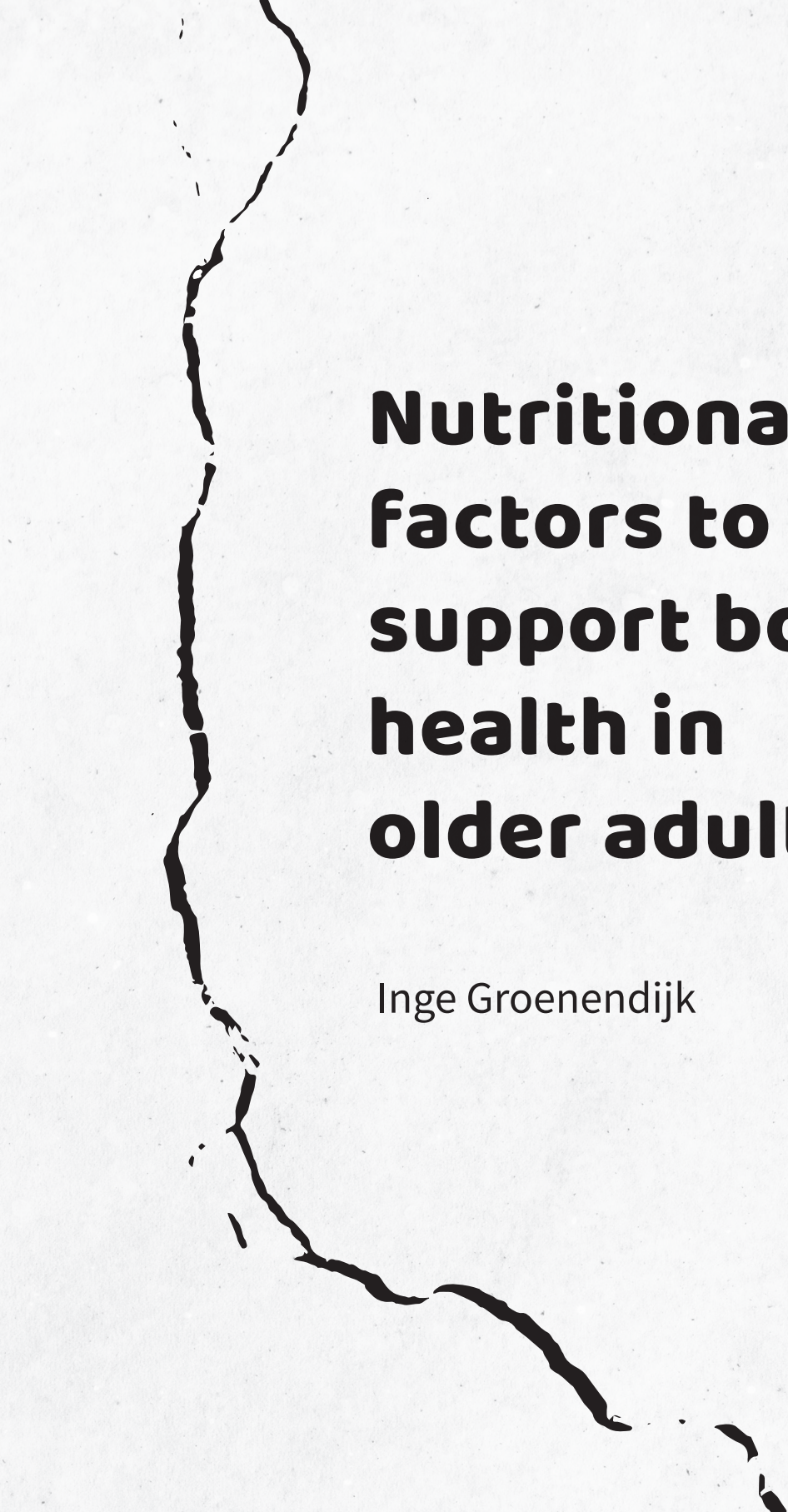

Nutritional factors to support bone health in older adults

Inge Groenendijk

PROPOSITIONS

1. Increasing calcium and protein intakes via the use of dairy products is an effective strategy to reduce the risk of falls and fractures in older adults. (this thesis)

2. Conducting research on bone health with only women is incorrect both scientifically and from a public health perspective. (this thesis)

3. Dancing is an undervalued nonpharmacological strategy to improve cognitive function in people with cognitive impairment.

4. Every statistical analysis should be performed independently by 2 researchers.

5. A key component of a successful PhD trajectory is knowing when to take a break.

6. The policy[1] to take the train if the journey time is within 6-8 hours is insufficient to reduce aviation emissions.

Propositions belonging to the thesis, entitled

Nutritional factors to support bone health in older adults

Inge Groenendijk
Wageningen, 27 June 2023

[1] Travel policy Wageningen University & Research. Accessed March 3, 2023. https://www.wur.nl/en/about-wur/our-values/sustainability/from-frequent-flying..-to-digital-first.htm

Nutritional factors to support bone health in older adults

Inge Groenendijk

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This research was conducted under the auspices of VLAG Graduate School (Bio-based, Biomolecular, Chemical, Food, and Nutrition sciences).

Nutritional factors to support bone health in older adults

Inge Groenendijk

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If you don't like how the table is set, turn over the table.

Frank Underwood

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Chapter 1

General introduction

THE AGEING BONE

The bones of the skeleton provide structural support for the body, protect vital organs and act as a storage site for minerals (such as calcium) [1]. Bone is a living tissue that changes over the course of a lifetime. From birth to adulthood, there is bone modeling in which bone resorption and bone formation may differ, leading to a gain or loss of skeletal bone mass and changes in skeletal form [2]. Peak bone mass is achieved around your mid-twenties [3]. In the adult skeleton, bone remodeling takes place in which there is replacement of old tissue by new bone tissue to maintain bone mass [2].

However, when getting older, bone mass decreases and bone will be more porous. If too much bone mass is lost, this leads to the development of osteoporosis. Osteoporosis is a chronic disease characterized by low bone mass and deterioration of bone microarchitecture [4]. It increases the risk of falls and fractures, which in turn leads to an increase in morbidity and mortality, loss of independence, and a decreased quality of life [5]. In Europe, the prevalence of osteoporosis in adults aged ≥50 years was estimated at 22.5% in women and 6.8% in men [5]. In addition, the risk of getting a hip fracture is estimated at 9.8 to 22.8% in women and 6.1 to 13.7% in men aged ≥50 years [5]. Hip fractures are associated with high morbidity and mortality. Osteoporosis develops more often in women than in men. This is mainly caused by the steep decline in estrogen levels after menopause, since this hormone inhibits bone resorption and stimulates bone formation [6]. Furthermore, men generally achieve a higher peak bone mass than women do.

BONE HEALTH ASSESSMENT

Bone health can be evaluated in various ways, namely via measuring bone mineral content (BMC), bone mineral density (BMD), fracture risk and bone turnover markers. BMC (g) is the amount of bone mineral in a specific area, while BMD (g/cm^2) reflects the amount of bone mineral in bone tissue and is derived by dividing BMC by the area (cm^2). These two outcomes can be assessed with a Dual-Energy X-Ray Absorptiometry (DXA) scan, which is regarded as the ''gold standard'' for diagnosing osteoporosis. From a DXA scan, a T-score for BMD assessed at the femoral neck can be derived, which reflects how many standard deviations (SD) the BMD differs from the mean BMD of a young adult [4]. A T-score ≤ -2.5 SD reflects osteoporosis, a T-score between -1 and -2.5 indicate osteopenia (stage before osteoporosis) [4].

Quantitative ultrasound (QUS) performed at the calcaneus is a convenient and practical approach to screen for osteoporosis. QUS provides an indication of both bone microarchitecture and BMD [7] by sending ultrasonic waves through the calcaneus. It has been shown that calcaneal QUS can predict incident fractures for both genders [8, 9]. However, QUS parameters may not change fast enough to monitor treatment effects.

Osteoporosis is one of the risk factors for fractures. Other factors that play a role are age, sex, low BMI, a previous fracture, a parent with osteoporosis or a hip fracture, smoking, excessive alcohol intake, steroid use, having rheumatoid arthritis or having secondary osteoporosis [10, 11]. These factors can jointly predict the 10-year probability of a major osteoporotic fracture using the risk assessment algorithm FRAX® [11].

Lastly, blood derived bone turnover markers may be used to reflect the metabolic activity of bone and can be divided in blood markers for bone formation and bone resorption [12]. Such markers of bone formation are produced by osteoblasts (cells responsible for bone matrix synthesis and its subsequent mineralization), while during the process of resorption of mineralized tissue by osteoclasts, resorption markers are present. Several bone turnover markers can be measured, each expressed during various phases and reflecting a different aspect of osteoblast or osteoclast function. For the clinical utility of bone turnover markers and the ability to pool studies more easily, procollagen type 1 N propeptide (P1NP) and C-terminal telopeptide of type I collagen (CTX) are recommended as the reference markers for bone formation and resorption, respectively [12].

PREVENTION AND TREATMENT OF OSTEOPOROSIS

Osteoporosis medication is effective in reducing fracture risk, but the occurrence of adverse events is also common [13]. This leads to low adherence to medication use; it has been shown that approximately half of the patients fail to comply with adhering to drugs prescriptions for osteoporosis within one year [14]. The non-pharmaceutical approach for both prevention as treatment of osteoporosis includes nutrition and exercise. The lifestyle and dietary guidelines for prevention of osteoporosis and fractures differ between countries. In 2022, the guidelines in the Netherlands were updated (**Box 1**) and these were in line with the guidance provided by the International Osteoporosis Foundation (IOF) and the European Society for Clinical and Economic Evaluation of Osteoporosis and Osteoarthritis (ESCEO) [10]. In short, an adequate vitamin D and calcium

status should be warranted, it is advised not to smoke and abstain from heavy drinking, and to perform regular (preferably weight-bearing) physical activity. It has been well established that consumption of sufficient calcium and vitamin D are required for proper bone health. Relatively high combined intakes of calcium and vitamin D may lead to a modest fracture risk reduction [15]. Physical activity is especially important to achieve a high peak bone mass. In later life, a combined resistance and impact exercise training program has been suggested to be the most effective for maintenance of BMD [16].

Box 1. Lifestyle and dietary guidelines for prevention of osteoporosis and fractures in the Netherlands, retrieved from [17].

- Daily 20 µg (=800 IU) vitamin D supplementation
- Calcium intake of 1000-1100 mg through the diet. If it is not possible through the diet:
  - 1000 mg/d supplementation when less than 2 standardized dairy products are consumed
  - 500 mg/d supplementation when 2-3 standardized dairy products are consumed
- Healthy and varied diet with sufficient dairy, vegetables, nuts, and fruit
- No smoking
- No or moderate alcohol consumption
- Engage in moderate-intensity physical activity for at least 2.5 hours a week including muscle- and bone-strengthening activities and for patients with osteopenia or osteoporosis: weight-bearing exercises of at least 2.5 hours per week, consisting of a combination of balance training plus training of muscle strength, mobility, and posture.

THE ROLE OF NUTRITION

Besides calcium and vitamin D, there are more nutrients that may play an important role in maintaining and improving bone health in later life. The most promising but not yet verified nutrient is protein. Protein-rich foods include meat, fish, milk(products), cheese, eggs, legumes and nuts. While the Recommended Dietary Allowance (RDA) for protein intake is 0.8 g protein/kg body weight/day [18], the ESPEN Expert Group recommends at least 1.0-1.2 g protein/kg body weight/day for healthy older adults and 1.2-1.5 g protein/kg body weight/day for older adults with an acute or chronic illness [19]. This recommendation is based

on evidence regarding preservation of muscle health. How this translates to bone health is not known and recommendations are ambiguous [10, 20]. Furthermore, it is suggested to avoid protein intakes >1.3 g/kg body weight/day in adults with CKD at risk of progression [21]. There are several mechanisms through which protein may impact bone health (**Figure 1**): protein can upregulate insulin-like growth factor 1 (IGF-1), improve intestinal calcium absorption and maintain muscle mass and strength [22]. A high protein intake also indirectly suppresses parathyroid hormone (PTH), which reduces bone resorption and, thereby, may improve bone density [22].

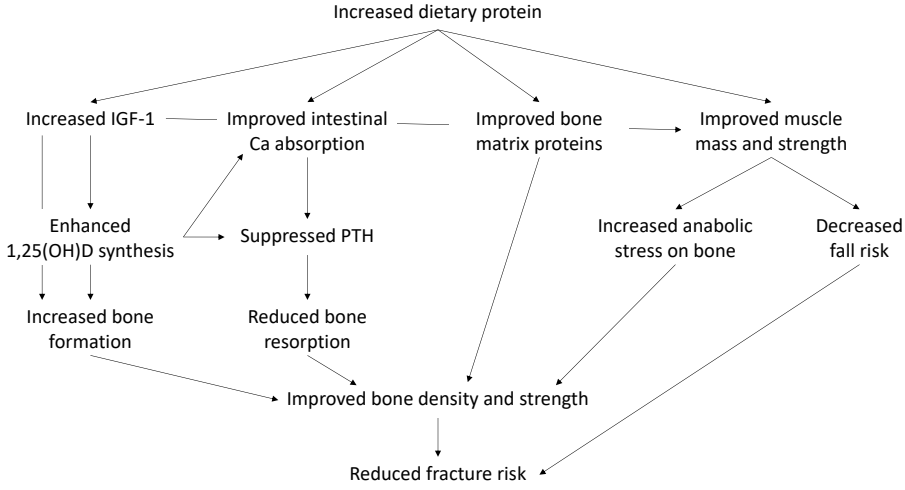

Figure 1. Proposed mechanisms of how increased dietary protein intake may improve bone health. Adapted from [22]. Abbreviations: IGF-1 = insulin like growth factor; PTH = parathyroid hormone; 1,25(OH)D = 1,25-dihydroxyvitamin D. Adapted from [22].

Another nutrient that plays a role in bone health is magnesium (**Figure 2**). Of the total magnesium content in the body, 50-60% is found in bones [23]. Magnesium is a co-factor for enzymes that play key roles in synthesis of the bone matrix [24], and it stimulates osteoblast proliferation [23]. A magnesium deficiency can result in larger hydroxyapatite crystals, which lowers bone stiffness [23]. In addition, magnesium deficiency increases the secretion of proinflammatory cytokines, which stimulate osteoclast activity, and lower 1,25-dihydroxycholecalciferol levels. Older adults may be at risk of magnesium deficiency due to a low dietary magnesium intake, decreased intestinal magnesium absorption or increased urinary excretion (e.g. reduced kidney function, diuretics, proton pump inhibitors) of magnesium [25]. Magnesium-rich foods include green leafy vegetables,

legumes, nuts, seeds and whole grains. An adequate magnesium intake is defined as 350 mg/day for adult men and 300 mg/day for adult women [26].

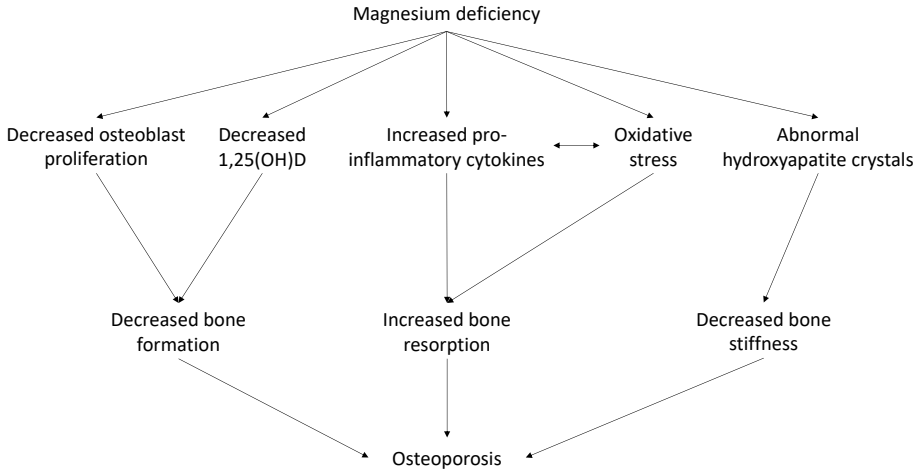

Figure 2. Proposed mechanisms of how a magnesium deficiency may compromise bone health. Abbreviations: 1,25(OH)D = 1,25-dihydroxyvitamin D.

An interesting food group for bone health is dairy. Since dairy products are rich in calcium and protein, intake of these products may be a good dietary strategy to support bone health. Observational studies in older adults have shown that the intake of dairy products are associated with a lower hip fracture risk, varying from 13 to 32% [27]. No controlled intervention trials with fractures as outcome parameters have been performed until recently. Dairy is also rich in vitamin B-12 and a low vitamin B-12 status has been associated with a low bone mineral density (BMD), increased bone turnover, and increased fracture risk [28, 29].

THESIS OUTLINE

Osteoporosis and osteoporotic fractures represent an increasing public health problem. The potential impact of nutritional strategies to delay or prevent the development of osteoporosis and, as such, support bone health remains understudied. The aim of this thesis is to investigate the role of nutritional factors in supporting bone health of older adults. The thesis is divided into three parts. In the first part, the focus lies on the role of dietary protein consumption in bone health. In the second part, other nutritional factors (magnesium, dairy and forti-

fied milk) which may also influence bone health are addressed. In the last part, insights emerging from studies in hip fracture patients are presented.

In **Chapter 2.1**, a systematic review and meta-analysis is presented on the impact of a dietary protein intake above the RDA of 0.8 g/kg body weight/day on multiple bone health outcomes in older adults compared to a lower dietary protein intake. In **Chapter 2.2**, individual data from four studies that included either (pre-)frail, undernourished or healthy older adults were combined to investigate the association between dietary protein intake (total, plant and animal) and BMD (spine and total body). In addition, the effects of protein supplementation on BMD were assessed. **Chapter 2.3** uncovers the effect of protein supplementation, alongside resistance exercise training, on BMD in a more vulnerable and clinical population, namely prostate cancer patients on androgen deprivation therapy. In a second systematic review and meta-analysis, the impact of magnesium on bone health in older adults is investigated (**Chapter 3.1**). In **Chapter 3.2,** results from a large intervention study in Australia are translated to the Dutch population to answer the question if increasing the intake of dairy products is beneficial for the bone health of aged care residents in the Netherlands. **Chapter 3.3** explores the effects of a combined nutrition (fortified milk supplement containing protein, calcium, cholecalciferol, and vitamin B-12) and exercise intervention on serum vitamin B-12 and 25-hydroxyvitamin D, bone turnover markers, and PTH concentrations in healthy Chinese middle-aged and older adults. **Chapter 4.1** assesses the nutritional status, dietary intake and muscle health of older Dutch hip fracture patients in geriatric rehabilitation wards. **Chapter 4.2** investigates the association between protein intake with bone markers, calcaneal QUS and BMD in older patients recovering from a hip fracture and investigates the association between nutritional status with QUS and BMD. Additionally, the change of bone turnover markers from post-surgery till 3 months is investigated. Finally, **in Chapter 5**, the findings of this thesis are discussed and directions for future research are proposed.

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Chapter 2

The role of protein in bone health

Chapter 2.1

High versus low dietary protein intake and bone health in older adults: a systematic review and meta-analysis

Inge Groenendijk, Laura den Boeft, Luc J.C. van Loon and Lisette C.P.G.M. de Groot

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ABSTRACT

Protein may play a beneficial role in the prevention of bone loss and in slowing down osteoporosis. The effect of dietary protein may be different in older adults compared to younger adults, since this population has a greater need for protein. The aim of this systematic review and meta-analysis was to investigate the impact of a dietary protein intake above the Recommended Dietary Allowance (RDA) of 0.8 g/kg body weight/day from any source on Bone Mineral Density (BMD)/Bone Mineral Content (BMC), bone turnover markers, and fracture risk in older adults compared to a lower dietary protein intake. A systematic search was conducted through October 2018 in 3 databases: CENTRAL, MEDLINE, and EMBASE. We included all prospective cohort studies and Randomized Controlled Trials (RCTs) among adults aged ≥65 years that examined the relation between protein intake on bone health outcomes. Two investigators independently conducted abstract and full-text screenings, data extractions, and risk of bias assessments. Authors were contacted for missing data. After screening of 523 records, twelve cohort studies and one RCT were included. Qualitative evaluation showed a positive trend between higher protein intakes and higher femoral neck and total hip BMD. Meta-analysis of four cohort studies showed that higher protein intakes resulted in a significant decrease in hip fractures (pooled hazard ratio: 0.89; 95% confidence interval: 0.84, 0.94). This systematic review supports that a protein intake above the current RDA may reduce hip fracture risk and may play a beneficial role in BMD maintenance and loss in older adults.

INTRODUCTION

Osteoporosis is an increasing public health problem worldwide [1]. The prevalence in nine industrialized countries is estimated at 9–38% for women and 1–8% for men, affecting up to 49 million people [2]. The rising prevalence of osteoporosis leads to an increase in the number of falls and fractures, which in turn affects mortality and morbidity, and increases the economic burden [1]. Protein may play a role in the prevention of bone loss and in slowing down osteoporosis [3].

An adequate intake of dietary protein is important for bone acquisition and maintenance. Older adults may become protein malnourished due to an inadequate intake of protein and a reduced ability to use available protein, because of age-related changes in metabolism, immunity, and hormone levels and sensitivity [4, 5]. At the same time there is a greater need for protein [5]. The current recommended dietary allowance (RDA) for protein is 0.8 g/kg body weight/day [6]. For the preservation of muscle function, evidence supports a protein intake of 1.0–1.2 for healthy older adults and 1.2–1.5 g/kg body weight/day for older adults suffering from acute or chronic illnesses [7].

Over the past years, the relation between dietary protein intake and bone health has received much attention. Safety concerns of a high dietary protein intake have been raised, but beneficial effects on bone health have also been found. As yet, several systematic reviews and meta-analyses [8-11] have been published investigating the effect of dietary protein intake on bone health. All of these publications pooled cohort studies and trials over a wide age range with specific exclusion criteria to find beneficial effects of protein intake on Bone Mineral Density (BMD), Bone Mineral Content (BMC), bone turnover markers, and/ or fracture risk. Some reviews include trials that have an intervention duration of <6 months [8, 10]. However, it is questionable if dietary interventions can already lead to a measurable change in BMD within such a short time span. To get a reliable estimate of the impact on changes in BMD, a minimum intervention duration of 6 months seems appropriate [12].

An expert consensus paper from 2018 summarized the systematic reviews and meta-analyses looking at the effects of dietary protein on bone health in adults [3]. It states that protein intakes above the RDA, in combination with an adequate calcium intake, is associated with higher BMD, a lower rate of bone loss, and a modestly reduced fracture risk. Furthermore, it was not proven that the acid load caused by a high protein intake is harmful for bone health.

None of the above reviews focused on older adults (age of 65 years and older) specifically. The effect of dietary protein may be different in older adults compared to adults, since this population has a greater need for protein. The aim of this systematic review and meta-analysis was to investigate the impact of a dietary protein intake above the RDA of 0.8 g/kg body weight/day from any source on BMD/BMC, bone turnover markers, and fracture risk in older adults compared to a lower dietary protein intake.

METHODS

The Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement is followed in reporting this systematic review [13].

Data Sources and Searches

A systematic search was conducted through October 29, 2018, in 3 databases: CENTRAL (http://www.thecochranelibrary.com), MEDLINE (https://www.ncbi.nlm.nih.gov/pubmed/), and EMBASE (https://www.ovid.com/). The searches were limited to the English language, and prospective cohort and human intervention studies that examined the relations of protein intake (food or supplemental sources) on bone health outcomes of interest. The search strategy per database is presented in **Supplementary Table A1**.

Study Eligibility Criteria

We included all prospective cohort studies and randomized controlled trials (RCTs) among older adults aged ≥65 years that examined the relation between protein intake from any source on several bone health outcomes (**Table 1**). Studies including both young and older individuals were still included if stratification was performed. Studies had to have an intervention duration of at least 6 months. Studies enrolling exclusively subjects with a diagnosed disease or where >20% of the baseline population was diagnosed with a disease were excluded. Furthermore, studies were excluded if they were designed to examine outcomes in response to protein type but not protein quantity and if protein was supplemented in the form of soy isoflavone. The reason for the latter is that these plant estrogens present in soy can independently have an effect on bone loss [14]. Also studies designed for weight loss were excluded.

Table 1. Bone health outcomes of interest.

Outcome	Sites or markers
BMC	Total body
BMD	Total body, total hip, femoral neck, lumbar spine
Bone turnover markers	Alkaline phosphatase, bone alkaline phosphatase, bone-specific alkaline phosphatase, collagen type I cross-linked C-terminal telopeptide, collagen type I cross-linked N-terminal telopeptide, C-terminal type 1 procollagen, N-terminal type 1 procollagen, deoxypyridinoline, hydroxyproline, pyridinoline, osteocalcin
Fracture	All sites

BMC = bone mineral content; BMD = bone mineral density.

Study Selection Process

First, citation duplicates across the 3 literature searches were removed. Second, titles were screened by a single investigator to exclude cross-sectional, animal, in vitro studies, and review articles. All abstracts were then independently screened by 2 investigators. When an abstract was regarded as potentially relevant, full-text articles were retrieved and independently screened by 2 investigators based on study eligibility criteria. All abstract and full-text articles screening conflicts were resolved through discussion between the 2 investigators and a final decision was made by the consensus of the entire research team.

Data Extraction

To capture data of interest from the eligible studies, a data extraction sheet was created in Excel. One investigator extracted the data from all studies, which was reviewed and confirmed by another investigator. The following items were extracted: study characteristics, baseline population characteristics, intervention details, relevant outcomes and their assessment methods, data details (including dropouts), confounders and effect modifiers used in statistical analysis, and results.

Risk of Bias in Individual Studies

The Newcastle-Ottawa Scale (NOS) was used to assess risk of bias of included prospective cohort studies [15]. For intervention studies, the Cochrane Collaboration's tool for assessing risk of bias was used to assess internal validity [16]. This tool addresses risk of selection bias, performance bias, detection bias, attrition bias, reporting bias, and other potential biases. Two investigators independently assessed the risk of bias in included studies. Disagreements were discussed among the research team and resolved via group consensus.

Data Synthesis

All included studies were summarized in narrative form and in tables. Items of the summary tables include study characteristics (first author, publication year, cohort name), participant characteristics, baseline mean age or age range, exposure assessment, follow-up period, and outcomes. Summary tables were organized by study type (cohort and RCT). Results were qualitatively and, if possible, quantitatively summarized by study type and outcome of interest. Meta-analysis was performed using R (v3.5.3; package meta) [17].

Qualitative Synthesis

Quality of the evidence was judged with the use of a grading system developed by the GRADE collaboration [18]. According to the GRADE approach, evidence was graded as 'High', 'Moderate', 'Low', or 'Very Low' depending on several criteria. Quality of evidence was downgraded based on five GRADE categories: risk of bias, imprecision, inconsistency, indirectness, and publication bias [19]. Quality was graded upwards when effects were sufficiently large, when all plausible biases would underestimate the effect, or when there was a dose-response gradient [20].

Quantitative Synthesis

If sufficient data were available and homogeneity in terms of participants, interventions and outcomes between studies were reasonable, a meta-analysis was conducted. Authors of relevant articles were contacted if required data were not reported. The methods described in the Cochrane Handbook for conducting meta-analyses was followed [16]. Results were pooled using a random-effects meta-analysis, with standardized mean differences for continuous outcomes and risk ratios (RR) or hazard ratios (HR) for binary outcomes. The extent of statistical heterogeneity was quantified using both the chi-squared test and the I-squared statistic. An I-squared value >50% was used as a threshold for indicating substantial statistical heterogeneity [16]. Random-effects model was used when studies were drawn from populations that differ from each other in such a way that it could influence the effect estimate. When heterogeneity was not present, a fixed-effect model was used. Sensitivity analyses were performed to explore the impact of excluding studies that were judged to be at high risk of bias and to detect the influence of a single study on the overall estimate.

RESULTS

The search yielded 659 citations and two additional articles [21, 22] were identified by searching in the reference lists of the other articles included in the analyses. After removal of 138 duplicates and exclusion of 53 articles because the title made clear that it was not a prospective cohort study or RCT, 470 articles were identified for dual abstract screening. A total number of 84 articles were identified for full-text screening, of which 13 were included for data extraction (12 prospective cohort studies and 1 RCT; **Figure 1**). The characteristics of the included studies are shown in **Table 2** and **Table 3**.

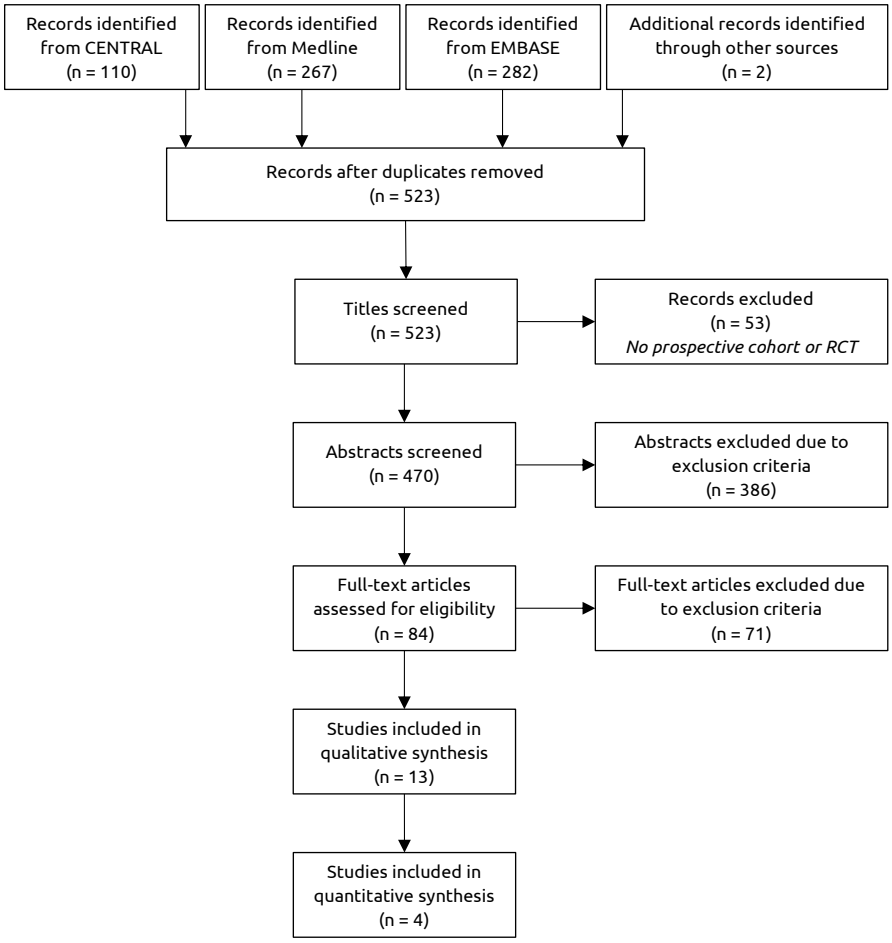

Figure 1. Flow diagram of study selection.

Table 2. Summary table of cohort studies included in the analysis.

First author, year [ref]	Cohort name (country)	Participants	N baseline/ analysed	Baseline mean age(SD) or age range (y)	Exposure assessment	Mean protein intake[a]	Follow-up (y)	Relevant outcomes	Effect sizes[b]
Beasley, 2014 [23]	Women's Health Initiative (US)	Post-menopausal women	161,808/ 144,580 (whole sample)	55-79; subgroups: 65 & 75	FFQ + calibrated with biomarkers	0.52, 0.75, 0.92, 1.11, 1.50 (quantiles, whole sample)	6	TB BMD	Per 20% increase in protein intake: 65 y: mean 0.003 (0.001, 0.005) ns 75 y: mean 0.003 (0.001, 0.007) ns
								Hip BMD	65 y: mean 0.003 (0.001, 0.005) ns 75 y: mean 0.004 (0.001, 0.007) ns
								Any fracture	65 y: HR 0.99 (0.96, 1.01) ns 75 y: HR 0.96 (0.91, 1.02) ns
								Hip fracture	65 y: HR 0.91 (0.82, 0.99) ns 75 y: HR 0.92 (0.83, 1.02) ns
Cauley, 2016 [24]	Osteoporotic Fractures in Men Study (US)	Men >65 y	5994/5876	No fracture: 73.5(5.8); fracture: 77.8(6.1)	Block semi-quantitative FFQ	No fracture: 16.1%; fracture: 15.3% of EI	8.6	Hip fracture	Per SD increase in protein intake (2.9% of EI): HR 0.82 (0.69, 0.97) p <0.05
Chan, 2011 [25]	- (China)	Men and women ≥65 y	2944/2217 (1225 men, 992 women)	Men: 71.6(4.6); women: 72.0(5.1)	FFQ	Men 88.8, women 65.7 g/d. Men 1.42; women 1.19 g/kg bw/d (estimated values)	4		Per unit increase in energy-adjusted protein intake:
								Hip BMD	Men: B -0.007 SE 0.005 p 0.147 Women: B 0.003 SE 0.009 p 0.744
								FN BMD	Men: B -0.013 SE 0.008 p 0.088 Women: B 0.010 SE 0.013 p 0.416
Dawson-Hughes, 2002 [26]	- (US)	Men and women ≥65 y	389/342	Supplemented group: 70(5), 71(4), 70(4); placebo group: 71(5), 71(5), 71(5) (tertiles)	Willett semi-quantitative FFQ	9.6-15.5, 15.5-18.2, 18.2-29.1% of EI (tertiles). Supplemented: 0.96, 1.07, 1.17; placebo: 0.90, 1.08, 1.20 g/kg bw/d (estimated values)	3		Supplemented group protein T3 vs T1:
								TB BMD	NR, less loss/gain p 0.042
								FN BMD	NR, less loss/gain p 0.011
								Spine BMD	NR, no difference ns
								Osteocalcin	NR, no difference ns
								N-telopeptide	NR, no difference ns
Devine, 2005 [27]	- (Australia)	Women >70 y	1077	75(3)	ACCV semi-quantitative FFQ	<0.84, 0.84-1.6, >1.6 (tertiles)	1	Hip BMD	Protein T3 vs T1: NR, higher p <0.05
								FN BMD	NR, higher p <0.05
Fung, 2017 [21]	Nurses' Health Study & Health Professionals Follow-Up Study (US)	Men ≥50 y and post-menopausal women	74,443 women; 35,439 men	Whole sample ≥50 y; stratification: <65, 65-75, ≥75	Semi-quantitative FFQ	Women 14.3, 18.6, 24.4% of EI; men 14.2, 18.3, 23.4% of EI (whole sample). Women 0.88, 1.12, 1.41; men 0.87, 1.12, 1.40 g/kg bw/d (estimated values)	32	Hip fracture	Protein Q5 vs Q1: Women 65-75 y: RR 0.92 (0.71, 1.18) Women 75+ y: RR 0.91 (0.69, 1.20) Men 65-75 y: RR 0.59 (0.33, 1.07) Men 75+ y: RR 0.77 (0.51, 1.15)
Hannan, 2000 [28]	Framingham Osteoporosis Study (US)	Men and women	855/615	75(4.4), 68-91	Willett semi-quantitative FFQ	0.21-0.71; 0.72-0.96; 0.97-1.23; 1.24-2.78 (quartiles)	4		Protein Q4 vs Q1:
								FN BMD	Mean -2.32(0.74)% vs -4.61(0.70)% p <0.001
								LS BMD	Mean -1.11(1.10)% vs -3.72(0.97)% p <0.05

Table 2. Continued

Isanejad, 2017 [29]	Osteoporosis Risk Factor and Fracture Prevention Study (Finland)	Women ≥65 y	750/544	68.1(1.9), 65-72	3 d food records	0.79, 0.90, 0.96, 1.18 (quartiles)	3	TB BMC	Per unit increase in energy-adjusted protein intake: B -0.16 SE 30.04 p 0.159
								TB BMD	B 0.04 SE 0.01 p 0.507
								FN BMD	B -0.01 SE 0.01 p 0.918
								LS BMD	B -0.31 SE 0.01 p 0.001
Langsetmo, 2017 [30]	Osteoporotic Fractures in Men Study (US)	Men ≥65 y	5994/5875	73.6(5.9)	Modified Block FFQ	0.67, 0.75, 0.83, 0.93 (quartiles)	10.5-11.2	Hip BMD	Per SD increase in protein intake (2.9% of EI): B 0.06 SE 0.01 p <0.001
								Spine fracture	HR 1.06 (0.92, 1.22) p 0.45
								Hip fracture	HR 0.84 (0.73, 0.95) p 0.01
Meng, 2009 [31]	- (Australia)	Women 70-85 y	1500/862	74.9(2.6)	ACCV quantitative FFQ	<0.84, 0.84-1.6, >1.6 (tertiles)	5	TB BMC	Protein T3 vs T1: 5.3% higher p <0.05
Misra, 2011 [22]	Framingham Osteoporosis Study (US)	Men and women ≥68 y	976/946	No fracture: 75(5.0); fracture: 76(5.2)	Willett semi-quantitative FFQ	46.5, 59.6, 67.7, 82.7 g/d (quartiles); 0.69, 0.88, 1.00, 1.22 g/kg bw/d (estimated values)	11.6 (median)	Hip fracture	Protein Q2-4 vs Q1: HR 0.63 (0.37, 1.09) p 0.04
Rapuri, 2003 [32]	Sites Testing Osteoporosis Prevention/ Intervention (US)	Women 65-77 y	489/92	71.3(0.8), 72.2(0.8), 70.1(0.8), 69.9(0.8) (quartiles)	7 d food diaries	0.95, 0.94, 0.98, 0.99 g/kg bw/d; 13.3, 15.2, 16.7, 19.5% of EI (quartiles)	3	TB BMD	Protein Q4 vs Q1: NR, no difference ns
								Hip BMD	NR, no difference ns
								FN BMD	NR, no difference ns
								Spine BMD	NR, no difference ns
								Osteocalcin	Mean -6.5(7.6)% vs -5.8(7.6)% p 0.042
								N-telopeptide	Mean 12.1(11.2)% vs 10.4(11.0)% p 0.226

BMC = bone mineral content; BMD = bone mineral density; EI = energy intake; FFQ = Food Frequency Questionnaire; FN = femoral neck; HR = hazard ratio; TB = total body; LS = lumbar spine; NR = not reported; ns = not significant; RR = risk ratio; SD = standard deviation; SE = standard error; US = United States.

[a] Unit is g/kg bw/d, unless stated otherwise. Values presented as mean or range.

[b] Values presented as mean(SE), mean (95% CI) or RR/HR (95% CI). BMD in g/cm^2.

Table 3. Summary table of the intervention study included in the analysis.

First author, year [ref]	RCT name (country)	Participants	N baseline/ analysed	Baseline mean age(SD) or age range (y)	Protein source	Control diet intake	High protein intake	Study length (y)	Relevant outcomes	Effect sizes
Zhu, 2011 [33]	- (Australia)	Healthy ambulant post-menopausal women 70-80 y	219/196 (after 1y) /179 (after 2y)	Protein: 74.2(2.8); placebo: 74.3(2.6)	Skim milk + whey protein isolate	2.1 g (skim milk); after 2y: 1.1 g/kg bw/d	30 g (skim milk + whey protein isolate); after 2y: 1.4 g/kg bw/d	2		Mean change(SD) protein vs placebo (mg/cm^2):
									Hip BMD	After 1y: -8.3(21) vs -5.1(22) p 0.31 After 2y: -10.8(25) vs -8.2(22) p 0.46
									FN BMD	After 1y: -5.7(22) vs -2.6(24) p 0.34 After 2y: -8.7(26) vs -7.9(27) p 0.84

BMD = bone mineral density; NR = not reported; ns = not significant; RCT = randomized controlled trial; SD = standard deviation.

Quality of Evidence

Risk of bias assessment using NOS in selected prospective cohort studies is presented in **Table 4**. Risk of bias was classified as high (score 1–3), potential limitations (score 4–6), or low (score 7–9). However, one study with a score of 4 was classified as having a high risk of bias due to a substantial large loss of follow-up (42.5%) [31]. Six cohort studies were classified as having a low risk of bias, five had potential limitations, and one study had a high risk of bias. With respect to adjustment of important confounders, 1 point was given if they controlled for age, gender, weight or BMI, energy intake, physical activity, smoking, alcohol, vitamin D, and calcium. Another point was given if family history of osteoporosis, fractures, (certain) illnesses, and (certain) drugs were included. Only one study took all those confounders into account [24]. Nine studies adjusted for a part of the relevant confounders [21-23, 25, 26, 29, 30, 32, 33] and in three studies

Table 4. Newcastle - Ottawa quality assessment scale for selected cohort studies.

First author, year [ref]	Representativeness of the exposed cohort	Selection of the non-exposed cohort	Ascertainment of the exposure	Outcome of interest absent at baseline	Control for important confounders	Outcome assessment	Adequate follow-up duration	Completeness of cohort follow-up	Total points out of 9	Risk of bias
Beasley, 2014 [23]	1	1	1	0	1	1	1	1	7	Low
Cauley, 2016 [24]	1	1	0	1	2	1	1	1	8	Low
Chan, 2011 [25]	0	1	0	0	1	1	1	0	4	Some concerns
Dawson-Hughes, 2002 [26]	0	1	0	1	1	1	1	1	6	Low
Devine, 2005 [27]	1	1	0	0	0	1	1	0	4	Some concerns
Fung, 2017 [21]	0	1	0	1	1	1	1	1	6	Some concerns
Hannan, 2000 [28]	1	1	0	1	0	1	1	0	5	Some concerns
Isanejad, 2017 [29]	1	1	1	1	1	1	1	0	7	Low
Langsetmo, 2017 [30]	1	1	0	0	1	1	1	1	6	Low
Meng, 2009 [31]	1	1	0	0	0	1	1	0	4	High
Misra, 2011 [22]	1	1	0	1	1	1	1	1	7	Low
Rapuri, 2003 [32]	1	1	1	0	1	1	1	0	6	Some concerns

A study can be awarded a maximum of one point for each numbered item within the selection and outcome categories. A maximum of two points can be given for comparability.

adjustment was inadequate [27, 28, 31]. The dropout rate was not clear in one cohort study [27] and another study stated that the rate was around 10% per 4 years with a total follow-up of 32 years [21]. Dropout rates were high (≥20%) in five studies varying from 24.7% to 81.2%, which (potentially) leads to attrition bias [25, 28, 29, 31, 32]. In the other five cohort studies, rates varied from 2.0% to 12.1%. Publication bias could not be assessed, because the numbers of studies in the meta-analysis were too small.

Using the Cochrane Collaboration's tool, overall risk of bias in the selected intervention study [33] was classified as 'low to some concerns' (**Table 5**). Those concerns were raised with respect to attrition and reporting bias. The dropout rate was 10.5% after 1 year and 18.3% after 2 years. An expected dropout rate of 30% was taken into account in the sample size calculation. Furthermore, there were no significant differences between the protein and control group in the number of people lost to follow-up.

According to the GRADE approach, evidence from observational studies should be rated as low quality (**Table 6**). Five cohort studies reported the protein intake per a defined unit increase in energy-adjusted protein intake (dose-response gradient). In absence of serious limitations in other categories, three of those cohort studies were rated up one level to moderate quality with respect to their BMD and fracture outcomes [24, 29, 30]. Quality of evidence for the outcomes total body BMC and bone turnover markers was rated down to 'very low' due to risk of bias, imprecision, and limited number of studies. In addition, quality of evidence for total fractures and spine fractures was rated 'very low', because both were assessed in one study only.

Prospective Cohort Studies

BMC – Total Body

Two studies assessed the effect of protein intake on total body BMC and showed different results [29, 31]. Meng et al. [31] found that postmenopausal women with a protein intake above the RDA (>1.6 g/kg bw/d) had a significant 5.3% higher whole body BMC compared to women with a low protein intake (<0.8 g/kg bw/d) after 5 years of follow-up. Isanejad et al. [29] found that protein intake was not significantly associated with total body BMC over 3 years of follow-up.

BMD – Total Body

Four studies assessed the effect of protein intake on total body BMD, of which one saw a beneficial effect [26] and three found no significant effects [23, 29, 32].

Table 5. Risk of bias in the selected intervention study using Cochrane Collaboration's tool.

	Risk of selection bias				**Risk of performance bias**				**Risk of attrition bias**			**Risk of detection bias**				**Risk of reporting bias**			**Overall risk of bias**
Author	Random sequence allocation	Allocation concealment	Baseline differences	Total	Blinding of participants	Blinding of personnel	Appropriate analysis	Total	Complete outcome data	Missing outcome data	Total	Inappropriate outcome assessment	Outcome assessment group differences	Blinding	Total	Analysis as pre-specified	Selection on multiple outcome measurements	Selection on multiple analyses	Total	
Zhu	L	L	L	Low	L	L	L	Low	PH	PL	Some concerns	L	L	L	Low	NI	L	NI	Some concerns	Low to some concerns

H = high; L = low; NI = no information; PH = probably high; PL = probably low.

Dawson-Hughes et al. [26] found that the highest tertile of protein intake (18.2–29.1% of total energy; estimated mean 1.17 g/kg bw/d) was associated with significantly higher total body BMD after 3 years of follow-up compared to the lowest tertile of protein intake (9.6–15.5% of total energy; estimated mean 0.96 g/kg bw/d; figure-derived: mean percent change 0.6% vs -0.2%, respectively). This effect was seen in a group who took calcium and vitamin D supplements for 3 years, but no association was found in the placebo group. Beasley et al. [23] found that each 20% increase in calibrated protein intake was associated with a non-significant increase in total body BMD in women aged 65 and 75 years at baseline after 3 years of follow-up. Isanejad et al. [29] found that protein intake was not significantly associated with total body BMD over 3 years of follow-up and Rapuri et al. [32] found no differences in total body BMD between four quartiles of protein intake above the RDA (0.95, 0.94, 0.98, 0.99 g/kg bw/d; 13.3, 15.2, 16.7, 19.5% of energy intake) after 3 years of follow-up.

BMD – Total Hip

Five studies assessed the effect of protein intake on total hip BMD, of which two found beneficial effects [27, 30] and three found no significant effects [23, 25, 32]. Devine et al. [27] found that women with a protein intake above the RDA (>1.6 g/kg bw/d) had a significantly higher hip BMD compared to women with a low protein intake (<0.8 g/kg bw/d) after 1 year of follow-up (figure-derived: mean 824 vs 798 mg/cm^2, respectively). Langsetmo et al. [30] showed that a higher protein intake (each standard deviation (SD) increase in total energy from protein intake) was associated with higher hip BMD in men after 10.5–11.2 years of follow-up (beta = 0.06, standard error = 0.01). On the other hand, Beasley et al. [23] found that each 20% increase in calibrated protein intake was associated with a non-significant increase in hip BMD in women aged 65 and 75 years at baseline after 3 years of follow-up. Chan et al. [25] found that protein intake was not associated with % change in hip BMD in men and women after 4 years of follow-up. Rapuri et al. [32] found no differences in hip BMD between four quartiles of protein intake above the RDA (0.95, 0.94, 0.98, 0.99 g/kg bw/d; 13.3, 15.2, 16.7, 19.5% of energy intake) after 3 years of follow-up.

BMD – Femoral Neck

Six studies assessed the effect of protein intake on femoral neck BMD, half of them showed beneficial effects [26-28] and the other half showed no significant effects [25, 29, 32]. Dawson-Hughes et al. [26] found that highest tertile of protein intake (18.2–29.1% of total energy; estimated mean 1.17 g/kg bw/d) was associated with significantly higher femoral neck BMD after 3 years of follow-up compared to the lowest tertile of protein intake (9.6–15.5% of total energy; esti-

Table 6. Quality of evidence per outcome of interest from selected studies.

Outcome	Number of cohort studies	Number of RCTs	Risk of bias	Imprecision	Consistency	Number of moderate quality studies	Overall quality rating
BMC - total body	2	0	Some concerns	Some concerns	1 p, 1 ns	0	Very low
BMD							
Total body	4	0	Low	Low	1 p, 3 ns	1	Low
Total hip	5	1	Some concerns	Low	2 p, 3 ns	1	Low
Femoral neck	6	1	Some concerns	Low	3 p, 3 ns	1	Low
Lumbar spine	4	0	Some concerns	Low	1 p, 1 n, 2 ns	1	Low
Bone turnover markers							
Osteocalcin	2	0	Some concerns	Some concerns	2 ns	0	Very low
N-telopeptide	2	0	Some concerns	Some concerns	2 ns	0	Very low
Fracture							
Total	1	0	Low	Low	1 ns	0	Very low
Spine	1	0	Low	Low	1 ns	1	Very low
Hip	5	0	Low	Low	4 p, 1 ns	2	Low

According to the GRADE approach, evidence was graded as 'High', 'Moderate', 'Low', or 'Very Low' depending on several criteria. Risk of bias is a combined judgement from risk of bias in the individual studies. Indirectness was rated low for all outcomes. BMC = bone mineral content; BMD = bone mineral density; n = negative; ns = not significant; p = positive; RCT = randomized controlled trial.

mated mean 0.96 g/kg bw/d; figure-derived: mean percent change 2.5% vs -0.4%, respectively). This effect was seen in a group who took calcium and vitamin D supplements for 3 years, but no association was found in the placebo group. Devine et al. [27] found that women with a protein intake above the RDA (>1.6 g/kg bw/d) had a significantly higher femoral neck BMD compared to women with a low protein intake (<0.8 g/kg bw/d) after 1 year of follow-up (figure-derived: mean 702 vs 679 mg/cm^2, respectively). Hannan et al. [28] showed that men and women with a protein intake above the RDA (1.24–2.78 g/kg bw/d) had significantly less femoral neck BMD loss than those with a protein intake below the RDA (0.21–0.71 g/kg bw/d) after 4 years of follow-up (mean percent change -2.32% vs -4.61%). With regard to the studies showing no effect, Chan et al. [25] found that protein intake was not associated with % change in femoral neck BMD in men and women after 4 years of follow-up. Isanejad et al. [29] found that protein intake was not significantly associated with femoral neck BMD over 3 years of follow-up and Rapuri et al. [32] found no differences in femoral neck BMD between four quartiles of protein intake above the RDA (0.95, 0.94, 0.98, 0.99 g/kg bw/d; 13.3, 15.2, 16.7, 19.5% of energy intake) after 3 years of follow-up.

BMD – (Lumbar) Spine

Four studies assessed the effect of protein intake on (lumbar) spine BMD, of which one found a beneficial effect [28], one found a negative effect [29], and two found no significant effects [26, 32]. Hannan et al. [28] found a beneficial effect, they showed that men and women with a protein intake above the RDA (1.24–2.78 g/kg bw/d) had significantly less lumbar spine BMD loss than those with a protein intake below the RDA (0.21–0.71 g/kg bw/d) after 4 years of follow-up (mean percent change –1.11% vs –3.72%). On the contrary, Isanejad et al. [29] found that in women with protein intakes ranging from 0.79 to 1.18 g/kg bw/d, protein was significantly negatively associated with total body BMD over 3 years of follow-up (beta = –0.31, standard error = 0.01). Dawson-Hughes et al. [26] found no association between the protein tertile of 18.2–29.1% of total energy (estimated mean 1.17 g/kg bw/d) vs 9.6–15.5% of total energy (estimated mean 0.96 g/kg bw/d) for spine BMD after 3 years of follow-up. In addition, Rapuri et al. [32] found no differences in spine BMD between four quartiles of protein intake above the RDA (0.95, 0.94, 0.98, 0.99 g/kg bw/d; 13.3, 15.2, 16.7, 19.5% of energy intake) after 3 years of follow-up.

Bone Turnover Markers

Two studies assessed the effect of protein intake on the bone turnover markers osteocalcin and N-telopeptide [26, 32]. Dawson-Hughes et al. [26] found no association between the highest and lowest protein tertile (18.2–29.1% vs

9.6–15.5% of total energy; estimated means 1.17 vs 0.96 g/kg bw/d) for both serum osteocalcin and urinary N-telopeptide cross-links after 3 years of follow-up. Rapuri et al. [32] also found no differences in serum osteocalcin and urinary N-telopeptides between four quartiles of protein intake above the RDA (0.95, 0.94, 0.98, 0.99 g/kg bw/d; 13.3, 15.2, 16.7, 19.5% of energy intake) after 3 years of follow-up.

Fractures

Only Beasley et al. [23] looked at total fractures and found that each 20% increase in calibrated protein intake was associated with a non-significant decrease in risk of total fractures in women aged 65 and 75 years at baseline after 3 years of follow-up.

With regard to risk of spine fracture specifically, only Langsetmo et al. [30] studied this outcome and found no association between a higher protein intake (each SD increase in total energy from protein intake) and risk of spine fracture in men after 10.5–11.2 years of follow-up.

More often investigated was the risk of getting a hip fracture. Five studies assessed the effect of protein intake on hip fracture risk, of which four showed a beneficial effect [21, 22, 24, 30] and one found no effect [23]. Cauley et al. [24] showed that each SD increase in total energy from protein was associated with an 18% decrease in risk of hip fractures in men after 8.6 years of follow-up. In the same cohort population but after 10.5–11.2 years of follow-up, Langsetmo et al. [30] found a significant association between each SD increase in total energy from protein intake and a decreased hip fracture risk of 16%. Fung et al. [21] showed an 8% lower risk of hip fractures in women aged 65–75 years consuming 1.4 g protein/kg bw/d (estimated value, 24.4% of energy intake) compared to a protein intake of 0.9 g/kg bw/d (estimated value, 14.3% of energy intake) and a 9% lower risk in women aged ≥75 years after 32 years of follow-up. In men aged 65–75 years, a 41% lower hip fracture risk was found between the highest vs lowest protein tertile (23.4% vs 14.2% of energy intake, estimated mean 1.4 vs 0.9 g/kg bw/d) and this reduction in risk was 23% in men aged ≥75 years. Misra et al. [22] found that a mean protein intake above the RDA (three quartiles: 59.6, 67.7, 82.7 g/d; estimated 0.88, 1.00, 1.22 g/kg bw/d) was significantly positively associated with a decreased hip fracture risk of 37% after 11.6 years of follow-up compared to a mean protein intake below the RDA (46.5 g/d; estimated 0.69 g/kg bw/d). However, Beasley et al. [23] found that each 20% increase in calibrated protein intake was associated with a non-significant decrease in risk of hip frac-

tures in women aged 65 and 75 years at baseline after 3 years of follow-up (9% and 8% respectively).

RCTs

No eligible RCTs were found investigating the effect of dietary protein intake on BMC, total body and lumbar spine BMD, and bone turnover markers. Only one RCT with total hip and femoral neck BMD as outcome was suitable for inclusion [33]. Zhu et al. [33] investigated the effect of a high-protein drink containing 30 g of protein compared to a placebo drink with 2.1 g of protein. Protein intake after 2 years was above the RDA for both groups (1.4 and 1.1 g/kg bw/d for protein and placebo group, respectively). No significant differences in hip and femoral neck BMD between women in the protein or placebo group after 1 and 2 years of protein supplementation were found. Hip and femoral neck BMD in both groups fell significantly from baseline.

Meta-analysis

A meta-analysis could be conducted for the outcome hip fracture among four cohort studies including eight different groups (**Figure 2**). The study of Cauley et al. [24] was excluded from the meta-analysis, since the same cohort population was used as in the study of Langsetmo et al. [30]. The latter was preferred because the follow-up duration was longer. The association between protein intake and risk of hip fracture was expressed as weighted HR (RR reported in one study was considered as HR). HR was transformed to its natural logarithm (ln) and the corresponding 95% CIs were used to calculate the standard errors. Inverse variance weighting was used to obtain the pooled HR for higher compared to lower protein intakes. Heterogeneity was not significantly present for hip fracture (I^2 = 0.0%, heterogeneity chi-squared p = 0.61) and a fixed-effect model was used. The meta-analysis of the cohort studies showed that high vs low protein intake resulted in a statistically significant decrease in hip fractures (pooled HR: 0.89; 95% CI: 0.84, 0.94; p<0.001).

Sensitivity analyses showed that there was no single study affecting the overall estimate considerably. A subgroup analysis was performed for gender. One study had to be excluded because no separate data was available for men and women [22]. A pooled HR of 0.82 (95% CI: 0.73, 0.93; p = 0.002) was found for men and 0.91 (95% CI: 0.86, 0.98; p = 0.007) for women. This difference was not statistically different (p = 0.13).

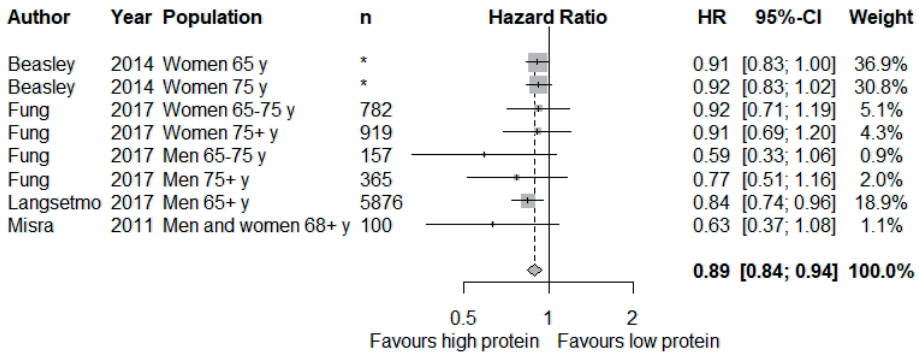

Figure 2. Effect of protein intake on hip fractures. Fixed-effect pooled hazard ratio (HR) analysis was used. Grey boxes represent the point estimates with the size of the box representing the weight of the study. Horizontal lines depict the length of the 95% CI. The diamond represents the pooled effect estimate.
* No exact sample size can be stated; the hazard ratio is the estimate of the effect at specific age levels (65 and 75 y) selected from a continuous distribution. Total sample size was 144,580 persons.

Meta-analyses with other outcomes of interest could not be performed, because missing quantitative data could not be provided for each relevant article after contact with authors.

DISCUSSION

The aim of this systematic review and meta-analysis was to investigate the impact of a dietary protein intake above the RDA of 0.8 g/kg body weight/day from any source on BMD/BMC, bone turnover markers, and fracture risk in older adults compared to a lower dietary protein intake. Our findings showed a positive trend between higher protein intakes and higher femoral neck and total hip BMD. Conflicting results were reported for lumbar spine BMD and no association was found for total body BMD. No conclusions could be drawn regarding BMC and bone turnover markers. Meta-analysis of four prospective cohort studies showed a statistically significant decrease of 11% in hip fracture risk.

Total body BMC was measured in only two cohort studies, of which one had a high risk of bias [31] and the other one had imprecise results [29]. Therefore, quality of evidence for total body BMC was rated as very low. Quality of evidence for the bone turnover markers was also rated as very low due to eligibility of only

two cohort studies, of which one was rated down due imprecision [32]. Risk of bias of the other cohort studies did not affect the quality of evidence.

The selected cohort studies compared varying levels of protein intakes, which makes comparisons between studies difficult and it can influence the magnitude of the measured effect. In addition, some studies looked at very high levels above the RDA, while others reported protein intakes closer to the RDA. For instance, two studies divided the protein intake in tertiles with the highest protein category being >1.6 g/kg bw/d [27, 31] and the highest category in the study of Fung et al. was 1.4 g/kg bw/d. While in three other cohort studies, the highest protein category was around 1.2 g/kg bw/d [22, 26, 29]. Rapuri et al. divided protein intake in four quartiles, which were very close to each other when transferred from percentage of energy intake to g/kg bw/d as unit. This minimal difference in protein intake may be one of the reasons why in this study no significant differences in bone health outcomes were found.

No sufficient evidence was available from intervention studies, only one RCT was eligible for inclusion in this review. This RCT showed no significant difference in hip and femoral neck BMD between protein and placebo group after 2 years. However, these outcomes fell significantly from baseline in both groups. Since protein intake after 2 years was above the RDA for both groups, this may indicate that a protein intake above the RDA is beneficial for hip and femoral neck BMD. This is in line with some of the cohort studies investigating hip BMD (3 studies showed no differences and 2 showed beneficial differences) and femoral neck BMD (3 studies showed no differences and 3 showed beneficial differences). The overall risk of bias in this RCT was classified as low to some concerns. Since this study was only done in women, representativeness is limited.

Over the years, several systematic reviews and meta-analyses assessing the impact of protein intake on bone health have been published. Darling et al. (2009) investigated the effect of varying protein intakes in healthy adults of 18 years and older and included 31 observational studies and 19 RCTs [8]. They concluded that there might be a small beneficial effect of protein supplementation on lumbar spine BMD (weighted mean difference = 0.02 g/cm^2; 95% CI: 0.00, 0.04), but that it is unknown if this also results in a reduced fracture risk. However, they also included studies with a duration of <6 months, leading to a less a reliable estimate of the impact on changes in BMD [12]. In comparison with the current review, we did not have enough data to assess lumbar spine BMD. We did find an effect on hip fracture risk, which can partly be explained by the differences between study populations (adults ≥18 years vs adults ≥65 years).

A more recent systematic review of Shams-white et al. [9] investigated the effect of varying protein intakes in healthy adults of 18 years and older and excluded studies with a duration of <6 months [9]. A total of 20 observational studies and 16 RCTs were included, which showed that there were positive trends of high compared to low protein intakes on BMD at different bone sites, especially the lumbar spine (net percentage change = 0.52%; 95% CI: 0.06, 0.97). Meta-analyses of RCTs assessing lumbar spine, total hip and femoral neck BMD were performed; no meta-analysis was done with hip fracture risk. Since insufficient evidence is available from RCTs in the current review, meta-analysis results cannot be compared.

The systematic review of Wallace & Frankenfeld [10] included healthy adults of 18 years and older and specifically focused on protein intakes above the current RDA [10]. They also excluded weight loss studies and studies in which women used hormone replacement therapy (HRT), resulting in the inclusion of 15 observational studies and 16 RCTs. They conclude that a protein intake above 0.8 g/kg body weight/day can potentially have a beneficial effect on hip fracture risk (pooled RR = 0.84; 95% CI: 0.73, 0.95) and BMD loss (qualitative evaluation). Although this meta-analysis included a younger population, the reduction in hip fracture risk is similar to what we have found. They also included studies with a duration of <6 months, leading to a less a reliable estimate of the impact on changes in BMD [12]. Therefore, results for BMD outcomes cannot be compared.

Lastly, results of the meta-analysis performed by Wu et al. [34] were the same as the current meta-analysis: a significant decrease in hip fracture risk of 11% (RR 0.89; 95% CI 0.82, 0.97) [34]. Two of the six included prospective cohort studies were also added to the current meta-analysis, the other four were performed with adults aged <65 years. Despite addition of recent publications investigating older adults in our analysis, no differences in hip fracture risk reduction could be observed.

The prevalence of osteoporosis and fracture risk are higher in women than in men [35, 36]. Therefore, the effect of a high protein intake on hip fracture risk may be gender-specific. However, subgroup analysis for gender showed no statistically significant difference in hip fracture risk reduction and therefore it was assumed that pooling the effect of men and women together was fair. Currently, much research is focused on postmenopausal women, but since men tend to have a higher mortality risk after a fracture, men should not be overlooked [36, 37].

Previously it was believed that a high dietary protein intake could have a negative effect on bone health by inducing chronic metabolic acidosis, which eventually leads to osteoporosis [38]. However, an increase in urinary calcium excretion observed after a high protein diet likely originates from an increased intestinal absorption instead of from bone calcium loss [39]. In addition, an expert consensus paper from 2018, assessing the risks and benefits of dietary protein for bone health, concluded that a protein intake above the RDA is beneficial for older adults [3]. A long-term protein intake of 2 g/kg body weight/day is reported safe for healthy adults, higher values may lead to digestive, renal, and vascular problems [40]. No conclusive evidence is available to set a new RDA for older adults in the light of bone health.

Two comments must be made with respect to the study eligibility criteria of the current review. In the pre-specified protocol, it was mentioned that studies including women using oral contraceptives or HRT would be excluded, except when stratification was done or when it was controlled for in the analyses. In principle, women aged ≥65 years do not take oral contraceptives anymore. Regarding HRT, a majority of the included studies did not report if women used this. Since we have older adults as study population, exclusion of this group of people might harm the generalizability. Therefore, we decided to remove this exclusion criterion. Secondly, it was stated that vegan individuals would be excluded, but this was not reported in the included studies. We assumed that the prevalence of vegan individuals in this generation is negligible [41] and therefore the expected impact on the results is minimal.

For the meta-analysis, standard errors were estimated from the HR or RR and its 95% CI, reported by the individual studies. When standard errors were back transformed to CIs, this indirect variance estimation resulted into a close but not similar value of the actually reported CIs. However, the differences were small (maximum interval difference of 0.02) and assumed not to influence the results.

A strength of this study was that only studies investigating older adults (aged ≥65 years) were included, making the recommendation specific for this more vulnerable group that has a greater protein need than younger adults. As a consequence, the review is limited by inclusion of only one intervention study. This makes clear that large and long-term RCTs in older adults are needed to judge if a protein intake above the current RDA can improve bone health and/or prevent osteoporosis. Another strength was the exclusion of studies with a duration of <6 months, studies that supplemented protein in the form of soy isoflavone, and

studies designed for weight loss. These exclusion criteria eliminate a proportion of confounding issues.

CONCLUSIONS

This systematic review supports that there is an association between a dietary protein intake above the current RDA of 0.8 g/kg body weight/ day and a reduced hip fracture risk in older adults. In addition, positive trends for total hip and femoral neck BMD were found. In comparison with younger adults, the body of evidence from the included studies is not strong enough to increase the protein recommendation for older adults with respect to bone health.

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SUPPLEMENTARY DATA

Supplemental Table 1. Literature search strategy per database.

Database	Search strategy
CENTRAL	#1 MeSH descriptor: [Dietary Proteins] explode all trees #2 MeSH descriptor: [Diet, Carbohydrate-Restricted] explode all trees #3 MeSH descriptor: [Meat] explode all trees #4 MeSH descriptor: [Dairy Products] explode all trees #5 protein intake #6 high protein #7 protein consumption #8 protein supplement #9 protein supplementation #10 dairy #11 #1 or #2 or #3 or #4 or #5 or #6 or #7 or #8 or #9 or #10 #12 MeSH descriptor: [Bone and Bones] explode all trees #13 MeSH descriptor: [Fractures, Bone] explode all trees #14 MeSH descriptor: [Bone Demineralization, Pathologic] explode all trees #15 MeSH descriptor: [Bone Density] explode all trees #16 MeSH descriptor: [Osteoporosis] explode all trees #17 MeSH descriptor: [Calcium] explode all trees #18 bone health #19 bone demineralization #20 bone density #21 bone mass #22 bone mineral #23 bone resorption #24 calcium excretion #25 acid-base load #26 bone turnover #27 calcium loss #28 #12 or #13 or #14 or #15 or #16 or #17 or #18 or #19 or #20 or #21 or #22 or #23 or #24 or #25 or #26 or #27 #29 MeSH descriptor: [Aged] explode all trees #30 elderly #31 #29 or #30 #32 #11 AND #28 AND #31
MEDLINE	(“dietary proteins”[MeSH] OR protein intake[TIAB] OR high protein[TIAB] OR protein consumption[TIAB] OR protein supplement[TIAB] OR protein supplementation[TIAB] OR “diet, carbohydrate-restricted”[MeSH] OR “meat”[MeSH] OR “dairy products”[MeSH] OR dairy[TIAB]) AND (“bone and bones”[MeSH] OR “Fractures, bone”[MESH] OR bone health[TIAB] OR “bone demineralization, pathologic”[MeSH] OR bone demineralization[TIAB] OR “bone density”[MeSH] OR “osteoporosis”[MeSH] OR bone density[TIAB] OR bone mass[TIAB] OR bone mineral[TIAB] OR bone resorption[TIAB] OR calcium excretion[TIAB] OR acid-base load[TIAB] OR bone turnover[TIAB] OR calcium loss[TIAB] OR “calcium”[MESH]) AND (“aged”[MeSH] OR elderly[TIAB]) AND (“clinical trial”[PT] OR “Randomized Controlled Trial”[PT] OR “cohort studies”[MeSH Terms])

Supplemental Table 1. Continued

Database	Search strategy	
EMBASE	#1	(Protein intake/ OR high protein.mp. OR protein supplement.mp. OR protein supplementation.mp. OR low carbohydrate diet/ OR meat/ OR dairy product/ OR dairy.mp.) AND (bone/ OR fracture/ OR bone demineralization/ OR bone density/ OR osteoporosis/ OR bone mass/ OR bone mineral/ OR osteolysis/ OR bone health.mp. OR bone turnover.mp. OR calcium/ OR calcium excretion.mp. OR calcium loss.mp. OR acid-base load.mp.) AND aged/
	#2	limit 1 to (clinical trial or randomized controlled trial)
	#3	prospective study/ or longitudinal study/ or cohort.mp. or cohort study.mp. or cohort studies.mp.
	#4	1 and 3
	#5	2 or 4

Chapter 2.2

Protein intake and bone mineral density: cross-sectional relationship and longitudinal effects in older adults

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ABSTRACT

Background. There are several mechanisms via which increased protein intake might maintain or improve bone mineral density (BMD), but current evidence for an association or effect is inconclusive. The objectives of this study were to investigate the association between dietary protein intake (total, plant and animal) with BMD (spine and total body) and the effects of protein supplementation on BMD.

Methods. Individual data from four trials that included either (pre-)frail, undernourished or healthy older adults (aged ≥65 years) were combined. Dietary intake was assessed with food records (2, 3 or 7 days) and BMD with dual-energy X-ray absorptiometry (DXA). Associations and effects were assessed by adjusted linear mixed models.

Results. A total of 1570 participants [57% women, median (inter-quartile range): age 71 (68–75) years] for which at least total protein intake and total body BMD were known were included in cross-sectional analyses. In fully adjusted models, total protein intake was associated with higher total body and spine BMD [beta (95% confidence interval): 0.0011 (0.0006–0.0015) and 0.0015 (0.0007–0.0023) g/cm^2, respectively]. Animal protein intake was associated with higher total body and spine BMD as well [0.0011 (0.0007–0.0016) and 0.0017 (0.0010–0.0024) g/cm^2, respectively]. Plant protein intake was associated with a lower total body and spine BMD [–0.0010 (–0.0020 to –0.0001) and –0.0019 (–0.0034 to –0.0004) g/cm^2, respectively]. Associations were similar between sexes. Participants with a high ratio of animal to plant protein intake had higher BMD. In participants with an adequate calcium intake and sufficient serum 25(OH)D concentrations, the association between total protein intake with total body and spine BMD became stronger. Likewise, the association between animal protein intake with total body BMD was stronger. In the longitudinal analyses, 340 participants [58% women, median (inter-quartile range): age 75 (70–81) years] were included. Interventions of 12 or 24 weeks with protein supplementation or protein supplementation combined with resistance exercise did not lead to significant improvements in BMD.

Conclusions. An association between total and animal protein intake with higher BMD was found. In contrast, plant protein intake was associated with lower BMD. Research is warranted to further investigate the added value of dietary protein alongside calcium and vitamin D for BMD improvement, especially in osteopenic or osteoporotic individuals. Moreover, more research on the impact of a plant-based diet on bone health is needed.

INTRODUCTION

Osteoporosis is a public health problem affecting the quality of life of 20 million older adults in Europe [1]. Calcium and vitamin D are well known to be key bone nutrients, and relatively high combined intakes can lead to a modest fracture risk reduction, especially in individuals with an insufficiency for these nutrients [2]. Dietary protein is also believed to play a role in combatting osteoporosis [3]. Protein intake is important for bone health via the up-regulation of anabolic hormones, improvements in intestinal calcium absorption and maintaining muscle mass and muscle strength [4]. However, evidence from studies investigating the association between protein intake and bone mineral density (BMD) or the effects of increasing protein intake on BMD is inconsistent [3]. For example, observational studies in older adults showed a positive trend between higher protein intakes and higher femoral neck and total hip BMD [3], although at the same time, no associations were observed between protein intake and lumbar spine BMD or total body BMD [3]. One large cohort from 1988 showed positive associations between animal protein intake with BMD at different sites, whereas plant-based protein was found to be negatively associated with BMD [5]. However, they did not adjust for vitamin D status.

Evidence from randomized controlled trials (RCTs) in older adults regarding the effect of protein on BMD is limited. One trial investigated the effect of consuming a high-protein drink containing 30 g of protein compared with a placebo drink with 2.1 g of protein for 2 years in healthy ambulant postmenopausal women [6]. No significant differences in hip and femoral neck BMD were found between females in the protein or placebo group after 1 and 2 years of protein supplementation [6]. In contrast, the PROVIDE study showed that 13-week vitamin D, calcium and leucine-enriched whey protein supplementation increased total body BMD (0.02 g/cm^2; ~2%) in sarcopenic non-malnourished older adults (intervention n = 184, control n = 196), possibly via suppression of parathyroid hormone (PTH) concentrations [7].

There are several potential mechanisms through which increased protein intake can maintain or improve BMD, but current evidence of an association or effect is inconclusive, and large trials are scarce. We therefore integrated data from four trials that included either (pre-)frail, undernourished or healthy older adults [8-11] to investigate the cross-sectional association between dietary protein and BMD and the effects of protein supplementation for 12–24 weeks on BMD. A further aim was to investigate if there were any differences between intakes of total, plant and animal protein in relation to BMD.

METHODS

This study included data from participants of four previously published trials under the acronyms NU-AGE (New dietary strategies addressing the specific needs of elderly population for healthy ageing in Europe), ProMO (Evaluating the Efficacy of a Novel Oral Supplement in Tackling Malnutrition in the Elderly), ProMuscle (Protein Supplementation and Exercise Strategy to Promote Muscle Protein Anabolism in Frail Elderly People) and PiP (ProMuscle in Practice) [8-11]. **Table 1** presents their inclusion and exclusion criteria and methods of dietary and BMD assessment. All four studies have been performed in accordance with the 1964 Declaration of Helsinki ethical standards and obtained medical and ethical approval from the South-East 6 Person Protection Committee (France), Independent Ethics Committee of the S. Orsola-Malpighi Hospital Bologna (Italy), the Wageningen University Medical Ethical Committee (Netherlands), the National Research Ethics Committee – East of England (UK), and the Bioethics Committee of the Polish National Food and Nutrition Institute (Poland). Informed consent of all participants was obtained prior to their inclusion in the study.

NU-AGE

The *NU-AGE* trial was conducted in five European study centres (Clermont Ferrand in France, Bologna in Italy, Wageningen in the Netherlands, Warsaw in Poland and Norwich in the UK). In total, 1296 participants were included, of which n = 1245 had sufficient data to be included in the current analysis. A complete overview of the study protocol is presented in previous papers [8, 12]. In brief, *NU-AGE* included adults aged 65–79 years who were living independently, were non-frail and non-malnourished and were free of dementia, major chronic diseases and diabetes. The *NU-AGE* trial was a 1-year intervention with a Mediterranean-style diet designed to meet the nutritional needs of older adults. As this intervention was not aiming at increasing protein intake, we only used the baseline data for the analyses presented in this paper.

Dietary intake was assessed via 7-day food records. Participants were trained in describing foods, portion sizes, preparation methods and complex recipes. During home visits or university visits, the food records were discussed and checked for missing data by trained dietitians or nutritionists. Dietary intake was coded via standardized coding protocols, and nutrient values were calculated by using country-specific food composition tables [13-18]. BMD was assessed via DXA [Discovery Wi; software version 2.3.1 Hologic, Inc. (Norwich, UK); Lunar iDXA; GE Health Care; enCORE 2011 software version 13.6 (Bologna, Italy); Discovery QDR, software version 3; Hologic, Inc. (Clermont-Ferrand, France); Lunar Prodigy;

Table 1. Overview of the four included trials.

	NU-AGE (n = 1296)	ProMO (n = 82)	ProMuscle (n = 127)	PiP (n = 168)
Trial registration number[a]	NCT01754012	NCT02683720	NCT01110369 NCT01109628	NTR6038
Years of enrollment	2012-2014	2016-2017	2009-2010	2016-2018
Inclusion criteria	65-79 years	≥65 years; Undernutrition (MNA-SF score <12)	≥65 years; Frail or pre-frail according to Fried criteria	≥65 years; Frail or pre-frail according to Fried criteria or physical inactive and experiencing difficulties in daily activities
Exclusion criteria	Frail according to Fried criteria; malnutrition; dementia; major chronic diseases; severe heart disease; insulin-treated diabetes	Resistance exercise >2h/week; life expectancy <12 months; eGFR <30 mL · min^{-1} · (1.73m²)$^{-1}$; use of diabetes medication; use of >21 alcohol units per week	Participation in resistance-type exercise programs in 2 years prior to study; eGFR <60 mL · min^{-1} · (1.73m²)$^{-1}$; any present form of cancer; COPD; diabetes	Recent surgery (<3 months); receiving terminal care; eGFR <30 mL · min^{-1} · (1.73m²)$^{-1}$; any present form of cancer; COPD; unregulated diabetes or hypertension
Dietary assessment	7-day food records	2-day food records	3-day food records	3-day food records
BMD assessment	DXA (Lunar Prodigy, Lunar iDXA, Discovery Wi, Discovery QDR)	DXA (Lunar Prodigy Advance)	DXA (Lunar Prodigy Advance)	DXA (Lunar Prodigy Advance)
Intervention	Two arms: Dietary intervention based on dietary recommendations for older adults and a vitamin D supplement (10 µg/d) versus control	Two arms: Supplementation with A (24 g casein, 4 µg vitamin D, 364 mg calcium), or B (11 g casein, 11 g whey, 7 g free BCAA, 11 µg vitamin D, 296 mg calcium)	Four arms: Supplementation with 31 g of milk protein concentrate or placebo, with or without resistance exercise training	Two arms: Increased protein intake of 25 g per main meal via conventional and enriched products in combination with resistance exercise training versus control
Duration	1 year	12 weeks	24 weeks	24 weeks
Primary outcome	C-reactive protein	Lean body mass	Lean body mass	Short Physical Performance Battery

BCAA, branched-chain amino acids; BMD, bone mineral density; COPD, chronic obstructive pulmonary disease; eGFR, estimated glomerular filtration rate; MNA-SF, Mini Nutritional Assessment short form; NU-AGE, New dietary strategies addressing the specific needs of elderly population for healthy ageing in Europe; PiP, ProMuscle in Practice; ProMO, Evaluating the Efficacy of a Novel Oral Supplement in Tackling Malnutrition in the Elderly; ProMuscle, Protein Supplementation and Exercise Strategy to Promote Muscle Protein Anabolism in Frail Elderly People.
[a]Trial registration numbers starting with NCT are registered at clinicaltrials.gov and numbers starting with NTR are registered at the Dutch Trial Register.

GE Health Care; enCORE 2011 software version 13.6 (Wageningen, Netherlands and Warsaw, Poland)] operated by trained nurses or researchers.

ProMO

The intervention trial *ProMO* (n = 82) included adults aged 65 years and above who were (at risk of being) malnourished. The full protocol of the trial has been described elsewhere [9]. The trial excluded those with an expected life expectancy of <12 months, performing over 2 h/week of resistance exercise, impaired kidney function, lactose intolerance or milk protein allergy and those who used corticosteroids (unless administered via inhaler or topically) or diabetes medications.

Nutritional intake was assessed by 2-day food records on consecutive days. Trained dietitians interviewed participants to maximize record completeness and to estimate portion sizes by using household measures. Food records were calculated into nutrients by using the Dutch Food Consumption Database 2011 [13]. DXA, performed by trained research assistants, was used to assess BMD (Lunar Prodigy Advance; GE Health Care, Madison, WI).

The participants in *ProMO* received 12 weeks of oral nutritional supplementation from two different brands. Brand 1 delivered, per day, 600 kcal, 23 g of fat, 74 g of carbohydrates, 24 g of protein (casein), 4.4 µg vitamin D3 and 364 mg calcium. The daily nutrient content of brand 2 was 586 kcal, 23 g of fat, 65 g of carbohydrates, 22 g of protein (whey and casein 1:1), 7 g of free branched-chain amino acids, 10.8 µg vitamin D3 and 296 mg calcium. The intervention did not contain any concurrent exercise programme, and all participants are coded as receiving a protein intervention in our analyses, regardless of the brand they received.

ProMuscle

The *ProMuscle* intervention included 127 frail or pre-frail adults aged 65 years and above [10, 19]. The trial excluded participants who were involved in resistance exercise training programmes in the 2 years prior to screening, who were diagnosed with chronic obstructive pulmonary disease (COPD), renal insufficiency, diabetes or cancer.

Dietary intake assessment was carried out via 3-day food records (one random weekday plus both weekend days). Trained dietitians discussed food records with participants and made use of household measures to estimate portion sizes. Foods were calculated into nutrients by using the Dutch Food Consumption

Database of 2006 [20]. BMD was assessed by DXA (Lunar Prodigy Advance; GE Health Care, Madison, WI).

The *ProMuscle* intervention consisted of four arms: placebo, protein, exercise and protein + exercise. We included all participants for the cross-sectional analyses and all participants except those admitted to solely exercise for the longitudinal analyses. The exercise intervention consisted of 24 weeks of supervised progressive resistance exercise twice per week. The protein intervention consisted of 24-week supplementation with two 250 mL beverages, which contained (per daily dose) 30 g of protein (milk protein concentrate), 14.2 g of lactose, 1 g of fat and 800 mg calcium. Beverages were consumed after breakfast and after lunch or after resistance exercise training. The control group received matched beverages with similar calcium and lactose content but without proteins. Protein and placebo beverages were matched on appearance and taste.

PiP

The intervention trial *ProMuscle in Practice*, or *PiP*, was a practice-based sequel to the more lab-based *ProMuscle* intervention. The protocol of *PiP* has been published before [21]. In brief, 168 community-dwelling older adults (aged 65 years and above) were recruited from five Dutch municipalities within the province of Gelderland. Participants were included when they were (pre-)frail or when they experienced difficulties in daily activities combined with physical inactivity. Excluded were those with COPD, cancer, unstable diabetes, unregulated hypertension, physical impairments or cognitive impairments.

Dietary intake was assessed via 3-day food records (three random days, of which one weekend day) for which participants received written and verbal instructions. Participants were visited by a trained research dietitian, who checked the records for completeness and used household measures to assess portion sizes. Nutrient intake was calculated by using the Dutch Food Consumption Database 2011 [13]. BMD was assessed via DXA (Lunar Prodigy Advance, GE Health Care, Madison, WI), which was operated by trained research assistants.

The *PiP* intervention consisted of protein + exercise. The protein intervention aimed to increase protein intake during each main meal moment to at least 25 g. Based on the food record, a dietitian gave tailored advice to each participant in the intervention group. No protein supplements were used; the protein intake was increased via protein-rich dairy products, such as yoghurt, quark and cheese. Progressive resistance exercise sessions (twice a week, 1 h per session) were supervised by physiotherapists. During the first 12 weeks of the intervention,

participants were supervised intensively and received protein-rich products for free. During the second 12 weeks, the supervision was less intense: The exercise had to be done without supervision at local gyms, and the participants did not receive free protein-rich products and dietary advice but could attend nutritional workshops. Participants assigned to the control group received no intervention and were asked to stick to their regular diet and exercise habits.

Other measurements

Physical activity was measured differently in each study: Physical Activity Scale for Elderly (PASE) questionnaire was used in *NU-AGE*, an accelerometer (ActiGraph GTX3, 2009, Pensacola, FL, USA) with data expressed in counts per minute in *ProMO* and *ProMuscle*, and total activity in min/day in *PiP*. In order to combine the physical activity variables, z-scores were constructed as follows: . Smoking status was classified as never, former or current smoker, and alcohol intake was calculated in g/d. The Mini Nutritional Assessment (MNA) was used in *NU-AGE* and *ProMO* to evaluate nutritional status. Details about fracture history were asked in *PiP* and *NU-AGE* and fall history in *NU-AGE* only. Serum 25 (OH)D concentrations, assessed by liquid chromatography-mass spectrometry, were measured in all studies except *PiP*. Vitamin B12 concentrations were only measured in *NU-AGE* by chemiluminescence.

Statistical methods

Descriptive statistics are presented as median (interquartile range) or as frequency (%). Linear mixed models were used to test for cross-sectional associations between protein and BMD while adjusting for covariance between participants within the same study cohort in a random intercept model. Three models of increasing complexity were built to adjust for confounding factors. The first model represented the crude association between protein intake and BMD. The second model was adjusted for age, sex, physical activity level, smoking status (never, former, current) and alcohol intake. The third model additionally adjusted for calcium intake, vitamin D intake and energy intake. Models 1–3 were also performed for males and females separately. In addition, we performed a subgroup analysis in which only participants with an adequate calcium intake (>950 mg, based on EFSA Population Reference Intake (PRI) [22]) and sufficient serum 25(OH)D concentrations (>50 nmol/L [23]) were included. For this analysis, we applied a variant on the third model in which we omit adjustment for calcium intake and vitamin D intake. Lastly, the ratio of animal protein to plant protein intake was divided in tertiles and evaluated with the third model as well. All cross-sectional analyses were carried out in SPSS 25 (IBM Corp., Armonk, NY,

USA), and graphs and figures were created using GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA, USA). Alpha was set at 5%.

The effects of protein or protein and exercise interventions versus control were analysed by using linear mixed models. Models were built with fixed effects for time, treatment, time*treatment, sex and age. Random effects were used to model subject-specific intercepts nested within the study these subjects participated in. Post hoc testing for differences between fixed effects was adjusted by Bonferroni correction. All longitudinal analyses were carried out in SAS 9.4 (SAS Institute, Cary, NC, USA), and graphs and figures were created using GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA, USA). Alpha was set at 5%.

RESULTS

In total, 1570 participants were included in the analyses (**Table 2** and **Supplemental Figure 1**). Participants had a median age of 71 (IQR 68–75) years, and 56% were female. Median protein intake was 1.03 g/kg/d (IQR 0.88–1.22) The percentage of participants with a protein intake below the recommended dietary allowance (RDA) of 0.8 and below the recommendation from the ESPEN Expert Group for healthy older adults of 1.0 and 1.2 g/kg/day [24] amounted to 17, 45 and 73%, respectively. Median total body BMD was 1.10 g/cm^2 (IQR 1.01–1.20), and 12% of the participants was diagnosed with osteoporosis. A calcium intake below the PRI was observed in 56% of the participants, 98% did not reach the estimated average requirement (EAR) of 10 µg vitamin D [25], and 31% had serum 25(OH)D concentrations below the recommendation for prevention of osteoporosis in postmenopausal women (>50 nmol/L) [23] and 64% below the suggested optimal concentration for a lower fracture risk and to support the skeleton (70–80 nmol/L) [26]. Characteristics of the study sample per study centre can be found in **Supplemental Table 1**.

Cross-sectional

A total of 1570 participants for which at least total protein intake and total body BMD were known were included in cross-sectional analyses (**Table 3** and **Supplemental Figures 2 and 3**). In fully adjusted models, total protein intake and animal protein intake were associated with higher BMD in the total body and spine (beta ranging from 0.0011 to 0.0017 g/cm^2). In contrast, plant protein intake was associated with a lower total body and spine BMD (beta ranging from –0.0019 to –0.0010 g/cm^2). Sex-stratified fully adjusted models showed a stronger association between total protein intake and spine BMD in females

Table 2. Characteristics of the total study sample.

	n	Median [IQR]	Freq. (%)
Age, year	1569	71 [68-75]	
Women			882 (56.2)
BMI, kg/m^2	1530	26.1 [23.8-28.9]	
Smoking	1565		
Never			827 (52.8)
Former			663 (42.4)
Current			75 (4.8)
Alcohol intake, g/d	1543	5.3 [0.2-13]	
Energy intake, kcal/d	1570	1852 [1578-2168]	
Calcium intake, mg/d	1569	901 [702-1153]	
Vitamin D intake from food, μg/d	1408	2.9 [1.8-4.1]	
Vitamin D intake from food + supplements, μg/d	1569	3.2 [2.0-4.8]	
Serum 25(OH)D concentrations, nmol/L	1363	61.0 [45.2-78.0]	
Vitamin B-12 intake, μg/d	1366	4.3 [3.0-6.2]	
Serum vitamin B-12 concentrations, pmol/L	1215	357 [281-443]	
Hip fracture past 12 months	1405		25 (1.8)
Fracture other than hip past 12 months	163		7 (4.3)
One or more falls past 12 months	1244		172 (12.3)
Diagnosis of osteoporosis	1401		172 (12.3)
MNA	1312	14 [12-14]	
Malnourished			13 (1.0)
At risk of malnutrition			184 (14.0)
Normal nutritional status			1115 (85.0)
Total protein intake, g/d	1570	74.5 [64.1-87.0]	
Total protein intake, g/kg/d	1566	1.03 [0.88-1.22]	
Plant protein intake, g/d	1570	26.5 [21.5-32.2]	
Animal protein intake, g/d	1570	45.7 [36.1-55.3]	
Total body BMD, g/cm^2	1570	1.10 [1.01-1.20]	
Spine BMD, g/cm^2	1369	1.04 [0.92-1.18]	

BMD, bone mineral density; BMI, Body Mass Index; IQR, Interquartile Range; MNA, Mini Nutritional Assessment; 25(OH)D, 25-hydroxyvitamin D.

Table 3. Associations between total, plant and animal protein intake with total and spine BMD[a].

			All			Males			Females		
Exposure	**BMD (g/cm²)**	**Model**	**B**	**SE**	**P value**	**B**	**SE**	**P value**	**B**	**SE**	**P value**
Total protein, g/d	Total body	1	0.0024	0.0002	<0.001	0.0009	0.0002	<0.001	0.0009	0.0002	<0.001
	(F: n=843,	2	0.0008	0.0002	<0.001	0.0009	0.0002	<0.001	0.0008	0.0002	<0.001
	M: n=649)	3	**0.0011**	**0.0002**	**<0.001**	**0.0009**	**0.0004**	**0.013**	**0.0014**	**0.0003**	**<0.001**
	Spine	1	0.0025	0.0003	<0.001	0.0010	0.0004	0.013	0.0012	0.0003	0.001
	(F: n=743,	2	0.0010	0.0003	<0.001	0.0010	0.0004	0.013	0.0012	0.0004	0.001
	M: n=549)	3	**0.0015**	**0.0004**	**<0.001**	0.0012	0.0006	0.062	**0.0022**	**0.0005**	**<0.001**
Plant protein, g/d	Total body	1	0.0032	0.0004	<0.001	0.0003	0.0004	0.52	-0.0002	0.0005	0.73
	(F: n=843,	2	-0.0002	0.0004	0.582	0.00002	0.0005	0.97	-0.0004	0.0005	0.50
	M: n=649)	3	**-0.0010**	**0.0005**	**0.026**	**-0.0013**	**0.0007**	**0.047**	-0.0009	0.0007	0.18
	Spine	1	0.0026	0.0006	<0.001	-0.0003	0.0008	0.68	-0.0010	0.0007	0.16
	(F: n=743,	2	-0.0006	0.0006	0.319	-0.0005	0.0009	0.59	-0.0007	0.0008	0.38
	M: n=549)	3	**-0.0019**	**0.0008**	**0.013**	**-0.0024**	**0.0012**	**0.037**	-0.0016	0.0010	0.10
Animal protein, g/d	Total body	1	0.0025	0.0002	<0.001	0.0012	0.0003	<0.001	0.0013	0.0003	<0.001
	(F: n=843,	2	0.0012	0.0002	<0.001	0.0012	0.0003	<0.001	0.0012	0.0003	<0.001
	M: n=649)	3	**0.0011**	**0.0002**	**<0.001**	**0.0011**	**0.0003**	**0.001**	**0.0014**	**0.0003**	**<0.001**
	Spine	1	0.0029	0.0003	<0.001	0.0015	0.0005	0.001	0.0019	0.0004	<0.001
	(F: n=743,	2	0.0016	0.0003	<0.001	0.0016	0.0005	0.001	0.0018	0.0004	<0.001
	M: n=549)	3	**0.0017**	**0.0004**	**<0.001**	**0.0017**	**0.0006**	**0.005**	**0.0022**	**0.0005**	**<0.001**

[a]Model 1: crude association. Model 2: adjusted for age, sex, physical activity level, smoking status and alcohol intake. Model 3: additionally adjusted for calcium intake, vitamin D intake and energy intake. Presented number of participants are based on model 3

(0.0022 g/cm^2; 95% CI: 0.0011 to 0.0032 g/cm^2) than in males (0.012 g/cm^2; 95% CI: –0.00006 to 0.0025 g/cm^2). However, plant protein intake had a stronger association with spine BMD in males (–0.0024 g/cm^2; 95% CI: –0.0047 to –0.00015 g/cm^2) compared with females (–0.0016 g/cm^2; 95% CI: –0.0036 to 0.0003 g/cm^2). In subgroup analysis of participants with an adequate calcium intake and sufficient serum 25(OH)D concentrations, the association between total protein intake with total body and spine BMD became stronger (**Table 4**). Likewise, the association between animal protein intake with total body BMD was stronger. Associations with plant protein intake became non-significant. Furthermore, older adults with a high ratio of animal to plant protein intake had a higher total body and spine BMD compared with those with a low ratio, while total protein intake was similar between the groups (**Supplemental Figure 4**). Lastly, results were similar to the total sample for persons with a low BMI, defined as lower than 24 kg/m^2 [27] (data not shown).

Longitudinal

In total, 340 participants were included in longitudinal analyses. Interventions of 12 or 24 weeks with protein or protein and resistance exercise did not lead to significant improvements in total body BMD (time*treatment interaction 0.66; **Figure 1**) or spine BMD (time*treatment interaction 0.67; **Figure 2**). Also within groups, post hoc contrasts did not reveal any significant changes. Notable was the 24-week increase in total body BMD after protein plus resistance exercise (0.006 g/cm^2; 95% CI: 0.001 to 0.011 g/cm^2), but this contrast lost significance after Bonferroni correction.

Table 4. Associations between total, plant and animal protein intake with total and spine BMD in participants with adequate calcium intakes and sufficient 25(OH)D concentrations[a].

Exposure	BMD	Model	B	SE	P value
Total protein, g/d	Total body	4	**0.0016**	**0.0005**	**0.001**
	Spine	4	**0.0021**	**0.0008**	**0.012**
Plant protein, g/d	Total body	4	-0.0007	0.0009	0.45
	Spine	4	0.0010	0.0016	0.56
Animal protein, g/d	Total body	4	**0.0015**	**0.0004**	**<0.001**
	Spine	4	**0.0014**	**0.0007**	**0.049**

[a]n = 355 for associations with total body BMD, n = 258 for associations with spine BMD. Model 4: adjusted for age, sex, physical activity level, smoking status, alcohol intake and energy intake.

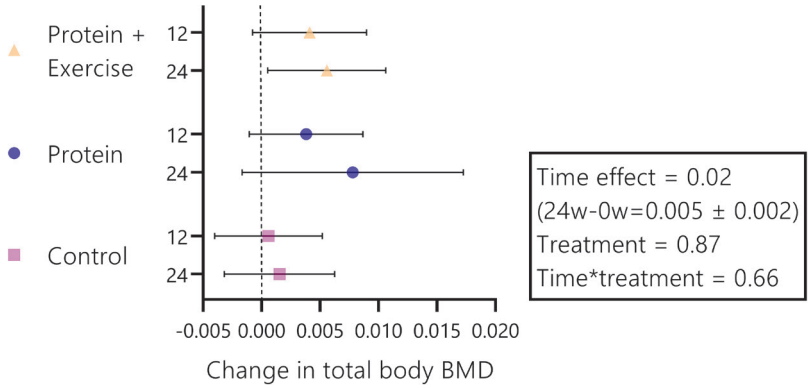

Figure 1. Change in total body bone mineral density (BMD) per treatment group and per time (after 12 and 24 weeks). Wicked bars represent 95% confidence intervals. The wide confidence interval at the 24-week time point in the protein group is a consequence of lower sample-size due to the 12-week duration of the *ProMO*-trial. The 24-week change in total body BMD after protein and exercise lost significance after Bonferroni-correction. For protein + exercise, protein and control, sample sizes were n = 112, n = 113 and n = 115 at week 12 and n = 111, n = 31 and n = 115 at week 24, respectively.

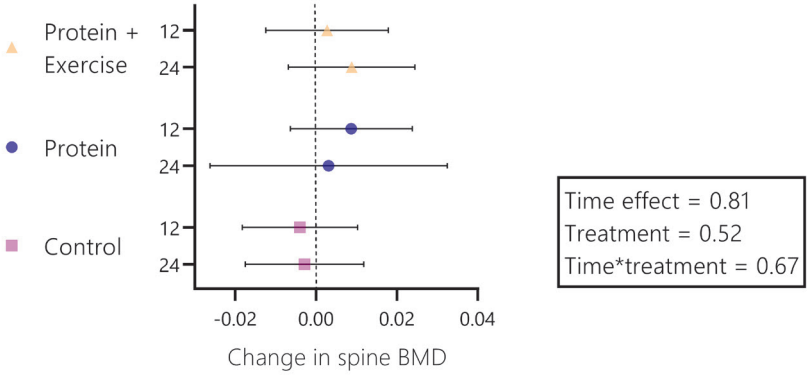

Figure 2. Change in spine bone mineral density (BMD) per treatment group and per time (after 12 and 24 weeks). Wicked bars represent 95% confidence intervals. The wide confidence interval at the 24-week time point in the protein group is a consequence of lower sample-size due to the 12-week duration of the *ProMO*-trial. For protein + exercise, protein and control, sample sizes were n = 112, n = 113 and n = 115 at week 12 and n = 111, n = 31 and n = 115 at week 24, respectively.

Sex-stratified analyses suggest that female participants responded better to the protein + exercise treatment on both total body and spine level (**Supplemental Figures 5 and 6**). However, no statistically significant differences were observed. After 24 weeks of protein plus resistance exercise in female participants, their total body BMD had increased by 0.010 g/cm^2 (95% CI: 0.002 to 0.017 g/cm^2) and their spine BMD by 0.011 g/cm^2 (95% CI: –0.003 to 0.025 g/cm^2).

DISCUSSION

This study included data from (pre-)frail, undernourished or healthy older adults [8-11] and showed that total and animal protein intakes were associated with higher BMD in the total body and spine. In contrast, plant protein intake was associated with a lower total body and spine BMD. We observed no significant improvements in total body or spine BMD after protein supplementation for 12–24 weeks, with or without a resistance exercise programme.

The possible explanation for our observation that only total protein and animal protein, but not plant protein, are associated with BMD is as follows. First, animal protein has a greater digestibility and a more complete amino acid profile than plant protein [28]. Second, animal foods typically contain calcium, vitamin D and/or vitamin B12, nutrients that have been linked to improved BMD [2, 29]. Indeed, earlier studies have linked vegetarianism and veganism to increased fracture risk [30, 31]. However, there are also reasons to argue that plant protein would lead to increased BMD. Consumption of plant protein sources typically result in a more bone-sparing alkaline metabolic environment, which may increase BMD [32]. In addition, animal foods have typically a higher sodium chloride (NaCl) content [33], and a high NaCl intake combined with a low calcium intake causes high calcium excretion, leading to bone resorption [34]. Previous cross-sectional research in older adults also found positive associations between animal protein [5, 35, 36] and plant protein [5, 35] with total body and spine BMD. A cohort from 1988 showed similar results in terms of direction and magnitude, for both plant and animal protein intake on spine and total body BMD [5]. On the contrary, one study observed a positive relationship between plant protein and spine BMD [37], but only in White women and not in any other sexes or ethnicities. Our finding that older adults with a high ratio of animal to plant protein intake had higher BMD conflicts with a study from Sellmeyer et al. [38], who found that older females with a high animal–plant protein ratio had lower BMD (not significant) and had a higher rate of bone loss over the year. However, total protein intake of the participants in this study from 1986 was much lower than in our study

and below the recommendation. In addition, no adjustments for vitamin D status were made.

Comparing the sexes, we found a stronger association between total protein intake and spine BMD in females, but a weaker association for plant protein intake with total body and spine BMD. The other associations were similar, suggesting that the association between protein intake and BMD is not sex specific. This was previously also found for hip fracture risk [3].

Subgroup analysis of participants with an adequate calcium intake (>950 mg) [22] and sufficient serum 25(OH)D concentrations (>50 nmol/L) [23] showed a stronger association between total protein intake with total body and spine BMD compared with all participants. Likewise, the association between animal protein intake with total body BMD was stronger. This suggests that animal protein has added value alongside calcium and vitamin D. Interestingly, the negative association between plant-based protein intake and BMD diminished in this subgroup analysis. Although the subgroup had a slightly higher median protein intake compared with the total sample, the proportion of protein from plant-based sources was equal (35.6% in both groups). A reason for the diminished associations could be inadequate power, as only 23% had both an adequate calcium intake and sufficient serum 25(OH)D concentrations.

Food products high in protein are generally also high in vitamin B6 and B12, which could play some role in bone health [29], but for which allowances have not been made. However, B12 concentrations were adequate (≥150 pmol/L) in >99% of our participants. Still, further analyses using other ways to control for nutrient–nutrient correlations in the light of bone health, such as network analyses, are warranted.

The advantage of the current study is that data from different trials were pooled, which increases the power and leads to a higher generalizability of the results. On the other hand, pooling of different studies has the disadvantage that data collection is often not unified. This limitation is mitigated by pooling studies performed by the same lab, with similar equipment and standard operating procedures. Also, correlated errors between participants from the same study have been modelled via a random factor in all models. In light of BMD, cross-sectional models do give valuable information despite their limitations regarding causality and temporal orders of exposure and outcome. BMD in older adults is affected by environmental factors, for example, diet, smoking and exercise. If dietary intake habits do not alter drastically over the life course,

the cross-sectional association between protein intake and BMD could very well represent the impact of lifelong high versus low protein intake on BMD. However, there are reasons to assume that in our cohort protein intake may have changed significantly during the life course. For instance, participants with malnutrition might have been advised to increase their intake of protein-rich products such as dairy. Alternatively, participants with poor mouth health, decreased appetite or anosmia might have lowered their intake of meat products. Therefore, in some participants, the protein intake assessed over the 2 or 3 days will not have been a good representation of their past habits.

To address the limitations of the cross-sectional analysis, we carried out analyses regarding changed protein intake over time. These longitudinal analyses still present limitations, as the duration of exposure (12–24 weeks) might be too short to induce changes in BMD. For the interpretation of our results, this short timeframe means that the direction of results might be more meaningful than the magnitude of the effects. We found no significant time*treatment effects in the models for total body BMD and spine BMD in the whole population and in the sex strata. Protein and exercise for 24 weeks seemed to increase total BMD in the whole population and in female participants, but in both cases, the significance of the post hoc comparison did not hold after Bonferroni correction. It is likely that a larger sample size or a longer exposure to a protein and exercise intervention would increase the magnitude of effect, but this hypothesis has to be tested in trials.

So far, well-designed studies on the effects of protein plus exercise on BMD in older adults are scarce. In general, BMD is only reported as a secondary outcome of protein and exercise interventions. One RCT by Kemmler et al. did look into the effects of 18 months of protein and exercise on bone health in older males [39]. In their study, the intervention group received high-intensity resistance exercise training combined with a protein intake increased by whey protein supplementation to 1.5–1.6 g/kg/d. The control group did not follow any exercise programmes, but did receive whey protein supplements to achieve a total protein intake of 1.2 g/kg/d. The authors reported significant between-group differences in lumbar spine BMD (MD = 0.012 mg/cm^2) and total hip BMD (MD = 0.013 mg/cm^2) in favour of the intervention group, fuelling our hypothesis that a longer training regimen is needed to observe effects from protein plus resistance exercise interventions on BMD.

To effectively increase BMD, mechanical loading is needed [40]. In short, impact and muscle forces cause strains on bones, thereby activating osteocytes,

which in turn signal osteoblasts and osteoclasts to adapt to the load [40]. The mechanical load needs to be strong enough, and combined resistance and impact exercise training are suggested to be the most effective [41]. Resistance exercise programmes that are progressive in nature could therefore stimulate BMD improvement for a longer period of time, as the mechanical loading keeps increasing throughout the programmes.

In the longitudinal analyses, only total body and spine BMD were available. Total body BMD may be incapable of capturing the effects of bone-loading activities. Exercise probably has the largest impact on femoral neck BMD, because the femoral neck is part of a weight-bearing joint. Furthermore, cancellous bone, which is found in the spine and femoral neck, is often more responsive to stimuli than cortical bone [42]. In addition, the BMD values were already at a sufficient level at baseline (median total body BMD = 1.10 g/cm^2, median spine BMD = 1.04 g/cm^2). A protein and exercise intervention may have a larger impact in osteopenic or osteoporotic individuals.

In conclusion, we found an association between higher total and animal protein intake with higher total body and spine BMD. In contrast, higher plant protein intake was associated with a lower total body and spine BMD. Research is warranted to investigate further the added value of dietary protein alongside calcium and vitamin D for BMD improvement, especially in osteopenic or osteoporotic individuals. Furthermore, more research on the impact of a plant-based diet on bone health is needed.

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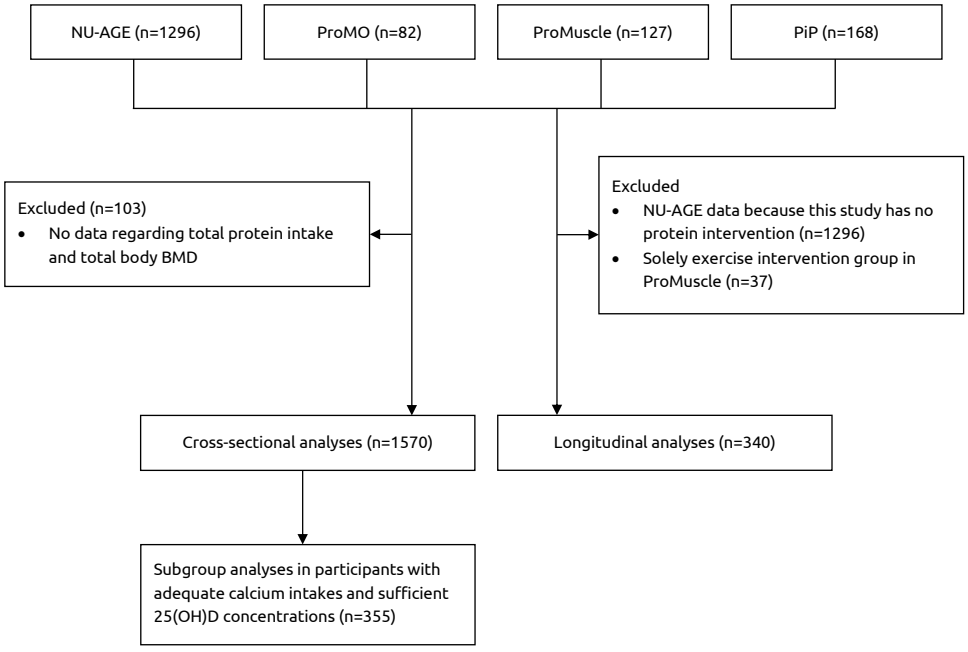
SUPPLEMENTARY DATA



Supplemental Figure 1. Flow-chart of the included data in the cross-sectional and longitudinal analyses from four previously published trials under the acronyms NU-AGE, ProMO, ProMuscle and PiP. 25(OH)D, 25-hydroxyvitamin D; NU-AGE, New dietary strategies addressing the specific needs of elderly population for healthy ageing in Europe; PiP, ProMuscle in Practice; ProMO, Evaluating the Efficacy of a Novel Oral Supplement in Tackling Malnutrition in the Elderly; ProMuscle, Protein Supplementation and Exercise Strategy to Promote Muscle Protein Anabolism in Frail Elderly People.

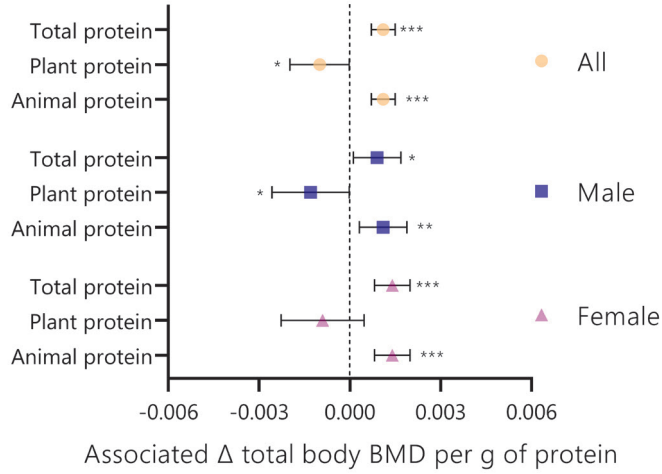
Supplemental Table 1. Characteristics of the study sample per study center.

	ProMO	ProMuscle	PiP	NU-AGE IT	NU-AGE UK	NU-AGE NL	NU-AGE PL	NU-AGE FR
Age, median [IQR], y	74 [69-78]	78 [70-84]	75 [70-80]	72 [68-75]	69 [67-72]	71 [67-74]	72 [68-74]	70 [67-73]
Females, n (%)	41 (50.6)	47 (58.0)	97 (59.5)	140 (51.7)	173 (64.1)	140 (55.6)	144 (57.4)	100 (50.0)
BMI, median [IQR], kg/m^2	21.4 [20.1-23.3]	27.4 [24.1-30.2]	26.5 [24.5-29.5]	26.6 [24.5-29.2]	26.2 [24.1-28.9]	26.0 [23.6-28.0]	27.4 [25.1-30.5]	24.9 [23.4-27.1]
Smoking, n (%)								
Never	33 (40.7)	73 (92.4)	59 (36.2)	120 (44.4)	161 (59.6)	127 (50.4)	123 (49.0)	131 (65.8)
Former	36 (44.4)	6 (7.6)	97 (59.5)	132 (48.9)	103 (38.1)	117 (46.4)	110 (43.8)	62 (31.2)
Current	12 (14.8)	0 (0.0)	7 (4.3)	18 (6.7)	6 (2.2)	8 (3.2)	18 (7.2)	6 (3.0)
Alcohol intake, median [IQR], g/d	8.0 [2.3-11]	6.7 [0.1-19.7]	4.6 [0.0-9.1]	5.0 [0.2-13]	8.3 [1.6-15]	10 [3.2-20]	1.1 [0.0-5.0]	5.6 [1.0-15]
Energy intake, median [IQR], kcal/d	2154 [1815-2434]	1916 [1612-2381]	1981 [1736-2265]	1701 [1434-1975]	1871 [1631-2113]	1843 [1621-2076]	1758 [1465-2145]	2001 [1656-2350]
Calcium intake, median [IQR], mg/d	1123 [851-1390]	1019 [822-1232]	1064 [890-1279]	715 [551-844]	1017 [870-1235]	933 [763-1132]	671 [516-848]	1109 [858-1341]
Vitamin D intake from food, median [IQR], μg/d	NA	NA	3.0 [1.9-4.4]	1.7 [1.0-3.2]	3.0 [1.9-4.2]	3.1 [2.3-4.4]	3.6 [2.4-5.4]	2.7 [1.9-3.6]
Vitamin D intake from food + supplements, median [IQR], μg/d	2.9 [2.0-4.4]	4.2 [2.8-5.8]	3.0 [1.9-4.4]	2.3 [1.1-5.1]	3.1 [2.0-4.5]	3.3 [2.4-4.7]	4.0 [2.7-7.3]	2.7 [2.0-3.8]
Serum 25(OH)D levels, median [IQR], nmol/L	86.0 [54.4-103]	52.0 [40.0-78.0]	NA	66.4 [51.8-82.7]	53.5 [39.3-67.3]	62.7 [49.0-75.0]	54.6 [42.7-67.1]	68.3 [49.9-84.7]
Vitamin B-12 intake, median [IQR], μg/d	4.2 [3.2-5.7]	4.0 [2.9-5.7]	4.3 [3.2-5.6]	2.7 [1.9-4.1]	5.9 [4.5-7.9]	4.3 [3.4-5.6]	4.1 [2.9-6.3]	NA
Serum vitamin B-12 levels, median [IQR], pmol/L	NA	NA	NA	319 [254-410]	327 [255-418]	386 [311-467]	357 [284-424]	399 [329-510]
Hip fracture past 12 months, n (%)	NA	NA	0 (0.0)	4 (1.5)	4 (1.5)	17 (6.7)	0 (0.0)	0 (0.0)
Fracture other than hip past 12 months, n (%)	NA	NA	7 (4.3)	NA	NA	NA	NA	NA
One or more falls past 12 months, n (%)	NA	NA	NA	32 (11.8)	29 (10.7)	37 (14.7)	49 (19.5)	25 (12.5)
Diagnosis of osteoporosis, n (%)	NA	NA	27 (16.6)	33 (12.2)	10 (3.7)	26 (10.3)	51 (20.3)	25 (12.9)

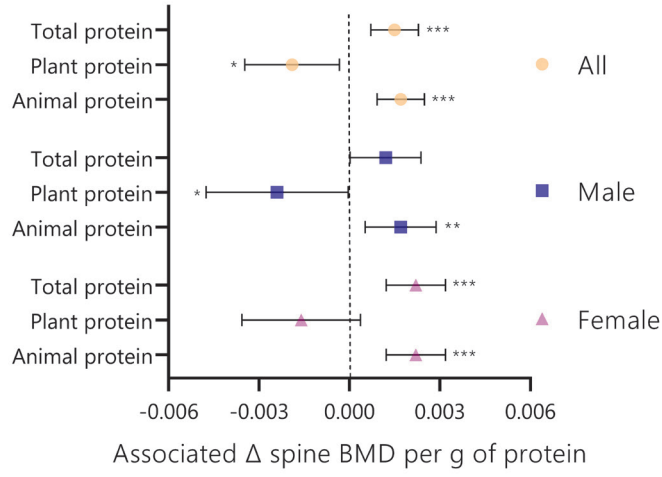
Supplemental Table 1. Continued

	ProMO	ProMuscle	PiP	NU-AGE IT	NU-AGE UK	NU-AGE NL	NU-AGE PL	NU-AGE FR
MNA, median [IQR], points	10 [9-11]	NA	NA	14 [12-14]	14 [13-14]	14 [13-14]	14 [13-14]	13 [12-14]
Malnourished, n (%)	9 (11.0)	NA	NA	1 (0.4)	1 (0.4)	1 (0.4)	1 (0.4)	0 (0.0)
At risk of malnutrition, n (%)	72 (88.9)	NA	NA	21 (7.8)	20 (7.4)	21 (8.8)	15 (6.0)	35 (17.5)
Normal nutritional status, n (%)	0 (0.0)	NA	NA	248 (91.9)	250 (92.3)	217 (90.8)	235 (93.6)	165 (82.5)
Total protein intake, median [IQR], g/d	82.7 [68.8-95.8]	75.7 [64.1-86.7]	78.5 [68.2-93.0]	65.8 [56.8-76.0]	75.1 [65.8-86.1]	74.5 [65.1-86.5]	73.6 [62.6-89.1]	77.3 [67.8-89.6]
Total protein intake, median [IQR], g/kg/d	1.32 [1.08-1.58]	0.97 [0.88-1.21]	1.07 [0.91-1.24]	0.92 [0.78-1.07]	1.04 [0.91-1.20]	1.03 [0.87-1.21]	1.01 [0.83-1.22]	1.13 [0.96-1.32]
Plant protein intake, median [IQR], g/d	31.6 [23.5-39.8]	25.6 [22.4-30.0]	30.1 [24.5-34.3]	23.6 [17.7-28.7]	26.9 [23.5-32.6]	28.6 [25.2-33.2]	24.6 [19.7-30.7]	24.4 [20.6-31.3]
Animal protein intake, median [IQR], g/d	46.4 [38.7-58.4]	49.7 [39.8-61.7]	48.9 [40.8-58.6]	31 [25.6-38.3]	48.5 [40.1-56.1]	45.9 [37.6-53.2]	49.0 [39.6-60.1]	51.5 [42.1-60.8]
Total body BMD, median [IQR], g/cm^2	1.07 [0.98-1.18]	1.11 [1.01-1.20]	1.13 [1.03-1.23]	1.06 [0.96-1.16]	1.05 [0.97-1.15]	1.14 [1.06-1.24]	1.14 [1.07-1.23]	1.09 [1.02-1.19]
Spine BMD, median [IQR], g/cm^2	0.99 [0.87-1.13]	1.14 [0.98-1.23]	1.08 [0.97-1.25]	1.04 [0.94-1.19]	0.95 [0.85-1.09	1.07 [0.94-1.19]	1.06 [0.96-1.20]	NA
Physical activity z-score	1.49	-0.35	0	-0.36	0.3	0.08	-0.03	0.01

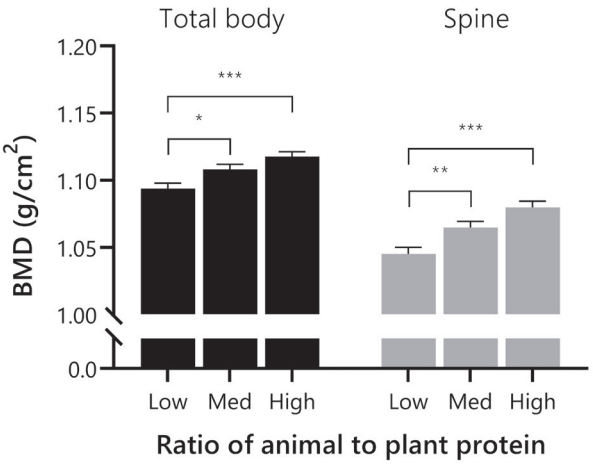
BMD, bone mineral density; BMI, Body Mass Index; MNA, Mini Nutritional Assessment; 25(OH)D, 25-hydroxyvitamin D.



Supplemental Figure 2. Associations between protein intake and total body bone mineral density (BMD). Markers represent betas and wicked lines represent 95% confidence intervals. All presented data stems from fully adjusted models (adjustment for age, sex, physical activity, smoking, alcohol, intake of calcium, vitamin D and energy). *, P<0.05; **, P<0.01; ***, P<0.001.

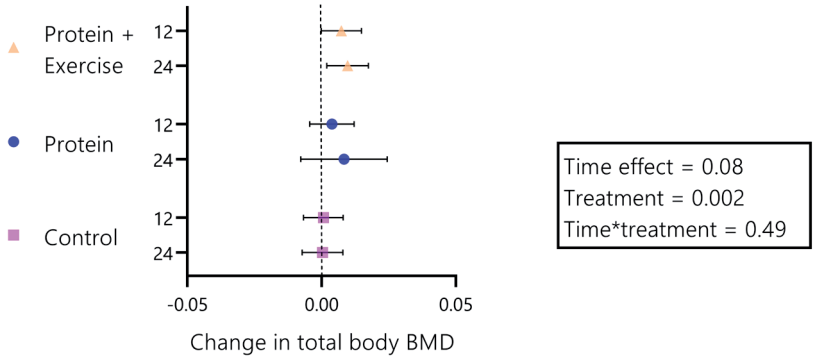


Supplemental Figure 3. Associations between protein intake and spine bone mineral density (BMD). Markers represent betas and wicked lines represent 95% confidence intervals. All presented data stems from fully adjusted models (adjustment for age, sex, physical activity, smoking, alcohol, intake of calcium, vitamin D and energy). *, P<0.05; **, P<0.01; ***, P<0.001.

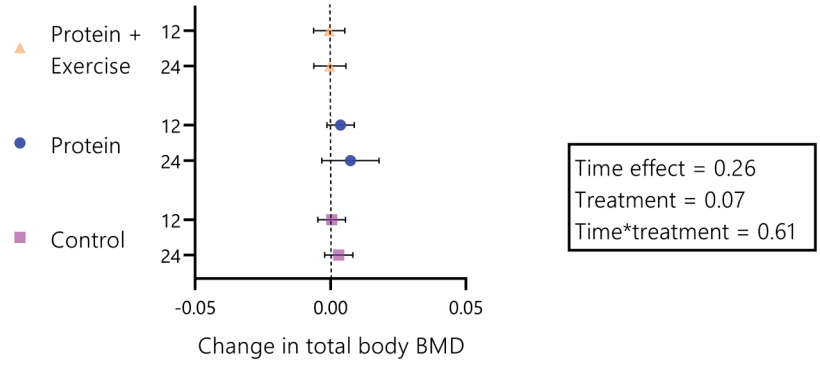


Supplemental Figure 4. Mean (+SE) bone mineral density per tertile of animal protein:plant protein ratio. All presented data stems from fully adjusted models (adjustment for age, sex, physical activity, smoking, alcohol, intake of calcium, vitamin D and energy). *, P<0.05; **, P<0.01; ***, P<0.001.

A Female

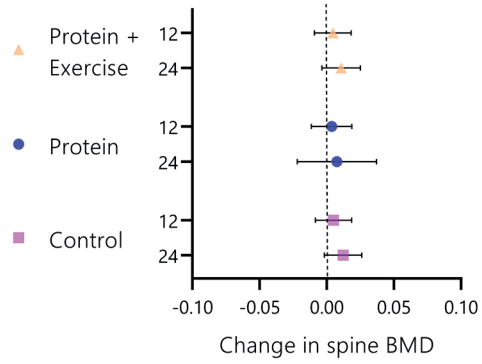


B Male

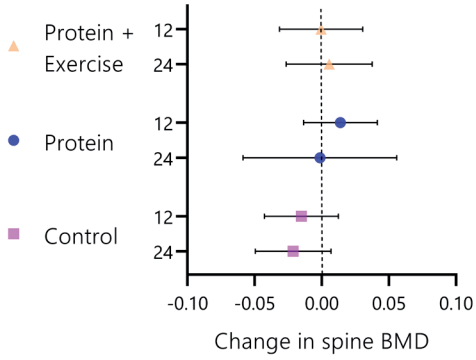


Supplemental Figure 5. Change in total body bone mineral density (BMD) per treatment group and per time (after 12 and 24 weeks) in [A] female and [B] male participants. Wicked bars represent 95% confidence intervals.

A Female



B Male



Supplemental Figure 6. Change in spine bone mineral density (BMD) per treatment group and per time (after 12 and 24 weeks) in [A] female and [B] participants. Wicked bars represent 95% confidence intervals.

Chapter 2.3

No effect of protein supplementation on bone mineral density in prostate cancer patients on androgen deprivation therapy

Inge Groenendijk, Lisanne H.P. Houben, Maarten Overkamp, Luc J.C. van Loon, Milou Beelen, Sandra Beijer and Lisette C.P.G.M. de Groot

Submitted for publication

ABSTRACT

Purpose. To assess the effects of 20 weeks of protein supplementation, alongside resistance exercise training, on bone mineral density (BMD) after 20 weeks and 1 year in prostate cancer (PCa) patients on androgen deprivation therapy (ADT).

Methods. Sixty PCa patients receiving ADT were randomly assigned to receive protein supplements for 20 weeks containing 31g whey protein (EX+PRO, n=30) or placebo (EX+PLA, n=30), immediately after exercise and every evening before sleep. Both groups followed a 20-week resistance exercise training program. At baseline, 20 weeks and 1 year, total body BMD was assessed with dual-energy X-ray absorptiometry and dietary intake with 3-day food diaries. Data were analyzed with linear mixed models.

Results. In EX+PRO, BMD decreased by -0.96 ± 1.98% from baseline till 20 weeks and -3.60 ± 3.31% from baseline till 1 year. In EX+PLA, BMD decreased by -0.99 ± 1.54% from baseline till 20 weeks and -2.25 ± 2.51% from baseline till 1 year. No significant differences between groups over time were found. At baseline, mean habitual protein intake was 1.0 g/kg/d in both groups. Protein supplementation increased protein intake in EX+PRO to 1.43 ± 0.35 g/kg/d at 20 weeks.

Conclusion. BMD decreased over time in PCa patients on ADT with reasonably good habitual protein intake. Protein supplementation for 20 weeks, alongside resistance exercise training, did not attenuate the decrease in BMD after 20 weeks and 1 year. Future trials should investigate the effect of combined resistance and impact exercise training on BMD at different sites.

INTRODUCTION

Prostate cancer (PCa) is the second most commonly diagnosed cancer in men worldwide [1]. The exact cause of PCa is still unknown, but risk factors include advanced age, ethnicity (more common in African than Caucasian men), genes and family history, and obesity [1]. Androgen deprivation therapy (ADT) is a frequently used treatment of localized high-risk, locally advanced, and metastatic PCa [2, 3]. ADT reduces androgen levels to castration level, which slows down PCa progression [4]. However, long-term ADT decreases bone mineral density (BMD) and increases fracture risk [5, 6].

Sex steroids play an important role in the maintenance of BMD. Ageing men have significant reductions in bioavailable estradiol and testosterone, contributing to lower BMD [7]. Following ADT, these levels will decrease even more. Testosterone and estrogen deficiency can enhance osteoclast activity, leading to bone loss [8]. It has been found that PCa patients initiating ADT have a 5- to 10-fold increased rate of bone loss compared to PCa patients with normal hormone levels [9].

Dietary protein can support BMD via the upregulation of anabolic hormones and enhancement of intestinal calcium absorption [10]. A systematic review and meta-analysis showed that protein intake above 0.8 g/kg/d may play a beneficial role in BMD maintenance and decreases hip fracture risk in healthy older adults [11]. Furthermore, improved muscle mass and strength through protein supplementation and exercise can lead to an increased anabolic stress on bone, which improves BMD as well [10]. A meta-analysis suggested that aerobic exercise is not suitable for inhibiting BMD loss in PCa patients receiving ADT, while a combination of resistance and impact exercise may preserve BMD in this population [12].

However, evidence for protein supplementation, alongside exercise, to ameliorate the adverse effects of ADT on BMD is limited. In a recent 12-month randomized controlled trial (RCT), the effect of daily whey protein (25 g), calcium and vitamin D supplementation combined with thrice weekly progressive resistance exercise training plus weight-bearing impact exercise on bone health in ADT-treated men was investigated [13]. Seventy PCa patients were randomized to exercise plus supplementation (n=34) or usual care (n=36). No effects of the intervention on BMD, bone structure and strength were found. However, adherence to the intervention was modest.

The current study was a secondary analysis. In the same study population, it was found that resistance exercise training improved lean and muscle mass (measured by total-body dual-energy X-ray absorptiometry [DXA] and upper leg computed tomography scan, respectively) and strength (leg press and leg extension strength) and diminished the ADT-induced decrease in aerobic capacity, compared to usual care [14]. The aim of this study was to assess the effects of 20 weeks of protein supplementation, alongside resistance exercise training, on BMD after 20 weeks and 1 year in PCa patients on ADT.

METHODS

Study population and design

The current study was a secondary analysis, in which two groups were compared: a group receiving resistance exercise training with protein supplementation (EX+PRO) and a group receiving resistance exercise training with placebo supplementation (EX+PLA). Details concerning participant recruitment, study design and methods have been described in detail elsewhere [14]. In short, a multicenter RCT was conducted in PCa patients receiving ADT. These patients were recruited in Maastricht University Medical Centre+ (MUMC+), Zuyderland Medical Centre (Zuyderland MC) and Máxima Medical Centre (MMC) between September 2017 and March 2020. Exclusion criteria included being unable to participate in the exercise training regimen, exposing a high risk for pathological fractures due to bone metastases, estimated life expectancy <1 year, lactose intolerance or whey protein allergy, cognitive disorders or severe emotional instability or unable to speak, and not understanding the Dutch language. Informed consent was obtained from all individual participants included in the study. Eligible patients were randomly allocated in a double blinded fashion to EX+PRO or EX+PLA. The intervention lasted for 20 weeks. At baseline, 20 weeks and 1 year, body composition (DXA) and habitual dietary intake (3-day food diary) were measured.

The study was approved by the local Medical Ethical Committee of MUMC+ and conducted according to the principles of the 1964 Helsinki declaration and its later amendments. This trial was registered at www.trialregister.nl as NTR6432.

Protein and placebo supplementation

Patients ingested either a protein or placebo supplement directly after each exercise session and every evening before sleep for 20 weeks. The protein supplement contained 31 g whey protein (Lacprodan® HYDRO.Rebuild, degree of hydrolysis 10%), 13 g carbohydrate and 1.0 g fat, providing 774 kJ of energy

(Arla Foods Ingredients Group P/S, Viby J, Denmark). The placebo supplement contained 1 g protein, 12 g carbohydrate and 0.4 g fat, providing 234 kJ of energy (Arla Foods Ingredients Group P/S, Viby J, Denmark). Both supplements were provided as powder in sachets, which needed to be dissolved in 250 mL water, and had a chocolate flavor. Adherence to supplement intake was assessed by counting returned (full and empty) supplement sachets.

Exercise intervention

Patients in both groups followed a supervised progressive whole-body resistance exercise training program (60 min, twice a week) for 20 weeks. The program consisted of six exercises targeting the large muscle groups: 1) leg extension 2) leg press 3) chest press 4) lateral pulldown 5) horizontal row 6) shoulder press. The program was divided into cycles of 4 weeks. During week 1-3 of the first cycle, the workload on each machine was gradually increased from 60% to 70% one-repetition maximum (1RM). In week 1-3 of the following cycles, the workload was increased from 65% to 70% 1RM. Every 4th week, workload was reduced to 60% 1RM to allow for proper recovery and minimize the risk of injury. After 4, 8, 12 and 16 weeks of training, 1RM was estimated using the multiple-repetition testing procedure (indirect 1RM) to progressively adjust the workload of the training sessions. Training load was adjusted for patients experiencing medical complications. The actual training load was registered in a personalized training log for every training session. Training adherence was calculated as the number of performed exercise sets divided by the number of prescribed sets.

Body composition

Body weight was measured wearing underwear and directly after voiding using a digital scale to the nearest 0.1 kg. Height was measured by a fixed stadiometer to the nearest 0.5 cm. Body mass index (BMI) was calculated as kilograms per square meter. Total body BMD was measured with DXA (Discovery A; Hologic, Marlborough, MA, USA).

Habitual dietary intake and physical activity

Habitual dietary intake was assessed with a food diary on two weekdays and one weekend day. Mean intake of energy (kcal), protein (g/kg/d, g/d), carbohydrate (g/d), fat (g/d) and calcium (mg/d) were calculated by web-based software (Eetmeter; Voedingscentrum, Den Haag, NL).

Habitual physical activity was measured over 7 days with a small triaxial accelerometer (ActiGraph wGT3X-BT; ActiGraph, Pensacola, FL, USA) worn on the waist during wakefulness. Accelerometer data were analyzed with ActiLife (version

6.13.4; ActiGraph, Pensacola, FL, USA) and mean daily step count, as well as percentage of time spent sedentary or in light, moderate, and (very) vigorous intensity were calculated. Data were included if the accelerometer was worn for ≥5 days and ≥10 h per day.

Statistical analysis

Data were analyzed using the intention-to-treat method and checked for normality. Continuous and normally distributed data are expressed as mean ± standard deviation (SD) and categorical data are presented as frequency (percentage). Baseline characteristics between groups were compared using independent samples t-test for continuous variables or a chi-square test for categorical variables.

Percentage changes over time were calculated for BMD and subsequently differences between groups over time were analyzed using linear mixed models (LMM) with participant as a random factor and time (baseline, 20 weeks, and 1 year), group (EX+PRO and EX+PLA) and time x group as fixed factors. Characteristics which differed between groups at baseline were added as confounders to the model. Since this had no effect on the outcome, only the crude model is showed. Changes in habitual dietary intake between groups over time were analyzed with LMM in a similar manner. All statistical analyses were performed using IBM SPSS Statistics (version 25.0; IBM Corp., Armonk, NY). A 2-sided P value of 0.05 was used for statistical significance.

RESULTS

In total, 84 patients were randomized to one of the two groups (EX+PRO n=43, EX+PLA n=41). After 20 weeks, both groups consisted of 30 patients. A total of 24 patients dropped out due to training and testing restrictions during the COVID-19-induced lockdown, 1 due to screening failure and 1 for medical reasons. Follow-up measurements of DXA after 1 year were available for 18 patients in EX+PRO and 19 patients in EX+PLA. Compliance to the training sessions was 84±18% and 79±23% in EX+PLA and EX+PRO, respectively. Supplement compliance was 94±8% and 86±23% in EX+PLA and EX+PRO, respectively. Baseline characteristics (**Table 1**) were different between groups for time since PCa diagnosis, Gleason score (system to grade the aggressiveness of PCa based on the appearance of cancer cells under a microscope, higher scores indicate a more aggressive cancer) and previous radiation.

Table 1. Baseline characteristics of the PCa patients in the intervention groups.

	EX+PRO (*n* = 30)	EX+PLA (*n* = 30)
Age, years, mean ± SD	73 ± 7	71 ± 7
Body weight, kg, mean ± SD	82.2 ± 13.5	84.5 ± 11.1
BMI, kg/m^2, mean ± SD	26.7 ± 3.4	27.5 ± 3.3
Time since PCa diagnosis, months, mean ± SD[a]	36 ± 52	12 ± 18
Gleason score (6-10), mean ± SD[a]	7.9 ± 1.1	8.4 ± 1.0
ADT duration, days, mean ± SD	107 ± 208	190 ± 282
Metastases, n (%)	11 (39.3)	14 (50.0)
Previous prostatectomy, n (%)	5 (16.7)	7 (23.3)
Previous radiation, n (%)[a]	9 (30.0)	4 (13.3)
Previous chemotherapy, n (%)	0 (0.0)	3 (10.0)
Number of comorbidities, n (%)[b]		
0	3 (10.0)	5 (17.2)
1	11 (36.7)	12 (41.4)
≥2	16 (53.3)	12 (41.4)
Step count, steps/d, mean ± SD	5586 ± 2774	6212 ± 2901
Time sedentary, %/d, mean ± SD	79 ± 7	77 ± 7
Time in light activity, %/d, mean ± SD	18 ± 6	19 ± 6
Time in moderate activity, %/d, mean ± SD	4 ± 3	5 ± 3

Data were not available for all patients: for bone metastases n=28 in both groups; for number of comorbidities n=29 in EX+PLA. EX+PLA, exercise training group with placebo supplementation; EX+PRO, exercise training group with protein supplementation; BMI, body mass index; PCa, prostate cancer; ADT, androgen deprivation therapy.

[a]*Significantly different between groups (P<0.05).*

[b]*Comorbidity assessed by the adapted Self-administered Comorbidity Questionnaire (SCQ).*

BMD

In the EX+PRO group, total body BMD changed -0.96 ± 1.98% from baseline till 20 weeks (n=30) and -3.60 ± 3.31% from baseline till 1 year (n=18; **Table 2**). In the EX+PLA group, total body BMD changed -0.99 ± 1.54% from baseline till 20 weeks (n=30) and -2.25 ± 2.51% from baseline till 1 year (n=19). LMM showed that BMD decreased over time (significant time effect), but this decrease was not different between groups over time (no interaction effect).

Dietary intake

Habitual dietary intake was similar between groups at baseline and over time, except for a mean decrease in habitual protein intake over time (-2.2 g/d in EX+PRO and -8.2 g/d in EX+PLA from baseline till 1 year; **Table 2**). At baseline, the percentage of patients with a protein intake below the recommended dietary

allowance (RDA) of 0.8 and below the recommendation from the ESPEN Expert Group for older adults of 1.0 and 1.2 g/kg/day [15] amounted to 15, 48 and 73%, respectively. Protein supplementation increased daily protein intake in EX+PRO from 1.04 ± 0.26 g/kg/d at baseline till 1.43 ± 0.35 g/kg/d at 20 weeks. The total protein intake including placebo supplementation in EX+PLA was 1.08 ± 0.23 g/kg/d at 20 weeks. A calcium intake below the EFSA Population Reference Intake (PRI) of 950 mg [16] was observed in 58% of the patients.

Table 2. Changes in bone mineral density and habitual dietary intake over time.

	EX+PRO (n=30)			**EX+PLA (n=30)**		
	Baseline	**20 weeks**	**1 year[a]**	**Baseline**	**20 weeks[b]**	**1 year[a]**
BMD, g/cm^2 [c]	1.139 ± 0.122	1.127 ± 0.116	1.077 ± 0.112	1.109 ± 0.134	1.098 ± 0.137	1.089 ± 0.154
Energy intake, kcal/d	2152 ± 462	2046 ± 435	2210 ± 568	2199 ± 476	2226 ± 516	2045 ± 408
Protein intake, g/kg/d[c]	1.04 ± 0.26	1.02 ± 0.29	0.96 ± 0.23	1.05 ± 0.31	1.06 ± 0.23	0.93 ± 0.24
Protein intake, g/d[c]	83.9 ± 21.9	84.1 ± 25.1	81.7 ± 22.3	87.6 ± 21.6	90.3 ± 20.7	79.4 ± 19.3
Carbohydrate intake, g/d	225.4 ± 55.0	206.0 ± 56.1	212.4 ± 67.5	210.3 ± 58.9	214.6 ± 56.9	188.2 ± 54.4
Fat intake, g/d	86.9 ± 23.4	85.8 ± 23.1	97.8 ± 38.8	92.7 ± 29.0	95.1 ± 32.0	93.8 ± 24.4
Calcium intake, mg/d	964.0 ± 368.1	910.1 ± 323.4	892.9 ± 396.9	927.6 ± 306.7	933.1 ± 386.3	782.9 ± 235.6

Values are means ± SD. No time x treatment interaction was observed for any of the variables. BMD, bone mineral density; EX+PLA, exercise training group with placebo supplementation; EX+PRO, exercise training group with protein supplementation.
[a]For BMD and dietary intake at 1 year, n= 18 or 19.
[b]For dietary intake at 20 weeks, n=28 in EX+PLA.
[c]Significant time effect (P<0.05).

DISCUSSION

The present study showed that 20 weeks of protein supplementation, alongside resistance exercise training, had no effect on BMD after 20 weeks and 1 year in PCa patients receiving ADT. Habitual protein intake did not differ between groups, but there was a mean decrease in dietary protein intake after 1 year. However, protein supplementation was successful in increasing daily protein intake till 1.43 ± 0.35 g/kg/d at 20 weeks.

Protein supplementation did not attenuate the decline in BMD in PCa patients on ADT. Even though the group receiving protein supplementation was able to improve their mean protein intake from baseline till 20 weeks (1.04 vs 1.43 g/kg/d), it decreased below baseline value (0.96 g/kg/d) at 1 year (supplementation stopped), after which there were no more differences in intake between the groups. An explanation for the absence of an effect on BMD could be the already reasonably good protein intake at baseline. Still, the percentage of patients with a protein intake below the recommendation from the ESPEN Expert Group for older adults of 1.0 and 1.2 g/kg/day [15] amounted to 48 and 73 %, respectively.

Another important finding was the high prevalence (58%) of an inadequate calcium intake from the diet at baseline, which did not change significantly over time. However, calcium supplementation was not recorded and it is, therefore, unclear whether the total calcium intake was indeed inadequate. Since it is proven that an adequate intake of calcium is needed for optimal bone health, health professionals should consider to advice their patients an increased calcium intake through the diet (or via supplementation if dietary intake is not sufficient) [17].

The rate of bone loss in older men is 0.5-1% per year [18, 19]. In men receiving ADT this can increase to more than 3% after 1 year of treatment [9, 20, 21]. In the current study, BMD had decreased after 1 year by 3.6% in exercise + protein group and 2.3% in the exercise + placebo group, which is in line with the expected decline. This suggests that the resistance exercise training had no effect on BMD attenuation. This can be explained by the fact that weight-bearing exercises were not included. Exercise can be beneficial for maintaining BMD as long as the mechanical load is sufficient and weight-bearing exercises are performed. Therefore, combined resistance and impact exercise training are suggested to be the most effective for BMD attenuation [12]. Indeed, a previous trial found that combined resistance exercise and impact loading attenuates lumbar spine BMD in PCa patients undergoing ADT, while no effect was seen of a resistance plus aerobic exercise program [22].

Strengths of this study include a good compliance (≥79%) to both the protein supplementation and exercise training program. The intervention was feasible, safe, and well-tolerated, and has therefore good potential to be implemented in clinical practice. Limitations of this study include the small sample size after 1 year and no registration of taking calcium supplements. Vitamin D status was also not measured in the current study, while this nutrient is known to be important for bone health [17]. In addition, different DXA-scanners were used in the various study centers and five participants had a different DXA-scanner at one

of the time points. However, adding type of DXA-scanner as a confounder to the model and running the analyses without these five participants did not affect the results. Lastly, only total body BMD was available, while larger effects may be seen in femoral neck BMD since this is part of a weight-bearing joint.

In conclusion, BMD decreased over time in PCa patients on ADT. Protein supplementation for 20 weeks did not attenuate the decrease in BMD after 20 weeks and 1 year. The decrease in BMD after 1 year of treatment was in line with the expected decline, suggesting that resistance exercise training was also not effective for BMD attenuation. Future trials should investigate the effect of combined resistance and impact exercise training on BMD at different sites, thereby considering an adequate calcium intake and vitamin D status.

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Chapter 3

Influence of other nutritional factors on bone health

Chapter 3.1

Impact of magnesium on bone health in older adults: a systematic review and meta-analysis

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ABSTRACT

Background. Magnesium plays a key role in bone health and may, therefore, represent an interesting nutrient for the prevention of bone loss and osteoporosis. The aim of this systematic review and meta-analysis was to investigate the impact of magnesium intake from any source on bone mineral density (BMD), bone mineral content (BMC), bone turnover markers, and fracture risk in older adults.

Methods. A systematic search was conducted using Embase, Medline Ovid and Cochrane Central from database inception to October 2020. All studies that related magnesium intake with bone health outcomes among adults aged ≥60 years were included. Two investigators independently conducted abstract and full-text screenings, data extractions, and risk of bias assessments. Authors were contacted for missing data.

Results. Once 787 records were screened, six cohort studies, one case-control study and five cross-sectional studies were included. Qualitative evaluation demonstrated a positive trend between higher magnesium intake and higher hip and femoral neck BMD. Meta-analysis of four studies showed a significant positive association between magnesium intake and hip BMD (pooled beta: 0.03, 95% CI: 0.01–0.06, $p < 0.05$).

Conclusions. This systematic review indicates that a higher magnesium intake may support an increase in hip and femoral neck BMD. Due to limited research no associations with BMD at other sites or fractures were found. There is a need for properly designed cohort studies to determine the association between magnesium intake and bone health in older adults. Next, large and long-term randomized controlled trials in older adults are needed to determine whether an increase in magnesium (supplementation) intake can improve bone health. The combination of several bone nutrients (calcium, vitamin D, protein, magnesium and potentially more) may be needed for the most optimal effect on bone health and to delay or prevent the development of osteoporosis.

INTRODUCTION

People from the age of 50 years are at risk of developing osteoporosis, a condition which causes 8.9 million osteoporotic fractures each year worldwide [1]. In order for bones to grow and to be maintained, several nutrients are needed [2]. For the prevention of bone loss, and thus the development of osteoporosis, calcium and vitamin D are well known. Another nutrient that plays a role in bone health is magnesium. This nutrient represents a crucial cofactor for enzymes necessary for the synthesis of bone matrix [3] and it plays a role in bone formation by stimulating osteoblast proliferation [4]. In addition, magnesium deficiency can lead to abnormal hydroxyapatite crystals (a major component of bone), to an increase in the secretion of proinflammatory cytokines which stimulate osteoclast activity, and to lower parathyroid hormone (PTH) and 25-hydroxyvitamin D [25 (OH)D] levels [3-4]. It is yet unclear if magnesium can have the same impact on the development of osteoporosis as calcium and vitamin D.

Results from studies investigating the relation between magnesium intake and bone health have been contradictory, according to the first and only systematic review and meta-analysis on magnesium intake and bone health in 2015 [5]. In this study, no restrictions on the study population were applied. A positive significant correlation between magnesium intake with hip bone mineral density (BMD) was found (pooled r: 0.16; 95% CI: 0.00–0.32) as well with femoral neck BMD (pooled r: 0.14; 95% CI: 0.00–0.28). However, no correlations were found between magnesium intake with lumbar spine BMD and risk of total and hip fractures [5]. More studies have been published since 2015, which may lead to new insights on the relation between magnesium and bone health.

In the present study, we addressed our hypothesis that an adequate total magnesium intake (350 mg/day for adult men and 300 mg/day for adult women [6]) results in higher bone mineral content (BMC) and BMD and suppresses bone turnover and subsequently reduces fracture risk in older adults (aged 60 years or older). Older adults specifically can be at risk of a magnesium deficiency due to a decreased absorption and increased excretion of magnesium [7]. In addition, magnesium intakes in older adults in multiple Western countries are found to be lower than the recommended intake for adults [8-9]. Considering the mechanisms by which magnesium can influence bone health, the higher risk of magnesium deficiency and age-related bone loss, we hypothesized that an adequate magnesium intake can contribute to the prevention of osteoporosis. Therefore, the aim of this systematic review and meta-analysis was to examine

the impact of magnesium intake from any source on BMC, BMD, bone turnover markers and fracture risk in older adults.

METHODS

The reporting of this systematic review and meta-analysis was conducted in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) guidelines [10]. This study was registered at Research Registry (identification number 1122).

Data sources and searches

The databases Embase, Medline Ovid and Cochrane Central were searched to identify relevant studies that examined the relations of magnesium intake (food and/or supplemental sources) with bone health outcomes of interest (**Table 1**) from database inception to July 2021. The searches were limited to the English language. The complete search strategy per database is available in **Supplemental Table 1**.

Table 1. Bone health outcomes of interest.

Outcome	Sites
BMC	Total body
BMD	Total body, hip, femoral neck, lumbar spine
Bone turnover markers	Bone formation and resorption markers
Fracture risk	Hip and total

BMC = Bone Mineral Content; BMD = bone mineral density.

Eligibility criteria

Studies were eligible for inclusion if they (1) evaluated the relationship between magnesium intake and bone health; (2) had an intervention duration of at least 6 months; (3) included older adults aged ≥60 years (or mean age ≥60 years if also younger individuals were included). There was no restrictive criterion on study design. Studies were excluded if no original data was presented or if published in the form of conference abstracts, letters, reviews, or meta-analyses. Animal studies and in vitro studies were excluded as well. Lastly, studies including solely participants with a diagnosed disease or with a baseline population of which >20% was diagnosed with a disease were also not included.

Study selection

First, duplicates across the three literature searches were removed. Second, titles and abstracts were independently screened for eligibility by two researchers, which were blinded to each other's decisions. Lastly, for articles that had passed the first screening, the full texts were retrieved to further verify eligibility. This was also done independently by two researchers and reasons for exclusion were collected in Excel. Disagreements between individual judgements were resolved by a third researcher.

Data extraction

From all eligible studies, the following information was extracted: study characteristics, intervention details, relevant outcomes and their assessment methods, data details and confounders. This information was organized by study type in a data extraction sheet in Excel. One researcher extracted the data, which was reviewed and confirmed by another researcher.

Risk of bias in individual studies

To assess risk of bias of included cohort studies and case-control studies, the Newcastle-Ottawa Scale (NOS) was used [11]. The NOS evaluates three parameters: selection, comparability and outcome/exposure. Each study was awarded with a score from 0 to 9 with higher scores reflecting lower risk of bias. Risk of bias in cross-sectional studies was assessed using an adapted version of the AXIS-tool, following the example of Weeda et al. [12], with only questions focusing on study design and conduct [13]. Each study was assigned a score from 0 to 8 with higher scores reflecting lower risk of bias. Two investigators independently assessed the risk of bias in included studies. Disagreements were discussed and resolved via group consensus.

Data synthesis

All eligible studies were summarized in tables including first author, publication year, cohort name (if applicable), participant characteristics, baseline mean age or age range, exposure assessment, mean magnesium intake, source of magnesium, follow-up period, relevant outcomes, and effect sizes. Summary tables were organized by study type (cohort, case-control and cross-sectional). Results were qualitatively and, if possible, quantitively summarized by study type and outcome of interest.

Meta-analysis

The Cochrane Handbook for conducting meta-analyses was pursued when adequate data were accessible [14]. If required data were not reported, authors of

relevant publications were contacted. Both the chi-square test and the I-squared statistic were used to address statistical heterogeneity across studies. A value ≥50% was used as a threshold for indicating statistical heterogeneity [14-15]. When heterogeneity was present, a random-effects model was applied, if not then a fixed-effect model was used. Results were pooled with standardized mean differences for continuous outcomes and hazard ratios (HR) for binary outcomes. Sensitivity analyses were conducted to identify the effect of a single study on the total estimate and of studies that were judged to be at high risk of bias. Meta-analysis was performed using R (v4.0.3; R Foundation for Statistical Computing, Vienna; packages meta and metafor).

RESULTS

Search results

The search resulted in 988 records (**Figure 1**). After removal of duplicates and exclusion based on abstract and title, a total of 62 publications were found eligible for full-text review. In total eleven articles were included for data extraction (five cohort studies, one case-control study and five cross-sectional studies). The characteristics of the included studies are presented in **Table 2** (cohort), **Table 3** (case-control) and **Table 4** (cross-sectional). Two cohort studies reported cross-sectional data as well and are, therefore, included in both **Tables 2** and **4** [16-17].

Risk of bias

The assessment of risk of bias in the selected cohort studies is presented in **Table 5**. Risk of bias was classified as high (score 1–3), some concerns (score 4–6), or low (score 7–9). Three cohort studies had some concerns [16, 18, 20] and two cohort studies were classified as having a low risk of bias [17, 19]. With respect to controlling for important confounders, one point was given if the study controlled for age, gender, weight or BMI, energy intake, physical activity, smoking, alcohol, vitamin D, and calcium. A second point was given if family history of osteoporosis, fractures, and illnesses were included. None of the cohort studies controlled for all these factors, but four studies adjusted for a large part of the relevant confounders [16-18, 20]. Dropout rates varied from 6% to 31%. A dropout above 20% and without a description of those lost was considered as high, which applied to two studies [16, 20].

The assessment of risk of bias in the selected case-control study is presented in **Table 6** [21]. Overall risk of bias was classified as 'some concerns', multiple

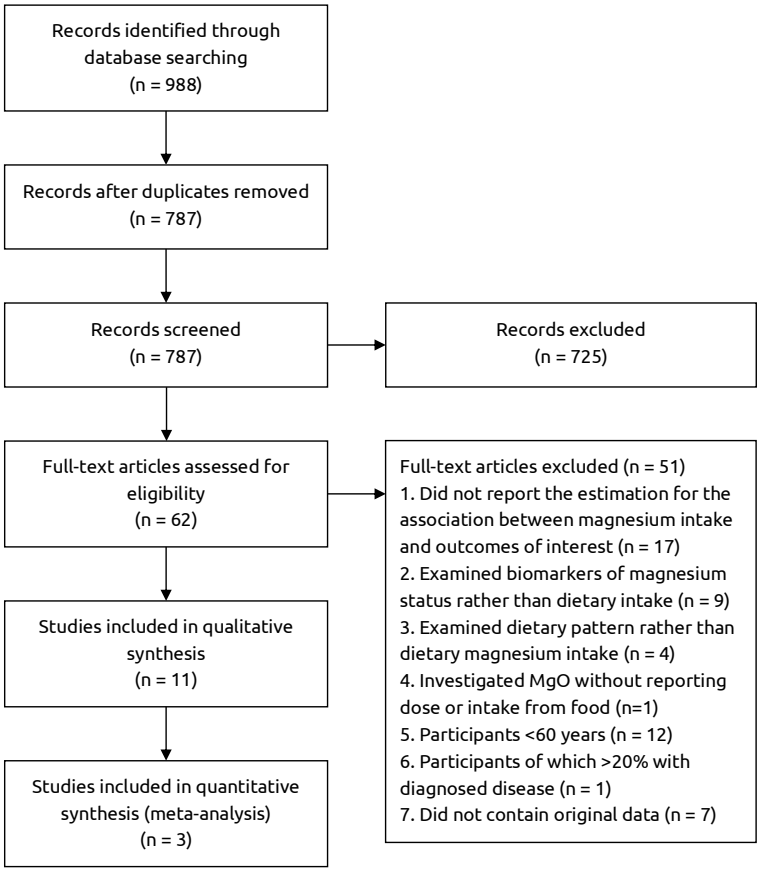

Figure 1. Flow diagram of study selection.

important confounders were not included and there was no information about hip fracture history for the controls.

For the cross-sectional studies (**Table 7**), only one scored 5 out of 8 points [24] and the other 6 studies scored 6 or 7 points. All cross-sectional studies failed on justification of sample sizes. In addition, half of the studies failed to receive points for the fact that the selection process was not likely to select participants that were representative of target/reference population under investigation [17, 23-24, 26].

BMC

No eligible studies were found investigating the effect of magnesium intake on BMC in older adults.

Table 2. Summary table of cohort studies included in the analysis.

First Author, year (ref)	*Cohort name (country)*	*Participants*	*N baseline/ analyzed*	*Baseline mean age(SD) (y)*	*Exposure assessment*	*Mean Mg intake*[a]	*Sources*	*Follow-up (y)*	*Relevant outcomes*	*Effect sizes*[b]	
Chan, 2011 [18]	- (China)	Men and women ≥65 y	2944/ 2217 (1225 men, 992 women)	Men: 71.6(4.6); women: 72.0(5.1)	FFQ	Men: 390; women: 387	Food	4	Hip BMD	Men: B -0.0002 SE 0.029 p 0.782 Women: B 0.036 SE 0.035 p 0.237	◯ ◯
									FN BMD	Men: B 0.001 SE 0.001 p 0.200 Women: B 0.022 SE 0.001 p 0.487	◯ ◯
Kaptoge, 2003 [19]	EPIC-Norfolk Study (US)	Men and women ≥65 y	944/ 892 (450 men, 442 women)	Mean (95%CI): Men: 72.0 (68.0, 77.4); women: 71.9 (67.9, 77.0)	7 d food records	Men: 304; women: 255	Food	2.8	Hip BMD	Men: B 0.082 SE 0.076 p 0.279 Women: B -0.020 SE 0.084 p 0.814	◯ ◯
Orchard, 2014 [16]	WHI Observational Study (US)	PM women 50-79 y	93,676/ 73,684	63	FFQ	<207, 207-270, 270-334, 334-422, ≥422 (quintiles)	Food and supplements	7.6	Hip fracture	Q2 vs Q1: HR 1.1 (0.90, 1.36); Q3 vs Q1: HR 0.90 (0.71, 1.12); Q4 vs Q1: HR 0.90 (0.71, 1.14); Q5 vs Q1: HR 1.04 (0.81, 1.34); p-trend 0.563	◯
									Total fracture	Q2 vs Q1: HR 1.02 (0.96, 1.08); Q3 vs Q1: HR 1.01 (0.95, 1.07); Q4 vs Q1: HR 1.00 (0.94, 1.06); Q5 vs Q1: HR 1.01 (0.95, 1.08); p-trend >0.999	◯
Tucker, 1999 [17]	Framingham Heart Study (US)	Men and women 69-97 y	907/ 628 (229 men, 399 women)	Men: 75.1(4.9); women: 75.3(4.8)	Semi-quantitative FFQ	Men: 300; women: 288	Food and supplements	4	FN BMD	Per 100 mg increase in Mg intake: Men: B 0.018 SE NR p<0.01 Women: B 0.002 SE NR p ns	 ⊕ ◯
Veronese, 2017 [20]	Osteoarthritis Initiative Study (US)	Men and women	4796/ 3765 (1577 men, 2071 women)	60.6(9.1)	FFQ	Men: 161, 239, 299, 359, 491; women: 144, 225, 281, 338, 454 (quintiles)	Food and supplements	6.2	Total fracture	Men: Q2 vs Q1: HR 0.53 (0.31, 0.90) p 0.02; Q3 vs Q1: HR 0.56 (0.33, 0.97) p 0.04; Q4 vs Q1: HR 0.65 (0.36, 1.17) p 0.15; Q5 vs Q1: HR 0.47 (0.21, 1.00) p 0.05; Women: Q2 vs Q1: HR 077 (0.52, 1.14) p 0.20; Q3 vs Q1: HR 0.62 (0.39, 0.99) p 0.05; Q4 vs Q1: HR 0.56 (0.32, 0.98) p 0.04; Q5 vs Q1: HR 0.38 (0.17, 0.82) p 0.01	 ⊕ ⊕

BMD = bone mineral density, HR = hazard ratio, Mg = magnesium, NR = not reported, ns = not significant, PM = postmenopausal, FFQ = food frequency questionnaire, FN = femoral neck, SE = standard error, US = United States, ⊕ = results show a significant positive association, ◯= results show no association.

[a] *Unit is mg/day. Values presented as mean or range.*

[b] *Values presented as mean(SE) or HR (95% CI), BMD in g/cm^2.*

Table 3. Summary table of the case-control study included in the analysis.

First Author, year (ref)	*Country*	*Participants*	*N*	*Mean age (y)*	*Exposure assessment*	*Mean Mg intake*[a]	*Sources*	*Relevant outcomes*	*Effect sizes*[b]	
Michaëlsson, 1995 [21]	Sweden	Women born in 1914-1948	1140 (247 fracture; 893 no fracture)	Fracture: 67.6; no fracture: 67.7	FFQ	<219, 219-256, 257-306, >306 (quartiles)	Food and supplements	Hip fracture	Q2 vs Q1: OR 1.48 (0.84-2.58) Q3 vs Q1: OR 2.65 (1.35-5.21) Q4 vs Q1: OR 2.74 (1.25-6.04) p-trend 0.007	⊖

FFQ = food frequency questionnaire, Mg = magnesium, OR = odds ratio, ⊖ = results show a significantly increased fracture risk.

[a] *Unit is mg/day. Values presented as range.*

[b] *Values presented as OR (95% CI)*

Table 4. Summary table of cross-sectional studies included in the analysis.

First Author, year (ref)	*Country*	*Participants*	*N*	*Mean age(SD) (y)*	*Exposure assessment*	*Mean Mg intake*[a]	*Sources*	*Relevant outcomes*	*Effect sizes*[b]	
Ilich, 2003 [22]	US	PM women	136	68.7(7.1)	3 d food records and FFQ	345	Food and supplements	FN BMD	B 0.000094 SE NR p 0.046	⊕
Gunn, 2014 [23]	New-Zealand	PM women	142	60.4; range 50-70	3 d food records	350	Food	FN BMD	r 0.14 p<0.05	⊕
								CTX	r -0.09 p ns	◯
								P1NP	r -0.20 p<0.05	⊖
McCabe, 2004 [24]	India	Men and women ≥60 y	745 (116 white men, 75 black men, 289 white women, 265 black women)	72.8(7.5)	Health Habits and History Questionnaire + (frozen) yogurt	White men: 317; black men: 262; white women: 326; black women: 212	Food	Hip BMD	Black and white men: r 0.18 p<0.05 Black and white women: r 0.03 p ns	⊕ ◯
								FN BMD	Black and white men: r 0.21 p<0.05 Black and white women: r 0.03 p ns	⊕ ◯
Orchard, 2014 [16]	US	PM women 50-79 y	4778	63	FFQ	<207, 207-270, 270-334, 334-422, ≥422 (quintiles)	Food and supplements	Hip BMD	Q5 vs Q1: 3% higher p<0.001 (adjusted least-squares mean: 0.830 vs 0.855)	⊕
								TB BMD	Q5 vs Q1: 2% higher p<0.001 (adjusted least-squares mean: 1.003 vs 1.021)	⊕
Ryder, 2005 [25]	US	Men and women 70-79 y	2038 (716 white men, 352 black men, 534 white women, 436 black women)	White men: 73.9(2.8); black men: 73.5(2.8); white women: 73.6(2.6); black women: 73.6(2.7)	Semi-quantitative FFQ	White men: 331; black men: 305; white women: 308; black women: 279	Food and supplements	TB BMD	White men: B 0.039 SE NR p 0.05 Black men: effect size NR p 0.55 White women: B 0.052 SE 0.019 p 0.005 Black women: effect size NR p 0.83	⊕ ◯ ⊕ ◯
								Hip BMD	White men: B 0.032 SE 0.024 p 0.19 Black men: NR White women: B 0.044 SE 0.020 p 0.03 Black women: NR	◯ ⊕
Tucker, 1999 [17]	US	Men and women 69-97 y	907 (345 men, 562 women)	Men: 75.1(4.9); women: 75.3(4.8)	Semi-quantitative FFQ	Men: 300; women: 288	Food and supplements	FN BMD	Per 100 mg increase in Mg intake: Men: B 0.023 SE NR p<0.1 Women: B 0.012 SE NR p ns	 ◯ ◯
Woo, 2009 [26]	China	Men and women ≥65 y	1098 (289 men, 809 women	72.3(5.3)	7 d FFQ	380	Food	Hip BMD	% difference per 196 mg increase in Mg intake: 0.1% (-0.9, 1) p ns	 ◯
								LS BMD	0.5% (-0.8, 1.7) p ns	◯

BMD = bone mineral density, CTX = C-terminal telopeptide of type I collagen, HR = hazard ratio, LS = lumbar spine, Mg = magnesium, NR = not reported, ns = not significant, P1NP = procollagen type I N propeptide, PM = postmenopausal, FFQ = food frequency questionnaire, FN = femoral neck, SE = standard error, TB = total body, US = United States, ⊖ = results show a significant negative association, ⊕ = results show a significant positive association, ◯ = results show no association.

[a] *Unit is mg/day. Values presented as mean or range.*

[b] *Values presented as mean (95% CI), partial r or HR (95% CI), BMD in g/cm^2.*

Table 5. Newcastle - Ottawa quality assessment scale for selected cohort studies.

	Selection				Comparability	Outcome				
First Author, year (ref)	**Representativeness of the exposed cohort**	**Selection of the non-exposed cohort**	**Ascertainment of the exposure**	**Outcome of interest absent at baseline**	**Control for important confounders**	**Outcome assessment**	**Adequate follow-up duration**	**Completion of cohort follow-up**	**Total points out of 9**	**Risk of bias**
Chan, 2011 [18]	0	1	0	1	1	1	1	1	6	Some concerns
Kaptoge, 2003 [19]	1	1	1	1	0	1	1	1	7	Low
Orchard, 2014 [16]	1	1	0	0	1	1	1	0	5	Some concerns
Tucker, 1999 [17]	1	1	0	1	1	1	1	1	7	Low
Veronese, 2017 [20]	1	1	0	1	1	0	1	0	5	Some concerns

A study receives a maximum of one point for each item within the category's 'selection' and 'outcome'. A maximum of two points can be awarded for the item within the comparability category.

Table 6. Newcastle-Ottawa quality assessment scale for the selected case-control study.

	Selection				Comparability	Exposure				
First Author, year (ref)	**Adequate case definition**	**Representativeness of the cases**	**Selection of controls**	**Definition of controls**	**Control for important confounders**	**Ascertainment of exposure**	**Same method of ascertainment**	**Non-response rate**	**Total points out of 9**	**Risk of bias**
Michaëlsson, 1995 [21]	1	1	1	0	0	0	1	0	4	Some concerns

A study receives a maximum of one point for each item within the category's 'selection' and 'outcome'. A maximum of two points can be awarded for the item within the comparability category.

Table 7. Risk of bias in the selected cross-sectional studies based on the AXIS-tool.

First Author, year [ref]	Study design	Justified sample size	Appropriate population base	Representative population	Appropriate measurements	Correctly usage of instruments	Discussion/ conclusion	Ethical approval	Total points out of 8
Ilich, 2003 [22]	1	0	1	1	1	1	1	1	7
Gunn, 2014 [23]	1	0	1	0	1	1	1	1	6
McCabe, 2004 [24]	1	0	0	0	1	1	1	1	5
Orchard, 2014 [16]	1	0	1	1	1	1	1	1	7
Ryder, 2005 [25]	1	0	0	1	1	1	1	1	6
Tucker, 1999 [17]	1	0	1	0	1	1	1	1	6
Woo, 2009 [26]	1	0	1	0	1	1	1	1	6

A study receives a maximum of one point for each item.

BMD – total body

Two cross-sectional studies assessed the effect of magnesium intake (food and supplements) on total body BMD, showing beneficial effects [16, 25]. Orchard et al. [16] found that total body BMD was 2% higher ($p < 0.001$), in women who consumed >422.5 mg/day compared with <206.5 mg/day (Q5 vs Q1) and Ryder et al. [25] found in white, but not black, men and women, that magnesium intake was significantly positively associated with total body BMD (white men: beta = 0.039, SE unknown; white women: beta = 0.052, SE = 0.019). For every 100 mg/day increase in magnesium intake, the total body BMD increased approximately with 2%. Furthermore, BMD was 0.04 g/cm^2 higher in white women and 0.02 g/cm^2 higher in white men in the highest compared to the lowest quintile of magnesium intake (247.8 and 394.2 mg/day, respectively; $p < 0.001$).

BMD – hip

Six studies assessed the effect of magnesium intake on hip BMD, including two cohort studies and four cross-sectional studies, of which three found beneficial effects [16, 24-25] and three found no association [18-19, 26]. Regarding the cohort studies, Chan et al. [18] found that dietary magnesium intake was not associated with % change in hip BMD in men and women after 4 years of follow-up. Kaptoge et al. [19] found no significant effect of dietary magnesium intake on hip BMD in both men and women after 2.8 years of follow-up (beta = 0.082, SE = 0.076 for men; beta = –0.020, SE = 0.084 for women). Four cross-sectional studies evaluated the effect of magnesium intake, from food only [24, 26] and combined with supplements [16, 25], on hip BMD. Orchard et al. [16] found that hip BMD was 3% higher ($p < 0.001$), in postmenopausal women who consumed >422.5 mg/day compared with <206.5 mg/day (Q5 vs Q1). Ryder et al. [25] found that magnesium intake was significantly positively associated with BMD at the hip in white women (beta = 0.044, SE = 0.020, $p = 0.03$), whereas in white men, the relationship was not as strong (beta = 0.032, SE = 0.024, $p = 0.19$). This association was not found in black men and women. McCabe et al. [24] found a significantly positive relation between magnesium intake and hip BMD for men (partial $r = 0.180$; $p < 0.05$). However, no correlation between hip BMD and magnesium intake was found in the women. Woo et al. [26] showed that magnesium intake was not associated with hip BMD in men and women.

BMD – femoral neck

Six studies assessed the impact of magnesium intake on femoral neck BMD, including two cohort studies and four cross-sectional studies, of which four studies found significant effects [17, 22-24] and two studies found no effect [17-18]. Tucker et al. [17] found a significant positive association between magnesium

intake, from both food and supplements, and change in femoral neck BMD in men after 4 years of follow-up (beta = 0.018, SE unknown, p < 0.01). In contrast with men, there was no association between magnesium intake and change in femoral neck BMD in women after 4 years of follow-up. Chan et al. [18] showed that dietary magnesium intake was not associated with % change in femoral neck BMD in men and women, after 4 years of follow-up. Four cross-sectional studies assessed the effect of magnesium intake, from food only [23-24] and combined with supplements [17, 22], on femoral neck BMD. Gunn et al. [23] found that magnesium intake was significantly positively associated with femoral neck BMD in postmenopausal women (partial r = 0.140; p < 0.05). McCabe et al. [24] found a significantly positive relation between magnesium intake and femoral neck BMD in black and white men (partial r = 0.210; p < 0.05) but not in black and white women. Ilich et al. [22] found a significant positive association between magnesium intake and femoral neck BMD in postmenopausal women (beta = 0.00094, SE unknown, p = 0.046). Tucker et al. [17] found no significant association in both men and women.

BMD – lumbar spine

Only one cross-sectional study by Woo et al. [26] looked at lumbar spine BMD and found that magnesium intake (from food only) was not associated with lumbar spine BMD in both men and women.

Bone turnover markers

Only Gunn et al. [23] looked at the association between dietary magnesium intake and the bone turnover markers C-terminal telopeptide of type I collagen (CTX) and procollagen type I N propeptide (P1NP) in postmenopausal women, in a cross-sectional study design. Magnesium intake was significantly inverse associated with P1NP (partial r = −0.20, p < 0.05), but not with CTX (partial r = −0.09, p ns).

Fractures

Two cohort studies looked at the association between magnesium intake and total fracture risk and reported different results [16, 20]. Veronese et al. [20] found that men and women in the highest quintile of magnesium intake (food and supplements) reported a significant lower risk for fractures, taking those in the first quintile as reference, after 6.2 years of follow-up (men: 491 vs 161 mg/day, HR 0.47, 95% CI 0.21–1.00, p 0.05, women: 454 vs 144 mg/day, HR 0.38, 95% CI 0.17–0.82, p 0.01). Orchard et al. [16] found no significant differences in HRs of total fractures across the quintiles of magnesium intake (food and supple-

ments) (Q5 ≥ 422.5 mg/day vs Q1 < 206.5 mg/day, HR 1.01, p-trend > 0.99) in postmenopausal women after 7.6 years of follow up.

Regarding hip fracture risk specifically, one case-control study and one cohort study examined this outcome. Michaëlsson et al. assessed the relation between magnesium intake (from food and supplements) and hip fracture risk in women, in a case-control study [21]. The study showed that high intakes of magnesium was significantly associated with an increased risk of hip fracture. The highest risk was calculated for the highest quartile of magnesium intake, taking the first quartile as reference (>306 mg/day vs <219 mg/day, OR 2.74, 95% CI 1.25–6.04, p-trend 0.01). Orchard et al. [16] found no significant differences in HRs of hip fractures across quintiles of magnesium intake (food and supplements) (Q5 ≥ 422.5 mg/day vs Q1 < 206.5 mg/day, HR 1.04, p-trend 0.56) in postmenopausal women after 7.6 years of follow up.

Meta-analysis

A meta-analysis could be performed for hip BMD among two cohort studies and two cross-sectional studies including seven different groups (**Figure 2**). Two cross-sectional studies could not be included because they reported the relation in a different effect size. Heterogeneity was not significantly present for hip BMD (I^2 = 0.0%, heterogeneity chi-squared p = 0.88). However, a random-effects model was used since different study designs were included. The meta-

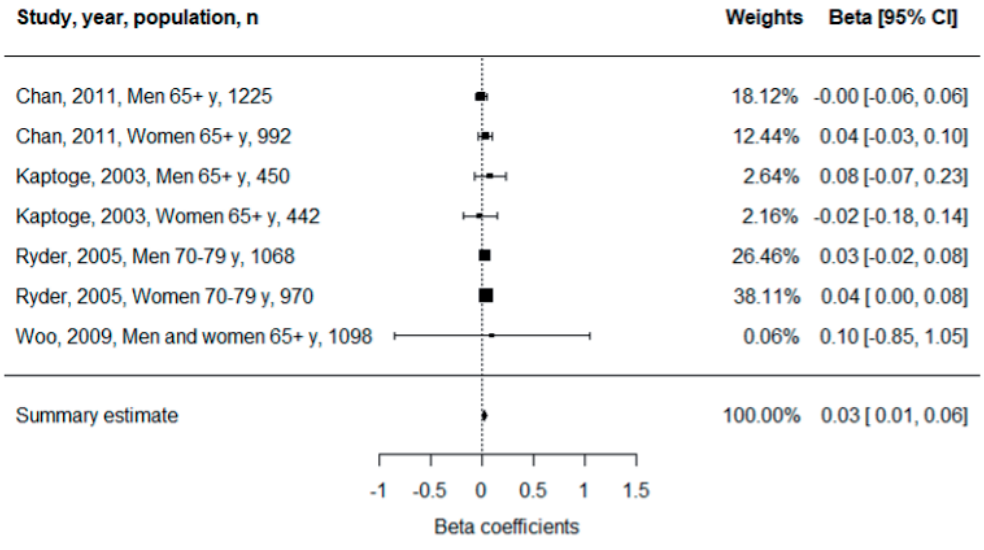

Figure 2. Forest plot illustrating the impact of dietary magnesium intake on hip BMD.

analysis showed a significant positive association between magnesium intake and hip BMD (pooled beta: 0.03, 95% CI 0.01, 0.06, $p < 0.05$). Sensitivity analyses demonstrated that there was no single study influencing the overall estimate substantially.

Since missing quantitative data could not be provided for each relevant publication after contact with authors, meta-analyses with other outcomes of interest could not be conducted.

DISCUSSION

The aim of this systematic review and meta-analysis was to examine the impact of magnesium intake from any source on BMC, BMD, bone turnover markers and fracture risk in older adults. Qualitative evaluation showed a positive trend between higher magnesium intake and higher hip and femoral neck BMD (**Table 8**). Meta-analysis of four studies including seven different groups showed a significant positive association between higher magnesium intake and higher hip BMD. No conclusions could be drawn regarding BMC, total body and lumbar spine BMD, bone turnover markers and fracture risk due to a limited number of studies assessing these outcomes.

Table 8. Summary of the evidence for the impact of magnesium intake on bone health.

Outcome	Number of studies	Positive impact	No impact	Negative impact	Conclusion
BMC	0	0	0	0	Not enough evidence
TB BMD	2	2	0	0	Not enough evidence
Hip BMD	6	3	3	0	Higher Mg intake potentially beneficial
FN BMD	6	4	2	0	Higher Mg intake potentially beneficial
LS BMD	1	0	1	0	Not enough evidence
BTM	1	1	1	0	Not enough evidence
Total fracture risk	2	1	1	0	Not enough evidence
Hip fracture risk	2	0	1	1	Not enough evidence

BMC = bone mineral content, BMD = bone mineral density, BTM = bone turnover markers, LS = lumbar spine, Mg = magnesium, FN = femoral neck, TB = total body.

Comparison of the included studies is complicated because varying levels of magnesium intake were studied and some looked at magnesium intake from both food and supplements, while others only took dietary magnesium into account. In studies reporting magnesium intake as a mean value, the intake ranged from 212 to 390 mg/day [17-19, 22-26]. Three studies divided the magnesium intake in quintiles with the highest magnesium category varying from 306 mg/day [21] to 422 mg/day [16] to 491 mg/day for men and 454 mg/day for women [20]. However, no difference in the number of significant results were seen in studies including participants with higher intakes or studies taking magnesium intake from both supplements and food into account.

Besides the different amounts of magnesium intake between the studies, the bioavailability of magnesium should also be considered. Magnesium absorption takes places in the small intestine, which can be affected by several factors, for example the dose, food matrix and dietary factors. Impairing dietary factors include high doses of other minerals, partly and non-fermentable fibers, phytate and oxalate [27]. Dietary factors enhancing magnesium uptake include protein, medium-chain-triglycerides, and low- or indigestible carbohydrates [27]. Factors that can increase magnesium requirements are gastrointestinal diseases [27], chronic alcohol abuse [28] and diseases that results in malabsorption, for example type 2 diabetes [29]. In addition, several drugs including proton pump inhibitors (PPI), diuretics and chemotherapeutic agents can lead to a magnesium deficiency [30]. Only two studies took drug use into account, but the investigated populations were mainly healthy older adults.

One study in the current review found that high intakes of magnesium were associated with an increased risk of hip fractures [21]. However, this case-control study had a higher risk of bias (scored 4 out of 9 points on the NOS) and they did not control for all important confounders. Only one other study investigated the impact on hip fractures and they found no association [16]. Hence, the impact of magnesium intake on hip fracture risk remains unclear.

There were some variations in the associations between magnesium intake and bone health outcomes between men and women across the studies. Veronese et al. found that the impact of magnesium on fracture risk was more important in women than in men (62% and 53% reduction, respectively) [20]. This pattern is in line with the findings of Ryder et al., who concluded that the associations between magnesium intake and total body BMD as well as hip BMD in men were not as strong as they were in women [25]. However, two studies found a significant association between magnesium intake and femoral neck and/or hip BMD for

men, while no significant association was found for women [17, 24]. It is known that women, particularly after menopause, have lower intakes of micronutrients than men have, making them more susceptible to the effects of nutritional deficiencies [31]. Furthermore, the prevalence of osteoporosis is higher in women than in men [32]. More research is needed to understand whether the effect of magnesium on bone health outcomes is gender specific.

The meta-analysis showed a significant positive association between magnesium intake and hip BMD (pooled beta: 0.03, 95% CI 0.01, 0.06) and is in line with the meta-analysis of Farsinejad-Marj et al. (2015) which included a younger population as well (r 0.16, 95% CI 0.001, 0.32) [5]. They also found that high intakes of magnesium were not associated with increased risk of hip and total fractures. In addition, they observed a positive marginally significant association between magnesium intake and BMD in femoral neck (r 0.14, 95% CI 0.001, 0.28), but no significant association was found with lumbar spine BMD. In comparison with current review, there was not enough data to assess femoral neck and lumbar spine BMD. Note that the results of the meta-analysis should be interpreted with caution due to the low number of included studies.

Since nutrients interact with each other, relationships among nutrients should also be considered. A review by Erem et al. (2019) looked at the interaction between magnesium and vitamin D in older adults [33]. They explained that magnesium is needed by several enzymes involved in vitamin D metabolism, for example for those involved in the conversion of vitamin D to the biological active form. Magnesium also interacts with calcium. As high calcium intake complicates magnesium retention and low magnesium levels can lead to excess calcium excretion, there is an optimal calcium-to-magnesium ratio (suggested to be 2–2.8: 1) [33]. Future studies are warranted to further explore the mechanisms.

Based on the current evidence, calcium and vitamin D remain the most important nutrients for the prevention of bone loss. However, magnesium may play an additional role, which also applies to protein [34]. For older adults, it is recommended to have a calcium intake of 1000 mg/d (inclusion of supplements only if needed), take vitamin D supplementation (800 IU cholecalciferol) to maintain serum 25(OH)D levels >50 nmol/L and have a dietary protein intake of 1.0–1.2 g/kg body weight/d [35-36]. Regarding magnesium, it is advised to avoid of a low intake via the diet, the adequate intake is set at 350 mg/day for adult men and 300 mg/day for adult women on the basis of balance studies [6].

This recommendation is not met for all older adults. The mean magnesium intake in healthy older adults in Western countries varies from 274 to 421 mg/d for males and 227 to 373 mg/d for females. This is lower in more frail older adults [37-38]. Dietary sources rich in magnesium include green leafy vegetables, legumes, nuts, seeds, and whole grains; the magnesium content in these products is presented in **Figure 3** [39]. Moreover, legumes, nuts and seeds also contain high amounts of protein and calcium and thus consist of multiple nutrients that have a positive effect on bone health. There is some more overlap between food products high in key bone nutrients. For example, dairy is rich in protein and calcium and although dairy is not in the top 5 of magnesium rich products, it still contributes to your daily magnesium intake. This makes clear that the effects of nutrients on bone health cannot be considered in isolation.

A strength of this review was that only publications studying older adults (aged ≥60 years) were eligible, making the recommendation specific for this more vulnerable group. Consequently, the review did not contain any randomized controlled trials, as existing human intervention studies with magnesium were focused on a younger population. However, a trial in twenty postmenopausal osteoporotic women found that oral magnesium supplementation (daily oral dose of 1830 mg magnesium citrate for 30 days) suppressed bone turnover [40]. All in all, this makes clear that large and long-term randomized controlled trials in older adults are needed to determine whether an increase in magnesium (supplementation) intake can improve bone health.

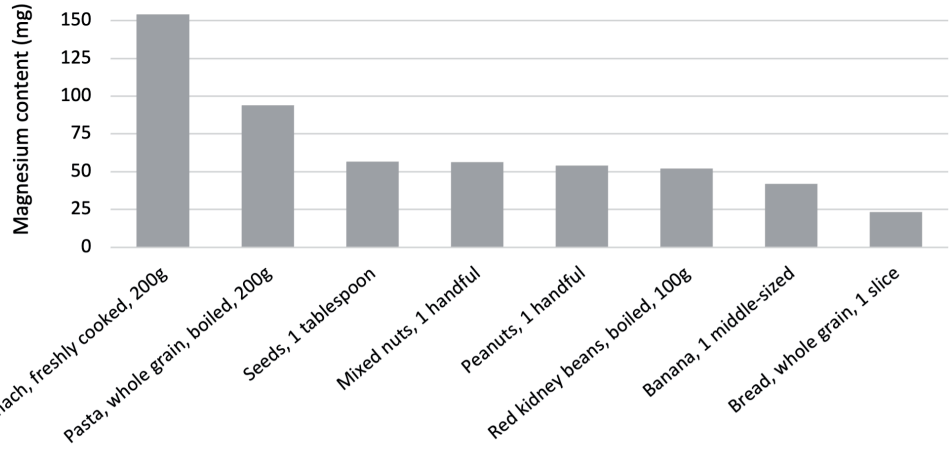

Figure 3. Food products rich in magnesium with content per portion size. Value for seeds is an average of pumpkin seeds, sunflower seeds and pine nuts.

CONCLUSIONS

This systematic review indicates that a higher magnesium intake may support an increase in hip and femoral neck BMD. Due to limited research no associations with BMD at other sites or fractures were found. We still miss properly designed cohort studies investigating the association between magnesium intake and bone health, adjusted for all relevant confounding factors. Understanding the relationship between magnesium and bone health is an important step toward finding preventive measures for age-related bone loss and prevention of osteoporosis. Moreover, we hypothesize that the combination of several bone nutrients (calcium, vitamin D, protein, magnesium and potentially more) are needed for the most optimal effect on bone health.

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SUPPLEMENTARY DATA

Supplemental Table 1. Literature search strategy per database.

Database	Search strategy	
EMBASE	#1	('magnesium'/exp OR 'magnesium intake'/exp OR 'magnesium consumption' OR 'magnesium supplement' OR 'magnesium supplementation') AND ('bone'/exp OR 'fracture'/exp OR 'bone density'/exp OR 'bone demineralization'/exp OR 'bone mineral'/exp OR 'osteoporosis'/exp OR 'bone mass'/exp OR 'bone health' OR 'bone turnover' OR 'calcium'/exp OR 'calcium excretion'/exp OR 'calcium loss') AND ('aged'/exp OR 'elderly')
	#2	limit #1 to (clinical trial or randomized trial)
	#3	'prospective study'/de OR 'longitudinal study'/de OR 'cohort' OR 'cohort study' OR 'cohort studies' OR 'cross-sectional study'/de
	#4	#1 AND #3
	#5	#2 OR #4
COCHRANE CENTRAL	#1	MeSH descriptor: [Magnesium] explode all trees
	#2	magnesium intake
	#3	magnesium consumption
	#4	magnesium supplement
	#5	magnesium supplementation
	#6	#1 OR #2 OR #3 OR #4 OR #5
	#7	MeSH descriptor: [Bone and Bones] explode all trees
	#8	MeSH descriptor: [Fractures, Bone] explode all trees
	#9	MeSH descriptor: [Bone Demineralization, Pathologic] explode all trees
	#10	MeSH descriptor: [Bone Density] explode all trees
	#11	MeSH descriptor: [Osteoporosis] explode all trees
	#12	MeSH descriptor: [Calcium] explode all trees
	#13	bone health
	#14	bone demineralization
	#15	bone density
	#16	bone mass
	#17	bone mineral
	#18	bone resorption
	#19	calcium excretion
	#20	bone turnover
	#21	calcium loss
	#22	#7 OR #8 OR #9 OR #10 OR #11 OR #12 OR #13 OR #14 OR #15 OR #16 OR #17 OR #18 OR #19 OR #20 OR #21
	#23	MeSH descriptor: [Aged] explode all trees
	#24	elderly
	#25	#23 OR #24
	#26	#6 AND #22 AND #25

Supplemental Table 1. Continued

Database	Search strategy
MEDLINE OVID	("magnesium"[MeSH] OR magnesium intake[TIAB] OR magnesium consumption[TIAB] OR magnesium supplement[TIAB] OR magnesium supplementation[TIAB]) AND ("bone and bones"[MeSH] OR "Fractures, bone"[MeSH] OR bone health[TIAB] OR "bone demineralization, pathologic"[MeSH] OR bone demineralization[TIAB] OR "bone density"[MeSH] OR "osteoporosis"[MeSH] OR bone density[TIAB] OR bone mass[TIAB] OR bone mineral[TIAB] OR bone resorption[TIAB] OR calcium excretion[TIAB] OR bone turnover[TIAB] OR calcium loss[TIAB] OR "calcium"[MeSH]) AND ("aged"[MeSH] OR elderly[TIAB]) AND ("clinical trial"[PT] OR "Randomized Controlled Trial"[PT] OR "cohort studies"[MeSH] OR "Cross-Sectional Studies"[MeSH])

Chapter 3.2

Dairy products help aged care residents to maintain strong bones

Inge Groenendijk, Lisette de Groot and Pol Grootswagers

Translated commentary.

Original title: Zuivel kan botten verzorgingshuisbewoner sterk houden.

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ABSTRACT

A well-performed and large intervention study by Iuliano et al. showed that protein and calcium supplementation via regular dairy products reduces the risk of fractures and falls in vitamin D replete older adults in aged care facilities. The study was performed in Australia, which raises the question whether their intervention might also be effective in aged care facilities in the Netherlands. Intake levels of protein and calcium are comparable between Australian and Dutch older adults. A higher protein intake, for example from dairy, is associated with higher muscle mass, muscle strength and bone density. Iuliano et al. showed that with accessible, regular food products, relevant health outcomes can be improved. These results are relevant for Dutch older adults as well. Future research should investigate whether sustainable dairy substitutes can be just as successful.

Frail older adults often have a low protein and calcium intake. A large Australian study in aged care facilities showed that an increased consumption of dairy products reduces the number of fractures and falls among residents.

According to an Australian study, protein and calcium supplementation via regular dairy products reduced the risk of fractures and falls in older adults [1]. The robust design of this cluster randomized controlled trial is compelling considering: the large study population, which consisted of more than 7000 frail older adults replete in vitamin D; the randomization of 60 different institutions; the excellent adherence to the intervention; and the long follow-up time of 2 years, which is highly desirable for research into bone health. By the end of the trial, the intervention group had a 33% lower overall fracture risk, a 46% lower risk of a hip fracture, and an 11% lower risk of falls compared to the control group. Based on these results, 65 people should receive protein and calcium supplementation via regular dairy products to prevent one fracture and 48 people to prevent one fall. In addition to the hard endpoints, improvements were also found for related biological parameters including an increase in insulin-like growth factor 1 and in bone mineral density.

The Dutch situation

The study was conducted in Australian aged care facilities. Since the Netherlands, like Australia, has a strong dairy consumption culture, the obvious question is whether this intervention might also be effective in Dutch institutions. The Australian participants had a daily protein intake of 0.9 g per kg of body weight and their calcium intake was only 700 mg per day. Though in the Netherlands, a protein intake of 1.0 g/kg per day is common in healthy older adults [2], protein intake is lower in institutionalized older adults: 0.8 g/kg per day [3]. The calcium intake of Dutch institutionalized older adults is approximately 640 mg [3]. So, regarding protein and calcium intake, Dutch and Australian residents of aged care facilities are comparable. A similar intervention might therefore be effective in the Netherlands as well.

Increased need, decreased intake

Older adults have an increased need for various nutrients due to illness, medication use, or absorption issues. At the same time, they generally eat less than younger adults do, whereby a decreased appetite may lead to unwanted weight loss and malnutrition. Accordingly, in the control group of the Australian study, we observed a weight loss of 1.4 kg during the two years in which these older adults were monitored.

Nutrient-dense foods such as enriched (medical) drinks or protein-enriched products are often considered a solution to bridge the gap between intake and requirements. However, the Australian study showed that with regular food products, which are available in every supermarket, highly relevant outcome measures could be improved. It has been previously shown that also the protein intake of Dutch older adults can be increased with protein-rich foods [4] or protein-enriched products [5], by 0.4 and 0.5 g/kg per day, respectively. The relevance of using recognizable products is in line with the findings of the so-called 'Ambiance' project of Wageningen University. This project showed that serving regular meals in an ambient family-style setting leads to a better nutritional intake among older adults [6].

Better muscle and bone health

The finding that an increased protein intake, combined with training, can lead to more muscle mass and strength in older adults was already observed in 2012 in the ProMuscle study. The effect remained visible after implementation in the practical setting [4], in which protein-rich foods were given similar to those in the Australian study. That such an increase in protein intake can also lead to a higher bone density and a reduced risk of fractures emerges from a recent meta-analysis of our research group, which showed that among healthy older adults a protein intake above 0.8 g/kg per day reduces the risk of a hip fracture by 11% [7]. Furthermore, we showed that there is an association between a higher protein intake and a higher bone density in frail, malnourished and healthy older adults [8].

In addition to protein, the investigated dairy products also provide other nutrients by nature which affect bone health, such as calcium, B vitamins and magnesium. An adequate calcium intake, in combination with vitamin D supplementation, can lead to fewer fractures [9]. And although the number of intervention studies is still limited, there seems to be a role for vitamin B12 in reducing fracture risk [10]. Magnesium plays a role in the synthesis of bone matrix and stimulation of cells that make bone tissue. A magnesium deficiency can actually lead to more bone resorption [11].

Animal or plant protein?

The downside of increased consumption of dairy products is that animal products contribute to greenhouse gas emissions. However, based on current knowledge, increasing the intake of plant protein is not a recommended strategy to reduce the fracture and fall risk among older adults. Plant proteins have a lower quality

than animal proteins because they often fall short in terms of amino acid profile and are less digestible and absorbable.

This caution is supported by results from an analysis of protein studies in older adults that our research group has conducted [8]. It showed that the intake of animal protein was positively associated with bone mineral density, while this was not the case for plant protein. This is in line with the finding that vegetarian and vegan diets have been associated with an increased risk of bone fractures [12]. In an ongoing research line, we are therefore investigating the effects of switching to a vegan diet later in life on muscle and bone health.

Conclusion

The results of the Australian study are relevant for Dutch institutionalized older adults as well. Regular dairy products seem helpful in increasing protein and calcium intake, thereby reducing the risk of falls and fractures. Future research should investigate whether sustainable dairy substitutes can be just as successful.

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Chapter 3.3

A combined nutrition and exercise intervention influences serum vitamin B-12 and 25-hydroxyvitamin D and bone turnover of healthy Chinese middle-aged and older adults

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ABSTRACT

Background. Hong Kong faces several public health problems including malnutrition and osteoporosis. Considering the typical Chinese diet and overall low physical activity levels of Chinese adults, timely interventions to improve nutritional status and bone health are needed.

Objectives. We examined the effects of a nutrition plus exercise intervention on serum vitamin B-12 and 25-hydroxyvitamin D [25(OH)D], bone turnover markers, and parathyroid hormone (PTH) concentrations in apparently healthy Chinese middle-aged and older adults.

Methods. In this 24-wk randomized controlled trial, 180 Chinese adults (85 women, mean ± SD age: 61 ± 6 y) were randomly assigned to receive a fortified milk supplement (2 × 30 g/d) and an exercise program (2 × 1 h/wk including resistance, balance, and aerobic training) or no intervention. The primary outcome was physical performance. In this article we analyzed the secondary outcomes serum vitamin B-12 and 25(OH)D concentrations, assessed at baseline, 12 wk, and 24 wk. Also, bone turnover markers and PTH concentrations were studied. Linear mixed models evaluated group differences over time.

Results. A significant time × group interaction ($P < 0.001$) was found for serum vitamin B-12 and 25(OH)D concentrations and the bone turnover markers, but not for serum PTH concentrations ($P = 0.09$). The intervention increased mean ± SD vitamin B-12 concentrations from baseline (345 ± 119 pmol/L) to 24 wk (484 ± 136 pmol/L), whereas concentrations remained stable within the control. For 25(OH)D concentrations, the intervention group had a greater increase from baseline (54.7 ± 14.2 nmol/L) to 24 wk (80.1 ± 19.2 nmol/L) than the control (60.6 ± 15.2 compared with 65.6 ± 14.6 nmol/L). The ratio of the net effect of bone formation and resorption was greater in the intervention group, suggesting less bone remodeling, irrespective of sex.

Conclusions. A fortified milk supplement and exercise intervention successfully improved vitamin B-12 and 25(OH)D concentrations as well as the balance of bone turnover markers of Chinese middle-aged and older adults.

INTRODUCTION

Approximately 31% of the population of Hong Kong is expected to be 65 y or older in 2036 [1]. Being one of the fastest-aging populations in the world, Hong Kong faces several public health problems [2], including osteoporosis and hip fracture incidence [3, 4]. In addition, there is a significant burden of malnutrition [5]. The onset or progress of aging-related pathologies may be delayed with nutrition and physical activity.

For optimal bone health, it is recommended to have a sufficient intake of calcium, vitamin D, and protein [6, 7]. However, the typical Chinese diet is low in calcium and due to the cultural habit of avoiding direct exposure to sunlight, vitamin D deficiency is highly prevalent among Chinese older adults (≥65 y) [8, 9]. Furthermore, older adults have a greater need for protein than younger healthy adults [10]. An inadequate intake of these key bone nutrients can increase the risk of falls and fractures and the prevalence of osteoporosis [7, 10, 11].

Vitamin B-12 deficiency is a common problem worldwide and its prevalence tends to increase with age [12]. However, data are missing on the vitamin B-12 status in middle-aged and older adults living in Hong Kong. Deficiency is often caused by inadequate dietary intake and/or malabsorption of vitamin B-12 [12, 13]. Dietary sources of vitamin B-12 are mostly animal-related such as milk. However, the ongoing cohort survey "China Health and Nutrition Survey" reported that milk intake was low in Chinese people aged 60 y and older [14]. This might lead to a low vitamin B-12 status, which in turn has been associated with a low bone mineral density (BMD), increased bone turnover, and increased fracture risk [15, 16].

Multiple trials have investigated the effect of nutritional supplementation on nutritional status and bone health, but these are mainly in Western populations. The Asian dietary pattern is different from Western diets, which may require other nutritional strategies. A limited number of studies in Chinese women have shown that milk supplementation for 24 mo increased calcium intake and led to less bone loss [17-19]. In addition, men should not be overlooked. Although BMD decreases at a faster rate in postmenopausal women than in men, the latter group tend to have a higher mortality risk after a fracture [20, 21]. Besides nutrition, exercise is beneficial for bone health and a stronger effect may be expected from a combined nutrition and exercise intervention [22, 23]. A survey conducted in 2014 showed low physical activity levels among the adult population of Hong Kong [24]. Therefore, addressing physical activity levels is of interest. To the

best of our knowledge, the current study is the first study targeting both Asian male and female middle-aged and older adults with a combined nutritional and exercise intervention to improve nutritional status and bone health.

This study is part of a large trial investigating the health impact of a nutrition and exercise program in Chinese adults. The aim of the present study was to assess whether a 24-wk multidomain lifestyle intervention including a fortified milk supplement and an exercise program had an effect on serum vitamin B-12 and 25-hydroxyvitamin D [25(OH)D] concentrations and markers of bone turnover in apparently healthy community-dwelling Chinese middle-aged and older adults, compared with a control group without intervention.

METHODS

Study population

A 24-wk randomized controlled trial (RCT) was conducted in 180 apparently healthy community-dwelling Chinese middle-aged and older adults aged 50 y or older living in Hong Kong (latitude 22.3°N). Exclusion criteria included recent (i.e., past 3 mo) or concurrent participation in any lifestyle intervention program, BMI ≥ 27.5 kg/m^2, self-reported allergy or intolerance to ingredients of the fortified milk supplement, poorly controlled or unstable chronic obstructive pulmonary disease/cardiovascular disease/hypertension, recent illnesses or fractures, undergoing treatment of cancer, and regular use of calcium or vitamin D supplements and traditional Chinese medicines. Eligible participants were randomly assigned, stratified by sex and age (50–64 compared with ≥65 y), to the intervention or control group. Further details concerning participant recruitment, study design, and methods have been described elsewhere [25]. The study was approved by the Clinical Research Ethics Committee of the Chinese University of Hong Kong (reference no. 2016.532) and registered at the Dutch Trial Registry (NTR6214). All participants provided written informed consent.

Treatment

The intervention consisted of a nutrition and an exercise component, both lasting for 24 wk. Participants in the intervention group had to drink 2 glasses of a fortified milk supplement (i.e., 2 sachets of 30 g each of unbranded OPTIMEL 60+ Diamond powder produced by FrieslandCampina, Netherlands; commercially available) daily providing 13.6 g protein, 1008 mg Ca, 30 μg cholecalciferol (vitamin D3), 2.9 μg vitamin B-12, and 212 kcal (**Supplemental Table 1** provides a complete overview of the nutritional composition). Cholecalciferol was provided

at 300% and 200% of the Chinese recommended nutrient intake (RNI) for persons aged 50–64 and ≥65 y, respectively; this was still below the tolerable upper limit [26]. Vitamin B-12 was provided at 121% of the Chinese RNI [26]. Participants also received a brief healthy lifestyle kit which highlighted the importance of a balanced diet and daily physical activity.

The exercise program consisted of 2 exercise sessions of 1 h each week, conducted in groups of 8–12 participants, for a total of 48 sessions. The exercise sessions consisted of a warm-up (5–10 min), resistance and balance training (20–30 min), an aerobic component (20 min), and a cool-down (5–10 min). Participants were asked to perform against 60%–80% of their estimated 1-repetition maximum load and resistance was progressively increased based on their Rating of Perceived Exertion (RPE) after each week of training. For the aerobic component, the aim was a minimum RPE of 13–15 at the beginning and to gradually proceed to an RPE of 15–18 during the later aerobic sessions. The exercises were designed to include variations and were conducted under the supervision of an exercise instructor.

Participants in the control group were asked to maintain their usual physical activities and dietary habits during the study period and were subjected to the same measurements as the intervention group.

Compliance

Participants were asked to return any unconsumed sachets of the fortified milk supplement every 2 wk. Subsequently, compliance to the nutritional intervention was assessed by dividing the number of sachets of fortified milk supplement consumed by the number of sachets provided. Compliance to the exercise intervention was assessed by dividing the completed exercise sessions by the total number of prescribed sessions.

Assessments

The primary outcome of the trial was physical performance, which was reported in another article [25]. In this study we analyzed the secondary outcome of nutritional status including serum vitamin B-12 and 25(OH)D concentrations, and the tertiary outcomes of serum bone turnover markers and parathyroid hormone (PTH) concentrations. For this, blood samples were collected in the morning (from 09:00 to 11:30) after fasting for ≥12 h and after 15-min rest at baseline, 12 wk, and 24 wk. Blood was collected in vacutainers with no added anticoagulant and was kept at room temperature for ~1 h in order to clot. Hereafter, serum was separated by centrifugation (at 2450 × g for 10–15 min at ambient tem-

perature) and stored at −80°C, according to the instructions of Pathlab Medical Laboratories Ltd and Quest. The laboratory was accredited by the College of American Pathologists. Testing standards were based on the requirements of ISO 15189:2012 and included inspection of policies, procedures, records, internal quality control, and external quality assurance programs.

Serum vitamin B-12 and 25(OH)D concentrations

Serum vitamin B-12 was measured using chemiluminescent immunoassay (IMMULITE 2000; Siemens Healthineers Global). Intra-assay and interassay CVs were 6.7%–7.0% and 6.0%–7.9%, respectively. A cutoff value of <150 pmol/L was used for vitamin B-12 deficiency [13, 27].

Serum 25(OH)D was measured using LC–tandem MS (Quest Diagnostics). This assay measures both 25-hydroxycholecalciferol [25(OH)D_3] and 25-hydroxyergocalciferol [25(OH)D_2]; total 25(OH)D concentrations were used for the results in the current study. Intra-assay and interassay CVs were between 3.7% and 6.9%. Cutoff values for serum 25(OH)D are <25 nmol/L for deficiency, ≥25 to <50 nmol/L for insufficiency, and ≥50 nmol/L for sufficiency [28, 29].

Bone turnover markers

Serum N-amino terminal propeptide of type I collagen (PINP) (marker of bone formation) was measured using immunoassay (Quest Diagnostics). Intra-assay and interassay CVs were <10%. Serum C-terminal telopeptide of type I collagen (CTX) (β-isomerized; marker of bone resorption) was measured using electrochemiluminescence assay (Quest Diagnostics). Intra-assay and interassay CVs were 1.7%–2.2% and 3.1%–5.7%, respectively.

PTH

Serum concentration of intact PTH was measured using chemiluminescent immunoassay (IMMULITE 2000; Siemens Healthineers Global). Intra-assay and interassay CVs were 4.2%–5.7% and 6.3%–8.8%, respectively.

Dietary intake

All participants were asked to fill in a 3-d dietary record including both weekdays and weekend days at baseline and after 24 wk, which was checked by trained staff for completeness. Data were processed by the Food Processor Nutrition Analysis and Fitness software version 8.0 (ESHA Research), with the addition of composition of local foods based on food composition tables from China and Hong Kong. Protein intake (g/d), calcium intake (mg/d), and vitamin D intake (μg/d) are reported in this article, because these are key nutrients for optimal

bone health. Vitamin B-12 intake (μg/d) is reported as well. More elaborate results on dietary changes will be described separately in another article.

Other measurements

Demographic, lifestyle, and medical history data were collected using standardized questionnaires. Physical activity was assessed with a validated Chinese version of the International Physical Activity Questionnaire (IPAQ-C) [30]. Anthropometric measurements were done using standardized methods. These additional measurements are described elsewhere [25].

Statistical analysis

The trial was powered based on the outcome of gait speed (primary outcome, described in another article [25]) and the secondary outcome of serum 25(OH)D. Based on a previous trial investigating the effect of milk supplementation on bone turnover markers and vitamin D status in healthy Chinese adults [28], a minimum sample size of 114 (57/group) was needed to detect a mean difference of 8 nmol/L in serum 25(OH)D between the control and intervention groups with an SD of 15 nmol/L, 80% power, and a 5% significance level. Accounting for a noncompliance rate of 20% and a dropout rate of 20%, a final sample size of 180 (90/group) participants was chosen.

Data were analyzed using the intention-to-treat method. Continuous variables are presented as mean ± SD for normally distributed data and as median [IQR] for nonnormally distributed data. Categorical variables are presented as number of subjects (percentage). Independent t test, chi-square test, or Mann–Whitney U test was used to compare values at baseline between groups. All data were checked for normality. In case of a nonnormal distribution, nonparametric tests or log transformations were applied. Extreme outliers in primary dependent variables were retained in final analyses when results including and excluding the outlier were similar.

Differences in serum vitamin concentrations and bone markers between groups over time were analyzed using linear mixed models with subject as a random factor and time (baseline, 12 wk, and 24 wk), group (control and intervention), and time × group as fixed factors. To determine differences in vitamin B-12 deficiency and vitamin D insufficiency between the 2 groups at each time point, a chi-square test was used. Within-group differences compared with baseline were tested by McNemar's test.

In addition to the crude concentrations of the bone turnover markers PINP and CTX, an uncoupling ratio was calculated as the ratio of percentage change from baseline [31]:

$$\text{Uncoupling ratio} = \frac{\text{end P1NP} - \text{baseline P1NP}}{\text{baseline P1NP}} \Big/ \frac{\text{end CTX} - \text{baseline CTX}}{\text{baseline CTX}}$$

A ratio <1 indicates net resorption, whereas a ratio >1 indicates net formation [31]. A Mann–Whitney U test was used to investigate if the ratio was different between groups.

Analyses of bone markers were not adjusted for potential confounders including age, sex, BMI, body weight, energy intake, physical activity, smoking, alcohol use, comorbidities, and fracture history [32], because these variables did not significantly differ at baseline and over time between the groups. For the analysis of serum 25(OH)D, season in which the baseline measurement was performed was added as a confounder to the model. Season was categorized as spring (March to May) and summer (June and July). The aforementioned analyses for serum vitamin concentrations and bone markers were also performed separately for men and women. These sex-specific results should be interpreted with caution, because the power of the study might be inadequate to detect a difference.

Changes in dietary intake over time and within groups were assessed with Wilcoxon's Signed Rank test.

All statistical analyses were performed using IBM SPSS Statistics version 25.0 (IBM Corp.). A 2-sided P value of 0.05 was used for statistical significance.

RESULTS

Participants

In total, 180 people were randomly assigned to either the control or the intervention group. At the end of the study, the control group consisted of 83 participants and the intervention group of 80. **Supplemental Figure 1** presents the flowchart of the numbers of participants at different study stages. The dropout rate was low in both groups (intervention: 8%; control: 11%). Furthermore, the intervention group showed a moderate to high compliance to the proposed intervention (80% achieved ≥80% of supplement compliance and 73% attended ≥80% of the exercise program). There were no significant differences in baseline characteris-

tics between participants who were not willing to receive the group allocation, lost to follow-up, or discontinued the study and those who remained in the data analyses (data not shown). The control and intervention groups significantly differed in education level and serum 25(OH)D concentrations at baseline (**Table 1**). Both groups maintained their weight during the study period.

Serum vitamin B-12 and 25(OH)D concentrations

A significant time × group interaction was found for serum vitamin B-12 concentrations ($P < 0.001$), independent of sex. The vitamin B-12 concentrations remained stable over time within the control group, whereas in the intervention group, the vitamin B-12 concentrations increased from baseline to 24 wk (**Table 2**, **Supplemental Figure 2A**). The mean vitamin B-12 concentration was sufficient at baseline in both groups. The prevalence of vitamin B-12 deficiency (<150 pmol/L) was low and similar in both groups over time (**Table 3**).

A significant time × group interaction was found for serum 25(OH)D concentrations as well ($P < 0.001$; adjusted for season), independent of sex. The 25(OH)D concentrations increased in both groups, with a greater increase in the intervention group (**Table 2**, **Supplemental Figure 2B**). The mean 25(OH)D concentration was sufficient at baseline in both groups. No vitamin D deficiency (<25 nmol/L) was seen in the study population over time. Prevalence of vitamin D insufficiency (≥25 to <50 nmol/L) decreased by 30% from baseline to 24 wk in the intervention group and in the control group by 12% (**Table 3**). The prevalence of insufficiency was significantly lower in the intervention group than in the control group at baseline and 12 wk ($P = 0.042$ and $P = 0.034$, respectively), but equal at 24 wk ($P = 0.42$).

Bone turnover markers

The interaction time × group was significant ($P < 0.001$) for both bone turnover markers PINP and CTX. PINP and CTX values in the control group did not change over time, whereas a decrease for both markers from baseline to 24 wk was seen in the intervention group (**Table 2).** CTX and PINP values were lower in men than in women, but followed a similar pattern over time (**Supplemental Figure 2C, D**). The uncoupling ratio was greater for the intervention group (median: 0.70, mean: 1.90) than for the control group (median: –0.11, mean: –0.10; $U = 1624$, $P < 0.001$) (**Figure 1**). Results were similar for men (median change: 0.52, $U = 403$, $P = 0.02$) and women (median change: 0.85, $U = 397$, $P = 0.002$) (**Figure 1**).

Table 1. Baseline characteristics of the middle-aged and older Chinese adults in the control and nutrition plus exercise intervention group[a]

	Control group			Intervention group			
Characteristic	**All**	**Men**	**Women**	**All**	**Men**	**Women**	***P* value[b]**
n	83	38 (45.8)	45 (54.2)	80	40 (50.0)	40 (50.0)	
Age, y	60.9 ± 6.0	62.9 ± 6.0	59.1 ± 6.0	61.7 ± 6.3	63.1 ± 7.0	60.3 ± 6.0	0.38
Age group							
50-64 years	58 (69.9)	22 (57.9)	36 (80.0)	53 (66.3)	23 (57.5)	30 (75.0)	0.62
65+ years	25 (30.1)	16 (42.1)	9 (20.0)	27 (33.8)	17 (42.5)	10 (25.0)	
Education level							
Secondary or below	65 (78.3)	29 (76.3)	36 (80.0)	49 (61.3)	18 (45.0)	31 (77.5)	0.02
Tertiary or above	18 (21.7)	9 (23.7)	9 (20.0)	31 (38.8)	22 (55.0)	9 (22.5)	
Smoking							
Never smoke	77 (92.8)	32 (84.2)	45 (100.0)	71 (88.8)	32 (80.0)	39 (97.5)	0.38
Ex-smoker/current smoker	6 (7.2)	6 (15.8)	0 (0.0)	9 (11.3)	8 (20.0)	1 (2.5)	
Alcohol use							
Never	72 (86.7)	29 (76.3)	43 (95.6)	69 (86.3)	32 (80.0)	37 (92.5)	0.93
Ex-user/current user	11 (13.3)	9 (23.7)	2 (4.4)	11 (13.8)	8 (20.0)	3 (7.5)	
Weight, kg	59.1 ± 9.3	65.1 ± 8.1	54.1 ± 7.1	59.5 ± 9.1	64.6 ± 6.9	54.4 ± 8.1	0.78
Height, cm	160.4 ± 8.4	166.7 ± 5.9	155.0 ± 6.1	162.7 ± 7.6	168.7 ± 4.6	156.8 ± 4.8	0.06
BMI, kg/m^2	22.9 ± 2.5	23.4 ± 2.4	22.5 ± 2.6	22.4 ± 2.4	22.7 ± 2.1	22.1 ± 2.6	0.18

Table 1. Continued

Characteristic	Control group			Intervention group			*P* value[b]
	All	Men	Women	All	Men	Women	
Self-reported major medical history							
Endocrinology diseases	4 (4.8)	2 (5.3)	2 (4.4)	1 (1.3)	0 (0.0)	1 (2.5)	0.37
Cardiovascular diseases	16 (19.3)	10 (26.3)	6 (13.3)	14 (17.5)	10 (25.0)	4 (10.0)	0.77
Bone, joint or muscular problems	13 (15.7)	6 (15.8)	7 (15.6)	13 (16.3)	4 (10.0)	9 (22.5)	0.92
Gastrointestinal problems	11 (13.3)	4 (10.5)	7 (15.6)	9 (11.3)	6 (15.0)	3 (7.5)	0.70
Cancer	4 (4.8)	2 (5.3)	2 (4.4)	2 (2.5)	1 (2.5)	1 (2.5)	0.68
Fracture history	2 (2.4)	1 (2.6)	1 (2.2)	3 (3.8)	1 (2.5)	2 (5.0)	0.62
Protein intake, g/d	76.0 [64.0-93.8)	88.9 [70.9-122]	70.1 [60.6-81.0]	85.0 [66.2-106]	91.4 [80.8-115]	73.1 [55.6-91.9]	0.14
Calcium intake, mg/d	505 [404-692]	516 [404-656]	479 [373-692]	520 [409-692]	568 [387-808]	511 [421-648]	0.66
Energy intake, kcal/d	1914 ± 531	2141 ± 541	1722 ± 444	1992 ± 475	2218 ± 425	1766 ± 415	0.33
Vitamin D intake, μg/d	1.9 [1.0-3.1]	2.3 [1.1-3.5]	1.6 [1.0-2.9]	1.9 [1.1-3.1]	2.7 [1.4-3.8]	1.7 [1.0-2.6]	0.74
Vitamin B-12 intake, μg/d	3.2 [2.2-4.7]	3.8 [2.4-4.9]	2.9 [2.2-3.8]	3.4 [2.2-6.0]	3.8 [2.6-6.2]	3.0 [2.0-5.5]	0.29
Serum vitamin B-12, pmol/L	343 ± 140	299 ± 116	378 ± 149	345 ± 119	345 ± 117	346 ± 122	0.89
Serum 25(OH)D, nmol/L	60.6 ± 15.2	65.2 ± 15.8	56.7 ± 13.7	54.7 ± 14.2	57.1 ± 14.3	52.2 ± 13.7	0.01
Serum CTX, pg/mL	295 [219-372]	255 [192-315]	310 [272-418]	276 [210-400]	256 [192-288]	348 [230-492]	0.55
Serum PINP, μg/L	41.0 [32.0-51.0]	34.0 [29.0-41.0]	44.0 [39.0-55.0]	41.0 [29.3-51.5]	32.5 [28.0-40.5]	49.0 [41.5-65.5]	0.88
Serum PTH, pmol/L	4.0 ± 1.7	4.2 ± 1.8	3.9 ± 1.6	4.2 ± 1.7)	3.9 ± 1.4	4.4 ± 2.0	0.65

[a]*Values are n (%), mean ± SD, or median [IQR]. CTX, C-terminal telopeptide of type I collagen; PINP, N-amino terminal propeptide of type I collagen; PTH, parathyroid hormone; 25(OH)D, 25-hydroxyvitamin D.*

[b]*P value by independent t test, Chi square or Mann-Whitney U test where appropriate to analyze differences between the entire control and entire intervention group.*

Table 2. Serum biochemistry in middle-aged and older Chinese adults in the control and nutrition plus exercise intervention group at baseline, 12 wk, and 24 wk[a]

Variable	Group	Baseline	12 wk	24 wk	P value for time[b]	P value for group[b]	P value for time x group[b]
Serum vitamin B-12, pmol/L	Control	343 ± 140	358 ± 138	357 ± 134	<0.001	<0.001	<0.001
	Intervention	345 ± 119	468 ± 140	484 ± 136			
Serum 25(OH)D, nmol/L	Control	60.6 ± 15.2	66.7 ± 15.7	65.6 ± 14.6	<0.001	0.001	<0.001
	Intervention	54.7 ± 14.2	81.1 ± 17.5	80.1 ± 19.2			
Serum PINP, µg/L	Control	41.0 [32.0-51.0]	42.0 [34.0-50.3]	40.0 [32.0-52.0]	<0.001	0.006	<0.001
	Intervention	41.0 [29.3-51.5]	34.0 [27.0-45.0]	29.0 [24.0-41.3]			
Serum CTX, pg/mL	Control	295 [219-372]	306 [228-394]	315 [232-428]	0.006	0.001	<0.001
	Intervention	276 [210-400]	234 [163-346]	239 [149-344]			
Serum PTH, pmol/L	Control	4.0 ± 1.7	4.1 ± 2.2	4.1 ± 2.3	0.02	0.50	0.09
	Intervention	4.2 ± 1.7	3.8 ± 1.5	3.7 ± 1.8			

[a]n = 83, control group; n = 80, intervention group. Values are means ± SDs or median [IQR]. CTX, C-terminal telopeptide of type I collagen; PINP, N-amino terminal propeptide of type I collagen; PTH, parathyroid hormone; 25(OH)D, 25-hydroxyvitamin D.

[b]P value for time, group and time x group effect were tested by linear mixed models. Logarithmic transformation was applied to the variables PINP and CTX for running the linear mixed model.

Table 3. Prevalence of vitamin B-12 deficiency and vitamin D insufficiency over time in middle-aged and older Chinese adults in the control and nutrition plus exercise intervention group[a]

Variable	Control (n = 83)	Intervention (n = 80)	P value[b]
Vitamin B-12 deficiency[c]			
Baseline	2 (2.4)	4 (5.1)	0.38
12 wk	4 (5.1)	1 (1.4)	0.20
24 wk	1 (1.4)	1 (1.4)	0.98
Vitamin D insufficiency[d]			
Baseline	19 (22.9)*	30 (37.5)*	0.042
12 wk	11 (14.1)[#]	3 (4.1)[#]	0.034
24 wk	8 (11.0)[§]	5 (7.1)[§]	0.42

[a]Values are n (%). Labeled percentages in a column without a common symbol differ, P < 0.05.
[b]P value by chi-square test to analyze differences between the control and intervention groups.
[c]Defined as <150 pmol/L or <200 pg/mL.
[d]Defined as ≥25 to <50 nmol/L. No vitamin D deficiency was present (<25 nmol/L).

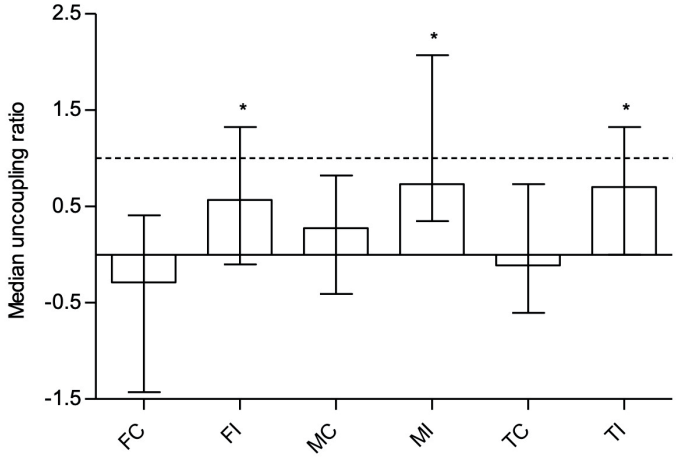

Figure 1. Uncoupling ratio in middle-aged and older Chinese adults in the control (n=83) and nutrition plus exercise group (n=80), separated by sex (FC, n = 45; FI, n = 38; MC, n = 40; MI, n = 40). Values are medians ± IQRs. The horizontal dotted line represents a balance between bone resorption and formation. *Different from corresponding control, P < 0.05. FC, females control; FI, females intervention; MC, males control; MI, males intervention; TC, total control; TI, total intervention.

PTH

The interaction time × group was not significant (P = 0.09) for serum PTH concentrations and there was no group effect (P = 0.50). Similar effects were found for men and women separately. Within the intervention group, PTH concentrations decreased from baseline to 24 wk, whereas they remained stable over time in the control group (**Table 2**). Thirty participants (36%) in the control group and 36 participants (50%) in the intervention group had elevated PTH concentrations at baseline (defined as >4.0 pmol/L).

Dietary intake

In the intervention group, median [IQR] calcium intake increased from 520 [409–692] mg/d at baseline to 1390 [1300 to 1540] mg/d after 24 wk (P < 0.001), median dietary vitamin D intake increased from 1.9 [1.1–3.1] μg/d to 31 [30–32] μg/d (P < 0.001), and median dietary vitamin B-12 intake increased from 3.4 [2.2–6.0] μg/d at baseline to 5.4 [4.2–7.3] μg/d after 24 wk (P = 0.001). In the control group, there was no significant change in calcium, vitamin D, and vitamin B-12 intake: from 504 [404–692] mg/d to 529 [417–678] mg/d, from 1.9 [1.0–3.1] μg/d to 1.8 [1.4–3.2] μg/d, and from 3.2 [2.2–4.7] μg/d to 2.8 [2.1–4.0] μg/d, respectively.

For protein intake, no statistically significant differences were observed between the 2 groups and over time. Protein intake was ≥0.8 $g \cdot kg^{-1} \cdot d^{-1}$ in almost all participants (≥96%), both at baseline and at 24 wk. In addition, protein intakes were ≥1.0 $g \cdot kg^{-1} \cdot d^{-1}$ and ≥1.2 $g \cdot kg^{-1} \cdot d^{-1}$ in the majority of the participants at 24 wk (in ≥84% and ≥70%, respectively).

DISCUSSION

The aim of this RCT was to investigate whether a 24-wk multidomain lifestyle intervention including a fortified milk supplement and an exercise program influenced serum vitamin B-12 and 25(OH)D concentrations and markers of bone turnover in apparently healthy community-dwelling Chinese middle-aged and older adults, compared with a control group without intervention. Our findings showed that the intervention was successful in improving vitamin B-12 and 25(OH)D concentrations and the balance of bone turnover markers.

Whereas no change was observed in the control group, the intervention group significantly improved in terms of serum vitamin B-12 concentrations. Overall, vitamin B-12 concentrations were adequate (i.e., ≥150 pmol/L) at baseline, with

low prevalence of deficiency in both groups, which may be attributed to the fact that the majority of the participants were younger than 65 y old. A difference in results may be expected when studies are conducted in older adults because vitamin B-12 deficiency is more prevalent in people aged 65 y and older [12]. The dietary vitamin B-12 intake was in line with the serum vitamin B-12 concentrations (increase in the intervention group and stable values in the control group). Both groups had intakes well above the Chinese RNI for vitamin B-12 of 2.4 μg/d [26]. In other parts of China, prevalence of low vitamin B-12 concentrations has ranged from 13.5% in Shanghai (deficiency defined as <139 pmol/L) [33] to 74.5% in rural North China (deficiency defined as <185 pmol/L) [34]. To our knowledge, there are currently no data available on the vitamin B-12 status in the general population aged 50 y or older living in Hong Kong. Therefore, the need for improving vitamin B-12 status in middle-aged and older adults in Hong Kong remains to be investigated.

The fortified milk supplement was effective in achieving and maintaining serum 25(OH)D concentrations >50 nmol/L, which is currently recommended for prevention of osteoporosis in postmenopausal women [7]. In addition, a serum 25(OH)D concentration of 70–80 nmol/L has been suggested as the optimal level for a lower fracture risk and to support the skeleton [35, 36]. The intervention group met this level as well; mean serum concentrations increased to 80 nmol/L. A similar improvement in serum 25(OH)D concentrations after milk supplementation was also found in other studies in postmenopausal Chinese women [17, 18].

The number of participants with vitamin D insufficiency decreased over time in both the intervention and control groups. As there were multiple participants in the control group with a baseline serum 25(OH)D concentration close to the cutoff value for vitamin D insufficiency (<50 nmol/L; 13 participants had a concentration between 50.0 and 55.0 nmol/L), it is likely that even a slight increase in serum concentrations might have caused a shift in vitamin D status classification. In addition, regardless of the treatment group, the participants were informed if they had low serum 25(OH)D concentrations at baseline together with being given very general lifestyle advice to improve their concentrations. It is, however, important to note that the dietary vitamin D intake stayed the same over time (baseline to 24 wk) in the control group. This suggests that although a lifestyle change may influence serum 25(OH)D concentrations, a combination of lifestyle and nutrition is more effective, as seen by the larger impact in the intervention group.

Human bone is continuously remodeled through a process of bone formation and resorption. Biomarkers of bone turnover can be measured to assess bone remodeling rates. A decrease in the serum concentrations of PINP and CTX was seen with the intervention, whereas the concentrations remained stable in the control group. Decreased PINP concentrations do not necessarily mean that there is less bone formation [31]. Likewise, decreased CTX concentrations do not automatically imply bone resorption. Therefore, an uncoupling ratio was calculated to arrive at an interpretation of the net effect [31, 37]. Although the median value for the uncoupling ratio was <1 for both groups, which would suggest net resorption, the ratio was significantly greater for the intervention group than for the control group. This suggests that there was less bone remodeling in the intervention group. Higher remodeling rates have been associated with an increased fracture risk and bone fragility [37-42]. Although CTX and PINP values were lower in men than in women, the uncoupling ratio was similar for both sexes. A reduction in CTX and PINP concentrations after milk supplementation was also found in other studies investigating Chinese postmenopausal women [28, 29, 43]. Long-term trials investigating milk supplementation alone confirm the beneficial effect on bone health. In the study of Lau et al. [17], milk supplementation for 2 y in apparently healthy postmenopausal Chinese women aged 55–59 y resulted in less bone loss at different sites. This was also found in the study of Chee et al. [18], who performed a 2-y milk supplementation study in apparently healthy postmenopausal Chinese women aged 50–65 y in Malaysia. Unfortunately, we had only indirect measures of bone health and no information about BMD.

PTH helps the body to maintain stable concentrations of calcium in the blood [44]. Suppression of PTH concentrations (by a high protein/calcium intake) may reduce bone resorption and thereby improve bone density [45]. In the current study, the interaction time × group for serum PTH concentrations was not significant but concentrations in the intervention group decreased significantly from baseline to 24 wk (mean change: 11%). In contrast, Chee et al. [18] found that serum PTH concentrations of their control group significantly increased over time (figure-derived mean change from baseline: 50%), whereas PTH concentrations in their intervention group did not significantly change over time. This difference in effect could be explained by the lower calcium intakes of participants in this study [46] and lower PTH baseline values. In the current study, 36% of the control group and 50% of the intervention group had elevated PTH concentrations at baseline (defined as >4.0 pmol/L).

The baseline values of PINP and PTH (41.0 µg/L and 4.1 pmol/L, respectively) were in line with the estimated reference concentrations for healthy Chinese adults aged 50–79 y (PINP: 36.9–52.7 µg/L; PTH: 3.7–4.0 pmol/L) [47]. However, mean CTX concentrations in our study were lower (320 and 311 pg/mL in the control and intervention, respectively) than the estimated reference concentration (445 pg/mL) [47]. This may indicate, keeping in mind interlaboratory variances, a lower rate of bone resorption in our study population, suggesting that a greater effect of the intervention may be expected in age-matched individuals.

To the best of our knowledge, there are no comparable studies looking at the effect of a combined nutrition and exercise intervention on bone turnover markers or BMD. As described above, studies in Chinese women investigating milk supplementation alone have shown a reduction in bone turnover markers [28, 29, 43] and less bone loss [17-19]. Multiple meta-analyses have assessed the effect of exercise alone on BMD in older adults [48-52]. All suggest that exercise can preserve or increase BMD to some extent, depending on the bone site and the duration, intensity, and type of exercises. There is a lack of studies looking at the effect of long-term exercise on PINP and CTX concentrations in comparable populations. There is no conclusive evidence available on the optimal exercise intervention, but it seems likely that resistance training, potentially combined with other forms of exercise, should be included. Because both nutrition alone and exercise alone can affect bone turnover markers or BMD, it remains to be investigated if a combined intervention has synergistic or additive effects.

The calcium intake at baseline was comparable with the mean daily calcium intake in men and women aged 60–84 y in Hong Kong (410 and 420 mg/d, respectively) [53]. The intervention increased calcium intake successfully (from 520 to 1390 mg/d) but no change was observed for protein intake. The milk supplement provided only an additional 13.6 g protein/d. Furthermore, protein intake ($g \cdot kg^{-1} \cdot d^{-1}$) was equal to or above the current RDA of 0.8 $g \cdot kg^{-1} \cdot d^{-1}$ in almost all cases. For healthy adults older than 65 y, a protein intake above the RDA may be beneficial for bone health (higher BMD, slower rate of bone loss, reduced bone turnover, and reduced hip fracture risk) [11, 54]. Still, the majority of the participants in both groups met the criteria of 1.0 and 1.2 $g \cdot kg^{-1} \cdot d^{-1}$. Note that our study population also included middle-aged adults (50–65 y). The already adequate protein intake, combined with the nutritional improvements and improved overall level of physical activity [25], could have contributed to the improved bone turnover [11, 55].

A limitation of this study is the absence of a nutrition or exercise group alone. Consequently, it is not possible to conclude which component or which combination of components of the intervention contributed the most to our positive findings regarding bone turnover. However, as stated before, a stronger effect may be expected when nutrition and exercise interventions are combined [7, 22, 23, 56]. Because there is little information available about the nutritional status of the general population aged 50 y or older living in Hong Kong, we are unable to conclude if our study population was a representative sample or that they were healthier. At least in this study population, the supplement was not needed to improve vitamin B-12 concentrations. Lastly, the sex-specific results should be interpreted with caution. The power of the study might be inadequate to detect sex-specific differences in serum vitamin concentrations and bone markers between the control and intervention groups.

Strengths of the current study include the low dropout rate and moderate to high compliance to the proposed intervention, which also indicates that this program may be sustainable over the long term. Furthermore, we were able to see improvements in nutritional status and bone markers in individuals with relatively good baseline values. This also shows the potential of the intervention program for more vulnerable groups, for example, frail older adults. Lastly, previous studies about nutritional and exercise interventions for improvement of bone health have largely focused on (postmenopausal) women. Osteoporosis in men is an underappreciated medical concern, even though it is known that hip fractures in men are associated with greater mortality than in women [57]. No remarkable sex differences were seen for the uncoupling ratio in the current study, showing that also male adults can benefit.

In conclusion, this study showed that milk supplementation in combination with exercise is effective in improving vitamin B-12 and 25(OH)D concentrations and bone turnover of apparently healthy community-dwelling Chinese middle-aged and older adults. Therefore, it contributes to the knowledge on how to prevent and reduce malnutrition and osteoporosis in the rapidly aging population of Hong Kong.

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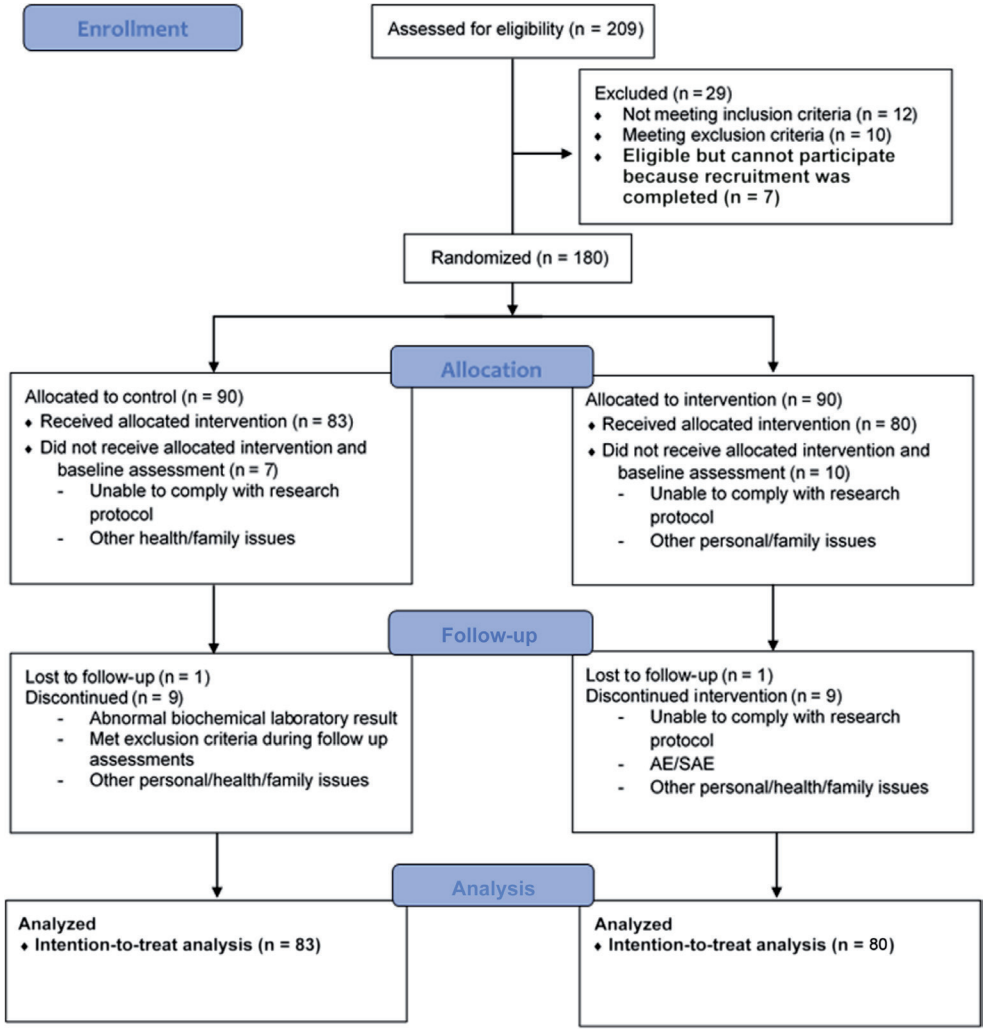
SUPPLEMENTARY DATA

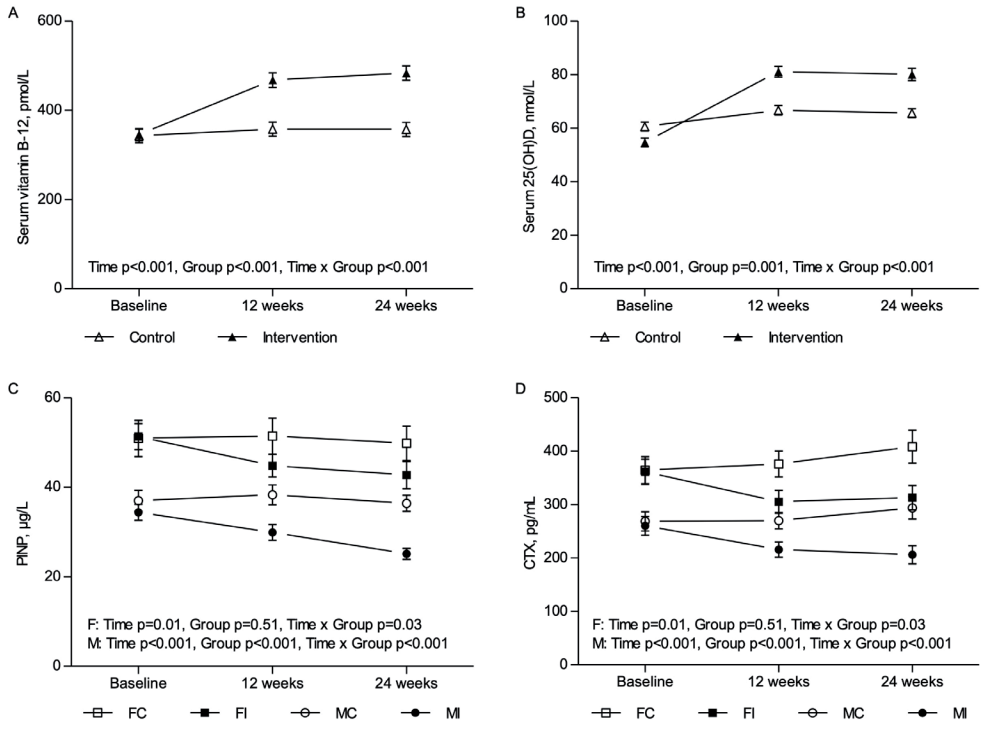
Supplemental Table 1. Nutritional composition of the OPTIMELTM 60+ Diamond® powder.

Component	per 100 g	per portion per day[a]
Protein (g)	22.5	13.6
Fat (g), of which	2.8	1.7
Milk fat (g)	2.6	1.6
Saturated fat (g)	2.0	1.2
Trans fatty acids (g)	<0.30	<0.30
DHA (mg)	50	30
Cholesterol (mg)	20	12
Carbohydrates, of which	60.1	35.2
Lactose (g)	33.5	20.0
Sugars (sum mono- and di-saccharides; g)	36.3	21.6
Fibre (g)	0.0	0.0
Minerals		
Calcium (mg)	1680	1008
Sodium (mg)	310	188
Potassium (mg)	1180	708
Magnesium (mg)	320	192
Selenium (µg)	11	6.8
Vitamins		
Cholecalciferol (D3) (µg)	50	30
Thiamin (µg)	2200	1320
Riboflavin (µg)	750	452
Vitamin B-6 (µg)	1500	900
Folic acid (µg)	800	480
Vitamin B-12 (µg)	4.8	2.9
Vitamin C (mg)	150	92
Energy (kcal / kJ)	361 / 1531	212 / 904

[a] *Two sachets of 30 g powder solved in 400 mL water.*



Supplemental Figure 1. CONSORT flow diagram of the present study. Reproduced from Woo et al. [25]. AE, adverse event; SAE, serious adverse event.



Supplemental Figure 2. Serum vitamin B-12 (A), 25(OH)D (B), PINP (C), and CTX concentrations (D) at baseline, 12 and 24 weeks in middle-aged and older Chinese adults in the control (n=83) and nutrition plus exercise intervention group (n=80). PINP (C) and CTX (D) concentrations are presented for females and males separately. Values, tested using linear mixed models, are means ± SEMs. CTX, C-terminal telopeptide of type I collagen; FC, females control; FI, females intervention; MC, males control; MI, males intervention; PINP, N-amino terminal propeptide of type I collagen.

Chapter 4

Insights of studies in hip fracture patients

Chapter 4.1

Hip fracture patients in geriatric rehabilitation show poor nutritional status, dietary intake and muscle health

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ABSTRACT

The aim of this study was to gain insight into the nutritional status, dietary intake and muscle health of older Dutch hip fracture patients to prevent recurrent fractures and to underpin rehabilitation programs. This cross-sectional study enrolled 40 hip fracture patients (mean ± SD age 82 ± 8.0 years) from geriatric rehabilitation wards of two nursing homes in the Netherlands. Assessments included nutritional status (Mini Nutritional Assessment), dietary intake on three non-consecutive days which were compared with Dietary Reference Intake values, and handgrip strength. Muscle mass was measured using Bioelectrical Impedance Analysis and ultrasound scans of the rectus femoris. Malnutrition or risk of malnutrition was present in 73% of participants. Mean energy, protein, fibre and polyunsaturated fat intakes were significantly below the recommendations, while saturated fat was significantly above the UL. Protein intake was <0.8 in 46% and <1.2 g/(kg·day) in 92%. Regarding micronutrients, mean intakes of calcium, vitamin D, potassium, magnesium and selenium were significantly below the recommendations. The prevalence of low muscle mass, low handgrip strength and sarcopenia were 35, 27 and 10%, respectively. In conclusion, a poor nutritional status, dietary intake and muscle health are common in older hip fracture patients in geriatric rehabilitation wards.

INTRODUCTION

Hip fractures (i.e., proximal femur fractures) are common injuries seriously affecting the health status and quality of life of older patients [1]. Within the following year, 22 percent of older hip fracture patients die and only 40 to 60 percent of the survivors regain their pre-fracture functional level [2, 3]. Furthermore, the risk of reoccurring fractures persists for at least 10 years following the initial fracture [4], meaning that not only the initial fracture but also subsequent fractures should be an important focus for prevention.

Three major risk factors for getting a hip fracture are sarcopenia [5], osteoporosis [6] and malnutrition [7]. Sarcopenia, defined by low levels of muscle strength, muscle quantity/quality and physical performance [5], is common in older hip fracture patients with higher prevalence rates compared to older adults without a hip fracture [8, 9]. Sarcopenia increases the risk of falls and fractures and has been associated with poorer functional recovery [10, 11], increased probability of long-term care placement, and mortality [11]. While sarcopenia might already have been present prior to the fracture, an acute period of disuse of muscles during hospitalization is likely to induce further and rapid decline of muscle mass, strength and function [12-14].

Osteoporosis is a chronic disease characterized by low bone mass and deterioration of bone microarchitecture [15]. It increases the risk of falls and fractures, which in turn leads to an increase in morbidity and mortality, loss of independence, and a decreased quality of life [6]. After a hip fracture, an increased loss of bone mineral density (BMD) can be observed, which can continue for at least 1 year [16-18].

Older hip fracture patients are often malnourished or at risk of malnutrition [19-21]. Malnutrition can be caused by multiple factors including a reduced dietary intake (due to a lack of appetite, inability to eat or oral health problems), malabsorption, increased nutrient losses or altered metabolic requirements [22]. Malnutrition increases the risk of post-fracture complications; it is associated with delirium, an increase in mortality and comorbidities, a decline in mobility, and it prolongs rehabilitation [21, 23-25].

Energy and protein requirements are increased in hip fracture patients; they often have a lower calorie intake plus an increased energy requirement due to an inflammatory state [21]. For protein, there is no official recommendation specifically for patients recovering from a hip fracture, but an intake of 1.2–1.5

g/(kg·day) for older people with a severe illness or injury can be derived from consensus papers [26, 27]. With respect to micronutrients, a sufficient intake of vitamin D and calcium are essential for musculoskeletal health [28-30]. Furthermore, there might be a role of vitamin K in reducing fracture risk, but there is no clear evidence [31].

Previous studies have shown that indeed energy, protein, vitamin D and calcium intakes can be low after a hip fracture [32-35]. However, these studies involve hospitalized patients and still little is known about inpatient geriatric rehabilitation. Furthermore, intakes may also be country-specific, therefore, results cannot always be translated. In addition, a broad overview of the characteristics of this population, from nutritional status to dietary intake to muscle health, is missing.

In order to prevent recurrent fractures and to create the most optimal rehabilitation program, more knowledge is needed on the characteristics of older patients recovering from a hip fracture. The aim of this study was to gain insight into the nutritional status, dietary intake and muscle health of older hip fracture patients in geriatric rehabilitation wards in the Netherlands.

METHODS

Study Population

A cross-sectional study was conducted at geriatric rehabilitation (GR) wards of two Dutch nursing homes. The study population consisted of 40 older adults (≥60 years) admitted to the GR ward after hospital treatment (conservative or surgical) for a hip fracture. A triage has taken place in the hospital to determine if the person was suitable for rehabilitation (i.e., trainable). No exclusion criteria were applied, and as a result, all patients admitted to these wards were invited to participate within the first three days after admission. Measurements were conducted during the first week of admission. The study was conducted in accordance with the Declaration of Helsinki, and the Medical Ethics Committee of Wageningen University gave ethical exemption for this study. All participants gave their informed consent for inclusion before they participated in the study.

Nutritional Status and Dietary Intake

Nutritional status was assessed using the 18-item Mini Nutritional Assessment (MNA). The MNA categorizes patients as either having a good nutritional status (24–30 points), being at risk of malnutrition (17–23.5 points) or as malnourished (<17 points) [36].

Dietary intake was recorded within the first week after admission on three non-consecutive days, including two weekdays and one weekend day. Trained researchers filled out food records through a combination of observations and weighing. The assortment available at the nursing homes for breakfast and lunch were weighed at the beginning of the study. Soup, desserts and each component of hot meals (and leftovers) were weighed using a kitchen scale (Kern EMB 2200-0, Kern & Sohn GmbH, Balingen, Germany) before and after the participant consumed them. Data were processed with Compl-eatTM (Department of Human Nutrition and Health, Wageningen University, Wageningen, the Netherlands), which was linked to the Dutch food composition database NEVO-online version 2016 [37].

Dietary intakes were compared with Dietary Reference Intake (DRI) values [38], the Estimated Average Requirements (EAR) and the Recommended Dietary Allowances (RDA). When these were not available, the Adequate Intake (AI) was used. Saturated fat, polyunsaturated fat and sodium did not have a recommended level of intake but only a Tolerable Upper Intake Level (UL). Macro- and micronutrient norms were based on guidelines of the Dutch Health Council [39-41]. The Dutch Health council only issued an advice for vitamin K1 and not total vitamin K. Therefore, the AI for total vitamin K intake were based on the recommendation of the American Institute of Medicine [42]. Energy intake (g/(kg·day)) and protein intake (g/(kg·day)) were compared with recommendations based on expert groups (European Society for Clinical Nutrition and Metabolism (ESPEN) and the PROT-AGE Study Group) [27, 43].

Protein intake per main meal was calculated to investigate protein distribution over the day. For older adults it is recommended to consume 25 to 30 g protein per main meal [27]. Since one nursing home served warm meals at dinner and the other nursing home at lunchtime, data were categorized as warm/cold meals instead of lunch/dinner moment.

Muscle Health

Sarcopenia was defined as the presence of both low Appendicular Skeletal Muscle Mass (ASMM) and low handgrip strength [5]. ASMM was measured using Bioelectrical Impedance Analysis (BIA) with a 50 kHz single frequency impedance meter (BodyExplorer PAD, Juwell Medical GmbH, Rheine, Germany) on the non-fractured side. The protocol by Kyle was used: in a fasted state (in the morning with patient still in bed), in a supine position with electrodes on the hand and foot, having rested with no exercise for >8 h and the bladder voided. Participants with pacemakers or implanted defibrillators were excluded from

the BIA measurement because of possible electromagnetic interference [44]. For the calculation of ASMM, the equation developed by Sergi was used [45]. As recommended by the European Working Group on Sarcopenia in Older People 2 (EWGSOP2), low ASMM was defined as <20 kg for men and <15 kg for women [5]. Subsequently, ASMM was divided by height squared (m^2) to adjust for body size. Corresponding cut-off points were <7.0 kg/m^2 for men and <5.5 kg/m^2 for women [5].

Handgrip strength was measured with a JAMAR hydraulic handheld dynamometer (model 5030J1, Sammons Preston, Bolingbrook, IL, USA) in seated position with the elbows flexed at 90 degrees. During three trials on each hand, participants were verbally encouraged to produce their maximum grip strength. The highest value was used for analysis [46]. EWGSOP2 cut-off points were used to indicate poor muscle strength: <27 kg for men and <16 kg for women [5].

Ultrasound scans of the rectus femoris were conducted using the HS-2200 (Honda, Toyohashi, Aichi, Japan) with the 7.5 MHz linear transducer probe. Patients were in supine position with extended knees. The middle between the anterior inferior iliac spine and the midpoint of the proximal border of the patella and the one-third point seen from the patella were marked using a measuring tape. The ultrasound was performed by positioning the probe at the one-third point with minimal pressure in a transversal position. The thickness of the rectus femoris was measured by hand using the measurement function of the HS-2200. The thickness was measured from the transition of fascia to muscle, to the transition of muscle to fascia at the muscle's thickest point. Cut-off points for low muscle mass were defined as 2SD below the gender-specific mean of a younger reference group: RF <19.9 and <15.9 mm in men and women, respectively [47].

Demographics, Medical and Physical Details

Demographic and medical data (comorbidities, number of medication use, estimated Glomerular Filtration Rate (eGFR), details about the fracture) were obtained from medical records. In addition, the Charlson Comobidity Index (CCI, 0–37 points) was used to determine the number and severity of comorbidities [48]. The higher the score, the more comorbidities the participant suffers from. The presence of a delirium was assessed according to Delirium Observation Screening (DOS, 0–13 points). A DOS score of ≥3 indicates delirium [49].

The Evaluative Frailty Index for Physical activity (EFIP) was used to determine pre-fracture frailty status [50]. The EFIP is a 50-item questionnaire that includes the physical, psychosocial and social domain and general health status. The score

is calculated by dividing the total score by 50. A cut-off score of 0.2 was used to indicate frailty [51].

The Barthel Index (BI, 0–20 points) was used to evaluate the level of independence in activities of daily living at the time of admission with higher scores indicating a higher level of independence [52]. Lastly, walking ability was measured using the Functional Ambulation Categories (FAC, 0–5 points) [53]. A score of 0 indicates no ambulation or non-functional walking and 5 indicates independent walking on all surfaces and able to climb stairs.

Body weight was measured to the nearest 0.1 kg using either a digital weighing scale (Seca 876, Seca Ldt, Birmingham, UK) or wheelchair scale (Seca 677, Seca Ldt, Birmingham, UK) depending on the participants ability to stand. Height was measured to the nearest 1 cm in standing position with a wall-mounted stadiometer (Seca, Seca Ldt, Birmingham, UK). In case the participant was not able to stand straight, total body height was calculated from knee height using the LASA formula, which is developed based on data from a Dutch cohort of older people [54].

Statistical Analysis

Continuous data are expressed as mean ± standard deviation (SD) or as median with interquartile range (IQR) in case of a non-normal distribution. Categorial variables are presented as number of participants with percentage. All data were checked for normality. Outliers (±3 SD from mean) in primary dependent variables were retained in final analyses when results including and excluding the outlier were similar.

To test whether the mean of a single dietary intake variable differed from the recommendation, a one-sample t-test was used. Micronutrient intakes which could not be compared with the EAR were classified as inadequate when statistically significant and <67% of the RDA or AI. In case of a non-normal distribution, Wilcoxon signed-rank test was used. Comparisons between the two study sites for patient characteristics and dietary intake were made using an independent sample t-test or the Mann–Whitney test.

All statistical analyses were carried out using IBM SPSS statistics (version 25.0; SPSS, Chicago, IL, USA). A two-sided p-value of 0.05 was used for statistical significance.

RESULTS

Study Population

Between October 2017 and April 2018, 44 hip fracture patients were admitted to the GR wards of which 40 patients (91%) were included. Reasons for not participating were not interested in participation (n = 3) and readmission to the hospital within two days (n = 1). Participant characteristics are described in **Table 1**. Before the hip fracture, 27 participants lived independently, 11 participants received home care and 2 participants lived with their family. The majority of participants suffered a fragility hip fracture (i.e., caused by a fall from standing height or less) and three participants suffered from a high impact fracture (i.e., traffic accident). Median length of stay in the hospital was five days. After admission to the GR wards, all assessments were completed in a median of four days (varying from 2 to 10 days). There were not statistically significant between-group differences in participant characteristics with regards to the two study sites.

Table 1. Characteristics of the hip fracture patients.

Characteristic	*n*	Value
Women, n [%]	40	29 [73]
Age, mean ± SD, year	40	81.6 ± 8.0
Weight, median (IQR), kg	40	68.0 (56.2–79.9)
Height, mean ± SD, cm	40	165 ± 9
BMI, median (IQR), kg/m^2	40	24.8 (21.5–28.5)
No weight bearing, n [%]	40	8 [20]
Surgical method	40	
Prosthetic replacement, n [%]		14 [35]
Internal fixation, n [%]		23 [58]
None, conservative, n [%]		3 [8]
Barthel index, mean ± SD, points	40	10.1 ± 3.9
FAC, points	40	
0, n [%]		18 [45]
1, n [%]		0 [0]
2, n [%]		2 [5]
3, n [%]		10 [25]
4, n [%]		10 [25]
5, n [%]		0 [0]
Delirium (DOS >3), n [%]	40	8 [20]
Frail (EFIP >0.2), n [%]	36	12 [33]

Table 1. Continued

Characteristic	*n*	Value
Kidney function [eGFR]	37	
Kidney failure (<15 mL/min), n [%]		0 [0]
Severe loss (<30 mL/min), n [%]		1 [3]
Moderate loss (30–60 mL/min), n [%]		8 [22]
Mild loss (60–90 mL/min), n [%]		19 [51]
Normal (>90 mL/min), n [%]		9 [24]
CCI, median (IQR), points	40	1 (0–2)
Comorbidity	40	
Diabetes Mellitus, n [%]		8 [20]
Cardiac, n [%]		17 [43]
Pulmonary, n [%]		8 [20]
Dementia, n [%]		5 [13]
Previous fracture due to fall	40	12 [33]
MNA	40	
Malnourished, n [%]		3 [8]
Risk of malnutrition, n [%]		26 [65]
Good nutritional status, n [%]		11 [28]
ASMM, median (IQR), kg	37	16.5 (15.2–18.9)
Low ASMM, n [%]		13 [35]
ASMM/height^2, mean (SD), kg/m^2	37	6.4 ± 1.0
Low ASMM/height^2, n [%]		13 [35]
Handgrip strength, mean ± SD, kg	37	22.5 ± 9.3
Low handgrip strength, n [%]		10 [27]
Sarcopenia, n [%]	35	4 [10]

Values are frequency [percentage], mean ± SD, or median (IQR). ASMM = Appendicular Skeletal Muscle Mass; BMI = Body Mass Index; CCI = Charlson Comorbidity Index; DOS = Delirium Observation Screening; EFIP = Evaluative Frailty Index for Physical activity; eGFR = estimated Glomerular Infiltration Rate; FAC = Functional Ambulation Categories; IQR = interquartile range; MNA = Mini Nutritional Assessment; SD = standard deviation.

Nutritional Status and Dietary Intake

Three participants (8%) were categorized as being malnourished and a further 26 participants (65%) as being at risk of malnutrition. Half of the participants had contact with a dietician at admission. Three-day records were completed for 31 participants and two days were completed for the remaining nine. The latter was due to discharge home (n = 3), illness (n = 3), readmission to the hospital

(n = 2) and closing of the GR ward because of gastroenteritis virus (n = 1). One participant had to be excluded because of outliers in the dietary intake variables.

Energy, protein, fibre and polyunsaturated fat intakes were below the recommendations, while saturated fat was significantly above the UL (**Table 2**). The mean daily protein was 0.82 ± 0.28 g/(kg·day)) and ranged from 0.25 to 1.55 g/(kg·day). The percentage of participants with an insufficient intake of <0.8, <1.0 and <1.2 g/(kg·day) amounted to 46, 74 and 92%, respectively. Mean protein intake (g) was below the recommended 25 to 30 g protein for each main meal (**Figure 1**). With respect to the micronutrient intakes, mean/median intakes of calcium, vitamin D, potassium, magnesium and selenium were significantly below the recommendations (**Table 3**).

Table 2. Daily mean macronutrient intake of 39 older hip fracture patients compared to the Dietary Reference Intakes from European Society for Clinical Nutrition and Metabolism (ESPEN) [43], the PROT-AGE Study Group [27] and the Health Council of the Netherlands (RDA, AI, UL) [39, 40].

Macronutrient	Intake	DRI		*p* value [b]
Energy, kcal	1319 ± 285	-		
Energy, kcal/kg bw	19.7 ± 6.1	30	ESPEN	<0.001
Protein, g	54.9 ± 14.3	60/51 [a]	RDA	0.033/0.095
Protein, g/kg bw	0.82 ± 0.28	1.0–1.2	ESPEN/PROT-AGE	<0.001
Protein, en%	17.5 ± 3.6	11	RDA	<0.001
Carbohydrates, g	128.1 ± 34.6	-		
Carbohydrates, en%	40.7 ± 7.3	40	RDA	0.56
Fibre, g	12.4 ± 3.9	-		
Fibre, g/MJ	2.3 ± 0.5	3.4	AI	<0.001
Fat, g	60.9 ± 15.0	-		
Fat, en%	41.4 ± 4.7	20–40	AI	<0.001–0.063
Saturated fat, g	29.3 ± 8.3	-		
Saturated fat, en%	19.9 ± 3.6	10	UL	<0.001
Monounsaturated fat, g	15.8 ± 4.6	-		
Polyunsaturated fat, g	8.4 ± 2.9	-		
Polyunsaturated fat, en%	5.8 ± 1.8	12	UL	<0.001

Data are presented as mean ± SD. AI = Adequate Intake; bw = body weight; DRI = Dietary Reference Intakes; EAR = Estimated Average Requirement; en% = energy percentage; UL = Tolerable Upper Intake Level; RDA = Recommended Dietary Allowance; - = no value established.
[a] Men and women, respectively.
[b] p value by one-sample t-test to analyse differences between mean intake and DRI.

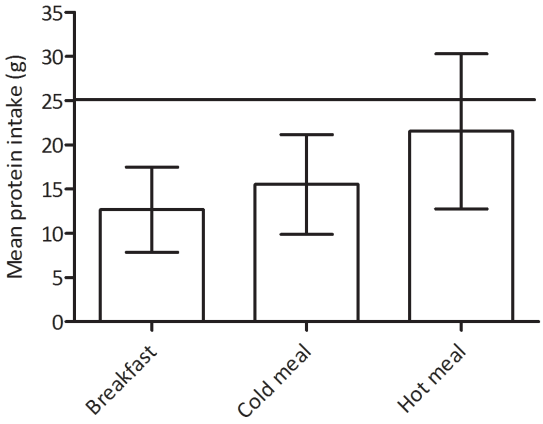

Figure 1. Protein intake of 39 older hip fracture patients per main meal. Values are means ± SD. Horizontal line represents the recommend lower limit of protein intake per main meal.

Muscle Health

BIA was performed in 37 participants; data were missing for three participants because of the presence of a pacemaker or implanted defibrillator. Low ASMM was present in 13 participants (35%). Handgrip strength measurements were completed for 37 participants, it was low in 10 participants (27%). Of the 35 participants with complete data, 10% had sarcopenia.

Mean muscle thickness of the rectus femoris measured with ultrasound was 10.5 ± 2.2 mm at the fractured site (n = 32) and 10.8 ± 2.0 mm and the non-fractured site (n = 34). For all participants with measurements, this was classified as being low. One outlier was removed from the ultrasound results of both the fractured as unfractured site.

Table 3. Daily micronutrient intake of 39 older hip fracture patients compared to the Dietary Reference Intakes from the Health Council of the Netherlands [41] and American Institute of Medicine (vitamin K) [42].

Micronutrient	Intake	DRI		Intake in % of DRI	*p* Value[b]
Calcium, mg	718 ± 287	1200	AI	60	<0.001
Vitamin D, µg	1.8 (1.2–2.4)	20	RDA	9	<0.001
		10	EAR	18	<0.001
Vitamin K, µg	109 (51–203)	120/90[a]	AI	91/121[a]	0.62/0.027
Phosphorus, mg	972 ± 271	550	AI	177	<0.001
Iron, mg	6.2 ± 1.9	11/16[a]	RDA	56/39[a]	<0.001
		6	EAR	103	0.49
Natrium, mg	1657 ± 455	2400	UL	69	<0.001
Potassium, mg	1875 ± 465	3500	AI	54	<0.001
Magnesium, mg	186 ± 46	350/300[a]	AI	53/62[a]	<0.001
Zinc, mg	7.5 ± 2.3	9/7[a]	RDA	83/107[a]	<0.001/0.19
		6.4/5.7[a]	EAR	117/132[a]	0.006/<0.001
Selenium, µg	27.3 (21.5–35.0)	70	AI	39	<0.001
Copper, mg	0.66 ± 0.16	0.9	RDA	73	<0.001
		0.7	EAR	94	0.11
Iodine, µg	118 ± 43	150	AI	79	<0.001
Vitamin B12, µg	3.0 ± 1.1	2.8	RDA	107	0.27
		2.0	EAR	150	<0.001
Vitamin C, mg	56.0 (35.3–74.7)	75	RDA	75	0.002
		60	EAR	93	0.76

Data are presented as mean ± SD or median (IQR). AI = Adequate Intake; DRI = Dietary Reference Intakes; EAR = Estimated Average Requirement; UL = Tolerable Upper Intake Level; RDA = Recommended Dietary Allowance.

[a] *Men and women, respectively.*

[b] *p value by one-sample t-test or Wilcoxon signed-rank test to analyse differences between mean intake and DRI.*

DISCUSSION

This study showed that along with a high prevalence of malnutrition (risk), nutrient intake was poor in hip fracture patients. Patients had low protein, energy, fibre and polyunsaturated fat intakes and a high saturated fat intake. In addition, intakes of several micronutrients were well below the recommendations. Approximately one third had a low muscle mass and a quarter showed low muscle strength.

The majority of the participants, 73%, were classified as either malnourished or at risk of malnutrition. In other studies that used the MNA to measure nutritional status in hip fracture patients, prevalence varied from 30 to 86% for malnourishment and risk of malnutrition together [20, 23, 32-34, 55-57]. Differences in prevalence may be explained by the inclusion of patients with dementia and delirium as we did in our study, because these patients have an increased risk of malnutrition [58-60]. Since most nutrient intakes were low relative to the recommendations, the prevalence of (risk of) malnutrition may even further increase if patients do not receive nutritional support.

One of the striking findings was that the percentage of participants with an insufficient protein intake of <0.8, <1.0 and <1.2 g/(kg·day) amounted to 46, 74 and 92%, respectively. Such low protein intake can further induce a decline of muscle and bone mass, a higher hip fracture risk and a poor nutritional status [26, 61]. Other studies in hip fracture patients found mean protein intakes ranging from 43 to 57 g [33-35, 62], which are comparable to our findings (55 g). In these studies energy intakes were also low (mean intakes ranged from 1025 to 1304 kcal versus 1319 kcal in the current study) [33-35, 62]. Since the intake of protein and energy were low (overall low intake of food), the intakes of many other nutrients were low as well. Therefore, nutritional support should primarily focus on increasing nutrient-dense foods. People with severe kidney problems should avoid a high protein intake, because this can be harmful [63]. In the current study, kidney failure was not present and only one participant had severe loss of kidney function (eGFR < 30 mL/min). Therefore, increasing the protein intake in this population seems (in general) feasible.

Micronutrients of concern include calcium, vitamin D, potassium, magnesium and selenium. Note that for these micronutrients, with the exception of vitamin D, intakes could only be compared with an adequate intake. However, these nutrients were <67% of this norm. Especially a sufficient intake of calcium and vitamin D are important in this population, because these nutrients are essential for musculoskeletal health [28-30]. A sufficient calcium intake may already be reached by increasing the protein intake through the consumption of more dairy products, vegetables and/or nuts. Median vitamin D intake (1.9 µg) was far below the EAR (10 µg) and RDA (20 µg). However, supplementation was not taken into account as this information was not available. The Dutch Health Council advices people aged ≥70 years to take a daily vitamin D supplement of 20 µg [64] and staff of the two nursing homes stated that indeed vitamin D supplements are given to hip fracture patients. If this is truly the case, vitamin D intake would be sufficient.

We also showed that low muscle mass was present in 35% of the participants according to BIA, while this was 100% according to the muscle thickness of the rectus femoris measured with ultrasound. One possible explanation for this discrepancy is that BIA is known to overestimate muscle mass (giving lower prevalence rates of low muscle mass) [65, 66]. Where BIA estimates muscle mass, ultrasound measures only the size of one muscle. Ultrasound shows good validity for measuring muscle size in older adults, but how this relates to overall muscle mass is unclear [67]. Considering that sarcopenia affects various muscles at different rates, sarcopenia seems more prevalent in the lower limb muscles [68]; multiple studies suggest that sarcopenia prevalence is higher when measuring thigh muscles than when measuring multiple body sites [69] and that the rectus femoris might decline specifically early [70]. Even though there are methodological considerations for each method, we can nevertheless conclude with relative certainty that the prevalence of low muscle mass was substantial (with a true prevalence being at least 35%).

Furthermore, handgrip strength was low in 27% of the participants, which is comparable to previous studies where it varied from 14 to 27% [10, 11, 32, 71]. Combining the low muscle mass measured with BIA and the low handgrip strength, 10% of the participants had sarcopenia. Although this percentage is not as high when considering muscle mass and strength separately, we should still be aware of the fact that patients are in a post-fracture catabolic state and may develop sarcopenia in a later stage. Besides pre-existing malnutrition, muscle mass may further decline by the inflammatory response after a hip fracture. The post-fracture catabolic state may continue for up to 3 months [72]. Prevalence of sarcopenia found in previous studies in hip fracture patients were quite diverse, ranging from 17 to 87% [10, 11, 32, 71, 73-76]. To minimize adverse outcomes like loss of muscle mass and mobility impairment, nutritional interventions in combination with resistance exercise may offer a solution.

In addition to this, the large variety in characteristics such as muscle mass and strength, cognitive and physical status and dietary intake in older hip fracture patients point to a compelling need for individually tailored interventions. For instance, 20% of participants experienced a delirium, 13% had dementia, 43% had a cardiac comorbidity, 20% had diabetes and 20% were not allowed to fully bear weight on the fractured leg. Therefore, some patients will need more supervision, modified exercises or different nutritional support. We advise that every new hip fracture patient admitted to a geriatric rehabilitation ward is guided by an interprofessional team of a physician, physiotherapist, nurse and dietician. Furthermore, monitoring dietary intake in geriatric rehabilitation is

important. Particular attention should be paid to the amount of protein intake and nursing staff should be made aware of the importance of sufficient protein intake in patients in a geriatric rehabilitation ward. Oral health should be taken into account as well, since poor oral health can lead to a reduced nutrient intake and malnutrition [77].

A limitation of this study is that there were missing data for some participants, because certain assessment tools, like BIA, were unsuitable for the participants. It remains a challenge to perform a variety of measurements on frail older adults. Sarcopenia cut-off points for low strength and low muscle quantity were according to the latest consensus EWGSOP2 [5], but we did not include a test to identify low performance levels (many patients are not able to perform such tests within the first couple of days after the hip fracture due to pain). Therefore, diagnosis of sarcopenia was not completely according to the consensus and we should be careful with comparing our sarcopenia prevalence with that in other studies. Lastly, this study was only conducted in two Dutch nursing homes, raising the question if this is representative of other centres in the Netherlands. The included population covered the entire target population of the two studied locations as 91% of hip fracture patients were willing to participate. Moreover, referral to geriatric rehabilitation wards is preceded by triage under the responsibility of an elderly care physician by using uniform criteria for all wards in the Netherlands [78]. This uniform selection, in addition to the high percentage of participation in the current study, suggests that the studied population is representative for hip fracture patients in geriatric rehabilitation wards in the Netherlands, despite the relatively small sample size.

In line with this, a strength of this study is that there were no exclusion criteria, which gives a better representation of the target population. Another strength is the accurate assessment of dietary intake by the combination of direct observations and weighing on three non-consecutive days. This method reduces the chance of measurement errors and recall bias compared to methods like 24-h recalls.

CONCLUSIONS

This study showed that hip fracture patients in geriatric rehabilitation have a poor nutritional status, dietary intake and muscle health. It is recommended to offer a postoperative personalized nutritional intervention with longitudinal follow-up to these patients with special attention to increase energy and protein intake. Such an intervention in combination with exercise may prevent recurrent fractures, reduce morbidity and optimise recovery after a hip fracture.

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Chapter 4.2

Protein intake, malnutrition, and its potential impact on bone health after a hip fracture: a 3-month prospective study

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ABSTRACT

Background. Adequate protein intake and being well-nourished may stimulate bone formation and may help to prevent bone mineral density (BMD) loss after a hip fracture. Objectives of this study were to investigate the association between protein intake with bone markers, calcaneal quantitative ultrasound (QUS) and BMD in older patients recovering from a hip fracture and to investigate the association between nutritional status with QUS and BMD. Additionally, the change of bone turnover markers from post-surgery till 3 months was investigated.

Methods. A 3-month prospective study in 96 adults aged ≥70 years with an acute hip fracture was conducted. Assessments, measured within 1 week after the fracture and after 3 months, included protein intake (questionnaire), nutritional status (Mini Nutritional Assessment Short Form [MNA-SF]), bone turnover markers procollagen type I N-terminal propeptide (PINP) and C-terminal telopeptide of type I collagen (CTX), insulin-like growth factor 1 (IGF-1) and parathyroid hormone (PTH) levels, QUS, and BMD (dual-energy X-ray absorptiometry).

Results. At baseline, half of the patients (mean age 84 years, 63% females) had a low protein intake (<0.8 g/kg/d) and this did not change over time. Patients had significant weight loss (median 3.6 kg) and percentage of patients at risk of malnutrition or being malnourished increased from 20 to 64%. Protein intake was only associated with QUS parameter broadband ultrasound attenuation in females (estimate 0.123, 95% CI 0.022-0.223). Higher pre-fracture MNA-SF was associated with higher BMD in the total body (estimate 0.048, 95%CI 0.015-0.080), spine (estimate 0.085, 95%CI 0.025-0.144), total hip (estimate 0.055, 95%CI 0.018-0.093) and trochanter (estimate 0.057, 95%CI 0.018-0.096). PINP and IGF-1 increased over time, while CTX remained stable and PTH decreased. IGF-1 was associated with PINP (estimate 1.215, 95%CI 0.363-2.066).

Conclusion. A good nutritional status comes with higher BMD in older hip fracture patients. The role of protein for bone health in these patients remains unclear and further studies are needed. After a hip fracture there is an increase in PINP, which is probably caused by IGF-1. Strategies seem warranted to prevent inadequate protein intake, malnutrition, and weight loss during rehabilitation.

INTRODUCTION

Hip fractures have a major impact on the health status and quality of life of patients [1]. Only half of the older hip fracture patients regain their pre-fracture functional level [2] and 24% die within the following year [3]. In addition, an increased loss of bone mineral density (BMD) can be observed after the sustained hip fracture [4, 5].

Since a higher dietary protein intake may reduce hip fracture risk, maintain BMD and prevent BMD loss in older adults [6, 7], this nutrient may play a beneficial role in the recovery of hip fractures. Previously, it was found that mean protein intake was insufficient in older hip fracture patients in geriatric rehabilitation wards (<0.8 in 46% and <1.2 g/kg/day in 92%) [8]. Similar findings have also been observed during hospital stay [9-11]. A low protein intake reduces insulin-like growth factor 1 (IGF-1) levels and may increase parathyroid hormone (PTH) levels, which leads to a reduction in bone formation and a stimulation of bone resorption, respectively [12-14]. So a high protein intake may relate to higher BMD.

BMD can be used as an indicator of bone health, but a dual-energy X-ray absorptiometry (DXA) scan is a costly and non-portable method which is difficult to perform in patients with a recent hip fracture. An alternative method to assess bone health is the use of bone turnover markers, which are produced during bone remodelling. Bone undergoes continuous remodelling through bone resorption by osteoclasts followed by bone formation by osteoblasts [15]. While in healthy young adults bone remodelling is in balance, an increased bone turnover rate is present in older adults, resulting in net bone loss [15, 16]. An increased bone turnover is also associated with a higher fracture risk [15, 16]. However, an increased bone metabolism is needed to re-establish bone structure following a fracture. A previous study in hip fracture patients found that procollagen type I N-terminal propeptide (PINP, bone formation marker) levels increased from baseline to 2 months while C-terminal telopeptide of type I collagen (CTX, bone resorption marker) levels did not change from baseline to 2 months [17]. However, the baseline measurement in that study was performed 2 weeks post-fracture, meaning that levels were probably already elevated. A study in Chinese hip fracture patients measured PINP and CTX before surgery and 30–60 days, 80–120 days, and 180–230 days after surgery [18]. Both markers peaked at 30-60 days after surgery. Since the follow-up measurements were determined within broad time ranges, the exact trajectory is unknown. The question remains what the levels of PINP and CTX are just after hip surgery and how they change within

3 months (catabolic state most pronounced [19]). Next to bone turnover markers, another non-invasive method that reflects bone health is the quantitative ultrasound (QUS) of the calcaneus. QUS parameters have been correlated with BMD and can predict the risk for a future fracture [20].

A limited number of studies investigated the effect of protein intake on bone turnover markers and QUS. Two studies in community-dwelling older adults found no differences in bone markers between high vs low protein intake after 3 years of follow-up [21, 22]. However, they used serum osteocalcin and urinary N-telopeptide cross-links which tend to change less than PINP and CTX [16]. In addition, no turnover rate was calculated. Regarding QUS, a study in Korean adults consuming relatively low protein diets found that meat protein intake was positively associated with one of the QUS parameters in older men, but not in older women [23]. To our knowledge, no studies have investigated the association between protein intake with bone turnover markers PINP and CTX, and QUS parameters in hip fracture patients.

Low dietary intake, including low protein intake, is one of the determinants of malnutrition [24]. Malnutrition is common in older hip fracture patients and increases the risk of post-fracture complications and mortality [25]. Body mass index (BMI) can be used to assess nutritional status and it has been shown that a low BMI is a risk factor for osteoporotic fractures [26]. However, BMI as a single measure of nutritional status is inadequate and it doesn't take into account sarcopenic obesity (co-existence of excess adiposity and low muscle mass/function) [27]. A more comprehensive tool to assess nutritional status is the Mini Nutritional Assessment Short Form (MNA-SF), which consists of questions regarding appetite, BMI, weight loss, mobility, acute psychological stress or diseases, cognitive impairment and depression [28]. Malnutrition may result in lower BMD as it can lead to deficiencies in essential nutrients for supporting bone health such as calcium, vitamin D, and protein [29, 30]. In addition, rapid weight loss is associated with a decrease in BMD [29, 31]. The association between MNA-SF with QUS and BMD has not been investigated in hip fracture patients, but there are indications that a higher MNA score is associated with higher QUS parameters in older adults [32, 33].

The first objective of this study was to investigate the association between protein intake with bone markers (IGF1, PTH, PINP, CTX) and bone health (QUS, BMD) in older patients recovering from a hip fracture. A second aim was to investigate the association between nutritional status with QUS and BMD in these patients.

Lastly, it was investigated how bone turnover markers change from post-surgery till 3 months.

METHODS

Study design and population

A 3-month prospective study was conducted at Rijnstate Hospital (Arnhem, NL). A total of 96 older adults (aged 70 years or older) with an acute hip fracture were recruited. In addition, patients were eligible if they were living at home and had a pre-fracture Clinical Frailty Scale (CFS) score ≥3 and <7. Exclusion criteria were a pathological or periprosthetic fracture, current participation in scientific research that interferes with the current study, history of dementia or no permission to request information about medical history, medication use, and liver and kidney values.

The study was conducted according to the principles of the Declaration of Helsinki (version 2013) and registered at ClinicalTrials.gov as NCT05039879. After being fully informed, patients gave explicit oral consent for participation in the study, which was recorded in the electronic patient record.

Study procedures

Measurements were performed at baseline on the traumatology ward (within 5 days after the hip surgery) and after 3 months during an outpatient clinic visit for evaluation of physical health and cognitive functioning.

Protein intake and nutritional status

Dietary protein intake was recorded using a protein screener developed by hospital Gelderse Vallei (Ede, NL). This tool aims to reflect protein intake (g/d) based on eating habits and specifically focuses on food products which are high in protein. For this study, patients were asked about the frequency and quantity they consumed of each food product per day or week considering their habitual intake in the past month.

Nutritional status was assessed with the MNA-SF [28]. Subjects were classified as having a normal nutritional status (12-14 points), being at risk of malnutrition (8-11 points), or being malnourished (0-7 points).

Calcaneal quantitative ultrasound

Quantitative ultrasound (QUS) parameters of the calcaneus (heel) were measured using the portable Achilles EXPII bone ultrasonometer (GE Healthcare, USA). This device emits sound waves at a high frequency (ultrasonic). The coefficient of variation (CV) was <2.0%. The measurement was performed in a seated position and both the right and left calcaneus were measured in duplo. From the parameters broadband ultrasound attenuation (BUA) and speed of sound (SOS), a Stiffness Index (SI) was calculated using the following formula: SI=0.67*BUA+0.28*SOS-420 [34]. Mean BUA, SOS and SI were calculated as the average of the left and right calcaneus. When only one side could be assessed, the mean of one side was taken.

Bone markers and vitamin D status

Bone turnover markers PINP and CTX were measured in serum in the hospital using chemiluminescent immunoassay (CLIA; Liaison XL, Diasorin) [16]. For CTX, intra-assay CV was 2.1-4.9% and interassay CV was 5.1-8.8%. For PINP, intra-assay and interassay CV were 2.6-3.0% and 4.2-5.3%, respectively. Since CTX levels are highly affected by circadian rhythm, time of blood sampling was in the morning before 10 AM for each patient and each time point [35, 36]. CTX is also affected by diet, therefore the patient needed to fast overnight before blood sample collection [15]. In addition to the crude levels, the ratio between the bone turnover markers were calculated to evaluate the net effect of changes in bone turnover. Furthermore, an uncoupling ratio was calculated as the ratio of percent change from baseline for PINP and CTX in which a ratio <1 indicates net resorption and >1 indicates net formation [37]. IGF-1 and PTH levels in the blood were measured using CLIA as well, interassay CV was 4.7-5.3% and 2.3-7.8%, respectively. Lastly, serum 25(OH)D was measured using Electrochemiluminescence immunoassay (interassay CV was 2.8-6.9%).

Other measurements

Two questionnaires were used to assess clinical frailty and activities of daily living. The CFS (version 2.0) [38, 39] is already used in daily practice to assess overall level of fitness and frailty in older patients. This scale is graded from 1 to 9 in which level 1 represents 'very fit' and level 9 'terminally ill'. An experienced clinician evaluated the frailty status. Activities of daily living were assessed with the Barthel Index [40, 41], which includes 10 different activities of daily living. A score between 0-20 is given, where 0 means fully dependent and 20 means totally independent.

The following outcomes were extracted from patient files: socio-demographic characteristics (gender, age, smoking and drinking habits, living situation), hip fracture details (cause, location, type of surgery), length of hospital stay and discharge destination, length of rehabilitation, complications, fracture history, eGFR (estimated glomerular filtration rate), C-reactive protein (CRP) and leukocytes, comorbidities using the Charlson Comorbidity Index (CCI) and mortality. Smoking and drinking habits and fracture history were also verified with the patient. In addition, education level was asked and classified as primary, lower secondary, upper secondary and higher education.

Medication that potentially affects bone health was recorded by the clinician at discharge, which included corticosteroids, bisphosphonates or other osteoporosis medication, hormones (i.e. estrogen, hormone replacement therapy), and vitamin D and calcium supplements.

Bodyweight (kg) was measured with a calibrated sitting weighing scale at baseline and at 3 months. Height was measured to the nearest 1 cm with a stadiometer. For patients who could not stand, knee length was measured while sitting or lying down with a straight ruler (SECA). With formulas specifically developed for older adults [42], total height was calculated. Subsequently, BMI was calculated as bodyweight divided by height in meters squared.

Two measurements were assessed at 3 months only: memory (Montreal Cognitive Assessment, MoCA [43]) and DXA (Lunar iDXA, GE Healthcare, USA). Using DXA, BMD (g/cm^2) of multiple sites (total body, spine, lumbar spine, total hip, femoral neck, Ward's triangle, trochanter and pelvis) and bone mineral content (BMC; g) were collected.

Statistical analysis

A power calculation for the associations between protein intake and bone health was based on the rule that there should be at least 10 observations per variable [44]. The intention was to make models with a maximum of 8 variables, leading to a sample size of 80 patients. With an expected drop-out of 20%, a sample size of 96 hip fracture patients was needed.

Data are expressed as mean ± standard deviation (SD), n (%), or as median with interquartile range (IQR) for non-normally distributed data. Data were checked for normality using histograms and the Shapiro-Wilk test. In case of non-normal distribution, nonparametric tests were used. Outliers (±3 SD from mean) in primary dependent variables were retained in final analyses when results including

and excluding the outlier were similar. All statistical analyses were performed using SPSS (IBM Corp., Chicago, IL, USA, version 28.0.1.0) and a two-sided p-value of 0.05 was used to determine statistical significance.

To assess differences between genders at baseline, an independent t test was used for continuous normally distributed data, Mann-Whitney U test for continuous nonparametric data and chi-square for categorical data. The change of continuous study parameters over time was assessed with a paired samples t test or Wilcoxon signed rank test.

Linear mixed models were used to test for associations between protein intake with bone markers (IGF-1, PTH, PINP, CTX) and QUS parameters. Three models of increasing complexity were built to adjust for confounding factors. In the first model only time was added as fixed factor. The second model was adjusted for age, sex, BMI, CFS, history of fractures, vitamin D status, and calcium supplementation. The third model additionally adjusted for number of drugs, smoking, and alcohol. Models 1–3 were also performed for males and females separately. BMD was only measured at 3 months, therefore, time was not added to the model and the association between baseline variables and BMD at 3 months was tested. Since changes in BMD are more likely to be detected after at least 6 months [16, 45], it was assumed that BMD was stable from baseline till 3 months, which justified to investigate the association between baseline variables with BMD at 3 months.

The associations between nutritional status with QUS parameters and BMD were also tested using linear mixed models with three models. These models were the same as described for protein, only BMI was not included as a confounder because BMI is part of the MNA-SF.

RESULTS

Study population

Between September 2021 and September 2022, a total of 385 older hip fracture patients were admitted to the emergency department. A total of 96 were included in the study, baseline measurements could be performed in 88 patients and follow-up measurements in 77 patients (**Figure 1**). Physical measurements were not performed for all 88 patients at baseline due to consent withdrawal (n=3) or since measurements were not possible (n=3). Likewise, at follow-up, consent was withdrawn for physical measurements (n=5) or patients were not

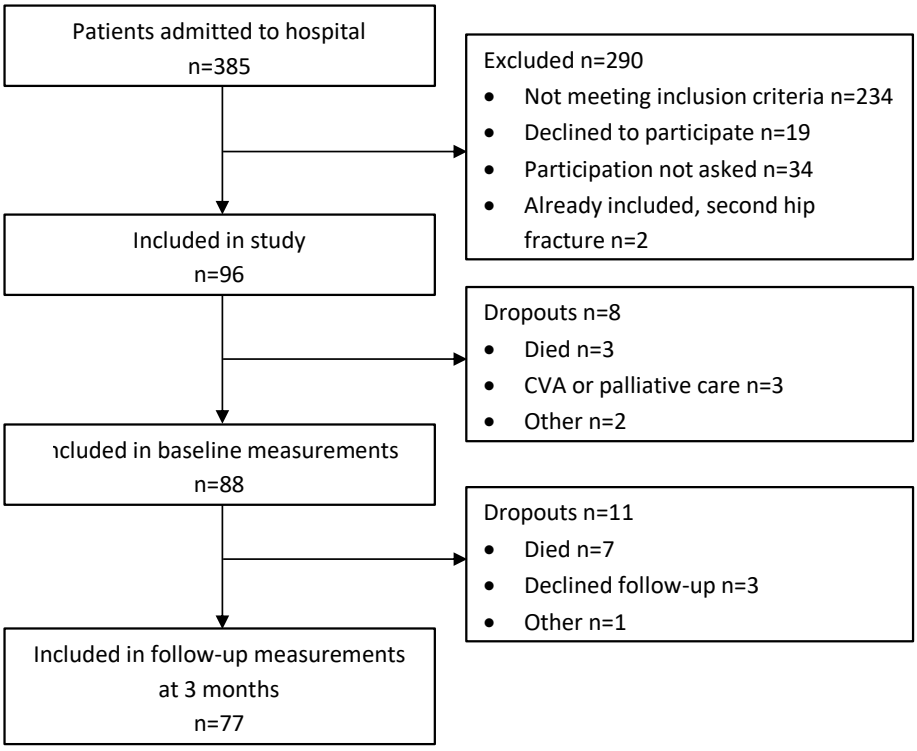

Figure 1. Flow diagram of participant selection.

able or not willing to come to the hospital (n=7). However, questionnaires could be completed using patients records in patients who declined follow-up but agreed to data retrieval, and in patients whose date of death was close to 3 months after hospital discharge and with recent data available. In total, 12.6% died before follow-up at 3 months.

Patients had a mean age of 84 ± 6 years with a median BMI of 27.2 kg/m^2 (IQR 24.0-30.1), and 63% was female (**Table 1**). Statistically significant differences between males and females were found for weight, smoking habits, alcohol use, having a partner, CFS, fractures in the past, osteoporosis diagnosis and CRP levels. Median duration from hospital admission until baseline measurements was 4 days and 80% of the patients had surgery within 1 day after admission. Median length of necessary hospital stay was 6 days. A small part of the study population used osteoporosis medication: 6% used corticosteroids, 3% bisphosphonates, 1% oestrogen or hormone replacement and 2% other osteoporosis medication. Cognitive assessment (MoCA) took place at follow-up (n=65); the median score

Table 1. Characteristics of the included hip fracture patients.

	n	All	Males	Females
Total participants, n (%)		95 (100)	35 (37)	60 (63)
Age, y, mean ± SD	95	84 ± 6	83 ± 7	84 ± 6
Weight, kg, median [IQR]	95	71.6 [62.5-80.0]	**78.5 (71.3-85.3)***	**69.7 (61.7-77.6)***
BMI, kg/m^2, median [IQR]	95	27.2 [24.0-30.1]	26.1 (23.6-28.9)	27.5 (24.8-30.7)
Education level	86			
Primary		13 (15)	5 (15)	8 (15)
Lower secondary, n (%)		27 (31)	7 (21)	20 (38)
Upper secondary, n (%)		29 (34)	14 (41)	15 (29)
Higher, n (%)		17 (20)	8 (24)	9 (17)
Smoking	94			
Never, n (%)		46 (49)	**13 (37)***	**33 (56)***
Former, n (%)		31 (33)	**18 (51)***	**13 (22)***
Current, n (%)		17 (18)	**4 (11)***	**13 (22)***
Alcohol use	94			
Yes, n (%)		43 (46)	**22 (63)***	**21 (36)***
Amount (glasses/day), median [IQR]		0.0 [0.0-4.7]	**1.5 [0.0-7.0]***	**0.0 [0.0-3.0]***
Fracture type	95			
Femoral neck fracture, n (%)		55 (58)	25 (71)	30 (50)
Pertrochanteric, n (%)		34 (36)	9 (26)	25 (42)
Subtrochanteric, n (%)		4 (4)	1 (3)	3 (5)
Other, n (%)		2 (2)	0 (0)	2 (3)
CFS, score	95			
3 - Managing well, n (%)		48 (51)	**25 (71)***	**23 (38)***
4 - Vulnerable, n (%)		24 (25)	**6 (17)***	**18 (30)***
5 - Mildly frail, n (%)		20 (21)	**3 (8)***	**17 (28)***
6 - Moderately frail, n (%)		3 (3)	1 (3)	2 (3)
Living situation	95			
At home without home care, n (%)		81 (85)	31 (89)	50 (83)
At home with home care, n (%)		14 (15)	4 (11)	10 (17)
Having a partner, n (%)	95	39 (41)	**21 (60)***	**18 (30)***
Surgical method	95			
Hemi-arthroplasty, n (%)		52 (55)	24 (69)	28 (47)
TFNA, n (%)		27 (28)	6 (17)	21 (35)
Extended TFNA, n (%)		12 (13)	4 (12)	8 (13)
Other, n (%)		4 (4)	1 (3)	3 (5)
Cause of fracture	95			
Accident inside, n (%)		58 (61)	19 (54)	39 (65)
Accident outside, n (%)		23 (24)	10 (29)	13 (22)
Fall after feeling unwell, n (%)		12 (13)	5 (14)	7 (12)
Other, n (%)		2 (2)	1 (3)	1 (2)

Table 1. Continued

	n	All	Males	Females
Fractures in the past, yes, n (%)	95	47 (49)	**12 (34)***	**35 (58)***
Hip fractures, yes, n (%)	95	10 (11)	4 (11)	6 (10)
Vertebral fracture, yes, n (%)	95	5 (5)	1 (3)	4 (7)
Comorbidities	95			
CCI, points, median [IQR]		1 [0-3]	2 [0-5]	1 [1-2]
Heart failure, n (%)		15 (16)	7 (20)	8 (13)
Peripheral vascular disease, n (%)		25 (26)	12 (34)	13 (22)
Pulmonary disorders, n (%)		14 (15)	6 (17)	8 (13)
Diabetes, n (%)		22 (23)	7 (20)	15 (25)
Kidney disease, n (%)		24 (25)	10 (29)	14 (23)
Cancer, n (%)		10 (11)	6 (17)	4 (7)
Osteoporosis, n (%)		15 (16)	**1 (3)***	**14 (23)***
Medication use at discharge	95			
Number of different drugs, median [IQR]		8 [6-12]	7 [5-13]	8 [7-11]
Polypharmacy[a], n (%)		87 (92)	32 (91)	55 (92)
Vitamin D supplementation, n (%)		74 (78)	26 (74)	48 (80)
Calcium supplementation, n (%)		47 (49)	18 (51)	29 (48)
Length of hospital stay, days, median [IQR]	92	7.0 [5.5-9.0]	8.0 [6.0-11.0]	7.0 [5.0-9.0]
Length of necessary hospital stay, days[b], median [IQR]	92	6.0 [5.0-8.0]	6.0 [5.0-9.0]	6.0 [5.0-8.0]
Complications during hospital stay	95			
Anemia, n (%)		23 (24)	8 (23)	15 (25)
Delirium, n (%)		11 (12)	4 (11)	7 (12)
Fever, n (%)		12 (13)	6 (17)	6 (10)
Hypotension, n (%)		16 (17)	4 (11)	12 (20)
Infections, n (%)		8 (9)	3 (9)	5 (8)
None, n (5)		38 (40)	16 (46)	22 (37)
Inflammation				
CRP, mg/L, median [IQR]	93	88 [50-128]	**108 [64-155]***	**74 [44-109]***
Leukocytes, 10^9/L, median [IQR]	92	10.6 [9.1-12.5]	11.4 [8.3-12.7]	10.6 [9.3-12.1]
eGFR, ml/min/1,73m^2, median [IQR]	87	66 [48-80]	65 [48-80]	67 [49-80]

BMI = Body Mass Index, CCI = Charlson Comorbidity Index, CRP = C-reactive protein, eGFR = estimated glomerular filtration rate, TFNA = TFN-Advanced Proximal Femoral Nailing System.
[a]When using at least 5 drugs at the same time.
[b]Days until hospital indication has ended, but who were forced to stay because there was not yet place in a rehabilitation center.
**Significant difference between sexes (p-value <0.05).*

was 25 (IQR 21-27) and 54% scored below the cut-off score of 26. At discharge, 5% went home without home care, 21% went home with home care, 68% went to a rehabilitation center, 1% to a hospice, and 5% died during hospital stay. Median length of stay in the rehabilitation centers was 35 days (IQR: 25-50). Barthel Index decreased significantly from baseline (median 20, IQR 18-20) till discharge (median 13, IQR 11-15).

Protein intake and nutritional status

At baseline, protein intake was <0.8 g/kg/d in 50% of the patients, between 0.8 and 1.2 in 43% and ≥1.2 g/kg/d in 7%. Protein intake in grams remained stable over time (**Table 2**), while protein intake in g/kg/d increased significantly (due to weight loss). Median weight change was -3.6 kg (IQR: -6.2 to -1.5; p<0.001 from baseline). At baseline, 6% consumed protein-enriched products, which increased to 13% at 3 months, protein content of these products was incorporated in the total protein intake. Nutritional status worsened over time. Percentage over patients at risk of malnutrition increased from 19 to 51% and those being malnourished from 1 to 13%.

Calcaneal quantitative ultrasound

QUS could not be performed in all patients at baseline (n=61), due to an impossible transfer from bed to chair or failure of the device. All three QUS parameters stayed similar over time (**Table 2**).

Bone markers and vitamin D status

Median PINP levels increased from 31.1 μg/L (IQR: 20.3-42.6) at baseline to 93.6 μg/L (IQR: 65.4-119.4) at 3 months, while CTX remained stable over time (**Table 2**). Its ratio (CTX/PINP) approached zero and median uncoupling ratio approached 1 (median: 0.982; IQR: -3.410-7.680), both indicating a balance between resorption and formation.

IGF-1 levels increased from baseline (median: 9.5 nmol/L; IQR: 7.6-12.4) to 3 months (median: 13.2 nmol/L; IQR: 10.6-18.4). PTH levels decreased over time and were above reference levels (>9.3 pmol/L) in 42% of the patients at baseline which declined to 8.8% of the patients at 3 months.

Serum 25(OH)D increased significantly over time. Patients with a deficiency (<25 nmol/L) changed from 26% at baseline to 3% at 3 months and patients with an insufficiency (≥25 to <50 nmol/L) from 46% to 16%. Patients with adequate levels (≥50 nmol/L) increased from 28% to 81% and those with levels ≥70 nmol/L increased as well (from 8.6% to 46%).

Table 2. Change in protein intake, nutritional status, QUS parameters and bone markers over time.

	n	Baseline	*n*	3 months	P value
Protein intake and nutritional status					
Total protein intake, g/d, mean ± SD	86	58.4 ± 14.7	75	60.9 ± 15.7	0.14
Total protein intake, g/kg/d, median [IQR]	86	0.79 [0.64-0.97]	75	0.84 [0.72-1.05]	**0.001**
MNA-SF, score (0-14), median [IQR]	95	13 [12-14]	84	11 [9-13]	**0.001**
Malnourished, n (%)		1 (1)		11 (13)	**<0.001**
At risk of malnutrition, n (%)		18 (19)		43 (51)	
Normal nutritional status, n (%)		76 (80)		30 (36)	
QUS parameters					
BUA, dB/MHz, mean ± SD	61	99.8 ± 11.6	60	101.7 ± 12.9	0.22
SOS, m/s, mean ± SD	61	1519.8 ± 32.3	60	1524.3 ± 34.2	0.51
SI, mean ± SD	61	72.0 ± 15.6	60	74.5 ± 17.0	0.63
Bone markers					
Serum PINP, mg/L, median [IQR]	90	31.1 [20.3-42.6]	67	93.6 [65.4-119.4]	**<0.001**
Serum CTX, mg/L, median [IQR]	88	0.466 [0.303-0.759]	67	0.540 [0.330-0.763]	0.69
Ratio CTX/PINP, median [IQR]	88	0.015 [0.011-0.025]	66	0.006 [0.004-0.007]	**<0.001**
Serum IGF-1, nmol/L, median [IQR]	90	9.5 [7.6-12.4]	67	13.2 [10.6-18.4]	**<0.001**
Serum PTH, pmol/L, median [IQR]	93	7.8 [6.3-12.3]	68	5.2 [4.1-6.7]	**<0.001**
Serum 25(OH)D, nmol/L, median [IQR]	86	42 [28-56]	70	68 [53-80]	**<0.001**
25(OH)D <10 nmol/L, n (%)[a]	93	7 (8)	70	0 (0)	

BUA = broadband ultrasound attenuation, CTX = C-terminal telopeptide of type I collagen, IGF-1 = insulin like growth factor, MNA-SF = Mini Nutritional Assessment Short Form, PINP = procollagen type 1 N propeptide, PTH = parathyroid hormone, 25(OH)D = 25-hydroxyvitamin D, SI = Stiffness Index, SOS = speed of sound, QUS = quantitative ultrasound.
[a]Patients below limit of detection.

DXA

Fifty-six patients underwent a DXA-scan at 3 months (**Table 3**). Lumber spine BMD was possibly overestimated in 30% of the patients due to scoliosis, sclerosis or condensed structure after a compression fracture. One extreme outlier for L2-L4 BMD was removed for this reason. While at baseline only 16% of the patients was previously diagnosed with osteoporosis (extracted from patient record), at 3 months, 34% were diagnosed with osteoporosis (T-score total hip ≤ -2.5 SD) and 52% had osteopenia (T-score total hip between -1 and -2.5 SD). These diagnoses at 3 months were not different between sexes.

Table 3. Bone mineral content and density of 56 patients at 3 months derived from DXA[a].

Outcome	Values
Total body BMD, g/cm^2	1.011 [0.870-1.076]
Total body BMC, g	2086 [1638-2533]
Spine BMD, g/cm^2	1.092 [0.985-1.194]
L2L4 BMD, g/cm^2	1.189 [1.017-1.298]
Total hip BMD, g/cm^2	0.806 [0.658-0.876]
T-score total hip	-2.1 (0.9)
Femoral neck BMD, g/cm^2	0.737 [0.667-0.792]
Ward's triangle BMD, g/cm^2	0.481 [0.427-0.583]
Trochanter BMD, g/cm^2	0.667 [0.549-0.727]
Pelvis BMD, g/cm^2	0.795 [0.718-0.898]

BMC = bone mineral content, BMD = bone mineral density, DXA = dual-energy X-ray absorption.
[a]Values are median [IQR] or mean (SD).

Associations

No adjusted associations were found between dietary protein intake with IGF-1, PTH, PINP, CTX, QUS parameters and BMD in the total sample (**Table 4, Supplemental Table 1**). Sex-stratified fully adjusted models showed that there was an association between protein intake and QUS parameter BUA (estimate 0.123, 95% CI 0.022-0.223) in females, but not in males. An additional analysis showed that higher IGF-1 was associated with higher PINP (fully adjusted model: estimate 1.215, 95%CI 0.363-2.066), but not with CTX (estimate 0.002, 95%CI -0.008-0.012). PTH was not associated with PINP and CTX.

In fully adjusted models, higher pre-fracture MNA-SF was associated with higher BMD in the total body (estimate 0.048, 95%CI 0.015-0.080), spine (estimate 0.085, 95%CI 0.025-0.144), total hip (estimate 0.055, 95%CI 0.018-0.093) and trochanter (estimate 0.057, 95%CI 0.018-0.096) but not with BMD at other sites or QUS parameters (**Table 5, Supplemental Table 2**).

Table 4. Associations between protein intake and bone health outcomes in older hip fracture patients.

Exposure	Outcome	Model	Estimate	SE	95% CI	P value
	Bone markers[a]					
Protein intake, g/d	IGF-1, nmol/L	3	0.019	0.030	-0.041-0.079	0.53
	PTH, pmol/L	3	-0.013	0.020	-0.052-0.027	0.53
	PINP, mg/L	3	0.17	0.16	-0.15-0.49	0.29
	CTX, mg/L	3	-0.001	0.002	-0.005-0.002	0.46
	QUS parameters[a]					
Protein intake, g/d	BUA, dB/MHz	3	0.044	0.053	-0.061-0.15	0.40
	SOS, m/s	3	-0.092	0.16	-0.40-0.22	0.56
	SI	3	0.017	0.062	-0.11-0.14	0.78
	BMD[b]					
Pre-fracture protein intake, g/d	Total body BMD, g/cm^2	3	-0.0003	0.001	-0.003-0.002	0.79
	Spine BMD, g/cm^2	3	0.003	0.002	-0.002-0.007	0.19
	L2L4 BMD, g/cm^2	3	-0.0004	0.002	-0.005-0.004	0.87
	Total hip BMD, g/cm^2	3	0.002	0.001	-0.001-0.005	0.11
	Femoral neck BMD, g/cm^2	3	0.003	0.002	-0.0005-0.006	0.092
	Ward's triangle BMD, g/cm^2	3	0.002	0.001	-0.001-0.004	0.18
	Trochanter BMD, g/cm^2	3	0.003	0.001	-0.0003-0.006	0.076
	Pelvis BMD, g/cm^2	3	-0.002	0.002	-0.005-0.002	0.34

BMD = bone mineral density, BUA = broadband ultrasound attenuation, CTX = C-terminal telopeptide of type I collagen, IGF-1 = insulin like growth factor, PINP = procollagen type 1 N propeptide, SI = Stiffness Index, SOS = speed of sound, QUS = quantitative ultrasound.
[a]Model 3: adjusted for time, age, sex, BMI, clinical frailty scale, history of fractures, vitamin D status, calcium supplementation, number of drugs, smoking, and alcohol.
[b]Model 3: adjusted for pre-fracture age, sex, BMI, clinical frailty scale, history of fractures, vitamin D status, calcium supplementation, number of drugs, smoking, and alcohol.

Table 5. Associations between MNA-SF with QUS and BMD in older hip fracture patients.

Exposure	Outcome	Model	Estimate	SE	95% CI	P value
	QUS parameters[a]					
MNA-SF, score (0-14)	BUA, dB/MHz	3	0.26	0.37	-0.48-1.00	0.48
	SOS, m/s	3	-0.96	1.02	-3.00-1.09	0.35
	SI	3	-0.29	0.41	-1.12-0.53	0.48
	BMD[b]					
Pre-fracture MNA-SF, score (0-14)	Total body BMD, g/cm^2	3	0.048	0.016	0.015-0.080	**0.005**
	Spine BMD, g/cm^2	3	0.085	0.029	0.025-0.144	**0.006**
	L2L4 BMD, g/cm^2	3	0.035	0.029	-0.024-0.095	0.24
	Total hip BMD, g/cm^2	3	0.055	0.018	0.018-0.093	**0.005**
	Femoral neck BMD, g/cm^2	3	0.029	0.020	-0.013-0.070	0.17
	Ward's triangle BMD, g/cm^2	3	0.028	0.016	-0.005-0.060	0.092
	Trochanter BMD, g/cm^2	3	0.057	0.019	0.018-0.096	**0.006**
	Pelvis BMD, g/cm^2	3	0.048	0.024	-0.0002-0.096	0.051

BMD = bone mineral density, BUA = broadband ultrasound attenuation, MNA-SF = Mini Nutritional Assessment Short Form, SI = Stiffness Index, SOS = speed of sound, QUS = quantitative ultrasound.

[a]*Model 3: adjusted for time, age, sex, clinical frailty scale, history of fractures, vitamin D status, calcium supplementation, number of drugs, smoking, and alcohol.*

[b]*Model 3: adjusted for pre-fracture age, sex, clinical frailty scale, history of fractures, vitamin D status, calcium supplementation, number of drugs, smoking, and alcohol.*

DISCUSSION

This study found no associations between protein intake with bone markers, QUS parameters and BMD in the total sample of older hip fracture patients, but there was an association between protein intake and QUS parameter BUA in females. Better pre-fracture nutritional status was associated with higher total body, spine, total hip and trochanter BMD. Regarding bone markers, PINP and IGF-1 levels increased, while CTX levels remained stable and PTH levels decreased. IGF-1 was associated with PINP.

Contrary to our hypothesis, no associations were found between dietary protein intake with bone markers, QUS parameters and BMD in the total sample. A possible explanation for this observation is that the results of the protein screener were affected by recall bias. Previous studies have found mixed results. Two studies in community-dwelling older adults also found no differences in bone turnover markers between high vs low protein intake after 3 years of follow-up

[21, 22]. Regarding QUS, protein intake was associated with BUA in females only in the current study. Previously, it was found that in Korean adults consuming relatively low protein diets, meat protein intake was positively associated with QUS parameter SI in older males, but not in older females [23]. The Korean adults were much younger than our study population (delta 24 years), consumed low protein diets and had lower BMI (delta 2 kg/m^2) and the studies are therefore difficult to compare. Regarding BMD, it was previously shown that total protein intake was associated with higher total body and spine BMD in older adults [46]. However, these older adults were younger, had higher total protein intakes and a better nutritional status, and consisted for a large part of healthy older adults. Recently, a prospective study following 26,318 women aged 35-69 years for a median time of 22.3 years found that a 25 g/d increment of protein was associated with a reduced hip fracture risk of 14% [47]. Although there are several trials in hip fracture patients investigating the effect of oral administration of protein on nutritional, clinical or functional outcomes [48], a well-designed trial (e.g. sufficient sample size, administration duration, dose, adherence, and follow-up period) on a broad variation of outcomes is missing.

This study found that a better pre-fracture nutritional status was associated with higher BMD at multiple sites. For every unit increase in MNA-SF, the predicted BMD increased with 0.048 g/cm^2 in the total body, 0.085 g/cm^2 in the spine, 0.055 g/cm^2 in the total hip, and 0.057 g/cm^2 in the trochanter. Using the measured BMD values in this study, this reflects an increase of 4.7%, 7.8%, 6.8% and 8.5% in the total body, spine, total hip and trochanter, respectively. Considering that the rate of bone loss is 0.5-1% per year in older men and about 0.5-2% in older women (depending on age since annual loss is faster in the first years after menopause) [49-52], this is a clinically relevant result. According to the European Society of Parenteral and Enteral Nutrition (ESPEN) guidelines, older patients with a hip fracture should receive nutritional supplements [53], which is not always achieved in clinical practice (as seen in the current study). Nutritional supplements can improve nutritional status and therefore may prevent BMD loss.

The need for nutritional support was also reflected in the data on protein intake and nutritional status. Protein intake was <0.8 g/kg/d in 50% of the patients and <1.2 g/kg/day in 93%, which is in line with previous studies [8-11]. Protein intake per g/kg/d increased significantly over time, which can be attributed to the median weight loss of -3.6 kg. Since weight loss is associated with bone loss and increased fracture risk [49, 54], this is an alarming finding. Even though the consumption of protein-enriched products increased over time (from 6 to 13%

of the patients), total protein intake in grams remained stable. The combined prevalence of risk of malnutrition and malnourishment even increased from 20% at hospital admission to 64% at 3 months. This, again [8], emphasizes the importance of better nutritional support in older hip fracture patients in order to increase their protein intake and to prevent weight loss and malnutrition.

The third aim was to investigate how bone turnover markers change from post-surgery till 3 months. It was found that PINP levels tripled over time, while CTX levels remained stable. These findings are in line with the study of Stewart et al., which found that PINP levels increased from baseline to 2 months in older hip fracture patients while CTX levels did not change [17]. However, the baseline measurement was performed at 2 weeks post-fracture, meaning that levels were probably already elevated. Indeed, baseline PINP was higher in the study of Stewart vs the current study: mean 117.9 vs median 31.1 μg/L, respectively. Likewise baseline CTX was higher (mean 0.91 vs median 0.47 μg/L, respectively). In Chinese hip fracture patients, PINP levels also tripled from baseline till 30-60 days and was still elevated at 180-230 days. CTX levels increased 1.5 times, was decreased by a large extent at 80-120 days and was almost back at baseline after 180-230 days. Since CTX was measured at 90 days in the current study, it could be that a peak in CTX was missed. The increase in PINP was probably caused by IGF-1, which is known to stimulate the proliferation and differentiation of osteoblasts [13]. The results of the current study were in line with this mechanism; higher IGF-1 levels were associated with higher PINP levels. Decreasing PTH levels were also found, which may relate to less bone resorption, but no association with CTX (or PINP) was found.

Another finding was that serum 25(OH)D increased over time leading to a remarkable decrease in patients with a deficiency or insufficiency. At 3 months, 81% of the patients had adequate levels (≥50 nmol/L) while this was only 28% at baseline. The reason for this improvement is that patients were prescribed vitamin D supplements after the laboratory results showed a vitamin D deficiency. Measuring serum 25(OH)D was not standard care but based on these results this might be implemented. Vitamin D supplementation of 800-1000 IU daily is recommended for this population [55, 56].

There were several limitations of this study. First, the tool to assess protein intake was not yet validated. This tool was chosen since it is not invasive, nor time-consuming and thus no burden for the patient, especially compared to the food frequency questionnaire and food records. Nevertheless, similar intakes at baseline were found as a previous study in a similar study population which filled

in food records through a combination of observations and weighing [8]. Second, results are generalizable to a part of the older hip fracture patients since patients with preexisting dementia were excluded and only community-dwelling older adults were included. However, still patients with cognitive impairment were included as reflected by the MoCA score at 3 months follow-up. Third, no adjustments were made for physical activity level and energy intake. However, these variables are correlated to BMI and frailty, for which was adjusted. In addition, instead of calcium intake, only adjustment for calcium supplementation was possible. Lastly, the actual dropout was in line with the expected dropout (both 20%) but not all measurements could be performed at 3 months, resulting in a higher dropout rate for some variables. However, this reflects the reality of everyday clinical practice in an older and fragile population. The dropout rate also had consequences for the power of the study. While the intention was to analyze models with a maximum of 8 variables for the associations with protein intake, model 3 was adjusted for 10 variables. However, multiple variables did not relevantly contribute to the model and there were no large differences in results between model 2 (adjusted for 7 variables) and 3.

To conclude, a good pre-fracture nutritional status may lead to higher BMD in older hip fracture patients. The role of protein in supporting optimal bone health in these patients remains unclear and well-designed trials are needed. After a hip fracture there is an increase in PINP, which is probably caused by IGF-1, and CTX remains stable over time. Strategies during rehabilitation seem warranted to prevent inadequate protein intakes, malnutrition, and weight loss.

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SUPPLEMENTARY DATA

Supplemental Table 1. Associations between protein intake and bone health outcomes in older hip fracture patients.

Exposure	Outcome	Model	Estimate	SE	95% CI	P value
	Bone markers[a]					
Protein intake, g/d	IGF-1, nmol/L	1	0.025	0.027	-0.030-0.079	0.37
		2	0.018	0.030	-0.041-0.077	0.55
		3	0.019	0.030	-0.041-0.079	0.53
	PTH, pmol/L	1	-0.013	0.018	-0.048-0.023	0.48
		2	-0.016	0.020	-0.057-0.024	0.43
		3	-0.013	0.020	-0.052-0.027	0.53
	PINP, mg/L	1	0.037	0.15	-0.26-0.33	0.80
		2	0.21	0.15	-0.094-0.51	0.17
		3	0.17	0.16	-0.15-0.49	0.29
	CTX, mg/L	1	-0.001	0.002	-0.005-0.002	0.47
		2	-0.001	0.002	-0.005-0.003	0.63
		3	-0.001	0.002	-0.005-0.002	0.46
	QUS parameters[a]					
Protein intake, g/d	BUA, dB/MHz	1	0.099	0.054	-0.009-0.21	0.072
		2	0.064	0.052	-0.040-0.17	0.22
		3	0.044	0.053	-0.061-0.15	0.40
	SOS, m/s	1	-0.056	0.14	-0.34-0.23	0.70
		2	-0.075	0.16	-0.40-0.25	0.64
		3	-0.092	0.16	-0.40-0.22	0.56
	SI	1	0.056	0.056	-0.055-0.17	0.32
		2	0.028	0.066	-0.10-0.16	0.68
		3	0.017	0.062	-0.11-0.14	0.78
	BMD[b]					
Pre-fracture protein intake, g/d	Total body BMD, g/cm^2	1	0.001	0.001	-0.002-0.003	0.58
		2	0.0004	0.001	0.001-0.002	0.64
		3	-0.0003	0.001	-0.003-0.002	0.79
	Spine BMD, g/cm^2	1	0.002	0.002	-0.002-0.005	0.32
		2	0.002	0.002	-0.002-0.005	0.27
		3	0.003	0.002	-0.002-0.007	0.19
	L2L4 BMD, g/cm^2	1	0.003	0.002	-0.006-0.001	0.15
		2	-0.002	0.002	-0.006-0.002	0.36
		3	-0.0004	0.002	-0.005-0.004	0.87
	Total hip BMD, g/cm^2	1	0.001	0.001	-0.001-0.003	0.43
		2	0.001	0.001	-0.001-0.003	0.29
		3	0.002	0.001	-0.001-0.005	0.11
	Femoral neck BMD, g/cm^2	1	0.002	0.001	0.0002-0.004	**0.036**
		2	0.002	0.001	-0.001-0.004	0.16
		3	0.003	0.002	-0.0005-0.006	0.092

Supplemental Table 1. Continued

Exposure	Outcome	Model	Estimate	SE	95% CI	P value
	Ward's triangle BMD, g/cm^2	1	0.002	0.001	-0.0002-0.004	0.078
		2	0.001	0.001	-0.001-0.003	0.26
		3	0.002	0.001	-0.001-0.004	0.18
	Trochanter BMD, g/cm^2	1	0.001	0.001	-0.002-0.003	0.53
		2	0.001	0.001	-0.001-0.003	0.30
		3	0.003	0.001	-0.0003-0.006	0.076
	Pelvis BMD, g/cm^2	1	-0.0003	0.001	-0.003-0.002	0.83
		2	0.0002	0.001	-0.003-0.003	0.91
		3	-0.002	0.002	-0.005-0.002	0.34

BMD = bone mineral density, BUA = broadband ultrasound attenuation, CTX = C-terminal telopeptide of type I collagen, IGF-1 = insulin like growth factor, PINP = procollagen type 1 N propeptide, SI = Stiffness Index, SOS = speed of sound, QUS = quantitative ultrasound.
[a]Model 1: adjusted for time. Model 2: additionally adjusted for age, sex, BMI, CFS, history of fractures, vitamin D status, and calcium supplementation. Model 3: additionally adjusted for number of drugs, smoking, and alcohol.
[b]Model 1: crude. Model 2: adjusted for pre-fracture age, sex, BMI, CFS, history of fractures, vitamin D status, and calcium supplementation. Model 3: additionally adjusted for pre-fracture number of drugs, smoking, and alcohol.

Supplemental Table 2. Associations between MNA-SF with QUS and BMD in older hip fracture patients.

Exposure	Outcome	Model	Estimate	SE	95% CI	P value
	QUS parameters[a]					
MNA-SF, score (0-14)	BUA, dB/MHz	1	0.33	0.34	-0.35-1.01	0.34
		2	0.45	0.37	-0.28-1.18	0.22
		3	0.26	0.37	-0.48-1.00	0.48
	SOS, m/s	1	-0.48	0.87	-2.22-1.25	0.58
		2	-0.25	1.05	-2.34-1.84	0.82
		3	-0.96	1.02	-3.00-1.09	0.35
	SI	1	-0.064	0.33	-0.73-0.60	0.85
		2	0.17	0.44	-0.71-1.04	0.70
		3	-0.29	0.41	-1.12-0.53	0.48
	BMD[b]					
Pre-fracture MNA-SF, score (0-14)	Total body BMD, g/cm^2	1	0.001	0.013	-0.25-0.027	0.94
		2	0.031	0.014	0.002-0.060	**0.035**
		3	0.048	0.016	0.015-0.080	**0.005**
	Spine BMD, g/cm^2	1	0.022	0.019	-0.016-0.059	0.25
		2	0.056	0.025	0.006-0.107	**0.029**
		3	0.085	0.029	0.025-0.144	**0.006**
	L2L4 BMD, g/cm^2	1	-0.017	0.019	-0.055-0.021	0.38
		2	0.004	0.026	-0.048-0.057	0.87
		3	0.035	0.029	-0.024-0.095	0.24
	Total hip BMD, g/cm^2	1	0.004	0.015	-0.025-0.034	0.77
		2	0.044	0.019	0.006-0.082	**0.025**
		3	0.055	0.018	0.018-0.093	**0.005**
	Femoral neck BMD, g/cm^2	1	-0.005	0.014	-0.032-0.023	0.73
		2	0.023	0.018	-0.014-0.060	0.22
		3	0.029	0.020	-0.013-0.070	0.17
	Ward's triangle BMD, g/cm^2	1	-0.003	0.012	-0.027-0.021	0.79
		2	0.022	0.016	-0.009-0.054	0.16
		3	0.028	0.016	-0.005-0.060	0.092
	Trochanter BMD, g/cm^2	1	0.017	0.015	-0.013-0.047	0.26
		2	0.044	0.019	0.005-0.082	**0.029**
		3	0.057	0.019	0.018-0.096	**0.006**
	Pelvis BMD, g/cm^2	1	0.001	0.015	-0.029-0.031	0.92
		2	0.026	0.021	-0.017-0.069	0.22
		3	0.048	0.024	-0.0002-0.096	0.051

BMD = bone mineral density, BUA = broadband ultrasound attenuation, MNA-SF = Mini Nutritional Assessment Short Form, SI = Stiffness Index, SOS = speed of sound, QUS = quantitative ultrasound.

[a]*Model 1: adjusted for time. Model 2: additionally adjusted for age, sex, CFS, history of fractures, vitamin D status, and calcium supplementation. Model 3: additionally adjusted for number of drugs, smoking, and alcohol.*

[b]*Model 1: crude. Model 2: adjusted for pre-fracture age, sex, CFS, history of fractures, vitamin D status, and calcium supplementation. Model 3: additionally adjusted for pre-fracture number of drugs, smoking, and alcohol.*

Chapter 5

General discussion

The aim of this thesis was to investigate the role of nutritional factors in supporting bone health of older adults. To address this comprehensive research objective, the thesis is divided in three parts. In the first part, the focus lies on the role of dietary protein consumption and in the second part, other nutritional factors (magnesium, dairy, and fortified milk) are addressed. The main findings of these parts (Chapters 2 and 3) are summarized in **Table 1**. In the last part, insights emerging from studies in hip fracture patients are presented. In this final chapter, the findings, methodological considerations, implications for clinical practice and existing dietary guidelines are discussed. Lastly, suggestions for future research are presented.

THE ROLE OF PROTEIN IN BONE HEALTH

Dietary protein is important for the growth and maintenance of bone tissue. Bone requires amino acids for collagen synthesis, osteoblast proliferation and differentiation, and bone mineralization [1, 2]. During childhood, protein is crucial for the growth of all organs systems and needed to reach peak bone mass (maximum amount of bone density and strength) [3]. However, there is less evidence available for the role of protein in later life. As shown in Figure 1 of Chapter 1, there are several mechanisms through which protein may impact bone health of older adults: a high protein intake can upregulate insulin-like growth factor 1 (IGF-1), improve intestinal calcium absorption, and maintain muscle mass and strength [4]. In Chapter 2, observational studies showed beneficial associations between dietary protein and bone health outcomes of older adults, except for plant protein. In addition, intervention studies reported null findings.

Chapter 2.1 was published in 2019 and since then new studies investigating the relationship between protein and bone health in older adults have been published. At least one observational study [5] and one intervention study [6] would have been included if the systematic review would be repeated. The observational study showed that older adults with higher protein intake (mean ± SD: 1.1 ± 0.4 g/kg/d) had 1.8%-6.0% higher mean hip and lumbar spine bone mineral density (BMD) at baseline compared to those with a lower protein intake (0.8 ± 0.3 g/kg/d), but this did not affect BMD change over 4 years of follow-up [5]. However, higher protein intake was associated with a reduced risk of vertebral fracture during 5 years of follow-up (HR 0.36). The intervention study investigated the effect of high intensity dynamic resistance exercise and whey protein supplementation for 18 months on BMD in older men with osteoporosis and sarcopenia [6]. Total protein intake was aimed at 1.5-1.6 g/kg/d in the intervention group and

Table 1. Main findings of Chapters 2 and 3 of this thesis.

	Outcome					
Exposure	**Total body BMD (g/cm²)**	**Spine BMD (g/cm²)**	**Total hip BMD (g/cm²)**	**Femoral neck BMD (g/cm²)**	**Hip fracture risk**	**Bone turnover markers**
Total protein	Chapter 2.1: NS Chapter 2.2: Beta 0.0011, 95% CI 0.0006-0.0015 Chapter 4.2: NS	Chapter 2.1: NS Chapter 2.2: Beta 0.0015, 95% CI: 0.0007-0.0023 Chapter 4.2: NS	Chapter 2.1: Qualitative evaluation shows positive trend Chapter 4.2: NS	Chapter 2.1: Qualitative evaluation shows positive trend Chapter 4.2: NS	Chapter 2.1: HR 0.89, 95% CI 0.84-0.94	Chapter 4.2: NS
Animal protein	Chapter 2.2: Beta 0.0011, 95% CI 0.0007-0.0016	Chapter 2.2: Beta 0.0017, 95% CI 0.0010-0.0024				
Plant protein	Chapter 2.2: Beta -0.0010, 95% CI -0.0020 to -0.0001	Chapter 2.2: Beta -0.0019, 95% CI -0.0034 to -0.0004				
Magnesium			Chapter 3.1: Beta 0.03, 95% CI 0.01-0.06	Chapter 3.1: Qualitative evaluation shows positive trend		
Intervention	**Daily 31 g whey protein for 20 weeks**				**Additional dairy products for 2 years**	**Fortified milk + exercise for 24 weeks**
	Chapter 2.3: total body BMD NS				Chapter 3.2: hip fracture risk HR 0.54, 95% CI 0.35-0.83	Chapter 3.3: Improved balance of bone turnover markers

Empty cells reflect that the outcome measure was not studied, or insufficient data was available to drawn conclusion (in the systematic reviews and meta-analyses). Meaning of text color: green = beneficial finding, red = adverse finding, grey = neutral finding. BMD, bone mineral density; HR, hazard ratio; NS, no significant finding.

1.2 g/kg/d in the control group. In the intervention group, BMD at the lumbar spine and total hip was higher than the control group (mean difference 0.012 and 0.013 mg/cm^2, respectively). Both studies confirm the relationship between a higher protein intake and an improved bone health in older adults, which was discussed in Chapter 2.1.

To identify relationships between dietary protein and bone health, a combination of different study types provides the most comprehensive understanding. Observational studies can explore and identify potential relationships between variables in real-world settings, while intervention studies can provide more rigorous evidence of causality. This approach was used in Chapter 2 of this thesis. While the meta-analysis (Chapter 2.1) and observational study (Chapter 2.2) showed that protein consumption plays a beneficial role in supporting bone health, this was not confirmed by the intervention studies (Chapters 2.2 and 2.3). However, the intervention studies were designed for improving muscle health and not for supporting BMD. In addition, while effects on muscle mass and strength can be observed after 12 weeks, effects on bone generally require a longer duration to be detected. Still, muscle and bone are interconnected to each other; osteoporosis and sarcopenia are two ageing-related morbidities that share risk factors [7]. Furthermore, low muscle mass and strength that occur with sarcopenia increase the risk of falling, and subsequently the risk of fractures [4]. This is partly explained by the mechanical relationship between muscle and bone. Mechanical loading of bones refers to the forces placed on bones through physical activity, i.e., during a muscle contraction, load is applied to bones [8]. This load can be higher than external loads, such as ground reaction forces during running [8]. Bones adapt and respond to mechanical loading, resulting in stronger bones. Although muscle and bone are interconnected, anabolic or catabolic changes within muscle and bone are not always occurring in parallel and improvements in muscle function may be inadequate to serve as an anabolic stimulus for bone [8]. Well-designed randomized controlled trials (RCTs) investigating the effect of protein (plus exercise) on bone health in older adults remain scarce, explaining the discrepancy between findings from observational and intervention studies.

Chapter 2.1 and other meta-analyses [9-11] showed that a higher protein intake can lead to higher BMD and can reduce hip fracture risk. On the other hand, an increase in physiological acidity may occur (blood pH below 7.35), which might be detrimental for the bones. The following nutritional components can increase physiological acidity: sulphur amino acids which are mainly found in animal protein, phosphate from dietary phytates in grains, and an inadequate potassium intake from mainly fruit and vegetables (or a high protein:potassium ratio) [12].

It is suggested that a long-term serious imbalance may lead to increased bone loss, because the bones are a source of alkaline mineral which can neutralize acid [12]. However, a high protein intake still benefits bone health as long as the diet contains enough calcium to help compensate for any sulphur amino acid-induced bone loss, and fruit and vegetables to increase potassium intake [12]. In Chapter 2.2, it was even shown that total and animal protein intake were associated with higher BMD in the total body and spine (beta ranging from 0.0011 to 0.0017 g/cm^2). In contrast, higher plant protein intake was associated with a lower total body and spine BMD (beta -0.0010 and -0.0019 g/cm^2, respectively). Compared to plant protein, animal protein has a greater digestibility and a more complete amino acid profile [13], and dairy products typically contain calcium as well [14]. However, plant-based food groups as legumes and nuts also contain adequate amounts of protein [14] and combining different plant-based protein sources may improve essential amino acid composition [15, 16]. More research is needed to investigate if a combination of plant-based protein sources may avoid adverse effects on bone health.

To conclude, protein plays a role in supporting bone health. Due to a lack of intervention studies showing a beneficial effect of protein on bone health, the body of evidence is not strong enough to increase the current protein recommendation. Therefore, trials are warranted to judge if a dietary protein intake above the current Recommended Dietary Allowance (RDA) can delay or prevent the development of osteoporosis in healthy older adults and osteopenic or osteoporotic individuals. Lastly, increasing the intake of plant protein is not (yet) a recommended strategy to support bone health of older adults.

INFLUENCE OF OTHER NUTRITIONAL FACTORS ON BONE HEALTH

Chapter 3.1 indicated that, in older adults, a higher magnesium intake may support an increase in hip and femoral neck BMD. Although magnesium has several mechanistic roles in bone formation and resorption (Figure 2 of Chapter 1), the added value of a high magnesium intake, or even magnesium supplementation, for improving bone health is questionable. In the systematic review and meta-analysis, limited evidence was available to assess the relationship between a higher magnesium intake and bone health. Furthermore, there were no RCTs. Since the systematic search in July 2021 until the writing of this discussion (February 2023), no new studies have been published on this relationship. Hence, there is no need to change dietary magnesium intakes beyond recommended values.

In the Netherlands, the adequate intake (AI) is set at 300 mg/d for females and 350 mg/d for males [17]. For future research, first well-designed cohort studies investigating the association are needed. If these show beneficial associations, then the causal relationship should be investigated with trials.

In contrast to the previous described single nutrients, dairy provide a combination of macro- and micronutrients. In Chapter 3.2, the results of the first controlled intervention trial with fractures as outcome parameter was translated from the Australian to the Dutch situation. These results suggested that for fracture and fall prevention, only 65 people should receive protein and calcium supplementation via regular dairy products to prevent one fracture to occur and 48 people to prevent one fall. Chapter 3.3 showed that a fortified milk supplement combined with exercise successfully improved the balance of bone turnover markers of Chinese middle-aged and older adults. These positive results on bone health are in line with a meta-analysis showing that a diet rich in dairy products was associated with a 41% lower risk of low BMD (note that studies were included with participants of all ages) [18]. Furthermore, observational studies have shown that dairy products are associated with lower fracture risk [19]. Based on Chapter 3.2, the consumption of 3.5 servings of dairy per day can be recommended to older adults for the reduction of fall and fracture risk.

Two remarks have to be made regarding this recommendation. First, not every person can tolerate dairy products due to a lactose intolerance (no digestion of lactose possible) or A1 beta-casein intolerance, leading to symptoms like abdominal pain and diarrhea [20]. Prevalence of lactose intolerance or malabsorption varies across the world, ranging from 28% in Europe to 70% in the Middle East [21]. In the Netherlands, it is estimated at 12% of the population [21]. Other guidelines are applicable for persons with lactose intolerance [22]. Second, the downside of increasing the consumption of dairy products is that animal-source foods can have a significant impact on the environment, including high levels of greenhouse gas emissions, water usage, and water pollution [23]. Currently, reducing the consumption of animal products, including dairy, and opt for plant-based alternatives with a lower environmental impact is considered a wise recommendation for healthy adults. However, as discussed earlier, this thesis gives the first indications (Chapter 2.2) that this cannot simply be extrapolated to older adults.

INSIGHTS OF STUDIES IN HIP FRACTURE PATIENTS

Hip fracture patients are more vulnerable than healthy older adults are. These patients often experience a reduced appetite, increased inflammation, and changes in metabolism [24, 25]. Therefore, the role of nutritional factors to support bone health may be different in this population. Findings of Chapter 4 give insights in this matter. **Chapter 4.1** showed that a poor nutritional status, dietary intake and muscle health were common in older hip fracture patients in geriatric rehabilitation wards. **Chapter 4.2** concluded that a good nutritional status comes with higher BMD in older hip fracture patients, but no association was found for protein with bone health.

Measuring habitual dietary intake in older adults can be challenging in nutrition research. Chapter 4.2 suggested that the estimation of pre-fracture habitual protein intake in older hip fracture patients may be even more difficult. The first days in the hospital can be an overwhelming situation for hip fracture patients [26, 27], affecting the answers to questionnaires. Since fractures happen unexpectedly, (3-day) food diaries (with or without being filled in through a combination of observations and weighing) are not possible before the fracture [28]. These methods can also not be used while the patient is in the hospital or rehabilitation center, because the menu at these locations is likely to differ from habitual pre-fracture intake. Usual intakes can also be assessed with a quantitative Food Frequency Questionnaires (FFQ) but these are subject to recall and social desirability biases [28]. Still, using a quantitative FFQ through direct questioning by a trained researcher or dietician seems the best option to assess pre-fracture habitual protein intake in older hip fracture patients.

Both studies in Chapter 4 showed that protein intake was insufficient in older hip fracture patients and that malnutrition or being at risk of malnutrition was common. Regarding micronutrients, mean intakes of calcium, vitamin D, potassium, magnesium, and selenium were significantly below the recommendations (Chapter 4.1). Time after time, studies show that nutritional status is inadequate in hip fracture patients and that this might have consequences such as an increased mortality risk, a reduction in activities of daily living (ADL), and more complications [29-33]. Existing guidelines encourage the use of nutritional strategies. For example, the guidelines of the European Society of Parenteral and Enteral Nutrition (ESPEN) on clinical nutrition and hydration in geriatrics state that older patients with a hip fracture should receive nutritional supplements for improving their dietary intake and clinical outcomes, and reduce the risk of complications [34]. In Dutch guidelines, it is advised to provide hip fracture patients

aged 70 years and older or at risk of malnutrition with extra protein and energy, using enriched foods, snacks and/or liquid nutritional supplements for at least eight weeks [35]. However, Dutch institutions may deviate from these guidelines if local policies are different [35]. Chapter 4 pointed out that still due attention for nutritional strategies is needed to improve nutritional status and clinical outcomes in hip fracture patients. According to prevailing guidelines and consensus of experts, such interventions should consider the following approaches (in no particular order):

1. A non-pharmacological intervention includes at least a nutritional and exercise component.
2. The nutritional component includes achieving an adequate calcium, protein and energy intake, and vitamin D status (via vitamin D supplementation) [35-38].
3. Protein intake should be at least 1.0 g/kg/d [34, 37].
4. Attention should be given to appetite [39].
5. An individualized approach should be used for both nutritional support as exercise training [34, 40].
6. A multidisciplinary team should be involved, including physicians, nurses, nutritional assistants, dieticians, physiotherapists, and other health care professionals [34, 40].
7. Regular visits from a dietician after hospital discharge should be implemented, independent of discharge location (rehabilitation center, nursing home, or home) [40].
8. The intervention should be multidimensional, as explained above [34].
9. Support should be especially present in the first 3 months after the fracture (due to a hypercatabolic state and inflammation [24]).
10. Impact of emotions, stress and social circumstances should not be underestimated [41-43].

Besides osteoporosis, hip fracture patients often encounter other geriatric conditions, such as malnourishment, sarcopenia, and frailty [44], which were also found in Chapter 4. Since these geriatric conditions are overlapping, interventions that are focused on one of these conditions may have a broader impact on clinical outcomes than expected [44]. Furthermore, this overlap makes it easier to tackle multiple geriatric conditions at the same time.

Besides improving clinical outcomes in hip fracture patients, prevention seems to have an important role. In the Netherlands, 388.000 females at high risk of fractures remained untreated for osteoporosis in 2019, which was a treatment gap of 56% [45]. Without a change in policy, it is projected that the number of

osteoporotic fractures will increase with 37% over the next 15 years (from 2019 till 2034) [45]. In Chapter 4.2, only 13% of the hip fractures was caused by a fall after feeling unwell, dizziness or loss of balance. Hence, many fractures could be prevented by adaptations to people's homes (e.g. no carpets, using non-slip bath mats, no furniture on wheels, no wires where you walk). A detailed approach for prevention and management of falls in older adults can be found in the recently published global guidelines [46]. Furthermore, it was found in Chapter 4.2 that at hospital admission only 16% of the patients was previously diagnosed with osteoporosis, while at 3 months follow-up 34% of the patients were diagnosed with osteoporosis and 52% had osteopenia (note that a dual-energy X-ray absorptiometry (DXA) scan could only be performed in 56 patients). Increasing the frequency of screening for low BMD in at-risk populations can be valuable to prevent fractures [36].

TAKEN TOGETHER, NUTRITIONAL FACTORS TO SUPPORT BONE HEALTH IN OLDER ADULTS

Based on this thesis, protein is suggested to have the greatest potential for improving bone health in older adults, next to the already established role of calcium and vitamin D [47]. Recommending a high protein intake via commercially available food products would lead to an increase in other key bone nutrients as well. This is due to an overlap between food products high in key bone nutrients. For example, dairy is rich in protein and calcium (but also still contains some magnesium), and whole grain pasta contains both protein and magnesium [14]. This is reflected in **Figure 1**, which shows the main food sources of calcium, protein, and magnesium calculated per portion size. Vitamin D is not shown since the contribution from the diet is limited [14]. Skin photosynthesis is the main source of vitamin D [48], while the main dietary source is fish; lean fish (0-5 g fat per 100 g) contains 3.1 μg vitamin D per 100 g and fatty fish (>5 g fat per 100 g) contains 5.5 μg vitamin D per 100 g [14]. Depending on the country, vitamin D fortified products can be found in the supermarkets [49]. However, for older and institutionalized populations, reaching an adequate vitamin D status seems only possible with vitamin D supplementation [38]. Next to the nutrients discussed in this thesis, there are more nutrients which have been proposed to influence bone health, for example vitamin K and vitamin A [50, 51]. It is not known to what extent these nutrients can contribute to healthy bones in older adults [50, 51].

The combination of several bone nutrients may be needed for the most optimal effect on bone health and to delay or prevent the development of osteoporosis.

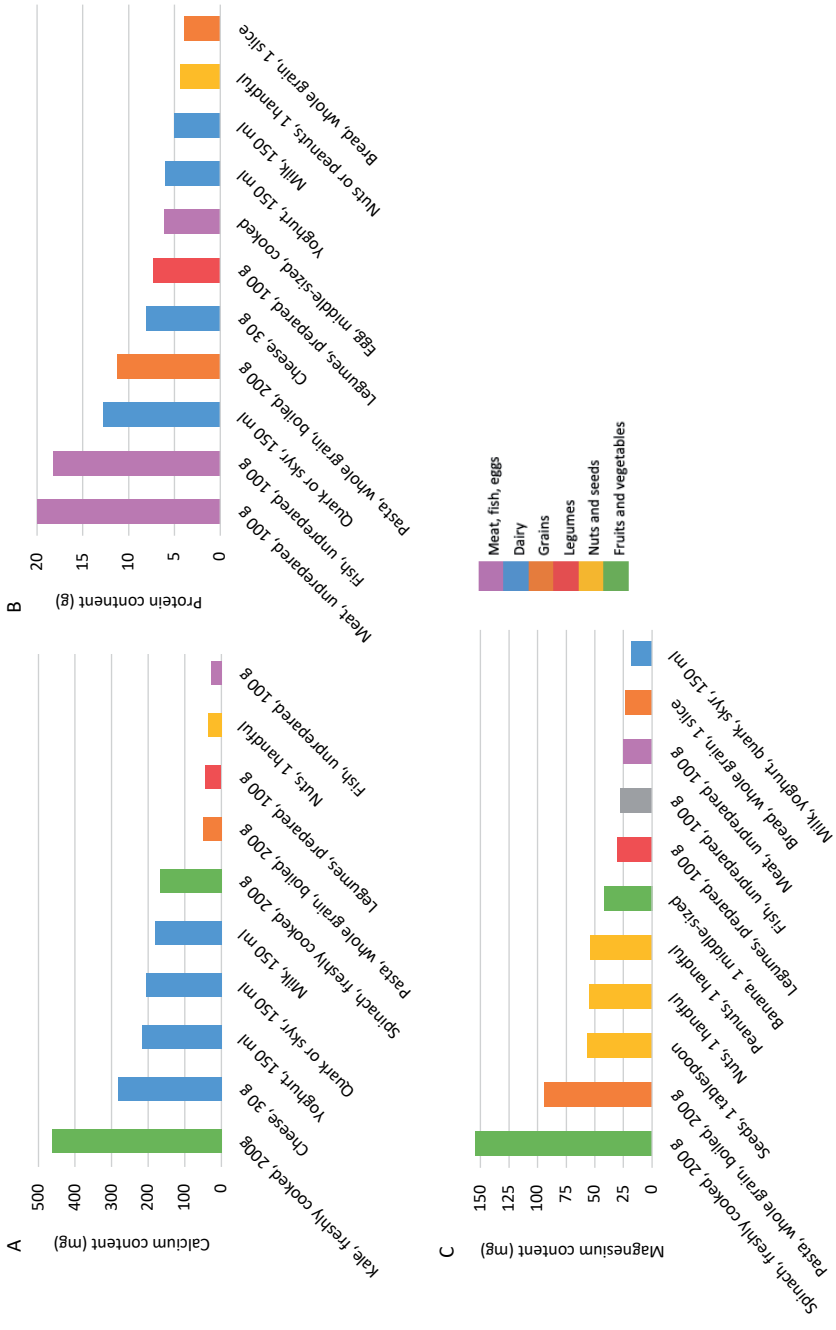

Figure 1. Calcium (A), protein (B) and magnesium (C) content of different food products per portion size. Values based on NEVO-online [14]. Details of the method can be found in Box 1.

Box 1. Method corresponding to Figure 1.

- For milk, yoghurt and quark, the mean was taken of the full, semi-skimmed and skimmed product.
- For cheese, the mean was taken of four different categories of ripening (young, mild matured, matured, old) and two different fat contents (30+ and 48+).
- For seeds, the mean of pumpkin seeds, sunflower seeds and pine nuts was taken.
- For nuts, the mean of almonds (peeled and unpeeled), cashew nuts, hazelnuts, Brazil nuts, walnuts, pecans and macadamias was taken.
- For legumes, the mean of fava beans, pinto beans, red kidney beans, chickpeas, brown lentils and red lentils was taken.
- Preparation methods may change the nutrient contents. This was the most pronounced for the calcium content in spinach (84 mg/100 g when freshly cooked vs 162 mg/100 g in frozen spinach) and in kale (231 mg/100 g when freshly cooked vs 150 mg/100 g in frozen kale).
- Calcium content varies also greatly between nuts. Cashew nuts, pecans and macadamias have the lowest contents and almonds, hazelnuts, Brazil nuts and walnuts have the highest contents.

Furthermore, one does not eat single nutrients and nutrients may interact with each other. Therefore, moving towards a concept of a 'bone healthy diet' has been explored.

Previously, a BMD-Diet Score has been developed, which reflects a diet that may be beneficial for BMD [52]. This score is composed of eight food groups associated with high BMD: vegetables, fruits, dairy products, whole grain products, fish, and legumes, and two food groups associated with low BMD: meat and confectionary. These food groups were based on studies investigating the effects of dietary patterns on BMD. A review assessing the impact of dietary patterns on bone health outcomes concluded that a dietary pattern with a high intake of fruits, vegetables, whole grains, poultry and fish, nuts and legumes, and low-fat dairy products and a low intake of soft drinks, fried foods, meat and processed products, sweets and desserts, and refined grains supports bone health [53]. However, in this review, studies with a diverse age range were included, while the impact may be different in older adults. A sub-analysis for older adults was performed in a meta-analysis investigating the dietary patterns: "Healthy" (veg-

etables, fruits, poultry, fish, and whole grains), "Milk/dairy", and "Meat/Western" (red meat, processed meat, animal fat, eggs, and sweets) [18]. A high adherence to the ''Healthy'' and "Milk/dairy'' pattern reduced fracture and low BMD risk compared to low adherence, whereas high intake of the "Meat/Western" pattern increased the risk of fractures and low BMD compared to low intake. Combining these three studies, a dietary pattern rich in vegetables, fruits, dairy, whole grains, poultry, and fish seems to fit in a bone health diet, which also matches with the findings of this thesis.

The dietary reference intakes for the nutrients presented in this thesis are shown in **Table 2**. The recommendation for protein from experts is higher than the RDA, and this is based on evidence regarding preservation of muscle health [54, 55]. Regarding bone health, European guidance provided by International Osteoporosis Foundation (IOF) and European Society for Clinical and Economic Aspects of Osteoporosis, Osteoarthritis and Musculoskeletal Diseases (ESCEO) in 2013 state that 1.0 g/kg/d of protein can be recommended in the general management of patients with osteoporosis [56]. However, in the updated guidance of 2019 this number is removed and changed to ''sufficient dietary protein'' [57]. In 2023, the first set of dietary recommendations in the prevention and treatment of osteoporosis have been published by the French Rheumatology Society and the Osteoporosis Research and Information Group [37]. For prevention and treatment of osteoporosis, a protein intake of at least 1.0–1.2 g/kg/day (with "high quality" animal proteins) as part of a balanced diet with adequate calorie, calcium and vitamin D intakes is advised. In addition, the French group recommend a Mediterranean-type diet and the daily consumption of 2 to 3 dairy products. Furthermore, they advise to avoid unbalanced Western diets, vegan diets, alcohol consumption and daily consumption of sodas. In non-overweight individuals specifically, weight loss diets were not recommended.

Table 2. Dietary reference intakes for calcium, vitamin D, protein and magnesium[a].

Nutrient	Recommendations for older adults (≥70 years)	Reference
Calcium	AI: 1000-1200 mg/d	[17]
Vitamin D	RDA: 800 IU/d (= 20 mg/d)	[17]
	Experts: 1000 IU/d (= 25 mg/d)	[58]
Protein	RDA: 0.8 g/kg/d	[59]
	Experts: 1.0-1.2 g/kg/d	[54, 55]
Magnesium	AI females: 300 mg/d	[17]
	AI males: 350 mg/d	[17]

[a]Derived from the Health Council of the Netherlands, except for the recommendation of the expert panels. AI, adequate intake; IU, international unit; RDA, recommended dietary allowance.

The national dietary guidelines of the Netherlands, issued by the Dutch Health Council, were last updated in 2015 [60]. These guidelines are based on available evidence with respect to nutrients, food products and dietary patterns in relation to 10 major chronic diseases in the Netherlands. Therefore, outcomes of bone health are not included. In addition, the guidelines are meant for the general adult population in the Netherlands. So do these guidelines fit within a bone healthy diet during ageing? In **Table 3**, the relevant guidelines for bone health (9 out of 15) from the Dutch Health Council are compared to evidence for supporting bone health and to the current intake in older adults. The findings of this thesis underpin the dietary guidelines of the Dutch Health Council, except for the guideline "Follow a dietary pattern that involves eating more plant-based and less animal-based food". Ongoing discussions will lead to new recommendations with regard to the preferred ratio between plant and animal

Table 3. Comparison of the dietary guidelines from the Dutch Health Council to evidence for supporting bone health and to the current intake in older adults.

Guidelines from Dutch Health Council [60]	Does it fit in a bone healthy diet during ageing?	Current intake in older adults (65-79 years)[a]
Eat at least 200 g of vegetables and at least 200 g of fruit daily	Yes, to reduce physiological acidity	Vegetables: mean 183 g/d and 36% ≥200 g/d Fruit: mean 170 g/d and 34% ≥200 g/d
Take a few portions of dairy products daily, including milk or yogurt	Yes, contain key bone nutrients	Dairy: mean 372 g/d ≈ 2.5 servings
Eat legumes weekly	Yes, contain key bone nutrients	0.4 times per week
Eat at least 15 g of unsalted nuts daily	Yes, contain key bone nutrients	Mean 8.2 g/d and 18% ≥15 g/d
Eat at least 90 g of whole-grain products daily	Yes, whole-grain products contain key bone nutrients	64% of total bread and grain products are wholegrain
Eat weekly one serving of fish, preferably fatty fish	Yes, contribute to dietary vitamin D intake	35% consumes fish once a week or more
Do not drink alcohol or do not drink more than one glass daily	Yes, heavy drinking is associated with low BMD and high fracture risk	27% consumes no alcohol and 20% 1 glass per day or less
Nutrient supplements only for specific groups for which supplementation applies— for example, groups that need extra vitamin D, folic acid or vitamin B12	Yes, vitamin D supplementation	Vitamin D supplements are taken by 37% of women aged ≥50 years and by 25% of men aged ≥70 years
Follow a dietary pattern that involves eating more plant-based and less animal-based food	May not support bone health	Ratio between plant and animal protein is 39:61

[a] *Based on the Dutch National Food Consumption Survey 2019-2021.*

protein in our diet. In Chapter 2.2, older adults with the lowest ratio of median animal to plant protein intake (52:48) had lower BMD values compared to those with higher ratios (63:37 and 71:29). Furthermore, previous literature found that vegan and vegetarian diets can lead to lower BMD values and a higher fracture risk [61, 62]. It remains to be studied which exact ratio should be advised without jeopardizing bone health. Another observation emerging from **Table 3** is that the current intake in older adults is often (far) below the guidelines. Therefore, the current dietary habits of Dutch older adults may not only have consequences for the 10 major chronic diseases, but also for bone health.

FUTURE RESEARCH

The following topics for future research emerge from this thesis:

- Well-designed, large, and long-term RCTs in older adults are needed to judge if a dietary protein intake above the current RDA can support bone health and/or prevent osteoporosis. The added value of dietary protein should be considered alongside calcium and vitamin D. These trials are warranted in healthy older adults, osteopenic or osteoporotic individuals, prostate cancer patients, and hip fracture patients.
- More research on the impact of a plant-based diet and sustainable dairy substitutes on bone health in older adults is needed.
- Properly designed cohort studies investigating the association between magnesium intake and bone health, adjusted for all relevant confounding factors, are preferred over conducting a trial.
- The reduction in risk of falls and fractures by consumption of regular dairy products (to increase protein and calcium intake) should be confirmed in other populations (than Australians living in aged care facilities).

Future research into bone health requires careful consideration of several methodological factors to ensure the accuracy and validity of the results. Points of attention are discussed below.

First, there are several parameters which should be assessed in bone health research. The most important ones are age, gender, weight or BMI, energy intake, vitamin D status, calcium intake, physical activity, smoking and alcohol use [57, 63]. Preferably, studies should also include family history of osteoporosis, fracture history, (certain) illnesses, and (certain) drugs [57, 63]. Furthermore, intervention studies investigating the effect of nutritional factors on bone health require large cohorts of subjects to achieve sufficient statistical power and to reduce

the risk of false positive or negative results. Moreover, the duration of follow-up must be sufficient in order to observe changes in bone health outcomes. The duration is dependent on which outcome parameters are investigated. Studies included in this thesis measured bone health using bone turnover markers, BMD, and/or fracture risk. Changes in bone turnover markers can already be measured within two weeks after the start of an intervention, whereas changes in BMD are more likely to be detected after at least 6 months [64-66]. However, the PROVIDE Study found an effect of a vitamin D, calcium and leucine-enriched whey protein supplement on total body BMD of older adults after only 13 weeks [67]. To date, no other studies have found effects on BMD of older adults in such a short time frame. Regarding assessing changes in fracture risk, it was found in Chapter 3.2 that the significant differences in hip fractures and falls became apparent after 5 and 3 months, respectively. Also in hip fracture patients, the first 3 months after the fracture are especially important for recovery due to a hypercatabolic state and inflammation [24].

Another possible measure is bone strength, which may have added value to assessing BMD. Bone strength refers to the ability of bones to withstand mechanical stress without fracturing. Specific parameters of bone strength include bone geometry, cortical thickness and porosity, and trabecular bone architecture [68]. BMD is one of the determinants of bone strength and it accounts for 60-70% of the variation in bone strength [68]. Therefore, an increase in BMD, due to a certain treatment, doesn't automatically lead to a reduction in fracture incidence. An *in vivo* method to estimate bone strength is using images obtained from high-resolution peripheral quantitative computerized tomography (HR-pQCT) subjected to finite element analysis (FEA). Previous studies in healthy postmenopausal women [69] and older men [70] have found that a higher protein intake was associated with higher bone strength. Measuring bone strength, in addition to assessing BMD at different sites, can have added value for bone health research. However, technical issues with this technique still exist (for example motion artifacts) and standardization is necessary [71]. In addition, HR-pQCT is a relatively expensive imaging technique and its availability is limited [71]. Therefore, widespread implementation of this technique is not yet feasible.

Literature on bone health has been dominated by studies in postmenopausal women. However, bone health research should also be focused on men. Osteoporosis and its consequences affect both sexes, but loss of bone mass starts at different ages and progresses at different rates [72]. Prevalence of osteoporosis and osteoporotic fractures are higher in females than in males, but mortality risk after a fracture is higher in males [72]. It has been proposed that infection

and poor medical treatment (males tend to receive less medical treatment after hip fractures) explain the higher mortality rate [72]. Indeed, a higher CRP level at baseline was found in males compared to females in Chapter 4.2. In multiple chapters of this thesis, due attention was given to differences between sexes:

- No difference in hip fracture risk reduction with higher protein intakes was found between sexes (Chapter 2.1).
- Association between protein intake and BMD was not sex specific (Chapter 2.2).
- The ratio of the net effect of bone formation and resorption in the group receiving the combined nutrition and exercise intervention was irrespective of sex (Chapter 3.3).

In these chapters, associations and effects on bone health were not different between sexes, suggesting that nutritional interventions should target both groups. In addition, everyone who experiences a fracture after the age of 50 should be screened for osteoporosis, irrespective of sex [36].

OVERALL CONCLUSION

To conclude, nutritional factors can support bone health in older adults. Based on this thesis, protein appears to have the greatest potential for supporting bone health in older adults, next to calcium and vitamin D. To achieve adequate protein and calcium intakes, dairy products are highly suitable. However, the added value of a high magnesium intake for supporting bone health remains unclear. Since protein intake is often insufficient in older hip fracture patients and malnutrition or being at risk of malnutrition is common, due attention for nutritional strategies in this population is needed. The work in this thesis adds important knowledge to the field of nutrition and bone health. Maintaining good bone health is essential for preventing osteoporosis, reducing the risk of fractures, and maintaining mobility and independence in later life. Therefore, research into bone health contributes to promoting healthy ageing and the overall well-being of older adults.

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Summary

Bone is a living tissue that changes over the course of a lifetime. During ageing, bone mass decreases and bone gets more porous. If too much bone mass is lost, this leads to the development of osteoporosis. Osteoporosis increases the risk of falls and fractures, which in turn leads to an increase in morbidity and mortality, loss of independence, and a decreased quality of life. It has been well established that consumption of sufficient calcium and vitamin D are required for proper bone health. However, there are more nutrients and nutritional strategies that may play an important role in maintaining and improving bone health in later life, including increasing the intake of protein, magnesium, and dairy. The potential impact of these nutritional strategies to delay or prevent the development of osteoporosis and, as such, support bone health remains understudied. The aim of this thesis was to investigate the role of nutritional factors in supporting bone health of older adults.

Chapter 2 focused on the role of dietary protein intake in bone health of older adults. The systematic review in Chapter 2.1 uncovered a positive trend between higher protein intakes and higher femoral neck and total hip bone mineral density (BMD), and the meta-analysis showed that higher protein intakes resulted in a significant decrease in hip fractures (pooled HR 0.89). In Chapter 2.2, total and animal protein intake were associated with higher BMD in the total body and spine (beta ranging from 0.0011 to 0.0017 g/cm^2). In contrast, higher plant protein intake was associated with lower total body and spine BMD (beta -0.0010 and -0.0019 g/cm^2, respectively). Interventions of 12 or 24 weeks with protein supplementation or protein supplementation combined with resistance exercise did not lead to significant improvements in BMD. Chapter 2.3 showed that supplementation of 31 g whey protein for 20 weeks had no effect on BMD after 20 weeks and 1 year in prostate cancer patients on androgen deprivation therapy.

In Chapter 3, the nutritional factors magnesium, dairy and a milk supplement were addressed. The systematic review in chapter 3.1 indicated that a higher magnesium intake may support an increase in hip and femoral neck BMD. The meta-analysis showed a significant positive association between magnesium intake and hip BMD (pooled beta 0.03). Evidence from randomized controlled trials was missing. In Chapter 3.2, it was concluded that the results from a large intervention study in Australia were translatable to the Dutch population; increasing the intake of dairy products (leading to a calcium intake of 1100-1200 mg/d and protein intake of 1.1 g/kg/d) may be beneficial for the bone health of aged care residents in the Netherlands. Chapter 3.3 showed that a 24-wk combined nutrition (fortified milk supplement containing protein, calcium, cholecalciferol, and vitamin B-12) and exercise intervention successfully improved vitamin B-12

and 25-hydroxyvitamin D concentrations as well as the balance of bone turnover markers of Chinese middle-aged and older adults.

In Chapter 4, insights emerging from studies in hip fracture patients are presented. Hip fracture patients are more vulnerable than healthy older adults are. Therefore, the role of nutritional factors to support bone health may be different in this population. Chapters 4.1 and 4.2 showed that protein intake was <0.8 g/kg/d in about half of the patients and <1.2 g/kg/day in more than 90% of the patients. Regarding micronutrients, mean intakes of calcium, vitamin D, potassium, magnesium, and selenium were significantly below the recommendations. The combined prevalence of risk of malnutrition and malnourishment increased from 20% of the patients at hospital admission to 64% at 3 months (Chapter 4.2). In geriatric rehabilitation wards, 73% of the older hip fracture patients were classified as either malnourished or at risk of malnutrition (Chapter 4.1). Furthermore, approximately one third had low muscle mass and a quarter showed low muscle strength. Chapter 4.2 concluded that good nutritional status comes with higher BMD in older hip fracture patients, but no association was found for protein with bone health outcomes.

Based on this thesis, protein appears to have the greatest potential for supporting bone health in older adults, next to calcium and vitamin D. Dairy products are a suitable source for achieving adequate protein and calcium intakes. However, the added value of a high magnesium intake for supporting bone health remains unclear. Due attention for nutritional strategies is needed to improve dietary intake, nutritional status, and clinical outcomes in hip fracture patients. This thesis provides valuable insights into the field of nutrition and bone health, which is essential to delay or prevent the development of osteoporosis, reducing the risk of fractures, and maintaining mobility and independence in later life. Therefore, research into bone health contributes to promoting healthy ageing and the overall well-being of older adults.

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Berber, Pol, Ellen, en **Marije**, mijn eerste congres was met jullie in Zweden. Met plezier denk ik terug aan de kilo's cottage cheese, posters strijken, en het Zweedse nachtleven. **Berber, Charlotte, Jacintha, Pol,** en **Tessa**, als een echte fan base zaten jullie in de zaal tijdens mijn presentatie op het ESPEN congres in Wenen. Bedankt voor jullie steun en de gezelligheid rondom het congres en het pretpark ernaast.

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Colleagues in **room 1059**, some shifts happened over the years, but thank you for creating a nice working environment. **Naomi**, **Tsitsi** and **Anniek**, good luck with the last steps of your PhD trajectories. Although it is not our room anymore, I will probably see you there.

Anniek, bij jou kan ik altijd aankloppen voor een advies of een praatje. Je oprechte nieuwsgierigheid in anderen is een mooie eigenschap. Bedankt voor alle kopjes koffie, wandelingen en spelletjes.

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About the author

On Sunday August 6, 1995, not far away from Wageningen, Inge Groenendijk was born. After completing secondary school at Pallas Athene College in Ede in 2013, she went to Wageningen University for the Bachelor Nutrition and Health. In the third year of the Bachelor, she did a minor in Public Health at the University of Eastern Finland in Kuopio. In 2016, she continued with the MSc program Nutrition and Health at Wageningen University, specializing in Nutritional Physiology and Health Status. For her MSc thesis, she conducted her first intervention study in young adult men, investigating the acute effects of a brain-healthy meal on working memory, episodic memory and salivary cortisol levels compared to a control meal. In 2018, she went for an internship to the Max Planck Institute for Human Cognitive and Brain Sciences in Leipzig, Germany. She investigated the association between the neuroimaging biomarker 'peak width of skeletonized mean diffusivity' and dietary patterns in the LIFE-Adult-Study. When she came back to the Netherlands, she started as a PhD candidate at the division of Human Nutrition and Health of Wageningen University & Research.

The scientific results of the PhD trajectory are described in this thesis. In addition, she was co-author of three other papers and wrote an article for the members of the Dutch Osteoporosis Foundation. She presented her work during international conferences in Canada, Sweden, France, Austria, and Spain. And yes, she travelled by train instead of the plane if the journey time was within 14 hours (in support of proposition number 6).

In March 2023, she started as a postdoctoral researcher in the lab of Lisette de Groot, where she will continue the investigation into the role of nutritional factors in supporting bone health of older adults.

LIST OF PUBLICATIONS

- Marco Mensink, **Inge Groenendijk**, Ellen Oosting. (2023). Wat eet een atleet en wat kunnen we daarvan leren voor revalidatie? *Nederlands Tijdschrift voor Revalidatiegeneeskunde*, 2.
- **Inge Groenendijk**, Pol Grootswagers, Aurelia Santoro, Claudio Franceschi, Alberto Bazzocchi, Nathalie Meunier, Aurélie Caille, Corinne Malpuech-Brugere, Agata Bialecka-Debek, Barbara Pietruszka, Susan Fairweather-Tait, Amy Jennings, Lisette C.P.G.M. de Groot. (2022). Protein intake and bone mineral density: Cross- sectional relationship and longitudinal effects in older adults. *Journal of Cachexia, Sarcopenia and Muscle,* 14(1), 116-125.
- **Inge Groenendijk**, Lisette de Groot, Pol Grootswagers. (2022). Zuivel kan botten verzorgingshuisbewoner sterk houden. *Ned Tijdschr Geneeskd,* 166, D6570.
- Charlotte S. Kramer, **Inge Groenendijk**, Sonja Beers, Hugo H. Wijnen, Ondine van de Rest, Lisette C.P.G.M. de Groot. (2022). The Association between Malnutrition and Physical Performance in Older Adults: A Systematic Review and Meta-Analysis of Observational Studies. *Current Developments in Nutrition*, 6(4), nzac007.
- **Inge Groenendijk**, Marieke van Delft, Pieter Versloot, Luc J.C. van Loon, Lisette C.P.G. M. de Groot. (2022). Impact of magnesium on bone health in older adults: A systematic review and meta-analysis. *Bone*, 154, 116233.
- **Inge Groenendijk**, Charlotte S. Kramer, Laura M. den Boeft, Hans S.M. Hobbelen, Gert-Jan van der Putten, Lisette C.P.G.M. de Groot. (2020). Hip Fracture Patients in Geriatric Rehabilitation Show Poor Nutritional Status, Dietary Intake and Muscle Health. *Nutrients*, 12(9), 2528.
- **Inge Groenendijk**. (2020). Gezonde botten? Eiwit is belangrijk! *Bot in Balans*, 2, 14- 15.
- **Inge Groenendijk**, Ruth Chan, Jean Woo, Sherlin Ong, Panam Parikh, Marjolijn C.E. Bragt, Lisette C.P.G.M. de Groot. (2020). A Combined Nutrition and Exercise Intervention Influences Serum Vitamin B-12 and 25-Hydroxyvitamin D and Bone Turnover of Healthy Chinese Middle-Aged and Older Adults. *Journal of Nutrition*, 150(8), 2112-2119.
- **Inge Groenendijk**, Laura den Boeft, Luc J.C. van Loon, Lisette C.P.G.M. de Groot. (2019). High Versus low Dietary Protein Intake and Bone Health in Older Adults: A Systematic Review and Meta-Analysis. *Computational and structural biotechnology journal*, 17, 1101–1112.

LIST OF SUBMITTED MANUSCRIPTS

- **Inge Groenendijk**, Hugo H. Wijnen, Diana G. Taekema, Lisette C.P.G.M. de Groot. (2023). Protein intake, malnutrition, and its potential impact on bone health after a hip fracture: a 3-month prospective study.
- **Inge Groenendijk**, Lisanne H.P. Houben, Maarten Overkamp, Luc J.C. van Loon, Milou Beelen, Sandra Beijer, Lisette C.P.G.M. de Groot. (2023). No effect of protein supplementation on bone mineral density in prostate cancer patients on androgen deprivation therapy.
- Lydia Bechraki, Ellen G.H.M. van den Heuvel, Lisette C.P.G.M. de Groot, **Inge Groenendijk**. (2023). The nutritional benefit of UV-exposed mushrooms for the Dutch population: Modeling the addition of UV-exposed mushrooms to the diet.

OVERVIEW OF COMPLETED TRAINING ACTIVITIES

Discipline specific activities	Organizer and location	Year
DUSRA Annual meeting	DUSRA, Leiden, NL	2018
NAV publiekslezing	NAV, Driebergen-Zeist, NL	2019
9th International Association of Gerontology and Geriatrics European Region congress	IAGG-ER, Gotenburg, SE	2019
Course Stable Isotope Methods in Nutrition Research	VLAG, Wageningen, NL	2019
Compl-eat™ coding training	HNH, Wageningen, NL	2019
DUSRA Annual meeting	DUSRA, Leiden, NL	2019
Food Summit on Personalised Nutrition: High Hopes from the Low Lands	TiFN, Wageningen, NL	2019
NAV publiekslezing	NAV, Driebergen-Zeist, NL	2020
International Conference on Frailty and Sarcopenia Research	ICFSR, Toulouse, FA	2020
SPADE training	RIVM, online	2020
NUTRITION 2020	ASN, online	2020
Ultrasound training for muscle assessment	Vallei Medical, Wageningen, NL	2020
42th European Congress on Clinical Nutrition & Metabolism	ESPEN, online	2020
Venipuncture training	MUMC+, Maastricht, NL	2020
Insertion peripheral intravenous cannula training	MUMC+, Maastricht, NL	2020
DUSRA Annual meeting	DUSRA, Leiden, NL	2021
DUSRA Annual meeting	DUSRA, Leiden, NL	2022
44th European Congress on Clinical Nutrition & Metabolism	ESPEN, Vienna, AT	2022

General courses and activities	Organizer and location	Year
VLAG PhD week	VLAG, Baarlo, NL	2018
Brain friendly working and writing	WGS, Wageningen, NL	2018
PhD Competence Assessment	WGS, Wageningen, NL	2019
Data Science: R Basics	HarvardX, online	2019
Pitch training	HNH, Wageningen, NL	2019
Statistics and R	HarvardX, online	2020
Introduction to Linear Models and Matrix Algebra	HarvardX, online	2020
Anatomy: Musculoskeletal and Integumentary Systems	MichiganX, online	2020
Communication with the Media and the General Public	WGS, online	2020
How to create impactful infographics and data visuals	YoungWUR, online	2020
Using Python for Research	HarvardX, online	2021
Career assessment	WGS, online	2021
Prince2 Foundation	LOI, online	2022

Optional courses and activities	Organizer and location	Year
Preparation of research proposal	HNH, Wageningen, NL	2018
PhD study tour to Canada	HNH, Canada	2019
Nutritional Biology research meetings	HNH, Wageningen, NL	2018-2022
VLAG PhD Council	VLAG, Wageningen, NL	10/2018-2/2020

Colophon

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