ASSOCIATION BETWEEN AMOUNT AND SOURCE OF DIETARY PROTEIN INTAKE WITH BONE MINERAL DENSITY AMONG ELDERLY WOMEN

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Several studies suggested that dietary protein may have impact on bone health. Result of a recent study indicated that protein intake was positively associated with all bone mineral density (BMD) sites, although longitudinal result showed that higher protein intake, was associated with greater bone loss.

Several hypotheses explained the effect of protein on BMD, from different angels including 1- Effect on calcium adequacy by increasing intestinal absorption. 2- Effect on insulin-like growth factor 1 (IGF-1) which is a bone-anabolic hormone. 3- Animal versus vegetable protein hypothesis indicating that animal sources may have inverse effect on BMD by increasing acidity of blood on the other hand. 4- Alkaline mechanism indicating that vegetable protein source can have protective effect on bone because of the buffering effect.

*Objective***:** Primary objective was to determine the association of protein intake (total, animal and vegetable) with BMD among elderly women.

*Design***:** Study participants were 554 elderly women from the Osteoporosis Risk Factor and Prevention-Fracture Prevention Study (OSTPRE-FPS). Study setting conducted for both cross-sectional at baseline and prospective with 3 years of follow-up. Inclusion criteria was, minimum aged 65 years by end of November 2002, and not participated in another OSTPRE bone densitometry sample. Dietary information ascertained by using 3-days food record at the baseline. BMD at the baseline and 3 years after, was measured with dual energy X-ray absorptiometry (DEXA) of the total body, lumbar spine (L2-L4), femoral neck, trochanter and ward's triangle. Association between dietary protein intake and BMD was analyzed by a linear mixed model, adjusted for all potential dietary and non-dietary confounders.

Results: In the cross-sectional analyses protein intake quartiles (total, animal, vegetable) was not associated with BMD. We analyzed total protein intake association based on current dietary recommendation with BMD, our result suggested that there is a negative trend (p=0.080) between total protein intake ≥1 g/kg- bw/d (40% of participants) and femur BMD. The same result was revealed between protein intake ≥1 g/kg- bw/d, and lower total BMD (p=0.055).

Moreover in the longitudinal analysis adjusted for age, hormone therapy (HT) and energy intake, first and second quartiles of vegetable protein intake was associated with greater femur BMD (p=0.045). After adjusting for all confounders, vegetable protein intake (17.33-24.90 g/d) was associated with higher femur BMD (p=0.053). Also vegetable protein intake, lower than median (21.05 g/d), was significantly associated with higher femur BMD (p=0.044), as compared to lower intake.

Conclusion: Present study suggests that total protein intake over than recommended level (≥1 g/kg-bw/d) and higher vegetable protein intake might have detrimental effect on BMD as compared to lower intake. Our finding does not support the dominant idea of beneficial effect of higher protein intake on BMD. Longer follow-up is required to examine the impact of protein on BMD.

Abbreviations

BMC: Bone mineral content

BMD: Bone mineral density

BMI: Body mass index

HT: Hormone therapy

LM: Lean mass

NEAP: Net endogenous acidity from protein

PA: Physical activity

PRAL: Protein renal acid load

RCT: Randomized control trial

“The real knowledge is to know the extent of one’s ignorance”

Confucius

To my parents Mr. M. Isanejad and Mrs. R, Ebarhimi. And thanks a million to my supervisors Arja, Erkkilä, PhD. Jaakku, Mursu, PhD.

CONTENT

1 INTRODUCTION

Protein plays an important role in body functions and also for maintaining bone health. For several years the dietary protein recommendation has been challenging and different estimations have been suggested from different studies; however, recommended dietary allowance (RDA) for protein is 0.80-1 g/kg- bw/d (Wolfe 2013).

The role of dietary protein on bone health remains controversial. On one side, plentiful characteristics of dietary protein are considered to be important for bone remodeling. On the other side protein may have negative effect on bone health, due to different mechanisms like hypercalciuria (Jesudason and Clifton 2011).

Several studies with different approaches have been conducted to evaluate the effect of protein on bone health; Most of them, but not all, suggested that dietary protein was affecting bone structure. Also several studies suggested that animal or vegetable protein source can have different effect on bone health. But still the quality of this protein effect remained inconclusive. The effect of protein on bone health has been determined by effect on muscle mass, BMD, BMC and fracture (Pedersen and Cederholm 2014).

Protein can have effect on muscle mass. In a health study by Houston and coworkers (2008), with 3 years follow up, total, animal and vegetable protein consumption was associated with lean mass (LM) loss. On the contrary in a prospective study with a 5 years follow-up in elderly women, higher protein intake was associated with higher LM. Overall evidences are more suggestive regarding a positive association between total protein intake and muscle mass.

Protein effect on bone health has been in several prospective and cross-sectional studies and also in few randomized control trials (RCT). In the RCT conducted by Dawson-Hughes (2004) 9 weeks of comparison between high protein (HP) diet (24- 32% of energy intake) and low protein (LP) diet (16-19% of energy intake) result showed that HP group had higher BMC as compared to LP group. Also in the Rancho Bernardo (2010) cohort study among men and women with 4 years of follow up, result indicated an inverse association between vegetable protein intake and BMD.

However, earlier findings regarding protein intake are mostly suggesting a positive association although in most of these studies amount of protein intake is unclear which cause difficulties for interpretation (Pedersen and Cederholm 2014).

Also some studies focused on the protein role in predicting or preventing fracture and bone loss. In 4 years osteoporosis study among men and women, total and animal protein intake was inversely associated with bone loss. On the other hand, Rancho Bernardo study (2003) did not show any statistically significant association at baseline between BMD and protein. Earlier findings regarding bone loss and bone fracture also are not in consistent with each other and most of the results are inconclusive (Pedersen and Cederholm 2014).

The main purpose of present study was to add knowledge to findings of previous studies. Results concluded by focusing on effect of protein from different sources (total, animal, vegetable) on BMD. Also study presented results by categorizing the protein effect based on RDA recommendation in addition to gram intake in quartiles, which was the gap of several previous studies. Also we assessed wide range of dietary protein sources (animal, vegetable, meat and dairy products) and measures like NEAP and PRAL which represent mostly the calciuric effect of protein.

2 LITERATURE REVIEW

2.1 Osteoporosis: definition and epidemiology

Osteoporosis is considered as the consequence of aging, which is an emerging medical and socioeconomic threat because of mobility limitation and decrease in the work force, in addition to considerable treatment costs. The etiology of osteoporosis is complex, and as modifiable factors and genetic determinants are known to have influence on it (Ahmadieh and Arabi 2011).

Osteoporosis simply can be defined as a disease of skeletal system and it is characterized by low bone mineral mass and losing matrix of bone tissue, which increase the risk of bone fractures. Until now the only tool for diagnosis of osteoporosis is to measure BMD, and the standard technique is Dual Energy X-ray Absorptiometry (DEXA) (Kung et al. 2005).

Number of hip fractures each year in the EU only is estimated to rise from current figures of 414000 to 972000 by 2050, representing an increase of 135% in fracture. Patients with hip fracture tended to have an overall mortality of 15–30%. The majority of excess deaths occurs within the first 6 month after the fracture. Third National Health and Nutrition Examination Survey (NHANES III) reported 50 -68% estimated national prevalence of low BMD among women aged 50 years or older (Looker AC et al. 1998).

From the economic point of view, the expenses of hospital rehabilitation and treatment for osteoporotic and fractures are considerable fiscal drain for the health care system. Osteoporosis hospital cares, costs treasuries over 3500 € million annually worldwide.

In addition to lifestyle modifications, there are different methods for osteoporosis therapy, such as quitting smoking, reduction of alcohol consumption, increase of physical activity and having an adequate diet. Vitamin D and calcium supplementation is recommended for patients with osteoporosis, combined vitamin D and calcium supplementation importance has been issued by American Society for Bone and Mineral Research. Other therapies are medication with antiresorptive drugs which slow down bone resorption and anabolic drugs which stimulate bone formation (Rachner et al. 2011).

Hormone therapy (HT) is a common treatment and prevention method, it is used for years and mostly it has been recommended to postmenopausal women. Results of a longitudinal study showed that among 80955 postmenopausal women, those who discontinued HT were at 55% greater risk of hip fracture as compared to those who continued HT (Karim et al. 2011).

However, according to several studies that implemented to examine the effect of different factors on bone health, prevention of osteoporosis is the health priority (Cashman 2007, Cashman 2002).

2.2 Osteoporosis Risk factors

Osteoporosis develops over the life course, about 90% of total adult bone mass is accrued by age 20, and the main risk factor for osteoporotic fracture is low BMD. Accordingly, any factor that can prevent bone to reach the peak of bone mass can be considered as osteoporosis risk factor and it may increase the fracture risk (Cashman 2007).

Several factors might influence BMD. They can be basically categorized into two main groups, factors that are not modifiable like gender, age, genetic, and modifiable factors including lifestyle determinants such as smoking, physical activity level, alcohol and diet (Cashman 2007).

2.2.1 Unmodifiable factors

2.2.1.1 Gender

Female gender itself is a significant risk factors for BMD loss, and bone fracture. Women are in greater risk of osteoporosis, because after menopause bone resorption intensifies. The reason is decrease in the secretion of estrogen and progesterone, as these anabolic hormones may effect on bone mineral resorption. As World Health Organization report (WHO) reported, women suffer more from hip fractures; their lifetime risk for osteoporotic fractures is at least 30%. In contrast, fracture risk is only 13% in men (WHO 2014).

2.2.1.2 Age

In the 21st century, life expectancy has increased significantly among general healthy population. During the aging process the balance between formation and resorption of bone skeleton change, thereby bone loss occurs. Additionally, some risk factors of osteoporosis are higher among elderly such as immobility, diseases, and therapeutic use of glucocorticoids (Adrawal and Verma 2013, Jasien et al. 2012).

2.2.1.3 Previous fracture

Previous fracture increases the risk of subsequent fractures regardless of the fracture location. Morin and coworkers (2014) found out that women with previous fracture were older than those without previous fracture, and also there was a significant difference in mean femoral neck scores between those with fracture, as compared to women without previous fracture. Also the lowest femoral neck T-scores BMD was seen in women who had hip fracture previously.

2.2.2 Modifiable factors

Osteoporosis prevention is the main health priority and strategy, as same as other public health issues. Strategy is either population-based project or targeting groups with higher risk. Addressing modifiable risks factors of osteoporosis, play an important role for both aforementioned approaches.

Although bone mass determined largely by heritability, other factors also play significant role in bone health. It is not easy to isolate the effect of each factor on bone health, several studies have examined the effect of different factors on bone health; such as dietary factors including nutrients calcium, vitamin D, vitamin K, phytoestrogens, alcohol, fatty acids and protein. Also some studies have focused on lifestyle determinants like physical activity or tobacco use (Levis and Lagar 2012).

2.2.3 Diet, nutrition and bone

Diet and nutritional factors have significant role in skeletal growth, moreover diet as a modifiable factor can prevent osteoporosis, and also take part in the osteoporosis treatment. Although bone health and BMD can be affected by substantially different dietary factors range of micronutrients like mineral, vitamins, and macro nutrients such as protein and fatty acids. Most of the studies stressed out the importance of calcium, vitamin D, fatty acid and protein effect on bone health, which will elaborate further (Maurel et al. 2012, Levis and Lagar 2012, Fenton et al. 2009).

2.2.3.1 Dietary calcium

Over the past decades relevant studies have been conducted about dietary calcium intake, and its' important role in development of skeleton structure (Cashman 2002). Besides, the amount of dietary calcium intake, gastrointestinal absorption of calcium is also an important factor to shape the mineral content of bone, because it indicates the availability of calcium for bone metabolism (Cashman 2003).

In food calcium exists in form of salt or bound to other dietary constituents, as calcium ions (Ca^{2+}). Before absorption, calcium should be released in soluble ionized form; in brief calcium is absorbed by 2 routes, transcellular and paracellular transport, the intracellular Ca^{2+} diffusion seems to be facilitated by a systolic calcium binding protein, calbindin D9K, which biosynthesis is dependent on vitamin D (Cashman 2003).

Moreover, some studies suggested that most of the calcium-bone benefits are derived just from dietary calcium, not supplementation. Ian in the recent meta-analysis (2014) indicated that there was no benefits of using calcium supplementation in fracture prevention. Also some evidences showed that calcium may acts as a weak antiresorptive, due to suppressing parathyroid hormone secretion.

2.2.3.2 Vitamin D

Vitamin D is an essential nutrient for human and has several important functions in body. The major source of vitamin D is from the skin exposure to UVB radiation of sunlight, also it can be found in limited food sources such as, wild mushrooms, margarine, lean fish, egg yolks and liver. In case of not enough skin exposure to sunshine, and low intake of food sources, vitamin D deficiency can happen and it may leads to osteoporosis (Cranney et al. 2007, Mattila et al. 2002).

Considerable number of studies and evidences indicated that vitamin D deficiency is an important risk factors for osteoporosis. Suggested mechanisms of vitamin D inadequacy effect on bone are, less efficient gastrointestinal absorption of calcium, loss of calcium from bone, and muscle weakness. It has been suggested that increasing vitamin D intake may significantly reduce risk of bone fracture in older people (Rizzoli and Bonjour 2004).

2.2.3.3 Vitamin K

Osteocalcin is known as non-collagenous protein in bone, and it has function as regulator of bone formation. Vitamin K play an essential role in converting 3-glutamic acid residues in osteocalcin to γ-carboxyl glutamic acid, which without this modification osteocalcin is not able to bind to calcium.

A systematic review and meta-analysis of randomized control trials (RCT) suggested heterogeneity in the effects of vitamin K on BMD. Meta-analysis of RCTs showed that supplementation with vitamin K was not associated with BMD increase at the femoral neck (Nakao et al. 1994).

Vitamin K might be a predictor for hip fracture and BMD. Studies which analyzed the effect of vitamin K on BMD like two large prospective cohort studies (the Nurses' Health Study and the Framingham Heart Study) reported an association between relative risk of hip fracture and vitamin K intake. Feskanich and coworkers conducted a prospective analysis in which, diet was assessed in 72327 women aged 38-63 years old. Result suggested that low intakes of vitamin K may increase the risk of hip fracture in women (Willett et al. 1995, Booth et al. 2000).

2.2.3.4 Dietary phytoestrogens

Estrogens are important hormones for body skeleton. In most women after menopause there is significant drop in blood estrogen concentration, which is resulting accelerated bone turnover and bone loss. Although hormone therapy prevents menopausal bone loss, recently there has been an emerging concern about possible effect of exogenous estrogen on increasing risk of breast cancer and cardiovascular disease. As a result it has been new perspective to shift to natural alternatives, phytoestrogens are a class of chemicals that have hormone like properties. Specifically, they can behave like the female hormone estrogen (Lagari and Levis 2010, Gallagher 2001).

A meta-analysis by Salari and coworkers (2011) among 1252 postmenopausal women selected from eleven RCTs showed that, low doses of phytoestrogen was associated with preventing of bone resorption, although the effect of bone formation was not significant.

Genistein is an edible source for phytoestrogen. Effects of purified genistein (a soy-based isoflavone) supplementation (56 mg/d) and continuous HT for 12 months on bone metabolism and BMD, have been analyzed by Morabito and coworkers (2002). Result showed that genistein supplements increased BMD which showed the same effect on estrogen as HT. However, further research is necessary to examine and clarify the role of dietary phytoestrogens as substitutive osteoporosis prevention

2.2.3.5 Dietary patterns

Dietary patterns can be related to BMD with several pathways. Although there are only few studies about the effect of dietary pattern on BMD. Canadian population based study, studied differences between two dietary patterns; nutrient dense diet with higher intake of fruits, vegetables and whole grains, was compared to energy dense diet, with more intake of soft drinks, potato chips, meats and desserts. Result showed that nutrient-dense diet was associated with lower risk of fracture (Langsetmo et al. 2011) .

Western diet components such as high consumption of sugar and fat, which is commonly accompanied by low consumption of dairy products, dark green vegetables, fish, and fruits have been found to be associated with low BMD. In a cross-sectional study, suggested that there is relation between Westernized diet and increasing bone fracture risk. Besides many of other determinants are involved in Westernized life style such as low physical activity, alcohol consumption and other nutritional factors (Massey 2003).

Vegetarian diet is an increasing trend in Western societies, estimations show that about 5 percent of western population is vegetarian. It has been suggested that vegetarian people have lower risk of coronary heart disease (CHD), cardiovascular disease (CVD) and mortality as compared to general population. In 2 years prospective study by Ho-Pham and colleagues (2012), among 210 Asians it has been presented, vegetarian diet did not have negative effect on BMD, but animal protein intake was negatively associated with bone fracture (Ho Pham et al. 2012) .

In vegetarian diet, on the other hand, intake of protein and calcium is lower than in mixed diet due to lower consumption of dairy and animal products, which can lead to lower BMD (Ballard et al. 2005).

2.2.4 Alcohol

The association of alcohol consumption and osteoporosis is controversial. Alcoholism (high alcohol intake) is a possible cause of secondary osteoporosis, although mild to moderate alcohol intake have suggestive protective effect on BMD (Jugdaohsingh et al. 2006).

The mechanism of alcohol effect on bone is not exactly known, it might be through decreasing of bone remodeling or effect on level of osteocalcin (Sripanyakorn et al. 2009) . In the result of meta-analysis conducted by Berg and coworkers (2012), those who consumed 0.5 to 1 drinks per day had lower risk of hip fracture, as compared to abstainers and heavy drinkers (Berg et al. 2008).

Du et al (2011), conducted a cross-sectional study on 703 Chinese women to explore the association of osteoporosis fracture regarding daily life habits. They showed that among different habits of daily life, only higher smoking and alcohol consumption were associated with greater fracture risk.

2.2.5 Medications

Long term medication with corticosteroids such as prednisone and cortisone, is related and interferes with bone-building process. Also those drugs which prescribed in treatment of seizures, depression, gastric reflux and cancer, may effect BMD. Muora and coworkers in CaMOS study found, increased

risk of fractures in individuals who used selective serotonin reuptake inhibitors (SSRI) or serotonin and noradrenaline reuptake inhibitors (SNRI), after controlling for multiple risk factors.

2.2.6 Physical activity

Physical activity (PA) is an important modifiable determinant of bone health. Bone is a dynamic tissue and both bone mass and geometry, will affect the bone strength and resistance to fracture. Exercise and daily activity, appear to modulate bone formation through simulative effects. Different studies showed positive effect of PA on bone health, either directly or by affecting body mass index (BMI) (Langsetmo et al. 2012).

Several studies have evaluated the association between PA and bone health, by using different methods and end points, like fracture, risk of falls and BMD. Among studied outcomes, hip fractures was studied more frequently. Most of the results showed, significant reduction of fracture risk among both women and men who had PA as compared to sedentary life (Moayyeri 2008) .

PA may have direct effect on BMD, results from RCTs that included different exercise and training for elderly women, showed preventive effect on bone loss and increased BMD (Prince et al. 1995, Pruitt et al. 1995). PA in adolescence is beneficial for increasing BMD, Baxte-Jones and coworkers (2008), in a prospective study investigated BMC indicator, between 151 physically active adult as compared to their peers. Result suggested that active groups had higher adjusted BMC, as compared to their peers (Baxter-Jones et al. 2008).

2.2.7 BMI

Body weight affect both bone turnover and bone density. Relevant evidences indicated that increasing body weight is a potential modifier, that can decrease the osteoporosis risk; and likewise, BMI recorded to show the same effect on BMD. Evidence among postmenopausal women suggested that, moderate obesity has positive effect on bone health (Siris et al. 2001) .

Both fat mass and lean mass are positively related to BMD. Numerous epidemiological studies have shown that, low body weight is a risk factor for fracture. In a meta-analysis among 6000 participants from 12 prospective studies, result demonstrated that each unit of increase in BMI, diminished total fracture risk by 2-3%. This result can explain the isolate effect of fat shock-absorbing, rather than effect of increasing total weight on BMD (De Laet et al. 2005) .

However, obesity may impact bone fractures differently. In particular observational study, conducted by Compston and coworkers (2011), it has been explored, higher risk of ankle fractures in postmenopausal women with greater BMI. These evidences suggested that closer look at the relationships between BMI and fracture risk is necessary (Compston et al. 2011).

There is no clear explanation for the mechanism of BMI on bone structure. However, it has been clarified that when weight is moderately high, consequently there is more weight-burden on bone frame which as cumulative effect can make the bone structure stronger. Therefore, other explanation are based on the endocrine connection between adipose tissue and bone. It has been revealed that adipocyte, can directly secrete cytokines and hormones and also indirectly affect number of endocrine glands (Reid 2013) .

Adipocyte cells, produce estrogen from adrenal precursors, this function has importance in postmenopausal women describes that why fat tissue and bone are associated. Adipocyte can also produce interlukin-6 which putatively is a bone active hormone (Reid 2013) . Some studies assessed the leptins' direct effect on bone; active leptin receptors have been found on osteoblasts. Leptin also can inhibit osteoclastogenesis, which can lead to increase bone mass (Gordeladze et al. 2002, Holloway et al. 2002).

2.2.8 Smoking

Smoking is known as risk factor for several health problems and also for osteoporosis. It has been recognized that smoking cause bone loss, among postmenopausal women. It has been reported that one in eight hip fractures, is related to smoking (Ward and Klesges 2001). Among elderly smokers, tendency and chance to fall seems to be higher. However, smoking relationship with bone fracture, is a controversial subject, and in some studies no association was found (Jacobsen et al. 1998, Valimaki et al. 1994).

Findings of the meta-analysis, conducted by Peter and coworkers (2007), showed that among post-menopausal women, BMD was adversely associated to number of smoking packs in year. In a Danish study among 2105 women aged 45-48 years, with followed up for 2 years, result showed negative association between lumbar bone mass and smoking (Hermann et al. 2000).

The pathophysiological mechanism of cigarette smoking and bone health, have not been fully explored. Vascular causes by smoking can increase risk of fall; Compared to non-smokers, smokers are weaker and they have impaired balance and neuromuscular function (Ward and Klesges 2001).

Also, smoking may alter calciotropic hormones, parathyroid hormones and vitamin D metabolism, which all affect calcium homeostasis. Two cross-sectional and cohort studies have demonstrated that level of serum 25-hydroxyvitamin D (25-OH-D), was lower in current smokers, as compared to non-smokers ; smoking may alter hepatic metabolism of vitamin D by effecting on 25 hydroxylase (CYP2R1). Evidences indicated, smoking decrease gastrointestinal absorption of calcium through changes in calciotropic hormone metabolism (Brot et al. 1999, Lorentzon et al. 2007).

Other possible explanation is that smoking can have impact on sex hormones and particularly estrogen, probably nicotine can reduce estrogen production. Smoking can enhance the hepatic metabolism of estradiol, also smokers have higher serum sex-hormone binding globulin (SHBG) compared to non-smokers which potentially can decrease estradiol level (Rapuri et al. 2000).

Moreover smoking can also affect directly on bone cells by modulating osteoclasts and osteoblasts, but this effect seems to be dose-dependent as nicotine at lower concentration may stimulate bone formation and at higher level can inhibit bone formation (Brand et al. 2011).

2.3 Dietary protein and bone health

Several studies suggested that dietary protein may plays an important role in maintaining bone health, however, the direction of this effect remained inconsistent . Several characteristics of dietary protein are considered to be important for bone remodeling. To the contrary protein may have detrimental effect on bone health due to different mechanisms (Jesudason and Clifton 2011).

Present study has skimmed the effect of dietary protein on bone health and what might be the underlying it, also we summarized possible mechanisms of this effect. Over last few years, several cross-sectional and longitudinal studies have been conducted to evaluate the effect of dietary protein on bone health, which are summarized in Table 1 and 2.

2.3.1 Cross-sectional studies

Selected cross-sectional studies, focused on comparing effect of dietary protein from different sources (total, animal and vegetable) on BMD, BMC or fracture; are summarized in Table 1. Although not all the studies are easily comparable, as they are different in number of subjects, age, sex and mean protein intake. In addition in most of the previous studies, protein intake was not reported as percentage of energy intake or gram intake, therefore it was difficult to interpret the results.

In a cross-sectional study among 1280 men and 1639 women, to examine the association of total protein intake and BMD, results indicated that protein intake was positively associated with all

BMD sites. Besides, in longitudinal analysis in men, higher protein intake was associated with greater bone loss (Sahni et al. 2013).

In addition, the association between dietary protein intake and BMD, was evaluated in a cross-sectional analysis among 560 females aged 14-40 years. Result showed that vegetable protein intake was adversely associated with lower BMD (Beasley et al. 2010).

Rapuri and coworkers (2003), analyzed the association of dietary protein intake with BMD among 489 women (aged 65-77years), cross-sectional analysis showed that higher intake of protein, was associated with higher BMD at the baseline. However, in longitudinal analysis with 3 years follow-up, no association was observed between protein intake and bone loss.

Among 161 postmenopausal women (mean of age 67), the association between dietary protein intake with BMD has been assessed, findings showed that increased protein intake, was beneficial to BMD. Although, the positive effect was offset by dietary acid load caused by protein (Thorpe et al. 2008).

In the other cross-sectional study among 946 participants, interaction between energy-adjusted protein intake and risk of hip fracture has been assessed; results indicated that increasing protein intake was associated with decreased risk of hip fracture (Misra, Berry et al. 2011) .

Association of total protein intake and bone fracture among 1628 women (aged 35-59 years), was examined in a cross-sectional study by Feskanich and coworkers (1996). Result showed that protein consumption over than 95g/d was associated with an increased risk of forearm fracture.

Dietary protein can affect BMD because of protein acid renal load (PARL). BMD association with PARL has been assessed among 543 community living women (aged 60 years and older); result suggested that none of the dietary nutrients nor PARL was associated with BMD (Pedone, Napoli et al. 2010).

Although higher number of cross-sectional studies, suggested positive trend between protein and bone health rather than adverse; the conclusion of findings is inconsistent, because there were not enough compatible studies to assess dietary protein effect on bone sites.

Table 1. Cross-sectional studies about total, animal and vegetable protein intake association with BMD, BMC or bone fracture. [a]

Study	Age (years)	Population	Exposure	Outcome	Result
Zoltick (2011)	67–93	807 men and women	Total protein intake by validated questionnaire at two time points	Falls were reported by participants	In subject with higher total protein intake odds of falling significantly reduced.
Misra (2011)	75	946 (576 women, 370 men)	Energy-adjusted protein intake by FFQ	hip fracture risk	Higher total protein intake was associated with decreased risk of hip fracture.
Basely (2010)	14–40	560 females	Protein intake measured by semi-quantitative FFQ	BMD	Low vegetable protein associated with low BMD and increase in protein portion of energy did not make any changes in BMD.
Pedone (2010)	60–96	497 Women(at the 6 years of follow up)	General dietary pattern, using EPIC questionnaire	BMD	Dietary approach and protein, none of the nutrients nor was PARL associated with BMD.
Rapuri (2003)	65–77	489 Women	Protein intake recorded by FFQ	BMD	The highest quartile of protein intake was associated with higher BMD at the baseline only when calcium intake was higher than 408 mg/day. But no longitudinal effect observed.
Feskanich (1996)	35–59	1628 women	Protein intake by food record	Fractures	Protein intake was associated with increased risk of forearm fracture.

[a] Abbreviations, BMD: bone mineral density; BMI: body mass index; FFQ: food-frequency questionnaire;

2.3.2 Longitudinal studies

Several prospective studies have been conducted to evaluate the association between dietary protein intake and bone health. Selective studies are summarized in Table 2. Prospective studies evaluated different bone outcomes, including BMD, BMC or fracture risk. Subsequently, usual adjusted

confounders for osteoporosis were age, weight, height, energy intake; and smoking, calcium intake; physical activity and hormone therapy.

Prospective analysis in 144580 women aged 50-79, showed an inverse association between higher intakes of biomarker-calibrated protein and fore-arm fracture. Further reported that each 20% increase in protein intake, was associated with significantly higher total body BMD (Beasley, Lacroix et al. 2014).

The association between veganism and bone loss, was studied in 210 post-menopausal women, with two years of follow-up. Results suggested that higher intakes of animal protein, fat, and corticosteroid use were associated with greater bone loss

Several studies focused on animal versus vegetable protein intake effect on bone health. Accordingly, Sahni and coworkers (2010), evaluated association of energy adjusted protein intake from different sources (total, animal, plant, animal/plant ration); with incident of hip fracture. Result showed that those with higher animal protein intake had an increased risk of hip fracture, in comparison with subjects with lower intake. Also those with lower vegetable protein intake tended to have fewer fractures.

Promislow and coworkers (2010), explored the association of total, animal and vegetable protein with BMD, in 572 women and 3878 men. Result of this study, indicated that for every 15 g/d increase in animal protein intake, BMD increased. Conversely, negative association between vegetable protein and BMD was observed in both sexes.

In cohort study among 1280 men and 1639 women, by Shivani (2011), protein intake association with BMD and bone loss, has been examined at baseline and 3 years after. Result showed that in women, protein intake has beneficial effect on bone, notably for those with lower calcium intake. However, in men higher protein intake lead to greater bone loss at trochanter. These findings were in agreement with finding of an observational study among middle-aged women. Protein intake was positively correlated with forearm and bone mineral mass, over the 4-years follow-up (Rizzoli and Bonjour 2004, Cao, Johnson et al. 2011).

Table2. Longitudinal studies about total, animal and vegetable protein intake association with BMD, BMC or bone fracture. [a]

Authors	Age (years)	Population	Exposure	Outcome	Result
Beasle (2014)	59-79	6 years of follow-up, 144,580 women	Biomarker-calibrated protein intake with FFQ	Fracture, BMD	Higher protein intake has positive effect on bone health in postmenopausal women.
Sahni (2013)	61	3 years of follow-up, 1280 men 1280 and 1639women	Protein intake recorded by FFQ at baseline and 3 years after	BMD and Bone loss	In women protein intake has beneficial effect especially for those with lower calcium intake, in men higher protein intakes lead to greater bone loss at trochanter.
Ho-Pham (2012)	over 50	2 years of follow-up, 210(105Vegans+105omnivores)	effect of vegan diet	Biomarkers of bone loss	Higher intakes of animal protein and lipid, were associated with greater rate of bone loss.
Sahni (2010)	55	4 years of follow-up, total of 1752 men and 1972 women	Energy adjusted protein intake by FFQ	Hip fracture	Higher animal protein intake may have protective effect against hip fracture when calcium intake is more than 800mg/day.
Thorpe (2008)	68 ± 6	one year of follow-up, among 161 postmenopausal women, experimented at baseline	Total protein intake/USDA multiple-pass 24-h dietary recall	BMD at lumbar spine and total hip	Increasing protein is beneficial to BMD of postmenopausal women, but this benefit is suppressed by the dietary acid load caused by sulfur containing amino acids.
Vatanparast (2007)	23	6 years of follow-up, 133 young adults male and female	Dietary intake, 24-h recalls (2–4 recalls per year)	Bone mass	When Ca intake is adequate, protein intake has beneficial effect on bone mass of young adults.
Promislow (2002)	55-92	4 year of follow-up, 572 women and 388 men	Total, animal and vegetable protein by FFQ	BMD	Higher BMD was associated with higher intake of animal protein and

					negatively associated with protein from vegetables.
Munger (1999)	55-69	3 years of follow-up, 44 Postmenopausal women	baseline Protein intake by FFQ	Hip fractures	Protein intake was associated with lower hip fracture risk.

[a] BMD: bone mineral density; BMI: body mass index; FFQ: food-frequency questionnaire; TP=total protein ; AP: animal protein; VP: vegetable protein

Effect of dietary protein and bone mass measures at different sites, has been also evaluated by Vatanparst and coworkers (2007), investigation among 133 young adults (59 males, 74 females) who were participating in Saskatchewan study, showed that protein intake in adulthood was positively associated with BMC.

In a cohort study among 1035 white women aged > 65 years, protein intake association with bone loss, was evaluated. Result showed that women with high ratio of animal to vegetable protein intake, had higher rate of bone loss and greater risk of hip fracture.

Hannan and coworkers (2000), examined the relationship between baseline dietary protein intake and result of 4-year change in BMD. Result presented that participants in the lowest quartile of protein intake, had greatest bone loss, similarly this effect has been observed for the overall protein effect (Hannan, Tucker et al. 2000).

It has been suggested that protein intake affect bone mass. Total protein intake was negatively associated with risk of hip among 44 women aged 55-69 years at baseline (Munger, Cerhan et al. 1999).

Is not easy to point out a clear conclusion from longitudinal studies, because results of studies are controversial, as they suggest different associations; either positive or negative. Similarly, result of the comparison of animal and vegetable protein effect on bone are different. Thus the evidence is inconclusive regarding the relation of protein intake with BMD or risk fracture.

2.4 Mechanisms of protein effect on bone

It has been suggested that dietary protein intake may have effect on BMD, the mechanism of this effect is not completely understood. Although the most possible explanations are, 1- Effect on calcium adequacy by increasing intestinal absorption. 2- Effect on IGF-1, which is a bone-anabolic hormone. 3- Dietary protein can affect bone cells function. 4- Animal versus vegetable sources, animal sources may have inverse effect on BMD by increasing acidity. 5- Acid- base theory, when pH falls, calcium crystal resorption will increase. 6- Alkaline-base theory, vegetable protein source can have protective effect on bone because of the buffering effect (Kelsey, Stephen et al. 2013).

2.4.1 Protein intake and calcium adequacy:

About half of the bone structure is protein and other half is calcium, phosphorus crystal and other elements. Normal function allows mineral and calcium, lose every day, so if dietary calcium intake does not offset the loss, calcium imbalance will occur, which can lead to lower BMD or in midrange to osteoporosis (Howard 1957) .

It has been suggested by Sherman and coworkers (1912) that dietary ingredients, can affect urine pH and acid-base homeostasis. The significance of this finding has been assessed further in German Vegan study, among 67 men and 87 women aged 21-75 years. Result showed that vegan diet did not affect acid-base hemostasis (Ströhle, Waldmann et al. 2011, McLean, Qiao et al. 2011).

Net endogenous acid productivity (NEAP), hypothesize that increasing protein intake, may lead to decrease in BMD, because of increasing urinary calcium excretion; which can lead to higher calcium resorption from bone and BMD loss (Noakes, Keogh et al. 2005, Heaney 2006).

Increase of protein intake from 75 to 125 g/d, caused 64 mg/d, increase in calcium excretion, which results up to 2% bone loss, annually . However, diet that provides about 30% of daily energy intake from protein (181-214 g/d), did not increase calciuria (Tang, O'Connor et al. 2014).

The detrimental effect of dietary acidity on bone health is relatively small, although it can have cumulative effect over time. Nurse's Health Study among women aged 35-59 years, with 12 years follow-up; showed that higher intake of total protein (>95g/d), was associated with greater risk of forearm fracture risk, as compared to whose intake was less than 68g/d.

Nevertheless, it should be taken into account that increase in calciuria, does not necessarily cause calcium and bone mass loss. Some studies indicated that protein intake, can increase BMD and decrease fracture risk (Hannan, Tucker et al. 2005). In study by Cooper and coworkers (1996), among premenopausal women, findings showed that, there was significant positive association between protein intake and BMC.

In addition protein can have effect on intestinal calcium absorption. Heaney (2000), studied 191 nuns (aged 48 years), over 20 years; no relationship between intestinal calcium absorption and dietary protein intake have been reported (Heaney 2000).

It is noteworthy that, calcium absorption can compensate the calcium urinary loss. RCT among 16 postmenopausal women, conducted by Cao and coworkers (2011), showed that higher level of dietary protein, increased urinary calcium excretion; although the net difference between calcium absorption and urinary excretion, did not differ.

Accordingly, intervention with high dietary protein intake for two weeks, increased both calcium absorption and urinary calcium excretion, thus no changes were observed in formation or resorption biomarkers; which indicated that high-protein intake did not have detrimental effect on calcium adequacy (Zamzam 2003).

2.4.2 Dietary protein and IGF-1 level

Insulin-like growth factor1 hormone, play role in transporting amino acids as well as protein synthesis in skeleton; IGF-1 is a potent anabolic hormone that stimulate formation of bone mass. Also is a key regulator in bone metabolism. In vitro experiments have been shown that IGF-1 can increase osteoblast and type1 of collagen activity (Bilezikian, Lawrence et al. 2008).

Furthermore IGF-1 is regulating calcium and phosphorus metabolism by effecting on renal transport of inorganic phosphate, in addition it can effect on kidney dihydroxycholecalciferol production (Mohan, Strong et al. 1992). Three studies have found out that increase in protein intake, will increase circulating level of IGF-1 and bone anabolic activity (Thorpe, Jacobson et al. 2008).

Evidences supported that IGF-1 can effect on bone, however, the association between dietary protein intake and level of IGF-1 is not clear. Result of a cohort study among 47 postmenopausal women showed that higher protein intake, was related to greater serum IGF-1, as compared to lower protein intake (Sukumar, Ambia-Sobhan et al. 2011).

2.4.3 Dietary protein and bone cell function

Osteoblast cells' function is to create bone matrix, which is the foundation of bone. Negative nitrogen balance derived from inadequate protein intake can cause detrimental effect on osteoblasts' function (Brandao-Burch, Utting et al. 2005). Other important role of protein is to maintain muscle strength, adequate dietary protein is necessary for keeping muscle strength and preventing sarcopenia (Paillaud, Bories et al. 2000, Cruz-Jentoft, Triana et al. 2011, Wachman and Bernsten 1986, Heaney and Layman 2008).

Protein supplementation showed positive effect on BMD, 20 g/d of protein supplementation, resulted better clinical outcome among hospitalized patients with diagnosed femoral neck fractures; they had shorter stay in hospital and lower rates of complications, as compared to patients without supplement therapy (Delmi, Rapin et al. 1990).

2.4.4 Animal versus vegetable protein

Different sources of protein have been suggested to have different effect on bone. Some observational studies showed that protein from animal sources, can have adverse effect on BMD by increasing acidity of blood; because animal protein contain edible amount of acid forming amino acids . On the other hand, vegetable protein source can have protective effect on bone, because of buffer-alkalizing effect (Heaney and Layman 2008). It is noteworthy that sulfur containing amino acids, also exists in vegetable sources of protein such as legumes and whole grains (Hanley and Whiting 2013).

As demonstrated in a systematic review conducted by Hanley and coworkers (2013), there was not enough evidence to support the causal relationship between acid/alkali composition of dietary foods and bone health. Also clinical studies did not support animal protein adverse effect on bone health, nor were vegetable sources beneficial for bone health.

Diet with adequate protein intake from vegetable and animal sources, can reduce the risk of bone fracture. In a cohort study by Hannan and coworkers (2008), among 855 participants, BMD and dietary data were analyzed at the baseline and 4 years after. Result showed that lower protein intake was related significantly to greater BMD. Also it was suggested that there was dose-response relationship between protein intake and greater BMD, along with reduce risk of fracture (Hanley and Whiting 2013).

Overall, different studies have different explanations to describe the mechanism of animal or vegetable protein effect on bone. However, the most common used explanations were acid base-theory and alkaline-potassium.

2.4.5 Acid-Base theory

Acid-base theory was proposed for the first time by Wachman and Bernstein in 1986, the first claim was based on the in vitro experiments, indicating that when pH falls, crystal form of calcium production will increase (hydroxyapatite). Acid-base theory focus is more to elaborate the differences between animal and vegetable protein sources on bone health (Rizzoli and Bonjour 2004, Wachman and Bernsten 1986).

Putatively, hepatic circulation will oxidize sulfur containing amino acids, like methionine and cysteine from protein (meat, egg, dairy products) and convert them to H_2SO_4, which can results decrease in blood pH level (Brandao-Burch, Utting et al. 2005). Studies also suggested that, calciuric effect can be more pronounced in elderly, because the glomerular filtration rate falls and kidneys' ability to excrete acid load is impaired (Frassetto, Morris et al. 1996, Heaney and Layman 2008, Cao, Johnson et al. 2011).

Bone matrix may act as substitutive buffer to maintain blood pH by eroding alkaline phosphate to blood, while normal body functions of excreting extra acid from blood drop off (Man 2000, Bonjour 2013). However, there is controversy over the bone-buffering mechanism, it has been documented that bone mineral resorption is more from the bone surface, where potassium and sodium bicarbonate exist; rather than calcium and phosphate. Conclusively losing calcium might happen only as cumulative bone-buffering response (Nicoll and Mclaren 2014).

Nonetheless, the role of bone skeleton as buffer system even in high acidosis remained refuted. Kidney and respiratory systems are the pivotal parts of body buffering mechanism, and it seems that animal protein acidosis, would not stimulate skeleton to act as pH buffer (Brandao-Burch, Utting et al. 2005). In pharmacological inhibition according bone resorption, buffering impairment was not caused by protein external acid load (Neuman, Diamond et al. 1980, H 2005, Promislow, Goodman-Gruen et al. 2002, Brandao-Burch, Utting et al. 2005).

Result of 16 weeks randomized crossover study, among healthy postmenopausal women showed that consuming high amount of animal meat (117 g/d), did not have adverse effect on urinary calcium excretion, as compared to low meat diet (68 g/d) of protein (Sellmeyer, Stone et al. 2001) . In spite of dietary protein, calciuric loss can be due to other factors, such as calcium intake amount, which may offset calcium excretion (Rizzoli and Bonjour 2004, Cao, Johnson et al. 2011).

2.4.6 Alkaline potassium hypothesis

Speculations about dietary vegetable protein effect on bone, describe that combination of high vegetable protein and high-alkaline load from fruits and green leafy vegetables known as Paleolithic diet, may show protective effect on bone health (Sebastian 2005).

Potassium can have alkalizing effect on blood pH, also potassium eliminates acids from blood including NH3 at proximal tubular cells (by adding proton and form NH4), which it can bind again with SO4 and form (NH4)2SO4. Ginty showed (2003) that low vegetable protein intake higher meat intake, was associated with higher calciuric and greater calcium loss.

To evaluate alkaline potassium hypothesis several RCTs have been established. Result of a randomized placebo-controlled trial showed that citrate supplementation, did not reduce the bone turnover neither increased BMD in healthy postmenopausal women (Macdonald et al. 2008).

Furthermore, Frassetto and coworkers assessed potassium bicarbonate effect (in doses of 30, 60, and 90 mEq/d) in women for 2 years. They found that overall changes in BMD, were not related to potassium, fruit and vegetable consumption (Frassetto et al. 2012). To the contrary in the RCT among 201 women aged 65-80 years, results indicated that treatment with potassium citrate (60 mEq/d) for one year increased BMD (Jehle et al. 2013).

A systematic review and meta-analysis conducted by Fenton and coworkers (2011), evaluated acid-base and alkaline diet hypothesis with bone related outcome. There was not enough supporting evidences supporting the association between dietary acid load and osteoporotic bone disease, also they did not found evidence that alkaline diet, would have protective effect on bone health.

3 AIMS OF THE STUDY

Tackling osteoporosis issue is integrated with prevention and changing lifestyle. Accordingly dietary factors are important for bone health status; decreasing risk factors in diet can make change to the large extent in prevention and treatment of osteoporosis. In present study has been tried to look over the association of protein effect from different sources on bone health.

The primary objective of the present study was to assess the association between protein intakes from different sources (total, animal and vegetable) with BMD. To explore this effect different analysis including relationship between proteins from meat, dairy products; protein renal acid load (PRAL) and net endogenous acid productivity (NEAP) have been conducted.

Also we assessed the effect of total protein intake with BMD, considering the current dietary recommendation for total protein intake with BMD, in order to cover the gap of the most previous studies, which they didn't report protein intake as gram intake for kg body weight.

4 MATERIALS AND METHODS

4.1 Study design and participants

This study has been based on the OSTPRE-FPS which started in 2003 in Kuopio, Finland. The primary idea of the study was to analyze the effect of vitamin D and calcium supplementation, in favor of finding preventive effect of supplementation on falls and fractures among postmenopausal women.

Target population (n=5407) was selected from the population-based OSTPRE cohort (n=13100) born in 1932-1941. Inclusion criteria was to have minimum age of 65 years by the end of November 2002, living in Kuopio region and have not been participated in other OSTPRE bone densitometry sample (Karkkainen et al. 2010).

In total 3432 women participated in intervention program, out of total population 750 was taken into the sample size that went through bone density and dietary measurements. Different clinical, physical indicators and laboratory tests were ascertained (Jarvinen et al. 2012).

4.2 Cross-sectional analysis

Dietary record information and BMD measurements of 554 postmenopausal women at the baseline, used for cross-sectional analysis.

4.3 Prospective analysis

In prospective setting 554 postmenopausal women were included, information of food record collected at the baseline and BMD compared for baseline measurements and after 3 years of follow-up.

4.4 Food record

Dietary assessment was collected using 3-days food record at the baseline. Form and instruction were sent to participants, they returned the questionnaire on the visiting day. It was recommended to the participants to fill the questionnaire for 3 consecutive days, including 2 days during week and one day in weekend (Saturday or Sunday). Nutrient intakes from food was calculated by using Nutrica program (version 2.5, Finnish social insurance institute, Turku, Finland) (Jarvinen et al. 2012).

4.5 BMD measurement

BMD was measured at the baseline and 3 years after using dual energy X-ray absorptiometry (DEXA) of the total body, lumbar spine (L2-L4), femoral neck, trochanter and ward's triangle. Technical quality of measurements was double checked and those with measurement error were not included in the statistical analysis (Jarvinen et al. 2012).

4.6 Potential confounders

All informations and variables was derived from self-administered questionnaire at baseline and 3 years after; included age, BMI (calculated from measured weight divided by height squared), duration and HT use (used, never used). Smoking status (never smoked, quitted and current smokers); also data about disease and use of medication affecting BMD was collected by questionnaire.

PAL variable was computed by using compiling of two variables of exercise times per week and mobility restriction. PAL categoriesed in three levels, restricted (restricted mobility and no exercise), passive (no mobility restriction and less than 2 times physical activity per week) and nrmal (participants had at least two times physical activity per week). Intake of calcium and vitamin D supplements also collected by self-questionnaire form (yes, no).

Protein renal acid load (PRAL) calculated by using the algorithm suggested by Remer (2003), [PRAL (mEq/d) = (mg P/d × 0.0366) + (g protein/d × 0.4888) – (mg K/d × 0.0205) − (mg Ca/d × 0.0125) − (mg Mg/d × 0.0263)]. This variable has been validated by dietary experiments and also proved that is significantly associated with urinary net acid excretion (Alexy et al. 2005).

4.7 Statistical analysis

All statistical analysis was executed using SPSS software version 19 for windows. Baseline characteristics were analyzed using one-way analysis of variance (ANOVA) for continuous variables, and chi square test for categorical variables. The distribution of characteristic was expressed as mean and standard deviation (SD). Participants were categorized into quartiles, regarding their gram intakes of protein from different sources. Characteristics of participants were compared in quartiles of protein intake (total, animal and vegetable) using ANOVA for continuous variables and chi square tests for categorical variables.

Mean of BMD was compared with quartiles of protein intake at the baseline cross-sectionally by using ANOVA. In the prospective setting, the association of total protein intake with BMD, was analyzed using mixed model. The initial model was just adjusted for age, HT, BMI (continuous) and total energy intake. Further, subsequent models were adjusted for all other potential confounders.

Adjusted covariates included both continuous variables; including age, BMI, energy intake, years since menopause and dietary intake of calcium and vitamin D; accordingly, categorical variables adjusted in model were smoking status (never, previous, current), physical activity level (restricted, passive, normal); calcium and vitamin D supplementation (yes, no), use of HT (yes, no), disease and the use of medication affecting BMD, alcohol consumption (yes, no) and intervention group.

Linear model tested the variables across quartiles of protein intake from different sources, animal (meat, egg, fish, and dairy products), vegetable (vegetables, cereals) and total. Also PRAL, NEAP, and vegetable protein intake for lower and higher than median intake was evaluated. Present study explored protein intake regarding current recommendation of 1g/kg- bw/d.

The analysis was also repeated after subcategorizing the participants for HT (no, yes) and BMI (<25, ≥25). Results in table 8 include all confounders and participants categorized base on HT use (no/yes) at the baseline. In table 9 participant stratified in to two groups BMI<25 and ≥25 kg/m^2.

In addition to result represented in table 10, according association of protein intake quartiles with BMD; by stratifying participants for their BMI and HT interaction in to four groups HT (no), BMI<25, HT (no) BMI≥25; HT (yes), BMI<25 and HT (yes) BMI≥25.

5 RESULTS

Characteristics of participants at the baseline and 3 years follow-up are presented in Table 3. Mean lumbar BMD was 1.09, femur 0.869 and total body 1.07 at the baseline, only femur BMD decreased (-1.89%) after 3 years follow-up, BMD at lumbar (+0.93%) and total body (+0.56%) increased. Participants' mean age was 68 years old. BMI was <25 kg/m^2 in 20% of participants and other 79% had BMI ≥25 kg/m^2.

Table 3. Baseline characteristics of the participants and bone mineral density after 3 years intervention. [a]

Variable	Baseline(n=556) [a]	At 3 years
Age(years)	67.9±1.9	
BMI (kg/m²)	28.8± 4.7	
Length of HT use (years)	11.0± 5.9	
Time from menopause (years)	18.4± 5.4	
Lumbar spine BMD (g/cm2)	1.096±0.186	1.107±0.187
Femoral Neck BMD (g/cm2)	0.869±0.125	0.853±0.126
Total body BMD (g/cm2)	1.077±0.093	1.083±0.098
Categorical variables (%)		
Smoking		
Never smoked N (%)	452 (81.3%)	
Previous smokers N (%)	67 (12.1%)	
Current smoker N (%)	26 (4.7%)	
Disease or medication that reduces BMD N (%)		
No	359 (63.7%)	
Yes	201 (36.2%)	
HT status N (%)		
Used HT	229 (41.2%)	
Not use HT	287 (51.6%)	
Physical Activity Level N (%) [b]		
Restricted	22 (4%)	
Passive	204 (36.7%)	
Normal	300 (54.0%)	
Dietary Variables		
Energy (kcal)	1569±373	
Total protein intake (g/d)	68,17±18.01	
Use of vitamin D supplement N (%)	128 (23.0%)	
Use of calcium supplement N (%)	144 (26.1%)	

Abbreviations: BMI, body mass index; HT, hormone therapy; BMD, bone mineral density

[a] Mean±SD

[b] Restricted: restricted mobility with no exercise/ Passive: no mobility restriction and less than 2 time - physical activity a week/ Normal: No mobility restriction and more than 2 times physical activity a week.

Table 4. Non-dietary and dietary factors in quartiles of total protein intake.

Quartile of total protein intake (g/ day)

	Mean±SD				
	1 (18.3-54.7)	2 (54.8-66.0)	3 (66.1-80.3)	4 (80.5-153.5)	p value
Age (years)	68.2± 2.0	67.9±2.0	67.7±1.8	67.7±1.7	0.084 [a]
BMI (kg/m²)	28.8±5.0	29.2±7.0	28.5±4.5	28.7±4.7	0.657[a]
Length of HT use (years)	5.5±7.0	5.7±7.0	6.7±7.5	6.5±6.5	0.447[a]
Time from menopause (years)	18.5±5.0	18.8±4.9	18.9±5.8	17.9±4.8	0.558[a]
Categorical variables (%)					
smoking					0.646 [b]
Never smoked N (%)	111 (24.5%)	116(25.6%)	111 (24.5%)	114 (25.2%)	
Previous smoker N (%)	12 (17.9%)	14 (20.8%)	22 (32.8%)	19 (28.3%)	
Current smoker N (%)	10 (38.4%)	6 (23.0%)	6 (23.0%)	4 (15.3%)	
Disease or medication that reduces BMD N (%)	55 (27.4%)	57 (28.4%)	46 (22.9%)	43 (21.4%)	0.119 [b]
HT status N (%)					0.199 [b]
Used HT N	130 (28.6%)	118 (25.5%)	108 (23.4%)	102 (22.5%)	
Not used HT	124 (42.3%)	142 (50%)	152 (49.4%)	156 (58.3%)	
Physical Activity Level N (%)[c]					0.131[b]
Restricted	8 (36.4 %)	7 (31.8%)	2 (9.1%)	5 (22.7%)	
Passive	40 (19.6%)	58 (28.4%)	53 (26.0%)	53 (26.0%)	
Normal	79 (26.3%)	67 (22.3%)	78 (26.0%)	76 (25.3%)	
Dietary Variables					
Energy (kcal/d)	1217±265	1470±244	1651±247	1932±295	0.138[a]
Calcium (mg/d)	665±234	893±231	1104±226	1375±334	<0.001[a]
Vitamin D (μg/d)	4.84±2,7	6.4±3,4	7.9±3,7	11.3±6,2	<0.001[a]
Use of vitamin D supplement N (%)	30 (23.4%)	30 (23.4%)	41 (32%)	27 (21.1%)	0.245[b]
Use of calcium supplement N (%)	29 (20.1%)	34 (23.6%)	49 (25.2%)	32 (22.2%)	0.044 [b]

Abbreviations: BMI, body mass index; HT, hormone therapy

[a] Analysis of Variance.

[b] Pearson Chi-square test

[c] Restricted: restricted mobility with no exercise/ Passive: no mobility restriction and less than 2 time -physical activity a week/ Normal: No mobility restriction and more than 2 times physical activity a week.

Time passed after menopause was 18 years, around half of the subjects had used HT at the baseline and 20% for specific time. The mean duration of taking HT was 11 years. 26% of participants used calcium supplementations and 23% vitamin D supplement, approximately 36% of women had disease or medication that could potentially have an effect on BMD. PA also presented, 54% were normal, 4% were mobility restricted and 36.7 % were passive (Table 3).

Table 5. Non-dietary and dietary factors in quartiles of vegetable protein intake.

	Quartiles of Vegetable protein intake (g /day)				
	Mean±SD				
	1 (6.7-17.7)	2 (17.3-21.0)	3 (21.0-24.9)	4 (24.9-44.2)	p value
Age (years)	68.1± 1.9	67.8±1.9	67.9±2.0	67.6±1.6	0.141[a]
BMI (kg/m²)	29.4±4.6	28.9±4.6	28.9±4.7	27.9±4.8	0.068[a]
Length of HT use (years)	6.1±7.2	5.9±7.5	6.6±6.6	5.67±6.5	0.702[a]
Time from menopause (years)	18.4±5.2	18.9±5.4	18.7±4.5	17.6±5.1	0.262[a]
Categorical variables (%)					
smoking					<0.001 [b]
Never smoked N (%)	96 (21.2%)	111 (24.5%)	119 (26.3%)	126 (27.8%)	
Previous smoker N (%)	25 (37.3%)	20 (29.8%)	12 (17.9%)	10 (14.9%)	
Current smoker N (%)	14 (53.84%)	6 (23.07%)	6 (23.07%)	0 (0%)	
Disease or medication that reduces BMD N (%)	50 (24.9%)	50 (24.9%)	50 (24.9%)	51 (25.4%)	0.266[b]
HT status N (%)					0.752[b]
Used HT N	58 (22.5%)	64 (27.7%)	51 (22.1%)	57 (24.7%)	
Not used HT	70 (47.6%)	69 (49%)	78 (55.3%)	70 (47.2%)	
Physical Activity Level N (%)[c]					0.663[b]
Restricted	8 (36.4%)	4 (18.2%)	5 (22.7%)	5 (22.7%)	
Passive	46 (22.5%)	58 (28.4%)	52 (22.5%)	48 (23.5%)	
Normal	73(24.3%)	70 (23.3%)	75 (25.0%)	82 (27.3%)	
Dietary variables					
Energy (kcal/d)	1253±296	1489±274	1628±275	1896±317	<0.001[a]
Calcium (mg/d)	858±323	972±326	1023±356	1185±389	<0.001[a]
Vitamin D (μg/d)	6.6±3.9	7.2±4.4	8.2±5.0	8.3±5.6	<0.001[a]
Use of vitamin D supplement N (%)	36 (28.1%)	34 (26.6%)	32 (25.0%)	26 (20.3%)	0.479[b]
Use of calcium supplement N (%)	36 (28.1%)	34 (26.5%)	32 (25.0%)	26 (20.3%)	0.729[b]

Abbreviations: BMI. body mass index; HT. hormone therapy

[a] Analysis of Variance.

[b] Pearson Chi-square test

[c] Restricted: restricted mobility with no exercise/ Passive: no mobility restriction and less than 2 time -physical activity a week/ Normal: No mobility restriction and more than 2 times physical activity a week.

Tables 3, 4 and 5 present the characteristics of participants in quartiles of protein intake (total, animal and vegetable). There was no significant trend among non-dietary characteristics across protein intake quartiles. However, energy intake significantly increased with increasing protein intake ($p<0.001$). Similarly dietary calcium and vitamin D significantly increased ($p <0.001$) with higher quartiles of protein intake; smoking was positively associated with the intake of vegetable protein ($p <0.001$).

Table 6. Non-dietary and dietary factors in quartile of animal protein intake.

	Quartiles of animal protein intake (g /day)				
	Mean±SD				
	1 (5.8-34.6)	2 (34.6-42.5)	3 (42.6-54.9)	4 (54.9-110.6)	p value
Age (years)	68.1±1.9	67.9±1.8	67.6±1.8	67.6±1.7	0.073[a]
BMI (kg/m²)	28.8±5.0	29.1±4.6	28.4±4.5	28.7±4.7	0.028[a]
Length of HT use (years)	5.5±7.2	5.6±6.7	6.7±7.5	6.4±6.5	0.443[a]
Time from menopause (years)	18.5±4.5	18.5±4.9	18.8±5.8	17.8±4.8	0.537[a]
Categorical variables (%)					
Smoking					0.221[b]
Never smoked N (%)	116 (25.7%)	115 (25.4%)	108 (23.9%)	113 (25.0%)	
Previous smoker N (%)	12 (17.9%)	12 (17.9%)	22 (32.8%)	21 (31.3%)	
Current smoker N (%)	5 (19.2%)	9 (34.6%)	8 (30.8%)	4 (15.4%)	
Disease or medication that reduces BMD N (%)	52 (25.9%)	56 (30.3%)	46 (22.9%)	42 (20.9%)	0.088 [b]
HT status N (%)					0.348 [b]
Used HT N	122 (26.6%)	126 (27.5%)	106 (23.1%)	104 (22.7%)	
Not used HT	128 (42.2%)	138 (51.3%)	154 (51.7%)	154(54.8%)	
Physical Activity Level N (%)[c]					0.426[b]
Restricted	7 (31.84%)	8 (36.4%)	3 (13.6%)	4 (18.2%)	
Passive	41 (20.1%)	56 (27.5%)	55 (27%)	52 (25.5 %)	
Normal	78 (26.0%)	71 (23.7%)	74 (24.7%)	77 (25.7%)	
Dietary variables					
Energy (kcal/d)	1217±265	1470±265	1651±265	1932±295	<0.001[a]
Calcium (mg/d)	648±215	911±221	1104±220	1373±344	<0.001[a]
Vitamin D (µg/d)	4.8±2.7	6.4±3.0	7.5±3.6	11.7±6.1	<0.001[a]
Use of vitamin D supplement N (%)	62 (24%)	58 (23%)	80 (31%)	56 (22%)	0.071[b]
Use of calcium supplement N (%)	29 (20.1%)	36 (25.0%)	45 (31.3%)	34 (23.6%)	0.203 [b]

Abbreviations: BMI. body mass index; HT. hormone therapy

[a] Analysis of Variance.

[b] Pearson Chi-square test

[c] Restricted: restricted mobility with no exercise/ Passive: no mobility restriction and less than 2 time -physical activity a week/ Normal: No mobility restriction and more than 2 times physical activity a week.

5.1 Cross-sectional association between protein intake and BMD

Result from cross-sectional analysis (data not shown) showed no significant association between quartiles of protein intake from different sources (animal, vegetable, total) and BMD (lumbar, femur, total) at baseline. However, those with lower intake of vegetable protein tended to have higher BMD at femur as compared to those with higher intake (p=0.080).

Table 7. Adjusted mean BMD at lumbar spine, femur and total body in quartiles of different dietary protein intake[a] among elderly women.

Variable quartiles limits (g /day)	Adjusted lumbar BMD [b] (g /cm²)			Adjusted Femur BMD [b] (g /cm²)			Adjusted Total BMD [b] (g /cm²)		
	All	HT- (n=183)	HT+ (n=225)	All	HT- (n=153)	HT+ (n=216)	All	HT- (n=214)	HT+ (n=273)
Total protein intake									
≤ 54.73	1.110	1.035 [b]	1.160	0.854	0.800	0.888	1.076	1.042	1.097
54.83-66.07	1.093	1.054 [b]	1.120	0.563	0.830	0.890	1.078	1.059	1.094
66.10-80.37	1.092	1.057 [b]	1.131	0.858	0.830	0.885	1.076	1.044	1.102
80.57+	1.114	1.074	1.154	0.871	0.833	0.905	1.088	1.042	1.126
p value [a]	0.742	0.872	0.628	0.848	0.505	0.871	0.825	0.737	0.362
Animal protein intake									
≤34.60	1.114	1.041 [b]	1.152	0.859	0.802	0.894	1.077	1.044	1.098
34.63-42.57	1.090	1.043 [b]	1.136	0.852	0.814	0.885	1.076	1.051	1.096
42.63-54.93	1.099	1.066 [b]	1.130	0.866	0.843	0.890	1.079	1.048	1.104
54.97+	1.106	1.071 [b]	1.146	0.868	0.835	0.900	1.087	1.042	1.122
p value [a]	0.802	0.817 [b]	0.927	0.755	0.274	0.933	0.843	0.957	0.461
Vegetable protein intake									
≤17.72	1.103	1.044	1.141	0.863	0.812	0.895	1.081	1.038	1.109
17.33-21.03	1.120	1.073	1.171	0.885	0.846	0.923	1.089	1.049	1.116
21.07-24.90	1.110	1.066	1.146	0.860	0.816	0.892	1.077	1.048	1.104
24.97+	1.076	1.036	1.108	0.837	0.821	0.958	1.073	1.052	1.091
p value [a]	0.408	0.652	0.432	0.045	0.371	0.074	0.700	0.936	0.596

Abbreviations: BMD, bone mineral density; HT, hormone therapy;

[a] mixed model analysis

[b] Adjusted just for body mass index, age, energy

5.2 Prospective association between protein intake and BMD

Total protein intake association was analyzed based on current dietary recommendation (0.8-1 g/kg-bw/d) with BMD. Our result suggested that there is a negative trend (p=0.08) between total protein intake ≥1 g/kg- bw/d (40% of participants) and femur BMD. The same result was revealed when protein consumption was ≥1 g/kg- bw/d total BMD was lower (p=0.055) (Data no shown).

In the longitudinal analysis adjusted for age, BMI, HT and energy intake with mean follow up of 3 years, higher vegetable protein intake tended to lower BMD (p=0.045). No other significant association was observed between quartiles of protein consumption and BMD sites.

Furthermore, we analyzed the association between quartiles of protein intake and BMD among participants with and without HT at the baseline (adjusted for BMI, energy intake and age). Results showed no significant association between protein intake and BMD (Table 7).

Table 8 shows the association of total, animal, and vegetable protein consumption with BMD. After adjusting the model for all covariates, there were no significant associations between quartiles of protein intake and BMD measurements (lumbar spine, femoral neck, and femur). Although vegetable protein intake (17, 33-24, 90 g/d) was associated with higher femur BMD (p=0.053) (Table 8). Also when vegetable protein intake was lower than median (21, 05 g/d), femur BMD was significantly higher (p=0.044) in comparison with lower intake.

Subgroup analyses according to HT use and BMI

Relationship between dietary protein intakes and BMD, adjusted for all confounders in women with and without HT at baseline has been explored. There were no significant associations among quartiles of protein intake and BMD measurements in the subgroups. Similarly, no relation between PRAL and AVR with BMD were found. Also a non-significant nonlinear correlation between vegetable protein intake and femur BMD was suggested (p=0.08) (Table 8). As data showed that femur BMD was higher when vegetable protein intake was 17, 33-24, 90 g/d among women without HT, which was similarly observed in all participants (p=0.053) (Table 8).

Table 8. Adjusted mean BMD at lumbar spine, femur and total body in quartiles of different dietary protein intake among elderly women.

Variable quartiles cut points (g/day)	Adjusted lumbar spine BMD [b] (g /cm²)			Adjusted Femur BMD [b] (g /cm²)			Adjusted Total BMD [b] (g /cm²)		
	All (n=408)	HT- (n=183)	HT+ (n=225)	All (n=369)	HT- (n=153)	HT+ (n=216)	All (n=487)	HT- (n=214)	HT+ (n=273)
Total protein intake									
≤ 54.73	1.095	0.976	1.196	0.833	0.756	0.883	1.038	1.014	1.041
54.83-66.07	1.013	1.048	1.211	0.853	0.803	0.889	1.048	1.036	1.048
66.10-80.37	1.109	1.061	1.172	0.848	0.805	0.892	1.043	1.042	1.031
80.57+	1.016	1.079	1.256	0.866	0.818	0.917	1.052	1.025	1.063
p value [a]	0.419	0.298	0.403	0.683	0.275	0.879	0.901	0.590	0.571
Animal protein intake									
≤34.60	1.107	0.992	1.205	0.842	0.763	0.892	1.042	1.044	1.074
34.63-42.57	1.116	1.036	1.222	0.834	0.777	0.884	1.043	1.052	1.069
42.63-54.93	1.116	1.077	1.171	0.856	0.822	0.882	1.044	1.047	1.065
54.97+	1.156	1.076	1.279	0.875	0.827	0.931	1.057	1.034	1.092
p value [a]	0.607	0.359	0.196	0.405	0.152	0.512	0.839	0.792	0.423
Vegetable protein intake									
≤17.72	1.118	1.032	1.212	0.847	0.765	0.908	1.054	1.016	1.047
17.33-21.03	1.137	1.052	1.234	0.884	0.832	0.933	1.060	1.034	1.061
21.07-24.90	1.139	1.065	1.241	0.851	0.793	0.896	1.058	1.037	1.063
24.97+	1.104	1.040	1.165	0.830	0.788	0.864	1.057	1.038	1.043
p value [a]	0.604	0.841	0.407	0.053	0.080	0.212	0.996[a]	0.824	0.734
Vegetable protein intake median									
≤ 21.05 (median)	1.125	1.040	1.217	0.866	0.811	0.915	1.045	1.027	1.049
≥ 21.05 (median)	1.123	1.057	1.201	0.834	0.785	0.877	1.048	1.035	1.035
p value [a]	0.944	0.637	0.655	0.044	0.210	0.122	0.811	0.659	0.965
Protein from dairy product									
≤ 15.47	1.087	1.021	1.136	0.859	0.813	0.890	1.046	1.015	1.056
15.63-21.87	1.125	1.043	1.215	0.859	0.802	0.914	1.048	1.030	1.055

21.90-28.57	1.132	1.056	1.218	0.842	0.788	0.890	1.040	1.027	1.033
28.63+	1.173	1.079	1.308	0.848	0.812	0.884	1.063	1.067	1.044
p value [a]	0.522	0.918	0.239	0.847	0.689	0.813	0.602	0.501	0.802
Protein from meat									
≤7.20	1.110	1.037	1.188	1.110	0.776	0.875	1.053	1.035	1.044
7.23-12	1.106	1.043	1.186	1.100	0.799	0.874	1.053	1.051	1.037
12.07-17.17	1.132	1.049	1.225	1.130	0.814	0.917	1.061	1.027	1.061
17.27+	1.132	1.058	1.245	1.130	0.792	0.912	1.059	1.018	1.060
p value [a]	0.595[a]	0.967	0.570	0.595[a]	0.437	0.375	0.867[a]	0.492	0.646
AVR									
≤ 0.07	1.107	1.023	1.174	0.833	0.787	0.869	1.049	1.016	1.05
0.07-0.10	1.135	1.053	1.231	0.854	0.806	0.895	1.052	1.039	1.066
0.10-0.14	1.122	1.055	1.204	0.861	0.811	0.899	1.050	1.035	1.047
0.14 +	1.124	1.044	1.215	0.854	0.791	0.911	1.060	1.023	1.042
p value [a]	0.847	0.873	0.714	0.524	0.705	0.690	0.573	0.712	0.766
PRAL									
≤-16.30	1.118	1.034	1.193	0.856	0.805	0.899	1.052	1.044	1.041
-9.16 - -3.01	1.108	1.018	1.201	0.852	0.814	0.887	1.037	1.019	1.037
-2.94- 2.78	1.127	1.071	1.203	0.855	0.798	0.895	1.043	1.033	1.039
2.83+	1.147	1.067	1.257	0.844	0.783	0.903	1.055	1.031	1.069
p value	0.602	0.505	0.566	0.918	0.594	0.963	0.608	0.732	0.458

Abbreviations: BMD. bone mineral density; HT, hormone therapy; BMI, body mass index; AVR, animal to vegetable protein ratio; PRAL, protein renal acid load

[a] Mixed model analysis

[b] Adjusting confounders include continues variables: age, body mass index, energy intake, years since menopause, dietary intake of calcium and vitamin D, categorical variables were: use of calcium and vitamin D supplementation (yes/no), smoking (never, quitted, current smoking), alcoholic beverages (yes/no), physical activity level (restricted, passive, normal), disease may affect BMD.

In addition, we analyzed the data adjusted for all the confounders separately for participants with BMI <25 and ≥25 kg/m^2. In general, no association was observed between quartiles of protein intake and BMD in the subgroup analysis (Table 9). Although non-significant association (p=0.066) between vegetable protein intake and femur BMD revealed.

Results also suggested a non-significant association (p=0.087) between lower vegetable protein intake (≤ 21.05) and higher BMD among women with BMI <25 kg/m^2. Moreover dairy protein intake in participants with BMI ≥25 kg/m^2 showed non-significant correlation with total BMD (p=0.095) (Table9).

Subgroup analysis according to both HT use and BMI

To explore the interactions of dietary protein intakes in quartiles with BMD we stratified participants regarding the interaction between HT use (no/yes) and BMI (< 25, ≥ 25 kg/m^2) (Table 10). Total protein intake tended to have association with femur BMD in women without HT and BMI<25 kg/m^2 (p=0.065). Also total protein intake was significantly associated with total BMD among women with HT and BMI<25 kg/m^2 (p=0.041) but the direction of association was not clear.

Results presented that amongst women with HT and BMI<25 kg/m^2, vegetable protein intake ≤21,30g/d was significantly associated with higher BMD at femur site (p=0.013), the same association was revealed for total BMD (p=0.018).

Table 9. Adjusted mean BMD at lumbar spine, femur and total body in quartiles of different dietary protein intake among elderly women. [a]

Variable quartiles cut points (g/day)	Adjusted lumbar BMD [b] (g /cm²)		Adjusted Femur BMD [b] (g /cm²)		Adjusted Total BMD [b] (g /cm²)	
	BMI<25 (n=96)	BMI≥25 (n=344)	BMI<25 (n=107)	BMI≥25 (n=417)	BMI<25 (n=101)	BMI≥25 (n=293)
Total protein intake						
≤ 54.73	1.096	1.116	0.827	0.839	1.052	1.054
54.83-66.07	1.133	1.149	0.856	0.856	1.032	1.063
66.10-80.37	1.026	1.154	0.816	0.864	0.979	1.063
80.57+	1.112	1.195	0.905	0.876	1.002	1.072
p value [a]	0.349	0.569	0.400	0.774	0.487	0.942
Animal protein intake						
≤34.60	1.099	1.138	0.834	0.849	1.005	1.064
34.63-42.57	1.082	1.151	0.826	0.837	0.99	1.063
42.63-54.93	1.056	1.146	0.815	0.869	0.972	1.062
54.97+	1.105	1.184	0.873	0.885	1.017	1.071
p value [a]	0.831	0.765	0.714	0.345	0.602	0.967
Vegetable protein intake						
≤17.72	1.001	1.164	0.816	0.859	0.967	1.068
17.33-21.03	1.119	1.164	0.895	0.888	1.032	1.062
21.07-24.90	1.100	1.161	0.823	0.861	1.009	1.072
24.97+	1.075	1.126	0.811	0.836	0.983	1.057
p value [a]	0.338	0.778	0.066	0.207	0.212	0.868
Vegetable protein intake median						
≤ 21.05 (median)	1.089	1.160	0.862	0.873	1.009	1.065
≥ 21.05 (median)	1.066	1.149	0.806	0.844	0.986	1.065
p value [a]	0.648	0.730	0.087	0.103	0.407	0.959
Protein from dairy product						
≤ 15.47	1.030	1.153	0.868	0.882	1.005	1.016
15.63-21.87	1.084	1.149	0.867	0.893	1.057	1.098
21.90-28.57	1.088	1.118	0.873	0.864	1.044	1.075
28.63+	1.082	1.135	0.862	0.884	1.045	1.106

p value [a]	0.757	0.678	0.993	0.509	0.431	0.095
Protein from meat						
≤7.20	1.037	1.119	0.877	0.855	1.029	1.089
7.23-12	1.093	1.126	0.835	0.879	1.019	1.099
12.07-17.17	1.020	1.158	0.882	0.894	1.016	1.105
17.27+	1.074	1.137	0.894	0.880	1.062	1.085
p value [a]	0.581	0.577	0.346	0.279	0.422	0.488
AVR						
≤ 0.07	1.058	1.124	0.794	0.835	0.969	1.057
0.07-0.10	1.134	1.135	0.827	0.854	1.022	1.063
0.10-0.14	1.089	1.131	0.809	0.875	1.010	1.060
0.14 +	1.020	1.155	0.839	0.859	0.981	1.051
p value [a]	0.413	0.878	0.755	0.421	0.371	0.917
PRAL						
≤-16.30	1.092	1.128	0.823	0.861	0.965	1.07
-9.16 - -3.01	1.084	1.117	0.802	0.862	0.976	1.043
-2.94- 2.78	0.984	1.156	0.813	0.859	0.943	1.059
2.83+	1.086	1.162	0.815	0.849	1.011	1.061
p value [a]	0.243	0.517	0.955	0.937	0.197	0.445

Abbreviations: BMD, bone mineral density; HT, hormone therapy; BMI, body mass index; AVR, animal to vegetable protein ratio; PRAL, protein renal acid load

[a] Mixed model analysis

[b] Adjusting confounders include continues variables: age, energy intake, years since menopause, dietary intake of calcium and vitamin D. categorical variables were: use of calcium and vitamin D supplementation (yes/no), smoking (never. quitted. current smoking), alcoholic beverages (yes/no), physical activity level (restricted, passive, normal), disease may affect BMD, HT(yes/no) .

Moreover, result suggested that vegetable protein intake tended to have positive association (p=0.068) with femur BMD in participants without HT and BMI<25 kg/m^2, also higher quartile of vegetable protein intake (>24.9g/d Vs ≤17.72g/d) was non-significantly related to higher lumbar BMD among participants with HT and BMI≥25 kg/m^2as compared to the lowest quartile (p=0.064) (Table 10).

To explore the previous suggested interactions between lower vegetable protein intake and higher BMD, additionally we analyzed the correlation between groups with vegetable protein intake lower than median amount (21.05 g/d) and higher. Result showed significant association between vegetable protein intake in lower median and femur BMD among participants without HT and BMI≥25 kg/m^2 (p=0.031), also with HT and BMI≤25 kg/m^2 (p=0.028) (Table 10). Moreover a lower intake of vegetable protein tended to be associated with higher lumbar BMD among women with HT and BMI≤25 kg/m^2.

Table 10. Adjusted mean BMD at lumbar spine, femur and total body in quartiles of different dietary protein intake among elderly women.

Variable quartiles cut points (g/day)	Adjusted lumbar BMD [b] (g /cm²)		Adjusted Femur BMD [b] (g /cm²)		Adjusted Total BMD [b] (g /cm²)	
	BMI<25 (n=96)	BMI≥25 (n=344)	BMI<25 (n=107)	BMI≥25 (n=417)	BMI<25 (n=101)	BMI≥25 (n=293)
Total protein intake						
≤ 54.73	1.096	1.116	0.827	0.839	1.052	1.054
54.83-66.07	1.133	1.149	0.856	0.856	1.032	1.063
66.10-80.37	1.026	1.154	0.816	0.864	0.979	1.063
80.57+	1.112	1.195	0.905	0.876	1.002	1.072
p value [a]	0.349	0.569	0.400	0.774	0.487	0.942
Animal protein intake						
≤34.60	1.099	1.138	0.834	0.849	1.005	1.064
34.63-42.57	1.082	1.151	0.826	0.837	0.99	1.063
42.63-54.93	1.056	1.146	0.815	0.869	0.972	1.062
54.97+	1.105	1.184	0.873	0.885	1.017	1.071
p value [a]	0.831	0.765	0.714	0.345	0.602	0.967
Vegetable protein intake						
≤17.72	1.001	1.164	0.816	0.859	0.967	1.068
17.33-21.03	1.119	1.164	0.895	0.888	1.032	1.062
21.07-24.90	1.100	1.161	0.823	0.861	1.009	1.072
24.97+	1.075	1.126	0.811	0.836	0.983	1.057
p value [a]	0.338	0.778	0.066	0.207	0.212	0.868
Vegetable protein intake median						
≤ 21.05 (median)	1.089	1.160	0.862	0.873	1.009	1.065
≥ 21.05 (median)	1.066	1.149	0.806	0.844	0.986	1.065
p value [a]	0.648	0.730	0.087	0.103	0.407	0.959
Protein from dairy product						
≤ 15.47	1.030	1.153	0.868	0.882	1.005	1.016
15.63-21.87	1.084	1.149	0.867	0.893	1.057	1.098
21.90-28.57	1.088	1.118	0.873	0.864	1.044	1.075
28.63+	1.082	1.135	0.862	0.884	1.045	1.106

p value [a]	0.757	0.678	0.993	0.509	0.431	0.095
Protein from meat						
≤7.20	1.037	1.119	0.877	0.855	1.029	1.089
7.23-12	1.093	1.126	0.835	0.879	1.019	1.099
12.07-17.17	1.020	1.158	0.882	0.894	1.016	1.105
17.27+	1.074	1.137	0.894	0.880	1.062	1.085
p value [a]	0.581	0.577	0.346	0.279	0.422	0.488
AVR						
≤ 0.07	1.058	1.124	0.794	0.835	0.969	1.057
0.07-0.10	1.134	1.135	0.827	0.854	1.022	1.063
0.10-0.14	1.089	1.131	0.809	0.875	1.010	1.060
0.14 +	1.020	1.155	0.839	0.859	0.981	1.051
p value [a]	0.413	0.878	0.755	0.421	0.371	0.917
PRAL						
≤-16.30	1.092	1.128	0.823	0.861	0.965	1.07
-9.16 - -3.01	1.084	1.117	0.802	0.862	0.976	1.043
-2.94- 2.78	0.984	1.156	0.813	0.859	0.943	1.059
2.83+	1.086	1.162	0.815	0.849	1.011	1.061
p value [a]	0.243	0.517	0.955	0.937	0.197	0.445

Abbreviations: BMD, bone mineral density; HT, hormone therapy; BMI, body mass index; AVR, animal to vegetable protein ratio; PRAL, protein renal acid load

[a] Mixed model analysis

[b] Adjusting confounders include continues variables: age, energy intake, years since menopause, dietary intake of calcium and vitamin D. categorical variables were: use of calcium and vitamin D supplementation (yes/no), smoking (never. quitted. current smoking), alcoholic beverages (yes/no), physical activity level(restricted, passive, normal), disease may affect BMD, HT(yes/no) .

6 DISCUSSION

Main findings of our study suggested a detrimental association between total protein intake≥ 1 g/kg-bw/d with BMD. This result was not in agreement with studies that suggested increasing protein intake, have protective effect on BMD. Our result was in consistent with result of cohort study conducted by Sahni (2013), which men with higher protein intake, had greater bone loss at trochanter.

Our results repeatedly suggested that vegetable protein intake was negatively associated with BMD, this finding was in consistent with observation from Promislow and coworkers (2010) study, which suggested negative association between vegetable protein and BMD in both sexes. In harmony with our result, Sahni (2010) showed that individuals with lower vegetable protein intake tended to have less fractures.

Results of the cross-sectional analyses showed no associations between protein intake and BMD. Overall in this prospective study of elderly women, after adjusting analyses for BMI, energy intake and age; total and animal protein intakes were not associated with BMD, however, vegetable protein consumption was negatively associated with femur BMD. However, after adjusting for all covariates aforementioned association was attenuated and not significant, or changed to different pattern. No evidence of association between total and animal protein intake with BMD were observed.

After stratifying participants for their HT status and further for BMI, no association were found except that vegetable protein consumption, tended to be associated with femur BMD in no HT group. The same trend was observed in women whose BMI was lower than 25 kg/m^2.

Moreover, no association for animal protein consumption was found, total protein intake tended to be positively correlated with femur BMD in women without and BMI< 25 kg/m^2. Conversely lower vegetable protein intake was negatively correlated with BMD at femur and total BMD (HT+ BMI< 25 kg/m^2) also a negative tendency of vegetable protein intake and BMD at lumbar site was suggested. (HT+ BMI< 25 kg/m^2). We evaluated the suggested effect of vegetable protein by looking at the result for lower and higher consumption than median, as expected from previous results lower consumption had protective effect on BMD.

6.1 Amount of total protein

Protein adequacy among participants in our study was compared to recent study conducted by Beasley (2014), which showed positive association between protein intake and BMD. Total protein intake among our participants was 17% of total energy which was relatively enough, similar to 15% in Besley's study (2014). However, gram protein intake was considerably lower in our study 67g/d as compared to 82 g/d. This fact may affect interpreting result in our study.

Dietary protein intake has historically been investigated in regard to its effect on BMD and still there is large controversy over this topic. Besides, protein is important part of bone structure, and can affect bone with different mechanisms (Sahni et al. 2013).

Most of the population-based, cross-sectional studies suggested that higher dietary protein intake, was associated with higher BMD (Hannan et al. 2000, Promislow et al. 2002, Sahni et al. 2013, Beasley et al. 2014), and usually some but not all bone sites showed an association with protein intakes. Our cross-sectional findings among postmenopausal women was consistent with the study by Beasley et al (2010), which found no association between protein intake and BMD cross-sectionally.

In addition it has been suggested that elderly people should meet the protein recommendation intake (0.8-1 g/kg- bw/d) or even higher to maintain skeletal mass. Or result was inconsistent with previous study, higher protein intake than recommendation was adversely associated with BMD (Tieland et al. 2012).

6.2 Animal protein

Previous longitudinal studies reported that animal protein intake, can affect BMD positively (Ho Pham et al. 2012, Sahni et al. 2010). Our prospective findings, were in agreement with one of the few neutral studies in elderly populations by Rapuri and coworkers (2003), which showed no association between animal protein intake and BMD.

6.3 Vegetable Protein

Although present study showed that higher intake of vegetable protein, was repeatedly associated with lower BMD, there are less evidences suggesting that vegetable protein, has detrimental effect on BMD, rather than beneficial (vegetable protein intake lower than median intake 21,05g/day or at the first and second quartiles).

Our result was in harmony with the result of the study by Promislow and coworkers (2002), this study suggested, adverse relationship between higher vegetable protein intake and BMD; conclusively our study accompanied with some others, do not support the dominant idea of acid-base theory (Young and Pellett 1994).

By no means is simple to predict the effect of vegetable or animal sources on bone, differences in the effect of vegetable and animal protein on bone, are integrated with the acid-base theory. Theoretically vegetable foods should protect bone, due to their alkaline-buffering effect, though it is noteworthy that sulfur-containing amino acids, which increase the acid load is markedly edible vegetable sources also, like legumes and most whole grains (Bonjour 2013, Hanley and Whiting 2013).

There are several mentionable reasons for the discrepancy between results of present study and previous cohort studies. First, comparison of mean BMD showed that only femur BMD decreased (-1.89%) after 3 years follow up, and BMD at lumbar (+0.93%) and total body (+0.56%) increased. Which making it difficult to detect the effect of protein, upon these small changes. Second, the pattern of nutrient intakes can be different between studies; for instance, mean calcium intake was relatively high (1010 mg/d) in this study, which is a significant indicator for BMD.

About 60% of our study population had protein consumption <1 g/kg- bw/d and 40% ≥ 1 g/kg-bw/d. This fact indicates that consumption of protein was lower than recommendation in most of the participants (0.8-1 g/kg- bw/d). Protein intake was 17% of energy intake, which can be interpreted as adequate amount; although gram intake was low (68±18 g/d) (Houston et al. 2008).

6.4 Strengths of the study

Current study has advantage to present results, both at cross-sectional and longitudinal settings. Also we specifically investigated the associations of each protein component (animal, vegetables, meat, dairy and total), with an absolute measure of bone mineral density among elderly women. Other positive point in this study was total energy intake variable was normally distributed among participants. Also the 3-days food record were used to collect dietary data, considerably is a good method to represent a person's actual diet (Karkkainen et al. 2010, Crawford et al. 1994).

Present study drew strength according that most of the previous studies, did not report the protein intake as g/kg- bw/d, or percentage of energy intake. Regarding to cover this gap, we reported protein intake for both g/kg- bw/d, and energy percentage.

A potential positive point is that, present study is unique in subcategorize analysis, no previous studies analyzed the association of protein intake with BMD, regards to stratifying the participant, for interaction between HT and BMI.

6.5 Limitations of the study

Although analysis was adjusted for several confounders that influence bone density, participants with higher intakes of protein, may have differed from those with lower intakes in ways that were not captured in this study. Also participants in an osteoporosis study, may have had a heightened awareness of their bone health, which may have led them to alter modifiable osteoporosis risk factors between the baseline and follow-up visits. Such an effect is unlikely to have influenced on protein consumption; since protein is not commonly perceived to be an osteoporosis risk factor, but the potential misclassification with respect to covariates could have biased the observed associations toward the null value.

This study was a relatively small study. Further follow up and longer period, may help to find changes in BMD. It is possible that strong effect of HT, dietary calcium and BMI could mask the effect of other factors including protein intakes.

7 CONCLUSIONS

Our study suggested that negative association of protein intake and BMD, can be more due to vegetable protein consumption rather than animal protein source. Although it is was possible to explain vegetable protein intake effects clearly. Further studies are required to explore the suggested interaction.

This prospective study did not support the possibility of dietary protein positive role for bone health among elderly women. On the contrary, we found that protein intake higher than recommendation ($\geq$ 1 g/kg- bw/d), may cause BMD loss.

Present study did not support the possible effect of animal protein consumption on BMD. Besides, effect of protein intake on BMD may only be recognizable when it sets for strong confounders such as HT and BMI.

Results also provide some information of an interaction between BMD and BMI. These findings, along with the intriguing observation of the negative association between vegetable protein consumption and BMD, provide implications for further osteoporosis study strategies and investigation in elderly cohort.

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