drug interactions with bone health, using other biomarkers such as PTH and bone turnover markers. Nutrient-drug interactions are largely understudied. The knowledge about this subject is scarce and awareness of potential adverse effect of nutrient-drug interaction, and also training for clinicians is needed (67). Guidelines for the prevention of bone loss due to use of loop diuretics might also be helpful. Future research should also focus on the association between polypharmacy and nutritional status in the older population due to the higher use of medication among this group and the possible association with reduced intake of some nutrients and potential food-drug interactions (70).

An early recognition of (colorectal) cancer

Clinical trials are not always achievable, as mentioned before. Thus to assess the effect of the B-vitamin supplementation on cancer an early diagnosis of malignant neoplasm is appreciated. Biopsies are the standard methods for diagnosis of lesions (71, 72), which are often invasive methods for the patient. Currently, research into microRNA (miRNA) and cancer is being conducted and has shown that the expression of miRNAs is dysregulated in different tumours and miRNAs is suggested as a potential biomarker for diagnostic and also prognostic targets in cancer (73, 74). In the follow-up to B-PROOF, we could have measured such potential biomarkers in order to detect a variety of common cancers earlier; however, due to budget constraints we were unable to do this, so we linked our data to data of the national cancer registration for the diagnosis of cancer. In other studies, the role of the miRNAs has been shown in the diagnostic and treatment of colorectal cancer (75), which could be used to investigate the effect of B-vitamin supplementation on cancer risk in the future.

Is folic acid fortification universally good ?

As we know, fertile women are advised to take folic acid pre- and peri-conceptionally in order to reduce the risk of neural tube defects (76). For this reason, folic acid fortification (folic acid added to enriched grain products) has been introduced in some countries. Due to fortification, the National Health and Nutrition Examination Survey Data determined a higher folic acid intake, which exceeded the upper limit intake (77). Now that we suspect a possible adverse effect of excess folic acid and/or vitamin B12 intake among older persons (presumably with malignant cells), the question arises as to whether it is still appropriate to add folic acid to food, since the majority of the population does not need extra folic acid or other micronutrients. On the other hand, there may be arguments that the older population may also benefit from fortification (due to their higher likelihood of deficiencies because of inadequate diet and changes in the absorption and excretion of micronutrients (78)). A recent study evaluated the effect of voluntary fortification of vitamin B12 and folic acid, and showed that in older adults, fortification was not an effective manner of preventing deficiencies (79). The B-PROOF study evaluated the effect of folic acid and vitamin B12 supplementation, and this study provides a further insight into the potential adverse effect of these micronutrients in the older population and contributes to evaluation and decision-making around the fortification of food. The suggestion from work presented in this thesis is not to stimulate widespread use but to limit the advice to consume micronutrients (through supplements) to pre- and peri-conceptional women and others who need additional micronutrients, such as those with a proven

deficiency. However, although it is conceivable that deficient individuals may be more likely to benefit from supplementation (80), studies addressing the specific subpopulations who may or may not benefit from supplementation are scarce.

Again, the B-PROOF study showed an adverse effect of vitamin B12 (and/or folic acid) on cancer, especially on colorectal cancer, and another recent study showed a potential association between higher vitamin B12 level and higher mortality, which has to be confirmed in other studies (81). Nevertheless, the adverse effects of high vitamin B12 is the subject of debate (with respect to both intake and level), and therefore, it can be argued that setting a maximum upper intake level for vitamin B12 (from supplements) is preferable in order to avoid the excessive intake of this micronutrient.

CONCLUSION

To conclude, the community-dwelling older population with obesity or excess body weight is at increased risk of deficiency in several micronutrients including vitamin D, vitamin B12 and folate. Those with obesity may need a higher intake of micronutrients. However, considering the potentially adverse effects of B-vitamins and the risks with respect to health outcomes, including more acute life-threatening ones such as cancer, dietary supplements may not always be beneficial to health in this population. Consequently, I do not recommend using B-vitamin supplementation for older persons as a population group, and micronutrients should preferably be obtained from a balanced diet. On the basis of the findings of this thesis it is suggested that supplements are recommended in case of proven deficiency but, even then, not in excessive amounts. The optimum dosage for supplementation needs to be further investigated. Furthermore, the use of micronutrients should be monitored carefully and the potential risks and adverse effects of dietary supplements should be investigated thoroughly. Possible nutrient-drug interactions also need to be explored in greater depth.

Finally, due to the impact of "frailty", *i.e.*, the combined effect of several chronic disabling and degenerating phenomena in elderly, in a community-dwelling older population, future interventions should focus on addressing factors that may accelerate the ageing process and exacerbate nutritional status, as described in the introduction. Such factors include quality of nutrition and improving physical activity to prevent frailty in the ageing population. Good quality of nutrition includes simply following the official guidelines for healthy nutrition and an achievable, personalized physical activity/exercise regime that suits the daily life of the ageing population under the supervision of a dietician.

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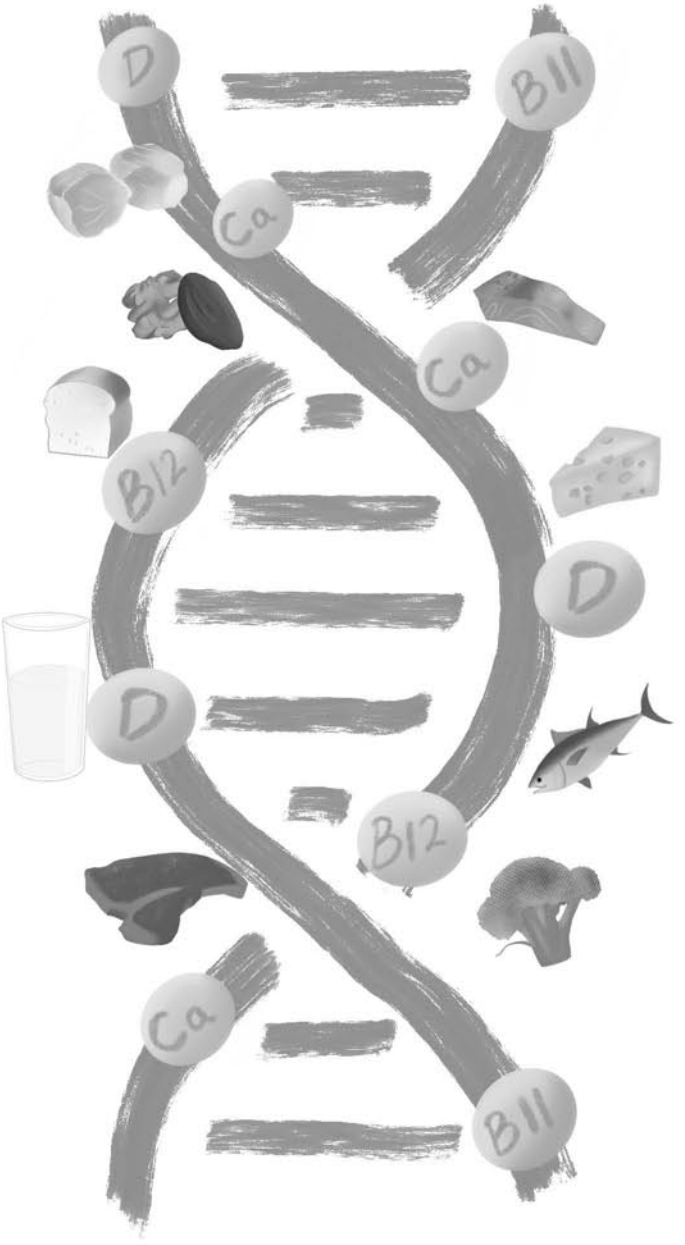
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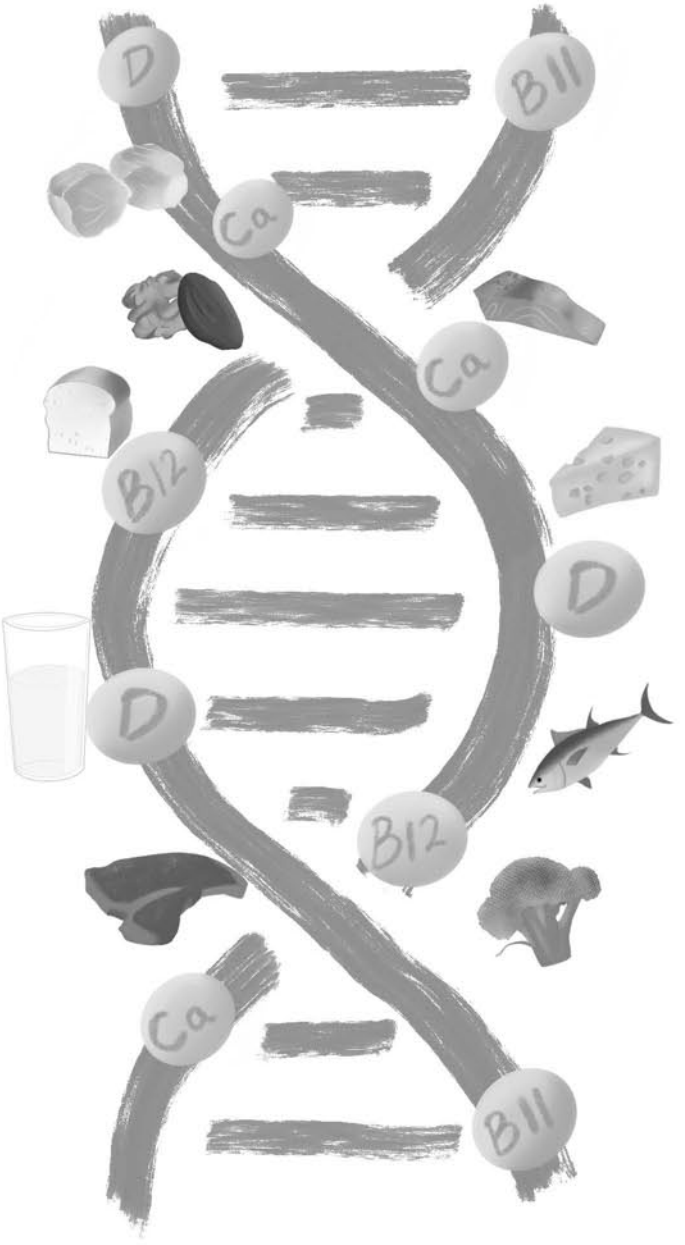
APPENDICES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Summary

Nederlandse samenvatting

SUMMARY

The growing interest in the role of micronutrients in optimizing health and prevention of diseases results in increasing in use of dietary supplements. Ageing is associated with higher risk on fracture, coronary heart disease, stroke, cancer and frailty. However, there is limited evidence to evaluate the role of the micronutrients in health and disease among older adults. Common used micronutrients are B-vitamins, calcium and vitamin D. Consequently, it is essential to identify the role of these micronutrients on the most common diseases in the ageing population. In this thesis we addressed the effect of these micronutrients on body composition, fractures, cardiovascular diseases and cancer. Furthermore, the role of the interaction of calcium and vitamin D in the association between diuretics and bone health, and the interaction of SNPs in the association between calcium and colorectal cancer is described in this thesis. Chapter 1 provides a general introduction on B-vitamins in the one carbon mechanism, calcium and vitamin D, the role of these micronutrients on health and disease and a description of the studies based in this thesis.

In all age categories, prevalence of overweight is growing, including the older population (1). Overweight has been associated with several diseases, such as increased risk of coronary heart disease, type 2 diabetes, strokes and certain types of cancers (2, 3). In chapter 2, we examined the association between vitamin D, B-vitamins and body composition in the B-PROOF study. We observed that a higher BMI and fat percentage were associated with lower serum 25(OH)D levels in older population. Also that overweight persons and persons with a higher fat percentage were at risk of vitamin D insufficiency. B-vitamins were associated with body composition, however, we found no effect of the B-vitamins intervention on body composition. This suggests that B-vitamins may not have a role in the etiology of obesity or changes in the body composition in this population.

In chapter 3 we showed that treating older persons with moderately increased homocysteine concentrations with combined folic acid and vitamin B12 supplementation was associated with a mild excess risk of overall cancer and statistically significant increased risk for colorectal cancer when compared with control group. This effect was even more pronounced in compliant participants. Furthermore, we assessed the long-term effect of micronutrients on other disease outcomes and found neither an effect on fracture risk, nor on cardiovascular disease in older individuals in the extended follow-up study of the B-PROOF trial. Individuals with high total homocysteine concentrations, probably with a higher chance of deficiency of B vitamins, might have

a decrease risk of fracture after supplementation. However, due to the low number of cases in the stratified analysis these findings should be interpreted with caution.

The interplay of micronutrients, SNPs and drug on diseases was evaluated in chapter 4. A positive association was shown between thiazide use (past use and current use) and LS-BMD and no association was found between thiazide use and LS-TBS. The associations were seen in participants older than 65 years old. In contrast to the thiazide use, we showed a decrease in LS-TBS if LD use was for a longer period and an increase in LS-BMD if LD use was for a short time period. Hence, our study prevents any strong conclusion on the impact of LD-use on bone health. Moreover, we found no consistently modified effect by vitamin D or dietary calcium intake in the association between LD-use and indices of bone health.

Finally we evaluated the association between calcium (intake and level) and colorectal cancer with the interplay of SNPs for calcium level in these associations. Dietary calcium intake higher than 1.485 mg/day compared to average calcium intake (1.100-1.485 mg/day) seems to be protective for CRC. However, after adjusting in a subgroup for albumin, we found an increased risk of CRC for dietary calcium intake. The weighted genetic risk score (GRS) from the calcium level SNPs were significant in the interaction analysis between calcium intake and calcium level and CRC risk. After stratification, we observed a lower CRC risk for subjects with a lower GRS. Overall, calcium homeostasis could contribute in other disease processes, i.e. neoplasms, and since other factors could play a role in calcium homeostasis and CRC risk which should investigated in the future.

Based on the results of this thesis, dietary supplementation is recommended for people with proven deficiencies and not above recommended daily doses. The optimal dose for dietary supplementation should be further investigated. Furthermore, the use of micronutrients should be monitored for the potential risks. Negative effects of supplementation should be investigated in the future. Also, the interaction between nutrition and medication is recommended to study thoroughly.

NEDERLANDSE SAMENVATTING

Door de grote interesse in de rol van micronutriënten bij het verbeteren van gezondheid en het voorkomen van ziektes, is het gebruik van voedingssupplementen toegenomen in de laatste jaren. De meest gebruikte micronutriënten zijn B-vitaminen, calcium en vitamine D.Veroudering is geassocieerd met een hoger risico op botbreuken, hart- en vaatziekten, kanker en andere problemen bij kwetsbare ouderen. Uit de geringe bewijzen uit de wetenschap is het lastig om de rol van micronutriënten bij gezondheid en ziekte(-ontwikkeling) van ouderen vast te Hierdoor is het belangrijk om de rol van micronutriënten (specifiek B-vitaminen, calcium en vitamine D) op de meest voorkomende ziektes bij ouderen te onderzoeken. In dit proefschrift behandelen we het effect van deze micronutriënten op lichaamssamenstelling, botbreuken, hart- en vaatziekten en kanker. Daarnaast, beschrijven we de rol van de interacties van calcium en vitamine D in de associatie tussen diuretica en botgezondheid, en de interactie van de SNPs in de associatie tussen calcium en colorectaal kanker. **Hoofdstuk 1** bevat een algemene introductie over B-vitaminen in het 'one-carbon' mechanisme, calcium en vitamine D. Daarnaast gaat hoofdstuk 1 nader in op de rol van deze micronutriënten op gezondheid en ziekten, en wordt een beschrijving gegeven van de studies die in dit proefschrift aan de orde komen.

In alle leeftijdscategorieën, zien we overgewicht toenemen, zo, ook bij ouderen. Overgewicht is geassocieerd met ziekten zoals een verhoogd risico op hart- en vaatziekten, type 2 diabetes, beroerte en sommige kankersoorten. In **hoofdstuk 2** hebben we de associatie tussen vitamine D en B-vitaminen en het lichaamssamenstelling onderzocht in de B-PROOF studie. In de populatie van de B-PROOF studie, vonden we dat een hogere BMI en een hoger vetpercentage geassocieerd zijn met lagere 25(OH) D waarden. Daarnaast zagen we dat mensen met overgewicht en een hoog vetpercentage mogelijk vitamine D insufficiënt waren. Overigens vonden we dat B-vitaminen geassocieerd waren met lichaamssamenstelling, maar we vonden geen effect van de interventie met de B-vitaminen op lichaamssamenstelling. Dit suggereert dat B-vitaminen mogelijk geen rol hebben in de etiologie van obesitas of veranderingen in lichaamssamenstelling van personen binnen deze populatie.

In **hoofdstuk 3** hebben we laten zien dat de oudere populatie met een mild verhoogde homocysteïne concentratie, die foliumzuur en vitamine B12 suppletie hadden gekregen (de interventie groep), een mild verhoogd risico hadden op ontwikkeling van alle kankersoorten en een statistisch significant verhoogde kans op colorectaal kanker, vergeleken met de controle groep. Dit effect was zelfs meer aanwezig bij trouwe deelnemers, die de suppletie bijna elke dag innamen. Verder hebben we het lange

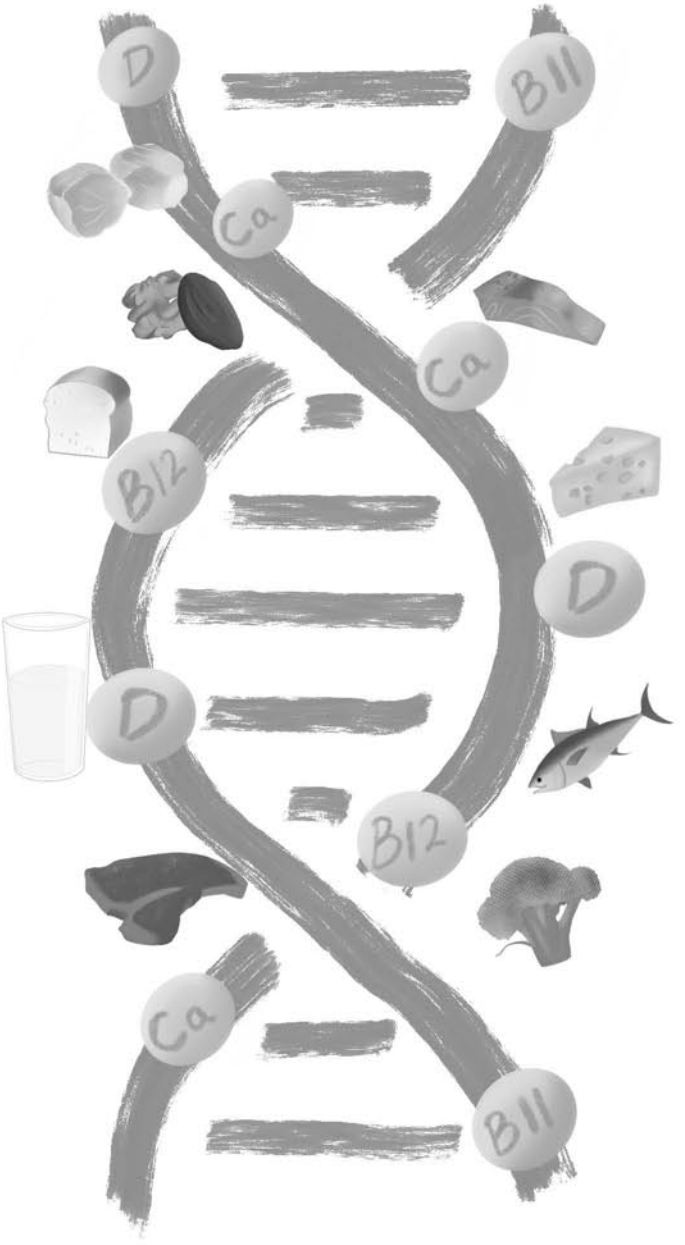
termijn effect van micronutriënten op andere ziekte-uitkomsten onderzocht. In de verlengde follow-up van de B-PROOF studie vonden we geen effect van de interventie op zowel botbreuken als op hart- en vaatziekten binnen de oudere populatie. In de groep ouderen met verhoogde homocysteïne concentratie (>15.1 mmol/l), die waarschijnlijk een hogere kans hebben op B-vitamine deficiëntie, vonden we dat suppletie risico op botbreuken mogelijk vermindert. Echter, door het lage aantal botbreuken in de gestratificeerde analyse, moeten deze bevindingen voorzichtig geïnterpreteerd worden.

De wisselwerking tussen micronutriënten, SNPs en medicatie op ziektes is geëvalueerd in **hoofdstuk 4**. Een positieve associatie tussen het gebruik van thiazide medicatie (zowel gebruik in het verleden als het huidig gebruik) en LS-BMD was gevonden. Daarnaast is er geen associatie gevonden tussen thiazide gebruik en LS-TBS. Deze associaties werden gezien bij participanten ouder dan 65 jaar. In tegenstelling tot thiazide gebruik, hebben we een verlaging in LS-TBS laten zien voor een andere diuretica, namelijk lis diuretica (LD), als deze voor een langere periode gebruikt was. Daarentegen, zagen we een verhoging van LS-BMD indien LD voor een kortere periode werd gebruikt. Daarom kunnen we met onze studie geen sterke conclusies trekken over de invloed van LD gebruik op botgezondheid. Daarnaast hebben we geen consequent effect modificatie van vitamine D, of inname van calcium gevonden in de associatie tussen LD gebruik en diverse metingen van botgezondheid.

Als laatst in hoofdstuk 4 hebben we de associatie tussen calcium (gemeten naar inname en via bloedwaarde) en colorectaal kanker geëvalueerd, rekening houdend met de wisselwerking van SNPs (die belangrijk zijn voor calcium in het bloed) in deze associatie. Hierin vonden we dat calcium inname uit voeding hoger dan 1.485 mg/dag mogelijk beschermend werkt tegen colorectaal kanker, dit vergeleken met de gemiddelde calcium inname (1.100-1.485 mg/dag). Echter, na de correctie voor de albumine waarden in een subgroep analyse, vonden we juist een verhoogd risico op CRC bij een hogere calcium inname uit de voeding. De gewogen genetische risico score van de 7 SNPs voor calcium waarden waren significant in de interactie analyse tussen calcium inname, calcium uit het bloed en CRC risico. Daarin vonden we een lager CRC risico bij mensen met een lagere GRS. Het lijkt dat er andere factoren, die een rol spelen in de calcium homeostase, betrokken zijn bij de associatie tussen calcium en het risico op colorectaal kanker. Dit vraagt om nader onderzoek.

Op basis van de bevindingen van dit proefschrift, wordt het innemen van voedingssupplementen enkel aangeraden bij een bewezen deficiëntie worden gebruikt, en bovendien niet in grote hoeveelheden. De optimale dosis voor voedingssupplementen

zou nog onderzocht moeten worden. Daarnaast dient het gebruik van micronutriënten nauw gemonitord te worden. Het potentiële risico en de negatieve effecten van voedingssuppletie moeten nauwkeuriger onderzocht worden. Dit geldt ook voor de mogelijke voeding-medicatie interactie.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Publications and manuscript

PhD portfolio

PUBLICATIONS AND MANUSCRIPTS

1. Oliai Araghi S, Kiefte-de Jong JC, van Dijk SC, Swart KMA et al. Long-term effects of folic acid and vitamin-B12 supplementation on fracture risk and cardiovascular disease: extended follow-up of the B-PROOF Trial. Clin. Nutr. 2020 Aug 5;S0261-5614(20)30398-8.
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PHD PORTFOLIO

Name PhD student: Sadaf Oliai Araghi Erasmus MC Department: Internal Medicine & Epidemiology Research School: NIHES/MolMed	PhD period: 2016 - 2019 Promotor(s): Prof. AG. Uitterlinden & Prof. B. Stricker, Jessica Kiefte & Nathalie van der Velde	
1. PhD training		
	Year	**Workload (Hours/ECTS)**
General courses		
Vena Punction	2012	2
Systematic Literature Research	2015	2.5
Biomedical English Writing and Communication	2015	4
Research Integrity	2018	0.3
BROK ('Basiscursus Regelgeving Klinisch Onderzoek')	2015	1
Specific courses		
NIHES Biostatistics for Clinicians	2015	2
NIHES Regression Analysis for Clinicians	2016	0.7
NIHES Pharmaco-epidemiology	2017	0.7
Molmed Basic course on R	2018	1.8
NIHES Joint Models for Longitudinal and Survival Data	2019	0.7
Seminars and workshops		
EMC seminars & Epi-IPCI & Nutrition & Lifestyle meetings		1
Journal clubs & 2020 meetings		1
Presentations		
Dutch Nutritional Science Days – Oral presentation on Vitamin D and Body Composition	2014	2
WEON – Poster Presentation on Vitamin B12/FZ and Cancer (ERGO)	2016	1
Dutch Nutritional Science Days – Oral presentation on Vitamin B12/FZ and Cancer (B-PROOF)	2017	2
Dutch Nutritional Science Days – Oral presentation on LD & Bone health (ERGO)	2018	2
Nutrition 2019 – Baltimore – Poster Presentation on Vitamin B12/FZ and Fractures/CVD (FU B-PROOF)	2019	2
Dutch Nutritional Science Days – Oral presentation on Vitamin B12/FZ and Fractures/CVD (FU B-PROOF)	2019	2
(Inter)national conferences		
Geriatrie dagen	2014	1
Dutch Nutritional Science Days	2014	1
WEON	2017	1
NAV public lecture, Driebergen-Zeist	2017	1
Dutch Nutritional Science Days	2018	1
Dutch Nutritional Science Days	2019	1
Nutrition 2019 - Baltimore	2019	1
Dutch Nutritional Science Days		
Supervising Master's theses	2018-2019	2

Other		
Reviewer of international peer-reviewed journal: Nutrition and BMJ open		1
Awards		
Third Price for 'Fop ten Hoor Prize' for Nutrition Science Days, The Netherlands	2017	
Firtst Price for 'Publication Prize' for Nederlands Academie van Voedingswetenschappen	2019	
TOTAL		38,7

ABOUT THE AUTHOR

Sadaf was born in 1982 in Arak in Iran. She left Iran when she was 12 years old and moved to the Netherlands. After learning Dutch she finished high school and started with the study of 'Voeding & Diëtetiek' at The Hague University of Applied Sciences in 2003. She obtained her degree in 2007 and continued with the pre-master and master program 'Nutrition in Health and Disease' at the VU University, which she finished in 2009. She worked from 2009 till 2016 as dietitian in diverse practices in The Hague and Rotterdam. From 2010 on she started at Erasmus MC as a research assistant for the B-PROOF trial (a multi-center study with Wageningen University and VU University Medical Center), helping with the inclusion of participants and other practical work. In the meantime she wrote her first published paper. After the end of the B-PROOF trial, Sadaf worked between 2013 and 2014 at the department of Neuroscience and Surgery, where she worked together with colleagues on systematic reviews on music intervention in surgery.

In 2015 she started a PhD project as a part-time job at the department of Internal Medicine at Erasmus MC on micronutrients in health and diseases. Sadaf worked mainly on the follow-up study of the B-PROOF study, and the Rotterdam Study (a large prospective cohort study in Rotterdam Ommoord). Since April 2020, Sadaf works as trial coordinator at the Flevoziekenhuis, department of Internal Medicine-Oncology.

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چند قبا بر قد دل دوختم
چند چراغ خرد افروختم

پیر فلک را که قراریش نیست
گردش بس بوتلعجب آموختم

گنج کرم آمد مهمان من
وام فقیران ز کرم توختم

حاصل از این سه سخنم بیش نیست
سوختم و سوختم و سوختم

بر مثل شمعم من پاکباز
ریختم آن دخل که اندوختم

بس که بسی نکته عیسی جان
در دل و در گوش خر اسپوختم

بس که اذا تم دنا نقصه
تا بنگوید صنم شوخ تم

مولوی