Proteins, dietary acid load, and calcium and risk of postmenopausal fractures in the E3N French women prospective study.

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Conflict of Interest: All authors have no conflicts of interest

ABSTRACT

Introduction: Excess dietary proteins and "acid ash" diets have been suspected to increase the risk of osteoporosis, but experimental and epidemiological evidence is mixed. We aimed to determine whether the association between protein intake and the overall acid-base equilibrium of the diet (as renal net acid excretion (RNAE) estimate) and fracture risk vary according to calcium intake.

Materials and Methods: During an average of 8.37 (± 1.73) years of follow up, 2408 women reported a fracture (excluding high-impact trauma) among 36,217 postmenopausal women from the E3N prospective study. We used Cox regression models to study the interaction between calcium and, respectively, proteins and RNAE, from the 1993 dietary questionnaire, for fracture risk determination, adjusting for potential confounders.

Results: There was no overall association between fracture risk and total protein or RNAE. However, in the lowest quartile of calcium (< 400 mg/1000 kcal), high protein intake was associated with a significant increased fracture risk (RR = 1.51 for highest versus lowest quartile; 95% CI 1.17-1.94). An increasing fracture risk with increasing animal protein intake was also observed (p trend <0.0001). A similar pattern of interaction for fracture risk was observed between RNAE and calcium.

Conclusions: In this Western population of post-menopausal women with normal to high protein intake and fairly high calcium intake, there was no overall association between total protein or RNAE and fracture risk. However, there was some evidence that high protein – high acid ash diets were associated with an increased risk of fracture when calcium intake was low (< 400 mg/1000 kcal).

Key words: nutrition, osteoporosis, epidemiology, population studies

INTRODUCTION

There is substantial evidence that insufficient dietary protein intake is deleterious for bone health, but there is a strong debate as to whether an excess of dietary protein may be associated with osteoporosis. It has long been known that increasing dietary protein increases urinary calcium excretion.[1-3] Until recently, the prevailing view was that bone was the source of the extra urinary calcium excreted during a high-protein diet. Several reviews have summarized the complex literature on the potential impact on bone of dietary protein.[4-6] One mechanism by which high dietary protein could induce bone loss may be related to the acid load generated by the protein metabolism. While renal metabolism represents the principal mechanism by which fixed metabolic acid loads are handled by the body, renal buffering may be incomplete, particularly with aging. Under those circumstances, the skeleton may be called on to act as a buffer to neutralize acid generated from high-protein diets. Liberation of buffer from bone comes at the expense of mineral dissolution and ultimately bone loss. Even mild acidosis could have profound effects: if bone is mobilized to buffer only 1 mEq of acid each day, 15% of the total body calcium in an average person would be lost in a decade. This theory has recently been challenged,[7-8] in particular following a series of short-term experimental studies that suggested that dietary protein might influence calcium intestinal absorption, and that most of the extra urinary calcium excreted during a high-protein diet originate from the intestine rather than from bone.[7] However, the relative contribution of diet and bone to protein-induced calciuria remains controversial, and the long-term impact of high protein diets on bone health is still unclear.[9]

Results of observational epidemiological studies have not helped to clarify the nature of the effect of high dietary protein intakes on the skeleton. Numerous studies showed a positive association with bone mass,[10-14] but others showed no association, [15, 16] while others still

showed a negative association with at least one bone site.[17-19] Fewer studies have evaluated the effect of protein intake on fracture risk. Two of them found a decreased risk of fracture with higher protein intake,[20, 21] while three others showed an opposite trend, in particular with proteins from animal sources.[22-24]

These discrepant results might be explained by the fact that the impact of protein on the skeleton is dependent on other components of the diet. In particular, it has been suggested that higher calcium intake, which results in more absorbed calcium, may help offset the urinary calcium loss induced by high protein intake.[5, 9, 25] At low calcium intake, the efficiency of intestinal absorption cannot be increased sufficiently to offset an increase in obligatory calcium loss. Thus, if protein exerts a negative effect on bone, it should be only under conditions of low calcium intake. Surprisingly, the potentially modifying influence of calcium on protein effect has not been thoroughly investigated.

Furthermore, although meat is considered to be the major source of acid ash in the diet, many grain products (such as white bread, white pasta, oat flakes, cornflakes, brown rice) also have a high potential renal acid load (PRAL).[26] Among dairy products, which are rich in both protein and calcium, cheeses (in particular hard ones) have very high PRAL (more than twice that of meat or fish), while yoghurt and milk have very low PRAL. Fruit and vegetables, on the other hand, have a negative PRAL, which means that they supply alkali-ash. Hence, the influence of protein may depend on whether the overall diet is balanced in terms of its acid-generating potential. Recently, several algorithms based on dietary intakes of key nutrients have been proposed for estimating the net acid load of the diet (i.e., acid minus base), which should help to further explore the association between dietary acidity and bone health.[27, 28]

Given the above considerations, we used data from a large cohort of postmenopausal women to assess the association between protein intake or the overall acid-base equilibrium of the

diet and fracture risk, and to explore whether the observed associations vary according to levels of calcium intake.

MATERIALS AND METHODS

The E3N cohort study

The E3N (Etude Epidémiologique de femmes de la Mutuelle Générale de l'Education Nationale, MGEN) prospective cohort was initiated in France in 1990 to study the risk factors for the most frequent sites of cancer in women.[29] The cohort includes 100,000 women, living in France, aged 40 to 65 years at baseline, and covered by the MGEN national teacher's health insurance plan.

Dietary habits, use of hormonal treatments, reproductive factors and other factors, such as tobacco consumption, anthropometrical measurements, personal history of a variety of diseases, or family history of cancer were recorded in self–administered questionnaires, completed approximately every 24 months. Each questionnaire investigated the occurrence of personal medical events, including fractures, since the last follow-up questionnaire. Women who replied to the dietary questionnaire constitute the French cohort of the European Prospective Investigation on Cancer,[30] named E3N-EPIC. After 15 years of follow-up, response rate to the latest questionnaire was still as high as 83 %.

Dietary data

Dietary data were collected between June 1993 and July 1995. The dietary questionnaire was composed of two parts, the first including questions on the consumption (quantity and frequency) of food groups, the second qualitative questions allowing detailing the food groups into food items. The questionnaire assessed dietary consumption of 208 items. It was sent

with a booklet of photos to facilitate the estimation of portion sizes. They were sent to 95,644 women and two reminders were sent to non-responders. Finally, 77,613 questionnaires were collected (81.1%).

Both the questionnaire and the booklet were validated.[31, 32] The validity of the dietary questionnaire was assessed using a sample of 115 women, taking as the reference the average of twelve 24-hr recalls obtained at monthly intervals over a 1-year period. The reproducibility of the dietary questionnaire was also tested. A high proportion of subjects (76% for foods and 72% for nutrients) were classified in the same or adjacent quintile for the dietary questionnaire and the 24-hr recalls. The Pearson's correlation coefficient for calcium intake was 0.38 for validity and 0.75 for reproducibility.

After exclusion of 985 questionnaires because of absence of consent for external health follow-up by the insurance plan in case of dropout, and of 2,132 questionnaires because of miscoded answers, 74,524 questionnaires were available for the analysis. Women with extreme values (in the bottom 1% or top 1%) of the ratio between energy intake and energy requirement (computed after taking into account age, weight and height) were excluded (n=1,492), leaving 73,032 women with analysable dietary data.

Studied population and identification of fracture cases

From the women with proper dietary data, only post-menopausal women at the dietary data assessment were considered eligible in the present study (n=40,224). In each follow-up questionnaire, women were asked to report on the occurrence of a fracture along with the date and the circumstances of the event. Women whose fracture was declared between the date of menopause and the dietary questionnaire were excluded (prevalent fractures, n=3,488) since dietary habits (in particular, calcium consumption) may have been modified after fracture occurrence. A specific mailing to women that had reported a fracture in one of the follow-up

questionnaires up to the 2002 questionnaire included was designed in order to record its date and circumstances. We excluded women with incomplete or missing data about events (n=185), those with fractures after the endpoint, or due to motor vehicle accidents, other high-impact trauma (fall from higher than one's standing height), or metastases corresponding to 334 women. Finally, our analyses were conducted on 2408 women with incident fracture and 33809 fracture-free women, i.e. 36,217 women in total.

Statistical analysis

We used relative risks (RR) from a Cox model regression, with age as time-scale, as measures of association, and calculated 95 percent confidence intervals (95% CI). To avoid problems of correlation between total energy intake and consumption of nutrients, we used the nutrient density method[33] for total proteins, animal proteins, vegetal proteins, and dietary calcium. Variables were studied as quartiles of nutrient intake, and tests for linear trend were performed considering categories as an ordinal variable. Additional analyses were also conducted in which protein intake was not energy-adjusted but expressed as the amount of protein per kg of body weight, according to current recommendations. Calcium intake was studied as a time-dependent variable in order to include an updated fifth category for calcium supplement users. Information on calcium supplement use was extracted from questions on treatment/prevention of osteoporosis and on dietary supplementation. To estimate dietary acid load, we calculated the renal net acid excretion (RNAE), using the formula proposed by Remer *et al*.[27].

We first evaluated the overall associations between fracture risk, and calcium and protein intakes (total, animal, and vegetal protein) as well as the estimate of the acid load (RNAE), in separate Cox models, with age as the time scale, adjusting for major potential confounders (BMI (continuous), physical activity at the dietary data assessment (in METs, continuous),

parity, maternal history of hip fracture (yes/no), ever use of post menopausal hormonal therapy (HT) as a time-dependent variable, smoking status (current, ex-, or never smoker) as a time-dependent variable, alcohol intake (in g of daily ethanol intake, continuous) and total daily non-alcoholic energy intake (Kcal/day)).

To assess interaction between protein and calcium for fracture risk, we then included both variables and the corresponding interaction terms in a single Cox model and calculated the RR associated with each combination of protein and calcium, taking the low/low combination (i.e., lowest quartile of both protein and calcium) as the reference category. A similar approach was used to assess the interaction between dietary acidity and calcium. All models were adjusted for potential confounders as described above.

All analyses were performed using the SAS© software, version 8·2.

RESULTS

The mean duration of follow-up was 8.37 ± 1.73 years. Characteristics of the population with and without fracture are presented in Table 1. The association between fracture risk and, respectively, dietary proteins, calcium and RNAE in the whole population is presented in table 2. There was no significant association between protein measures or RNAE and fracture in age-adjusted models or in multivariate models adjusting for major potential confounders. Similarly, there was no clear association between dietary calcium and fracture, although use of calcium supplements was associated with a significant decreased fracture risk (RR = 0.83; 95% CI: 0.72 – 0.96).

Table 3 shows RRs of fracture associated with each combination of calcium and, respectively, total proteins and RNEA. High protein intake, expressed as energy density (in g/1000 kcal), was associated with a statistically significant increased fracture risk in the lowest quartile of calcium (RR = 1.51, 95% CI: 1.17-1.94) for the high protein – low calcium group compared

to the low protein – low calcium group, taken as the reference; p for linear trend across protein quartiles < 0.0001), whereas there was no association between protein and fracture risk at higher calcium levels. We also examined the interaction of calcium and, respectively, animal and vegetal proteins (results not shown). A significant trend of increasing fracture risk with increasing protein was found for animal protein in the lowest calcium quartile (RR = 1.66 for highest versus lowest protein quartiles, 95% CI: 1.29-2.13; p trend < 0.0001) whereas a trend in the opposite direction was found for vegetal protein (RR = 0.68 for highest versus lowest protein quartiles, 95% CI: 0.53-0.87; p trend < 0.0001). With regard to RNAE, we found a pattern of interaction similar to that with total protein and animal protein: there was a significant trend of increasing fracture risk with increasing RNAE in the lowest calcium quartile (RR = 1.44 for highest versus lowest RNAE quartile, 95% CI: 1.11 – 1.86; p trend < 0.0001), whereas there was no clear association at higher calcium levels.

We also examined the association between fracture risk and total protein intake expressed in grams per kg of weight rather than in terms of density. The results were similar to those observed with total protein density : no significant association in the overall population, but a significant trend of increasing fracture risk with increasing protein intake in the lowest quartile of calcium (RR = 1.46, 95% CI: 1.03-2.06 for the highest protein quartile versus the lowest; p for linear trend across quartiles < 0.0001). Noteworthy, the lowest quartile bound was 1.15 g/kg and the mean intake in that quartile was 0.94 g/kg, i.e., higher than the currently recommended intake of 0.8 g/kg; [35] the highest quartile bound was 1.71 g/kg, which means that more than 25% of our population had an intake twice higher than the current recommendations.

DISCUSSION

In this prospective study of postmenopausal women, there was no significant association between protein intake and fracture risk in the whole population. However, an elevated fracture risk was found in women with a high intake of protein in the presence of low calcium intake. The same pattern of interaction was observed when total protein intake was expressed in grams per kg of weight . A significant trend of increasing fracture risk with increasing protein was also found for animal protein in the lowest calcium quartile whereas a trend in the opposite direction was found for vegetable protein. The difference between animal and vegetal proteins is unlikely to be explained by inherent differences in the effects of animal and vegetal proteins per se.[8, 34] A more likely explanation is related to the fact that vegetal protein is present in alkalinogenic foods (i.e., fruits and vegetables) whereas animal protein is not. In our cohort, vegetal protein was negatively correlated with RNAE ($r = -0.10$; $p < 0.0001$) whereas animal protein was positively correlated with RNAE ($r = + 0.36$; $p < 0.0001$), and the 2 types of proteins were inversely correlated ($r = - 0.36$; $p < 0.0001$). Hence, in our cohort, a higher vegetal protein intake can be considered as an indicator of a diet that is globally less acidic. In the low calcium group, fruits and vegetable intakes increased across quartiles of vegetal protein (from 468 g/d in the lowest quartile to 582 g/d in the highest quartile of vegetal protein).

Similarly to what was observed for proteins, there was no significant association between RNAE and fracture risk in the overall population, but higher RNAE was associated with a significant increased risk of fracture in the lowest quartile of calcium. The consistency of the pattern of the observed interactions supports the hypothesis that high protein - high acid ash diets can lead to chronic metabolic acidosis and subsequent bone loss unless calcium intake is also high. It must be emphasized that these results apply to a population with relatively high

intakes both of calcium and of protein. Indeed, average calcium intake was 1034 mg/day; and for 89% of women in the cohort, protein intake represented more than 15% of their total alcohol-free caloric intake, i.e. the upper limit of recommendations for the French population.[35] Almost two thirds of proteins came from animal sources. Because protein and calcium intakes are positively and significantly correlated ($r = + 0.38$; $p < 0.0001$) and the two nutrients are hypothesized to have countervailing effects, it should not be surprising, as noted by Heaney,[5] that there is no appreciable effect of protein intake on bone health at the population level.

Few studies have examined the effect of protein intake on the skeleton as a function of calcium intake, but their results also suggest an interaction between calcium and protein intakes. In a large cohort of middle-aged Norwegians,[22] there was no clear association between calcium intake or non-dairy animal protein intake and hip fracture. However, women with both low calcium intake (< 623 mg/d) and high non-dairy protein intake (> 21.6 g/d) had approximately twice the risk of hip fracture than women with higher calcium intake and lower non dairy protein intake. In the Nurses' Health Study, high protein intake was associated with an overall 22% increased risk of forearm fracture, and the association seemed to be more pronounced at low calcium intake (below 540 mg/d).[23] No increase in risk was observed for hip fractures, but as noted by the authors, the power to detect a similar increase was limited by the smaller number of such fractures in the study.

In a secondary analysis of data from a 3-yr randomised controlled trial of calcium and vitamin D supplementation,[36] high protein intake was associated with a favourable change in total body and femoral neck BMD (bone mineral density) in the supplemented group but not in the placebo group (mean calcium intake of 1346 and 871 mg/d, respectively). These results

suggest that a high calcium intake may compensate for protein-induced urinary calcium losses, and even tilt the balance of positive and negative effects of proteins on bone towards a net positive effect. Dietary protein intake supplies the amino-acids necessary for maintaining bone matrix, and there is evidence that it also promotes the production of IGF-1, a factor that stimulates bone growth.[25] A high calcium intake may therefore enable the full expression of the dietary protein-mediated anabolic drive on bone. Because the calcium supplement used in that study provided alkali source (citrate and malate), an alternative hypothesis, as noted ty the authors, is that the beneficial effect of protein on bone in the supplemented group may be due, at least in part, to the provision of sufficient alkali to neutralize the protein-derived acid.

In our study, we found no clear evidence that high protein intake is associated with a reduced fracture risk when calcium intake is high, although fracture risk tended to decrease with increasing protein intake in the subgroup of women who were taking calcium supplements. We did not have precise information on the type and dosage of calcium supplement used, nor on the reasons for taking the supplement. If prescription was based on an increased osteoporosis risk, the potentially beneficial effect of a high calcium / high protein intake may be underestimated. We found no significant difference in the prevalence of common risk factors for osteoporosis (i.e., HT use, BMI, physical activity, parity, maternal history of hip fracture, smoking) in the supplemented group as compared to the non-supplemented group. However, we cannot be sure that the two groups did not differ on some unmeasured characteristics related to osteoporosis risk. It should also be noted that in Dawson-Hugues' study, calcium supplement was associated with vitamin D supplement (700 UI daily). Hence, the effect of calcium cannot be separated from the effect of vitamin D. In this study, we don't have precise information on the type of calcium supplement used, in particular we don't know if it was associated or not with vitamin D.

Few epidemiological studies have examined the association between RNAE and bone mineral density and, to the best of our knowledge, no study examined its association with fracture risk or the interaction of RNAE and calcium on fracture risk. Results from the Aberdeen Prospective Osteoporosis Screening Study (APOSS) have shown that lower estimates of the net endogenous acid production were associated with greater lumbar spine and hip bone mineral density, and with lower levels of biomarkers of bone resorption.[37, 38] Other indirect evidence that dietary acid-base balance plays a role in bone health has come from epidemiological studies that found a positive association between potassium intake or fruit and vegetables intake (which represent the major source of buffer in the diet) on axial and peripheral bone mass and on bone metabolism.[39, 40] In the Framingham cohort, for instance, high intakes of potassium, and of fruit and vegetables were associated with lower rates of bone loss in men and greater bone mass in women.[41] A later analysis in the same cohort provided evidence that dietary protein is beneficial to bone mass: subjects with higher total and animal protein intakes had lower rates of bone loss than did subjects consuming less protein.[12] These results suggest that in the Framingham cohort, dietary protein intake was beneficial to bone, at least in part because its potential acidifying influence was balanced by the alkalising effect of the dietary potassium intake.[42]

Our results suggest that the effect of dietary protein on bone is best considered in the context of the entire diet of each individual, particularly in terms of balance between acid-forming and base forming foodstuffs as well as overall calcium intake. There was no association between high protein – high acid ash diets and fracture risk in the highest quartile of calcium density (> 600 mg/1000 kcal) which corresponds to a mean intake of 1389 mg/d, whereas a significant trend of increasing fracture risk with increasing protein or RNAE was observed in

the lowest quartile of calcium (< 400 mg/1000 kcal) which corresponds to a mean intake of 749 g/d. This suggests that currently recommended calcium intake of over 1200 mg calcium per day for postmenopausal women may usually be sufficient to compensate for urinary calcium losses induced by high protein – high acid ash diets. On the other hand, it should also be noticed that when protein intake was within recommendation (our low group) or RNAE was low, increasing calcium intake above 400 mg/1000 kcal yielded no additional benefit in terms of fracture risk reduction, which suggests that optimum calcium requirements for bone health may depend on protein intake and, more generally, on the overall acid-base balance of the diet.

Our findings suggest that there may be two alternative strategies to reduce fracture risk in our Western population where protein intake is generally high and diets are predominantly 'acidogenic': either increase calcium intake up to at least 1200 mg/d, or decrease protein intake to moderate (recommended) amounts while increasing foods rich in base precursors such as fruits and vegetables and ensuring moderately high calcium intake. This last strategy is particularly appealing as a global health policy since it may reduce the burden of several other chronic diseases in addition to osteoporosis.[40] We should also keep in mind that if the effect of a high protein – high calcium diet is similar on the bone to the effect of a normal protein – normal calcium diet, a recent controlled trial suggested a potential adverse effect of calcium supplements on risk of cardiovascular events.[43]

Our study had several limitations. First, fracture data could not be verified through radiographic or surgery reports. However, a specific questionnaire was addressed to women who had reported a fracture during follow-up to ask them the date and circumstances of the fracture as well as the type of fracture, and women with incomplete or missing data about events were excluded. In addition, we should emphasize that our cohort is mostly composed

of educated women, and validation studies of other endpoints have demonstrated that our participants were very reliable in their answers.[44, 45, 31] Second, we did not have reliable information on corticotherapy or osteoporosis treatments use, and thus could not exclude from the analysis women using such treatments. However, given the mean age of the cohort at baseline (56 years) and the study period (1998 – 2002), the proportion of women who had been using effective osteoporosis treatments such as biphosphonates was probably small. Another study limitation is that we do not know how well our nutritional data described the diet in the long term. However, imprecision in the estimation of nutrients intake probably does not account for the relation found between high protein / low calcium intake and fracture since any misclassification would be expected to be nondifferential and thus would tend to reduce the strength of a true relation. On the other hand, the observed association do not necessarily imply a causal relation. Although adjustment was made for a number of risk factors for fracture in the multivariate analyses, we cannot exclude the possibility that other factors associated with protein and/or calcium could be responsible for the association. For instance, vitamin D is known to promote calcium intestinal absorption and could also decrease the risk of falling by improving lower extremity muscle function. Several reports indicate that vitamin D insufficiency is very common among post-menopausal women, and a recent randomized controlled trial showed that vitamin D supplementation reduced fracture risk among subjects living in the community.[46] In this study, we didn't have information on sun light exposure and questions concerning fish did not distinguish between fatty fish, rich in vitamin D, and other types of fish. Phytoestrogens may also affect the risk of fracture, but we didn't have information on consumption of soy products or use of dietary supplements including phytoestrogens (although both were rather uncommon in France before the year 2002).

Finally, it should be emphasized that our results apply to a population with normal to high protein intake, and that it is the combination of excess protein / low calcium that was associated with an increased fracture risk. Our results do not apply to elderly subjects at risk of undernutrition, in particular. Given the studied range of intakes, we could not examine the risk of fracture associated with low protein intake. But there is already considerable evidence that low protein intake is detrimental to bone. Protein is a major bulk constituent of bone and must be regularly supplied by the diet throughout life. In the elderly, low protein intakes are often observed in patients with hip fracture. In these patients, a randomized controlled study after orthopedic management demonstrated that protein supplementation attenuated post-fracture bone loss, increased muscles strength and reduced medical complications and hospital stay.[8]

In summary, in this population of post-menopausal women with normal to high protein intake and fairly high calcium intake, we found no overall association between total protein or RNAE and fracture risk. However, there was some evidence that high protein – high acid ash diets were associated with an increased risk of fracture in the lowest calcium group (< 400 mg/1000 kcal; lowest quartile bound), which is compatible with the acid-ash hypothesis. On the other hand, in normal protein – low RNAE subgroups, there was no association between calcium and fracture risk.

Acknowledgment:

Funding/support :

The E3N study is supported by the French League against Cancer, the European Community, the 3M Company, the "Mutuelle Générale de l'Education Nationale", the French Institute of

Health and Medical Research (Inserm), the Gustave Roussy Institute and several General Councils in France.

The present analysis has been supported by a grant from Inserm.

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Table 1. Characteristics of the study population

	No fractures (n= 33809)	Fractures (n= 2408)	p value*
Mean (sd)			
Age (years)	56.1(5.5)	57.1 (5.6)	<.0001
BMI (kg/m²)	23.2 (3.3)	23.3 (3.4)	0.01
Physical activity (METs/day)	52.7 (36.6)	53.7 (36.1)	0.18
Number of children	2.1 (1.2)	2.0 (1.3)	0.03
Alcohol intake (g/day)	11.0 (14.0)	11.5 (14.4)	0.12
Energy (alcohol excluded) (Kcal/day)	1988.1 (532.2)	1973.3 (526.5)	0.19
Total proteins (g/1000Kcal/day)	45.7 (7.3)	46.0 (7.6)	0.09
Animal proteins (g/1000Kcal/day)	28.4 (8.7)	28.8 (8.9)	0.04
Vegetal proteins (g/1000Kcal/day)	12.2 (3.1)	12.1 (3.2)	0.39
Proteins by kg of weight (g/kg/day)	1.45 (0.43)	1.44 (0.43)	0.16
Dietary calcium (mg/1000Kcal/day)	526.4 (161.3)	520.7 (155.2)	0.08
Dietary potassium (mg/1000Kcal/day)	1916.4 (451.6)	1922.6 (441.1)	0.51
RNAE (mEq/day)	45.46 (16.57)	45.76 (19.03)	0.07
N (%)			
Maternal history of hip fracture	2928 (8.7)	240 (10.0)	0.03
HT use (% ever)	24072 (71.2)	1516 (63.0)	<.0001
Current smokers	4082 (12.0)	297 (12.3)	0.71
Calcium supplement users	7019 (20.8)	291 (12.1)	<.0001

* Student's t test for quantitative variables, Chi^2 for qualitative variables

Table 2. Overall association between dietary proteins, calcium (density of nutrients in g or mg/1000 kcal), and RNAE and fracture risk (separate Cox models for each nutritional variable, adjusted for BMI, physical activity, parity, maternal history of hip fracture, HT use, smoking status, and alcohol intake)

Variables	Model	Quartile 1	Quartile 2	Quartile 3	Quartile 4
Total proteins (g/1000 kcal)	Cut-off	< 40.75	40.75 – 45.16	45.16 – 50.11	> 50.11
	Age-adjusted RR	1	1.05 (0.94-1.18)	1.00 (0.89-1.12)	1.10 (0.98-1.23)
	Multivariate RR	1	1.05 (0.93-1.17)	0.99 (0.88-1.11)	1.06 (0.94-1.19)
Animal proteins (g/1000 kcal)	Cut-off	< 22.42	22.42 – 27.75	27.75 – 33.52	> 33.52
	Age-adjusted RR	1	1.07 (0.96-1.20)	1.05 (0.94-1.18)	1.14 (1.02-1.27)
	Multivariate RR	1	1.07 (0.95-1.20)	1.04 (0.93-1.16)	1.10 (0.98-1.24)
Vegetable proteins (g/1000 kcal)	Cut-off	< 10.07	10.07 – 12.01	12.01 – 14.12	> 14.12
	Age-adjusted RR		0.94 (0.84-1.06)	0.95 (0.85-1.06)	0.94 (0.84-1.05)
	Multivariate RR	1	0.95 (0.85-1.06)	0.96 (0.85-1.07)	0.95 (0.85-1.06)
Total proteins by weight (g/kg)	Cut-off	< 1.15	1.15-1.41	1.41-1.71	>1.71
	Age-adjusted RR	1	0.96 (0.86-1.07)	0.97 (0.87-1.08)	0.92 (0.82-1.03)
	Multivariate RR	1	1.00 (0.88-1.12)	1.03 (0.90-1.18)	1.02 (0.86-1.20)
RNAE (mEq)	Cut-off	< 34.2	34.2 – 45.7	45.7 – 57.1	> 57.1
	Age-adjusted RR	1	1.06 (0.95-1.19)	1.10 (0.98-1.23)	1.03 (0.92-1.15)
	Multivariate RR	1	1.06 (0.95-1.19)	1.11 (0.99-1.24)	1.05 (0.93-1.19)

Variables		Quartile 1	Quartile 2	Quartile 3	Quartile 4	Supplement use
Calcium	Cut-off	< 417.3	417.3 – 500.6	500.6 – 604.3	> 604.3	
(mg/1000kcal)	Age-adjusted RR	1	1.06 (0.94-1.19)	1.01 (0.90-1.14)	0.92 (0.82-1.04)	0.85 (0.73-0.98)
	Multivariate RR	1	1.05 (0.94-1.19)	1.00 (0.89-1.13)	0.91 (0.80-1.03)	0.83 (0.72-0.96)

Table 3. Relative risk of fracture associated with combinations of animal protein or RNAE and calcium categories (Cox model adjusted on BMI, physical activity, parity, maternal history of hip fracture, HT use, smoking status, and alcohol intake)

Variables		Quartile 1	Quartile 2	Quartile 3	Quartile 4
Total proteins (g/1000 kcal)	Calcium intake:				
	1st quartile	1	1.16 (0.93-1.44)	1.09 (0.86-1.39)	1.51 (1.17-1.94)
	2nd quartile	1.20 (0.97-1.48)	1.31 (1.07-1.61)	1.11 (0.89-1.39)	1.10 (0.85-1.41)
	3rd quartile	1.05 (0.83-1.34)	1.03 (0.82-1.30)	1.12 (0.90-1.39)	1.29 (1.04-1.60)
	4th quartile	0.91 (0.65-1.26)	0.95 (0.73-1.23)	1.00 (0.80-1.26)	1.10 (0.90-1.34)
	Supplements	1.11 (0.86-1.44)	1.02 (0.78-1.34)	0.89 (0.68-1.17)	0.72 (0.53-0.97)
Proteins by weight (g/kg)	Calcium intake:				
	1st quartile	1	1.00 (0.78-1.28)	1.08 (0.84-1.38)	1.46 (1.03-2.06)
	2nd quartile	0.98 (0.77-1.25)	1.06 (0.84-1.34)	1.09 (0.85-1.39)	1.24 (0.95-1.60)
	3rd quartile	1.07 (0.85-1.35)	1.09 (0.86-.38)	1.04 (0.81-1.32)	0.91 (0.69-1.19)
	4th quartile	0.94 (0.74-1.19)	0.85 (0.66-1.09)	0.97 (0.76-1.25)	0.97 (0.74-1.26)
	Supplements	0.82 (0.62-1.09)	0.84 (0.63-1.12)	0.89 (0.67-1.18)	0.87 (0.64-1.18)
RNAE (mEq)	Calcium intake:				
	1st quartile	1	1.18 (0.92-1.51)	1.49 (1.18-1.90)	1.44 (1.11-1.86)
	2nd quartile	1.23 (0.96-1.57)	1.39 (1.10-1.76)	1.29 (1.01-1.65)	1.40 (1.09-1.81)
	3rd quartile	1.26 (0.98-1.61)	1.32 (1.03-1.68)	1.46 (1.15-1.85)	0.98 (0.75-1.27)
	4th quartile	1.13 (0.87-1.47)	1.07 (0.82-1.40)	1.08 (0.83-1.40)	1.23 (0.97-1.56)
	Supplements	1.13 (0.85-1.49)	1.06 (0.79-1.42)	0.91(0.67-1.24)	1.05 (0.78-1.41)