

ORIGINAL COMMUNICATION

Bone and nutrition in elderly women: protein, energy, and calcium as main determinants of bone mineral density

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Objective: Nutrition is an important factor in the prevention and treatment of osteoporosis. Our goal was to examine the relationship between various nutrients and bone mass of several skeletal sites in elderly women, taking into account possible confounding variables.

Design/methods: A cross-sectional study in 136 healthy Caucasian, postmenopausal women, free of medications known to affect bone was carried out. Bone mineral density (BMD) and body composition (lean and fat tissue) were measured by dual X-ray absorptiometry using specialized software for different skeletal sites. Parathyroid hormone (PTH) and vitamin D, 25(OH)D, as possible confounders, were determined in serum samples. Dietary intake, including all supplements, was assessed by 3-day dietary record and analyzed using Food Processor[®]. Past physical activity and present walking were examined as well and accounted for as potential confounders. Simple and multiple regression models were created to assess the relationships between nutrients and BMD. To examine the co-linear variables and their possible independent association with bone, subgroup analyses were performed.

Results: Showed independent influence of calcium, energy, and protein, examined separately and in multiple regression models on BMD of several skeletal sites. Magnesium, zinc and vitamin C were significantly related to BMD of several skeletal sites in multiple regression models (controlled for age, fat and lean tissue, physical activity and energy intake), each contributing more than 1% of variance. Serum PTH and 25(OH)D did not show significant association with bone mass.

Conclusions: Despite the cross-sectional nature of our study we were able to show a significant relationship between BMD and several critical nutrients: energy, protein, calcium, magnesium, zinc and vitamin C. The exact involvement of these nutrients and their clinical significance in bone health need to be further elucidated in humans and conclusions about the effects of a single nutrient on bone mass must be given cautiously, taking into account its interaction and co-linearity with others. Understanding relationships among nutrients, not just limited to calcium and vitamin D, but others that have not been investigated to such extent, is an important step toward identifying preventive measures for bone loss and prevention of osteoporosis.

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Introduction

Once 'nobody's disease', and almost unrecognizable in medical establishments (unless a fracture occurred), osteoporosis

recently became the focus of much interest and the last decade has brought about a wealth of information on bone health. This increased interest, advanced technology for bone mass measurements, and newly identified markers of bone turnover have enhanced our understanding of osteoporosis risk factors, its causes and routes of its prevention and management. Nevertheless, osteoporosis remains a complex, multi-factorial condition leading to increased risk of fractures. Despite the fact that up to 80% of the bone strength (including bone density and quality) might be genetically determined (Pocock *et al*, 1987), many other nutritional and lifestyle factors, as well as the host's physiological condition—age, weight, height, fat and muscle mass, and hormonal status—are other determinants of bone

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Contributors: JZI is a principal investigator and was responsible for analyzing the data and writing the manuscript; RAB was responsible for collecting all data and analyzing nutritional and physical activity; LT was responsible for helping with the study and collecting information on nutritional supplements.

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health whose effects and interactions still need to be elucidated.

Nutrition could be an important modifiable factor in the development and maintenance of bone mass and the prevention and treatment of osteoporosis. Roughly 80–90% of bone mineral content is comprised of calcium (Ca) and phosphorus. Protein is another crucial nutrient and is incorporated into the organic matrix of bone for collagen formation, upon which mineralization occurs (Baron, 1999). In addition, protein seems to be involved in regulation of Ca absorption (Kerstetter *et al*, 1998). Although Ca has been studied most extensively, other minerals such as magnesium, fluoride, zinc, copper, iron, selenium and vitamins D, A, C, K and folate are required for normal bone metabolism. Total energy consumption, fat, carbohydrates and fiber intake, and the electrolytes, sodium and potassium, may also, for different reasons, affect bone health (Ilich & Kerstetter, 2000). Many nutrients are co-dependent and at the same time under the influence of genetic and hormonal factors and/or in reciprocal interaction with various lifestyle modifiers. Due to the complexity of these interactions and to a dominant influence of biological factors, the effect of nutrients might be masked and hard to distinguish. It is also important to note that some of the statements about the potential role of micronutrients (minerals and vitamins) in bone health are either based on studies in animals or just theoretical presumptions, not actually tested and proven in human studies. All these are probable reasons why many studies have controversial or inconsistent findings regarding the contribution of a single or a group of nutrients in bone health.

This controversy is particularly present in the observational studies and cross-sectional assessments, which capture just the association between a presumed causal factor and a variable of interest. In addition, studies that evaluate nutrient relationships suffer from a weakness inherent in the methods for data collection and analysis. Regardless of what screening tool is used (dietary histories, dietary records, food frequencies, to name just a few), the subjective errors in self-reporting, changes in nutrient intake over time, and limitations of nutritional data bases used for calculation are, more or less, always present. These flaws have been addressed earlier (Heaney, 1997; Ilich *et al*, 1998). Despite these obvious disadvantages, a rather reliable trend in nutrient intakes could still be gathered and useful information obtained from cross-sectional studies with a large enough number of participants to provide for statistical power. In addition, in many instances, this is the only way to obtain information about possible health-related associations with nutrients, generate hypotheses for testing in stronger designs, and/or identify potentially harmful factors that may be unethical to test otherwise.

Therefore, our specific goal was to examine whether it is possible to establish and show a relationship between various macro- and micro-nutrients and bone mass of

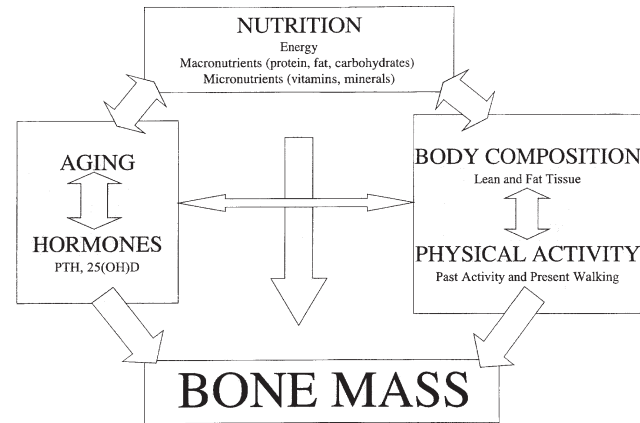


Figure 1 Path diagram with possible interactions among bone mass and various biological, and lifestyle variables examined in this study. The arrows indicate direction of the interaction.

several skeletal sites, despite the strong effect of biological factors. Nutrients considered were those either scientifically proven or theoretically presumed as capable of affecting bone health. Many of the likely confounding variables including age, body composition (lean and fat tissue), energy intake and hormones critical in bone metabolism, as well as present walking and past activity (measures of physical activity), were evaluated as well. Figure 1 presents a path diagram of possible interactions among bone mass and variables discussed in this report. Our hypothesis was that the model based on a thorough assessment of nutrients and careful statistical analysis could provide useful information about their relationship with bones, despite the strong influence and/or possible masking of biological factors.

Methods

Study design and subjects

This was a cross-sectional study in 136 generally healthy Caucasian women, 68.7 ± 7.1 y (mean \pm s.d.), each at least 5 y postmenopausal and recruited as part of a larger, longitudinal study. All variables discussed in this report were assessed during the first visit. Inclusion criteria consisted of subjects being able to live independently, non-smokers, free of chronic disease (kidney stones, hypertension, diabetes, severe osteoporosis, cancer) and not taking diuretics or medications known to affect bone metabolism, including but not limited to hormone replacement therapy, bisphosphonates, corticosteroids, insulin or anticonvulsants for the past 3 months. The subjects were recruited through senior centers and newspaper advertisements throughout the eastern part of Connecticut and came from a variety of socio-economic backgrounds. The study protocol was approved by the Human Subjects Review Board at the University of Connecticut and an informed consent was signed by each participant.

Anthropometry body composition and bone densitometry

Weight (WT) in kg and standing height (HT) in cm were recorded in light, indoor clothing without shoes. Body mass index (BMI; kg/m²) was calculated to screen for obesity. Bone mineral density (BMD) was measured by dual X-ray absorptiometry (DXA) with a Lunar DPX-MD instrument (GE Medical Systems, Madison, WI, USA) using specialized software for different skeletal regions, as described earlier (Ilich *et al*, 2000). The measured skeletal sites were: whole body (which yields the analysis of body composition—lean and fat tissue), lumbar spine (antero-posterior), femur (neck, trochanter, Ward's triangle, shaft and total femur), forearm (ulna and radius at ultradistal and one-third distal region measured from the styloid process), and hand. Although hand BMD is not typically measured in the clinical setting, it was the skeletal site most sensitive to aging, as reported previously in this population (Brownbill & Ilich, 2002). The quality assurance of a densitometer was performed daily and *in vitro* and *in vivo* stability, as well as the coefficients of variation in our laboratory have been reported previously (Ilich *et al*, 2000).

In the evaluation of nutrients and their impact on BMD, it is important to select appropriate skeletal sites with respect to their utilization for the diagnostic purposes and/or ability to reflect either predominantly trabecular or cortical bone. These two types of bone tissue change differently with age, as well as with other environmental factors, and are present in different proportions in skeletal sites prone to osteoporotic fractures. For example, lumbar spine, trochanter of the femur and ultradistal forearm are characterized primarily by the trabecular bone tissue, while total body, other femoral regions and proximal forearm are characterized as containing more cortical bone tissue (Mundy, 1999). Although DXA cannot distinguish between cortical and trabecular bone, it is possible to obtain a general idea about the predominant character of one or another by measuring different skeletal sites.

Blood collection and analysis

Serum calcium, vitamin D and parathyroid hormone (PTH) are major factors involved in Ca homeostasis, bone metabolism, and the pathogenesis of age-related bone loss. When evaluating nutritional factors in relation to bones, particularly dietary Ca and vitamin D, it is important to examine their serum levels and detect possible deficiencies or abnormalities otherwise common in the elderly, eg low circulating vitamin D (Jacques *et al*, 1997) or hyperparathyroidism (Heaney, 1996). Therefore, we collected blood samples and analyzed them for serum Ca, 25-hydroxy vitamin D metabolite, 25(OH)D (indicator of vitamin D status), and PTH. Serum 25(OH)D was measured by a radiobinding assay with a sensitivity of 5 ng/ml, while the carboxy-terminal fragment of the PTH molecule was measured by Allegro radioimmunoassay with a sensitivity of 39 pg/ml, by Nichols Institute (San Juan Capistrano, CA, USA).

Dietary assessment

Dietary intake was assessed by a 3-day dietary record (2 week and 1 weekend day). A registered dietitian instructed participants individually how to complete the records and advised them to choose typical days for reporting. Food models and pictures were used for instruction. After the records were completed, the same dietitian followed up and rechecked every record with each participant, particularly about additional food or snacks consumed, portion sizes and ways of preparation. The same dietitian analyzed nutrient intake from the records using Food Processor[®] (ESHA Research, Salem, OR, USA). Mean daily intake, including total energy (kJ/day) and all other macro- and micronutrients, was calculated. The Ca/phosphorus and Ca/protein ratios were also calculated, as well as the nutrient densities with respect to energy intake (eg Ca/kJ) to correct for the different amounts of food consumed between bigger and smaller individuals. In addition to 3-day dietary records for the assessment of the present typical diet, participants also completed a food frequency questionnaire (NOF, 1998) for the assessment of typical Ca intake (Ca-FFQ) over a longer period of time (at least for a past year). This questionnaire was completed upon entering the study, within about a week prior to the dietary records, and under the supervision and instructions of the same dietitian.

Special emphasis was placed on estimating the frequency and amount of consumption of vitamin and/or mineral supplements. According to some recent surveys, about 30–40% of Americans over the age of 60y take vitamin and mineral supplements and that number is on the rise (Blendon *et al*, 2001; Freeman *et al*, 1998). In our study, more than two-thirds of participants were taking various supplements—most often one of the common multivitamin–mineral preparations. Excluding those women from the study would substantially reduce potentially eligible candidates. Therefore, the intake of supplements was carefully recorded and included in the nutrient analysis. Each participant was asked to either bring in the supplements and/or give their names, amount and frequency of intake. When only the name/brand of supplement was given, we checked for its nutrient content in supermarkets and health food stores. All nutrients from the supplements were calculated to a per day amount and added as a separate (supplement) column in the food analysis. Also, the amount of each nutrient taken as a supplement was added to the amount obtained from the 3 day dietary record analysis for that nutrient. Therefore, each nutrient was expressed as dietary, supplemental and/or total (diet + supplement).

With these extensive measures and effort implemented to properly collect the data, we are confident that our estimate of nutritional intake either from food or supplements was fairly reliable.

Physical activity assessment

Past physical activity and present walking were assessed using interview format with a modified version of the

Allied Dunbar National Fitness Survey for older adults (Fenton *et al*, 1994). We used this questionnaire in our previous studies in postmenopausal women and found it reliable and easy to complete (Ilich *et al*, 2002a). The questionnaire was filled in with the participants and with the help of a dietitian. Data collected included frequency and duration of each activity, as described previously (Ilich *et al*, 2002a).

Data analysis and calculation

All data are presented as mean \pm s.d. (unless otherwise noted), and analysis was performed using Data Desk[®] (Data Description Inc., Ithaca, NY, USA) and Statistica[™] (StatSoft Inc. Tulsa, OK, USA). Pearson's correlation coefficient, r (unadjusted), was calculated as a part of preliminary analysis to examine overall association among various variables and further analyses were pursued only with those nutrients that showed significant relationship. To examine the co-linear variables (eg lean body mass, WT, kcal, protein and Ca) and whether their association with bone mass was independent, the subgroup analyses were performed. The participants were stratified into groups below and above the median for each variable, and their simultaneous relationship with BMD was evaluated. For the evaluation of 25(OH)D and PTH the subjects were stratified into groups according to age (< 69.9 and ≥ 70 y) and season during which the blood was drawn. ANOVA, with *post-hoc* two-sample t -tests was conducted to determine the group differences.

Stepwise multiple regression models were utilized to assess relationships between nutrients and bone mineral density or content of various skeletal sites. Diagnostics and residual plots for these models were analyzed to examine for heteroskedasticity and/or non-normality. When appropriate, the nutritional variables were transformed or examined as a ratio (eg Ca/kJ), but in either case the results were similar. Therefore, the results are presented with the variables in their original forms. Partial regression to remove collinearity among predictors and Hadi's influence method with potential-residual-plots analyses to identify influential data points were performed (Velleman, 1997). The regression was performed in an interactive way and at each step the plots were generated to check if an extraordinary case had unduly influenced regression and if other linear regression assumptions were violated. Each model was controlled for age, body size, including lean and fat tissue, HT (for TBBMC, only), past physical activity, present walking and energy intake. Lean and fat tissue were better determinants for BMD of most of the skeletal sites than WT, while HT was significant only in TBBMC models, as reported previously (Ilich *et al*, 2002a). After each model was created, the potential-residual-plot of a model was analyzed and any influential data point was recorded as the '0/1 indicator' variable and forced into the model to isolate its influence (Velleman, 1997). This process improved the overall model and the significance of

explanatory nutrients. We found this interactive approach to building regression models more appropriate than computerized stepwise models built without human interaction and intelligent selection among variables.

The overall accepted level of significance was set at $P < 0.05$. However, since we measured several skeletal sites, our interpretation of the results from multiple regression models took into account those sites and how much each explanatory variable contributed to the variance of the model. For example, if $P < 0.05$ was found at three or more skeletal sites, and if the explanatory variable in question contributed 1% or more to the variance, it was taken as evidence that the variable was associated with the overall BMD. If $P < 0.05$ was found in only one or two sites, it was interpreted as a trend, and considered not strong enough to characterize that variable as being associated with bone mass.

Results

Bone mineral and nutritional status

Descriptive characteristics (mean \pm s.d.) of participants are presented in Table 1. Twenty-two subjects were overweight (BMI > 30), and 16 were taking non-diuretic anti-hypertensive medications. For the reasons of obtaining better generalizability and mimic the health profile of today's women, these subjects were not excluded. Forty-six subjects reported having mild to moderate osteoarthritis in their hands, knees and/or spine, diagnosed previously by their physicians. Their BMD was not different from those without osteoarthritis in any of the measured skeletal sites (Brownbill & Ilich, 2002). The computer-generated T -scores, comparing each individual's BMD with that of young, normal, adult women (reference population) and used for diagnosing osteoporosis by the World Health Organization (WHO, 1994), are also presented in Table 1 for the applicable skeletal sites. On average, BMD in our population was within the normal range and comparable to that of healthy postmenopausal women at a national level (Black *et al*, 1992). This was to be expected, as one of our exclusion criteria was presence of osteoporosis. In few individuals we did find one or two skeletal sites with osteoporotic changes (eg one or two vertebral bodies or regions of hip), but other sites were either within the normal range or just osteopenic.

Anthropometrics (HT, WT and/or BMI), and even more so body composition (lean and fat tissue), parameters showed strong positive association with BMD or BMC of various skeletal sites, while age and years since menopause showed negative when examined in simple correlation analyses. Therefore, in our further analyses in multiple regression models, we controlled for those variables, specifically for age, lean and fat tissue, which comprise WT, and HT in TBBMC, only.

Table 2 illustrates energy and nutrient intake from food, supplements, and total. The corresponding Dietary Reference Intakes (DRI) or other official recommendations in US for this age group are presented as well (Institute of

Table 1 Descriptive characteristics (mean \pm s.d.) for anthropometrics, body composition and bone variables with minimum and maximum and with T-scores of skeletal regions used for diagnosis of osteoporosis, $n=136$

Variable	Mean \pm s.d.	Min–Max	T-scores ^a
Age (y)	68.6 \pm 7.1	57.4–88.6	
YSM (y)	18.5 \pm 8.4	5–41	
WT (kg)	68.0 \pm 11.3	44.8–104.9	
HT (cm)	161.7 \pm 6.8	143.9–179.0	
BMI (kg/m ²)	26.0 \pm 3.8	17.2–38.0	
LBM (kg)	38.0 \pm 4.3	27.8–48.9	
TBF (kg)	26.1 \pm 7.7	7.3–50.2	
TBF (%)	40.0 \pm 6.2	15.7–54.1	
TBBMD (g/cm ²)	1.077 \pm 0.095	0.770–1.302	–0.6 \pm 1.2
TBBMC (g)	2291 \pm 397.3	1281–3338	N/A
Lumbar Spine BMD (g/cm ²)	1.075 \pm 0.196	0.786–1.900	–1.1 \pm 1.6
Total femur BMD (g/cm ²)	0.857 \pm 0.132	0.581–1.269	–1.2 \pm 1.1
Forearm UD BMD (g/cm ²)	0.275 \pm 0.048	0.159–0.389	N/A
Forearm $\frac{1}{3}$ BMD (g/cm ²)	0.591 \pm 0.079	0.372–0.789	–1.9 \pm 1.1
Hand BMD (g/cm ²)	0.385 \pm 0.04	0.306–0.486	N/A

^aT-scores present World Health Organization criteria for diagnosing osteoporosis by measuring bone density. The bone density value measured in a patient is compared with the mean young adult value and the difference is expressed in standard deviation. According to the criteria, bone status in a patient is normal with T-score = 0 to –1, osteopenic with T-score = –1 to –2.5 and osteoporotic with T-score > 2.5 (WHO, 1994).

YSM, years since menopause; WT, weight; HT, height; BMI, body mass index; BMD, bone mineral density; LBM, lean body mass; TBF, total body fat; TBBMD, total body bone mineral density; TBBMC, total body bone mineral content; forearm UD BMD, BMD of the ultra-distal region of the forearm; and forearm $\frac{1}{3}$ BMD, BMD of one-third the distal region of the forearm (radius and ulna) measured from the styloid process.

Medicine, 1997, 1998, 2000, 2001; National Research Council, 1989). The basal metabolic rate (BMR), using Harris–Benedict equation (Grant & DeHoog, 1999), was calculated and reported energy intake (EI) and BMR ratio evaluated to assess for accuracy of reporting. Mean protein intake was about 16% of total EI and close to the recommendation of 1.0 g of protein/kg WT in older adults. Fat and carbohydrate intakes of 31 and 52%, respectively, of the total energy, and cholesterol of 218 mg/day, were in accordance with the dietary guidelines, while the dietary fiber intake was lower than the dietary goal of 22 g/day or more. With the exception of Ca, magnesium (Mg), potassium (K), vitamin D, vitamin K and folate, the levels of all other nutrients consumed with food were at or above the DRIs. However, since 76.5% of participants were taking some kind of vitamin/mineral supplement, average levels of all nutrients increased to at or above DRIs except K, vitamin D, and vitamin K, which remained below recommendations (Table 2).

The values for Ca intake assessed from food frequency questionnaire, as long-term typical and from 3-day dietary records as current typical intakes, were close: 834.0 \pm 364.8 and 872.8 \pm 364.8 mg/day, respectively with $r=0.634$, $P<0.0001$. The ratios of dietary Ca/protein (mg/g), Ca/P (mg/mg), and Ca/energy intake (mg/kcal) were 12.3, 0.8 (about half of the optimal ratio of 1.7) and 0.5, respectively.

Biochemical indices: serum Ca, vitamin D and PTH

The mean \pm s.d. values for serum Ca, 25(OH)D and PTH were all within normal range and normally distributed: 9.5 \pm 0.4 mg/dl (2.4 mmol/l), 21.1 \pm 5.1 ng/ml (52.8 \pm 12.8 nmol/l), and

175.5 \pm 46.2 pg/ml (61.9 \pm 16.3 pmol/l), respectively. The participants were stratified into groups according to season when the blood was drawn, as well as below and above 70 y, as that age was shown to be critical for the development of hyperparathyroidism and decrease in circulating 25(OH)D (Jacques *et al*, 1997; Riggs & Melton, 1986). The participants in the spring/summer groups were older, had higher Ca and vitamin D intakes ($P<0.05$), but there were no statistically significant differences in their serum 25(OH)D or PTH levels compared with those in the fall/winter groups. The difference in 25(OH)D (20.9 \pm 5.2 vs 21.5 \pm 5.1 ng/ml) and PTH (174.3 \pm 46.6 vs 177.8 \pm 45.9 pg/ml) across the two age groups was not statistically significant. There was also no significant relationship between 25(OH)D and PTH. Neither 25(OH)D nor PTH showed significant relationship with BMD or BMC of any of the skeletal sites analyzed in simple regression/correlation. Therefore, they were not pursued in further analyses.

Associations between nutrients and bone parameters

Each nutrient was examined as dietary (from food only), total (from food and supplements) and/or corrected for EI. There was a strong correlation among some of the nutrients, examined either as dietary or total. Some examples are between Ca and phosphorus ($r=0.825$), Ca and Mg ($r=0.618$), Ca and K ($r=0.635$), Ca and vitamin D ($r=0.621$), phosphorus and Mg ($r=0.756$), as well as protein and phosphorus ($r=0.718$), and protein and Ca ($r=0.607$), all with $P<0.0001$. Similarly, but in a lesser extent, other minerals from the diet—copper (Cu), iron (Fe), selenium (Se), zinc (Zn), sodium (Na) and vitamins C and K—showed positive

Table 2 Dietary, supplemental and total intake of nutrients per day (mean \pm s.d.), with appropriate recommendations (Institute of Medicine, 1997, 1998, 2000, 2001; National Research Council, 1989). Nutrients in bold are below recommendations

Nutrient	From food	Min-max	Supplements	Total (food + suppl)	% ^a	Recommendations
Energy Intake (kJ/day)	7068 \pm 1596	3336–11282				—
Energy Intake/BMR	1.3 \pm 0.3	0.7–2.2				—
Protein (g)	70.7 \pm 18.8	24.4–125.4				68.0 ^b
Protein (Percentage of energy)	16	—				—
Total fat (g)	57.9 \pm 23.9	16.8–138.5				56.4 ^b
Total fat (Percentage of energy)	30.9	—				30 or less ^b
Cholesterol (mg)	218.9 \pm 106.7	41.3–645.5				200 or less ^c
Carbohydrate (g)	221.8 \pm 55.6	101.3–399.8				—
Carbohydrate (Percentage of energy)	52.4	—				50 or more ^c
Dietary fiber (g)	18.1 \pm 6.1	6.7–50.5				22 or more ^c
Calcium (mg)	872.8 \pm 364.8	283.6–2203.9	722.7 \pm 490.8	1377.8 \pm 631.9	69.9	1200 ^d
Ca FFQ (mg)	834.0 \pm 364.8	250–1857	—	—	—	—
Phosphorus (mg)	1049.4 \pm 348.9	386.4–2313.9	105.4 \pm 52.3	1077.2 \pm 351.7	26.5	700 ^e
Magnesium (mg)	254.5 \pm 85.7	99.5–620.1	163.9 \pm 141.6	344.9 \pm 157.8	55.1	320 ^e
Zinc (mg)	8.9 \pm 5.7	2.6–41.2	18.3 \pm 9.0	19.1 \pm 13.5	55.1	8 ^e
Iron (mg)	13.4 \pm 4.8	6.8–37.9	16.4 \pm 6.8	18.6 \pm 10.4	31.6	8 ^e
Selenium (μ g)	69.8 \pm 26.9	15.8–142.8	30.0 \pm 32.8	85.7 \pm 38.2	52.9	55 ^e
Copper (mg)	1.0 \pm 0.3	0.4–1.9	1.9 \pm 0.6	2.0 \pm 1.1	52.2	0.9 ^e
Sodium (mg)	2367.7 \pm 769.7	559.5–5159.9	—	—	—	500 ^g
Potassium (mg)	2747.7 \pm 846.9	1117.8–7053.8	67.4 \pm 22.1	2779.9 \pm 848.3	47.8	3500 ^h
Folate (μ g)	289.2 \pm 119.3	90.1–713.9	446.8 \pm 175.5	565.1 \pm 294.7	61.8	400 ^e
Vitamin A (RE)	893 \pm 580	102–2748	517 \pm 225	1197 \pm 657	58.8	700 ^e
Vitamin C (mg)	127.5 \pm 70.1	23.0–401.5	314.5 \pm 354.4	324.1 \pm 335.8	62.5	75 ^e
Vitamin D (IU)	185.9 \pm 128.9	5.9–695.7	500.6 \pm 208.7	495.1 \pm 317.7	61.8	600 ^d
Vitamin K (μ g)	73.5 \pm 85.4	2.4–531.4	19.5 \pm 15.7	82.5 \pm 85.9	46.3	90 ^d

BMR, basal metabolic rate. ^aPercentage of participants taking supplements. ^bRecommendation for protein (1.0 g/kg weight), and fat (30% of total energy). ^cDietary goal. ^dAdequate intakes (AI). ^eRecommended dietary allowances (RDA). ^fEstimated safe and adequate intake. ^gEstimated minimum requirements. ^hDesirable intake.

relationships with Ca and with each other. As expected, most of the nutrients (except vitamins A, K and C) showed relatively high correlation with EI, with r ranging from 0.217 for Zn to above 0.700 for fat and carbohydrates. The co-linearity among nutrients did not change much when adjusted either for energy or protein intake. EI was also significantly associated with WT ($r=0.259$, $P=0.0023$) and lean body mass (LBM; $r=0.313$, $P=0.0002$), but not with total body fat (TBF; $r=0.156$, $P=0.0712$).

The simple association between BMD and nutrients that might possibly have some effect on bone (either scientifically proven, metabolically plausible, or theoretically presumed) was examined by calculating Pearson's correlation coefficients (unadjusted). The highest correlation coefficients were for energy, Ca and protein for most of the skeletal sites. Ca, assessed by food frequency as an indicator of a long-term typical Ca intake, was significantly positively related to sites in femur (strongest with trochanter, $r=0.207$, $P=0.0158$) and spine (L2–L4, $r=0.201$, $P=0.0192$). This relationship was also present when computer generated T -scores for hip and spine BMD, otherwise used for the clinical diagnosis of osteoporosis and derived from the young normal adult women (WHO, 1994), were regressed on Ca-FFQ. Other nutrients (either alone or adjusted for energy)

that showed weak, but statistically significant relationship with bone mass were: phosphorus, Mg, Fe, Se, Zn, Cu, K, Na, vitamins C, D, K and folate (r between 0.170 and 0.200; $P<0.05$). In these analyses total nutrients had in some instances lower statistical significance, but never higher than just nutrients from food.

Energy, protein and Ca consistently showed the highest correlations with bone mass of almost all measured skeletal sites. Also, when these nutrients were stratified in groups below and above the median intake, a lower BMD and/or BMC were associated with the below-median intakes. However, since there was interrelationship among some variables, eg energy, protein, Ca, LBM, WT, we conducted a subgroup analyses by categorizing subjects into groups below and above median values and simultaneously examining the interaction of the sets of two variables on each skeletal site. Figure 2 presents energy and Ca interaction on BMD of Ward's triangle and TBBMD. Higher Ca intake was associated with higher BMD irrespective of energy and vice versa, implying independent effect of each. This was true for all other skeletal sites. A similar relationship existed between protein, Ca and BMD of various skeletal sites. Figure 3 presents the interaction of protein and Ca and Figure 4 that of protein and energy on BMD of Ward's triangle. Although the latter relationship was

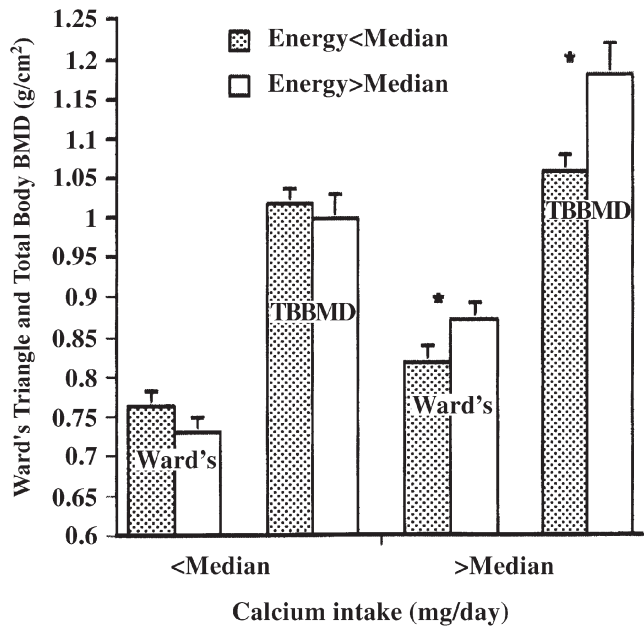


Figure 2 Interaction between energy and calcium on bone mineral density (BMD) of Ward's triangle and total body. Values, mean \pm s.e. (g/cm^2), are adjusted for below and above median of energy (7089 kJ/day) and calcium (750 mg/day) intake, and controlled for age, total body fat and lean tissue, past physical activity and present walking. *Both set of bars are statistically different from the corresponding groups at below median for energy and calcium intake, $P < 0.05$. Note the independent effect of both calcium and energy on BMD.

of a lesser magnitude, the trend still implied the independent effect of protein and energy. Table 3 shows energy and LBM interaction on BMD of various skeletal sites. There was a dominant effect of LBM and the added effect of energy on BMD. A similar pattern was seen between energy, body WT and BMD of various skeletal sites, although the magnitude of relationships was smaller, indicating that LBM had a higher influence on BMD than WT, but both effects were augmented by higher energy intake.

Multiple regression results

To examine closer the above relationships and to control for the potential confounders, stepwise regression models were constructed with BMD and/or BMC of various skeletal sites as dependent variables. The selected models are presented in Table 4, with the coefficients of determination, r^2 (adjusted), for the models with confounding variables only, and with those after each explanatory variable had been added. The slope coefficients, t -ratios and P -values are presented as well.

We considered that the relationship between BMD and a particular nutrient was biologically meaningful if that nutrient showed statistically significant association with BMD in three or more skeletal sites and contributed to more

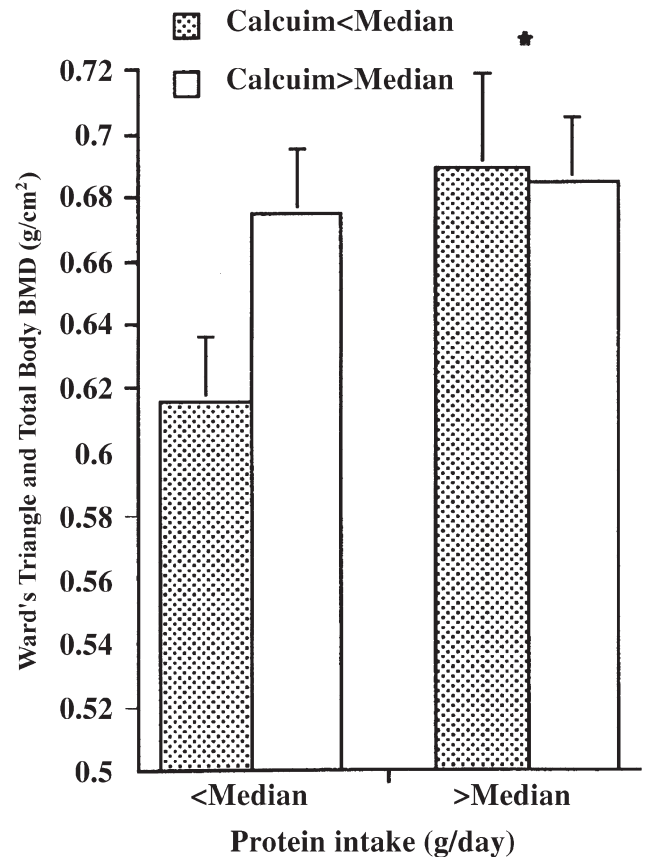


Figure 3 Interaction between protein and calcium on bone mineral density (BMD) of Ward's triangle. Values, mean \pm s.e. (g/cm^2), are adjusted for below and above median of protein (68.9 g/day) and calcium (750 mg/day) intake, and controlled for age, total body fat and lean tissue, past physical activity, and present walking. *Both bars statistically different from the group at below median for energy and calcium intake, $P < 0.05$. Note the independent effect of both calcium and protein on BMD, and augmented by the higher intakes of both.

than 1% of the variance when added to a model. These nutrients were as follows: Ca, either assessed by FFQ or as a total from 3-day dietary records, was a significant predictor for TBBMD, hand and femoral Ward's triangle and shaft regions; protein was a significant predictor in the models with TBBMD, TBBMC, Ward's, and hand; Mg was a predictor in the models with trochanter, neck, shaft and total femur; Zn was a predictor in models with trochanter, shaft, total femur and ultradistal forearm; vitamin C was a predictor in the models with total femur, trochanter, Ward's and shaft (Table 4). Vitamin K and folate and minerals Cu, Fe, Se, K and Na showed significant positive association (or in the case of Na, negative) with one, or at most, two skeletal sites. This was considered to be a trend, however it was not strong enough to warrant their inclusion in Table 4.

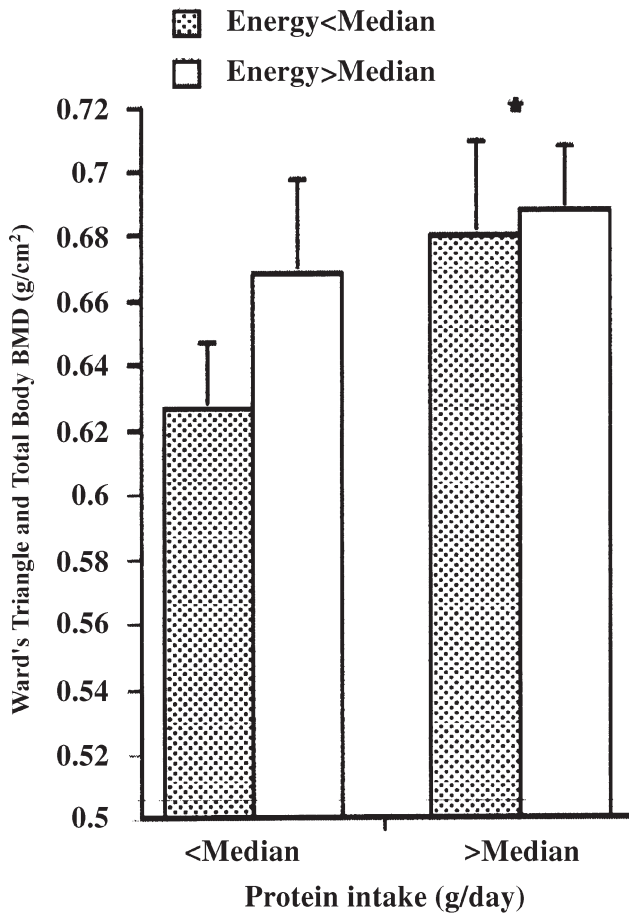


Figure 4 Interaction between protein and energy on bone mineral density (BMD) of Ward's triangle. Values, mean \pm s.e. (g/cm²), are adjusted for below and above median of protein (68.9 g/day) and energy (7089 kJ/day) intake and controlled for age, total body fat and lean tissue, past physical activity, and present walking. * Both bars statistically different from the group at below median for energy intake, $P < 0.05$. Note the independent effect of both protein and energy on BMD.

Discussion

Overall

The goal of this cross-sectional study was to assess bone and nutritional status in a group of healthy postmenopausal women and discern any possible relationship between nutrients and bone mass of clinically important skeletal sites. The study is distinct with respect to: careful nutritional assessments with large number of nutrients evaluated in relation to bone mass; number of measured skeletal sites, identifying predominantly trabecular and/or cortical bone tissue; subgroup analyses identifying independent influences of LBM, energy, protein and Ca on BMD of various skeletal sites; creation of interactive multiple regression models that enabled more certain identification of nutrients related to various skeletal sites; and precise identification of and control for confounding variables.

In simple correlation analyses we found a weak but positive relationship between several nutrients and BMD/BMC of various skeletal sites. These nutrients were then pursued further in multiple regression analyses. Our subgroup analyses, conducted to overcome the effect of co-linearity among some of the nutrients and components of body size on BMD, showed that energy, protein and Ca were independently related to BMD (Figures 2–4). In addition, energy augmented the effect of LBM and/or WT (Table 3). Nutrients that showed statistical significance in multiple regression models and were related to three or more skeletal sites were protein, Ca, Mg, Zn, and vitamin C (Table 4). Other nutrients, significant in the models, but related to only one or two skeletal sites, were vitamin K, folate and minerals Fe, Cu, Se, P and Na (negative relation with hand BMD). The nutrients with borderline significance ($P < 0.1$ but > 0.05) related to one or two skeletal sites were potassium and vitamin D. The association of these nutrients with bone mass was considered to be without biological importance in this context.

It also needs to be noted that, in general, many of our participants seemed to be health-conscious women, taking care of their diets and lifestyles (although not always following the scientific recommendations, but rather media hypes), which might have brought some bias into the overall generalizability of our data.

Confounding factors

Although some previous studies showed that 25(OH)D decreases (Jacques *et al*, 1997) and PTH increases with age (Riggs & Melton, 1986), this was not noted in our study. We did not observe a significant relationship between BMD and 25(OH)D and/or PTH analyzed in simple regression. Moreover, the conclusions from the cross-sectional assessment presented here, were confirmed further in our 1y follow-up of the same population (Ilich *et al*, 2002b). Despite that the mean dietary vitamin D intake was below the current recommendations, even with the supplements, serum 25(OH)D (indicator of vitamin D status) was within the normal range. Estimating accurately vitamin D intake is hard, as its fortification in milk (main dietary source in US) is unreliable, resulting in variable amounts per serving. This may explain (in addition to the unknown amount formed in the skin) why we only observed a trend and not a statistically significant relationship between vitamin D intake and serum 25(OH)D levels and how the subjects had normal vitamin D status despite the apparently low intake. Some other researchers also found poor correlation between the two (Thomas *et al*, 1998). Based on the serum indices of Ca homeostasis, our subjects appeared to be in good condition, without signs of hypovitaminosis D or secondary hyperparathyroidism, each of which could interfere with bone status.

One of the biological factors researchers unequivocally agree upon as being influential on bones is body weight. Since WT includes lean and fat tissue (as well as the bone itself), it is not always clear which one has a more dominant

Table 3 Energy and lean body mass (LBM) interaction with BMD of various skeletal sites

Energy intake	Lean body mass		
	< Median	> Median	
Total body BMD			
< Median	A 1.039 ± 0.09	C 1.087 ± 0.07	A vs C, <i>P</i> = 0.0183; A vs D, <i>P</i> < 0.0001
> Median	B 1.036 ± 0.08	D 1.135 ± 0.09	B vs C, <i>P</i> = 0.0174; B vs D, <i>P</i> < 0.0001 C vs D, <i>P</i> = 0.0242
Femoral neck BMD			
< Median	A 0.761 ± 0.10	C 0.817 ± 0.08	A vs C <i>P</i> = 0.0145; A vs D, <i>P</i> < 0.0001
> Median	B 0.728 ± 0.09	D 0.871 ± 0.12	B vs C, <i>P</i> = 0.0004; B vs D, <i>P</i> < 0.0001 C vs D, <i>P</i> = 0.0315
Total femur BMD			
< Median	A 0.813 ± 0.10	C 0.864 ± 0.10	A vs C <i>P</i> < 0.0483; A vs D, <i>P</i> < 0.0001
> Median	B 0.794 ± 0.11	D 0.932 ± 0.15	B vs C, <i>P</i> = 0.0183; B vs D, <i>P</i> < 0.0001 C vs D, <i>P</i> = 0.0285
Lumbar spine (L2–L4) BMD			
< Median	A 1.017 ± 0.14	C 1.058 ± 0.12	A vs D, <i>P</i> = 0.0007; B vs D, <i>P</i> = 0.0005
> Median	B 1.000 ± 0.14	D 1.179 ± 0.25	C vs D, <i>P</i> = 0.0112
Forearm $\frac{1}{3}$ region BMD			
< Median	A 0.564 ± 0.08	C 0.598 ± 0.08	A vs C, <i>P</i> = 0.0785; A vs D, <i>P</i> = 0.0002
> Median	B 0.561 ± 0.07	D 0.629 ± 0.07	B vs C, <i>P</i> = 0.0770; B vs D, <i>P</i> = 0.0006

Values, mean ± s.d. (g/cm²), of total body, femoral neck, total femur, lumbar spine, and forearm (both ulna and radius) are adjusted below and above median for energy intake (7089 kJ/day) and LBM (37.7 kg) and controlled for age, total body fat, past physical activity, and present walking. Note the dominant effect of LBM and the augmented influence of energy only in the > median LBM group (C vs D).

role on bone and under what circumstances. In our previous study in women of wide age range (22–85 y) we found that both fat and lean tissue affect bone mass, with the stronger influence of LBM for most of the skeletal sites. The dominance of LBM was even stronger than that of WT itself (Ilich *et al*, 2002a). This is in accordance with observations made by several other authors (Lindsay *et al*, 1992; Takada *et al*, 1997). Based on these reports and our own findings, we included both lean and fat tissue into the regression models, which in each case contributed more to the overall variance than WT and HT or BMI.

The evaluation of bone health and its relationship with certain nutrients should be performed in concert with the overall, or at least some aspects of physical activity exerted by subjects, since it might affect bones directly and indirectly (via muscle and fat tissue). That is particularly true for the less strenuous everyday activities as part of a regular life style (Coupland *et al*, 1999), as well as past activity (Greendale *et al*, 1995), especially in older individuals. Since we found earlier a strong relationship between past physical activity and bone mass in part of this cohort (Ilich *et al*, 2002a), we included past physical activity, as well as the modes of present walking, as important variables to control for in evaluating bone status.

Calcium, energy, and protein in relation to bone mass

We used 3 day dietary records for the assessment of the typical recent intake of nutrients and, additionally for Ca, the food frequency method to assess the typical longer time intake. The average Ca intake assessed by the two methods matched closely (*r* = 0.634, *P* < 0.0001), indicating that intake of Ca was relatively stable over time. In addition, we

separately examined the influence of each nutrient from food and total (supplements and food) on bones. The typical amount of a nutrient consumed from food probably reflects more constant and regular intake, and that is particularly true for Ca. However, with today's health-consciousness and influence of dietary and health messages, many people are taking various supplements. This phenomenon is a relatively new one, at least in the magnitude we noticed (over 70% of participants) and the amount of any supplement may vary, depending on the regularity and duration of supplement consumption. Nevertheless, this supplemental intake needs to be taken into consideration. Therefore, each nutrient was examined separately: from food and total, and the form showing a stronger relationship with BMD was noted as such.

In all our analyses (correlational, subgroup, or multiple regressions), Ca, protein and energy showed the strongest and independent relationships with BMD of different skeletal sites. There is an abundance of studies examining the effects of Ca on bone in populations of all age groups. Heaney recently gave a monumental review of the available evidence regarding the relationship between Ca and bone mass in women of all age groups (Heaney, 2000). Overall evidence from randomized, placebo-controlled clinical trials with Ca supplementation suggests a positive relationship between bone mass and calcium in pre- and late postmenopausal women and a smaller effect in women around the onset of menopause. However, it needs to be emphasized that the effect of Ca on bones is relatively weakly supported by cross-sectional and observational studies, particularly in older population. Therefore, the results from this study contribute to the clarification of this relatively obscure aspect of the Ca–bone relationship.

Table 4 Stepwise regression models for different bone variables as dependent ones. The models were controlled for age, lean body mass, total body fat, and height (in TBBMC model, only), as well as for the past physical activity, present mode of walking, and energy intake

Dependent variable	r ² _(adj) for a model	Explanatory variables	Coefficient	t-ratio	P-level	r ² _(adj) with added variable
TBBMD	50.0	Calcium	4.7×10 ⁻⁵	2.58	0.0111	52.5
		Protein	1.0×10 ⁻³	2.24	0.0268	51.8
TBBMC	72.5	Protein	2.9	2.17	0.0325	73.4
Femoral neck BMD	48.3	Magnesium total ^a	9.4×10 ⁻⁵	2.01	0.0464	49.6
Ward's BMD	44.9	Protein	1.4×10 ⁻³	2.34	0.0210	47.0
		Calcium	5.7×10 ⁻⁵	2.32	0.0224	47.0
		Vitamin C	2.6×10 ⁻⁴	1.97	0.0515	46.3
Trochanter BMD	56.9	Zinc total	1.6×10 ⁻³	2.70	0.0080	59.3
		Magnesium total	1.2×10 ⁻⁴	2.45	0.0160	58.9
		Vitamin C	2.4×10 ⁻⁴	2.01	0.0473	58.1
Shaft BMD	47.4	Magnesium total	1.7×10 ⁻⁴	2.59	0.0109	50.0
		Calcium total	3.9×10 ⁻⁵	2.25	0.0262	49.3
		Vitamin C	3.6×10 ⁻⁴	2.24	0.0270	49.3
		Zinc Total	1.8×10 ⁻³	2.26	0.0261	49.3
Total femur BMD	53.8	Magnesium total	1.4×10 ⁻⁴	2.88	0.0049	56.7
		Vitamin C	3.2×10 ⁻⁴	2.55	0.0122	56.0
		Zinc Total	1.6×10 ⁻³	2.36	0.0200	55.6
Forearm UD-BMD	46.5	Zinc	1.3×10 ⁻³	2.06	0.0422	48.0
Hand BMD	49.7	Calcium	2.0×10 ⁻⁵	2.86	0.0051	52.7
		Protein	4.1×10 ⁻⁴	2.35	0.0206	51.6

TBBMD, total body bone mineral density; TBBMC, total body bone mineral content; BMD, bone mineral density; forearm UD-BMD, BMD of ultradistal region of the forearm (both ulna and radius) measured from the styloid process.

^aTotal refers to the nutrient from food and supplements vs just from food.

The nutrients in each model are listed in descending order of their contribution to the overall variance.

Obviously, when examining nutritional factors, energy intake should be taken into account since it also determines the amount of most other nutrients. Generally noted, the positive effect of excess EI, resulting in overweight, on BMD may be due to weight-bearing forces exerted on the skeleton (Harris & Dawson-Hughes, 1996). Likewise, moderate weight loss often results in some bone loss (Svendson *et al*, 1993). However, it is much harder to distinguish between the effects of energy *per se* and that of either increased or decreased weight as a result of over- or under-consumption. In our study, EI was related to BMD of all skeletal sites in simple correlation, as well as in subgroup analyses, augmenting the effect of muscle (Table 3) and WT. It was therefore included in the multiple regression models as a confounder.

It is well established that protein increases urinary Ca excretion (Kerstetter & Allen, 1994), leading to a speculation that with a higher protein intake the bone loss will increase. However, the epidemiological and clinical data addressing this hypothesis are controversial (Cooper *et al*, 1996; Feskanich *et al*, 1996; Meyer *et al*, 1997). Conversely, there is evidence that a low-protein diet might decrease Ca absorption. Kerstetter *et al* showed that short-term intake of a moderately low-protein (0.7 g/kg) diet decreased urinary Ca excretion and was accompanied by secondary hyperparathyroidism (as a consequence of reduced Ca absorption), while the levels of ≥ 0.9 g/kg with the same intake of Ca (800 mg/day) did not interfere with Ca absorption in healthy young women (Kerstetter *et al*, 1997, 2000a). Findings from

the epidemiological studies imply that a low-protein diet may adversely affect bone and significantly contribute to reduced BMD (Hannan *et al*, 2000; Kerstetter *et al*, 2000b). Such trends were observed in the present study. Although the average protein intake of 70.7 ± 18.8 g/day in our cohort was close to the recommendations of 68 g/day (based on 1.0 g/kg), it was still possible to detect significant difference in BMD of various skeletal sites between groups divided by median intake (68.9 g/day). This might suggest that recommendations for protein in elderly women should be higher.

Multiple regression results

In our multiple regression models some nutrients emerged as statistically significant for several skeletal sites, contributing to more than 1% of the variance in the model (Table 4). For example, Ca, either from food or as total, was significantly associated with TBBMD, femoral Ward's and shaft, proximal forearm and hand, all predominantly cortical bone. A similar situation was found with protein (TBBMD, TBBMC, Ward's, hand), Mg (neck, Ward's, shaft, total hip), and vitamin C. It seems that the effect of these nutrients is systemic. This fits the current understanding of their action, since all except vitamin C are involved in Ca homeostasis and regulation of calcitropic hormones (Mundy, 1999), thereby affecting cortical bone more than trabecular.

It is known that Mg deficiency alters Ca metabolism resulting in hypocalcemia, vitamin D abnormalities, and neuromus-

cular hyperexcitability, probably due to the impaired PTH secretion (Rude, 1998). Although the national and other surveys consistently show low Mg intakes among females of all age groups (FASEB, 1995; Ilich *et al*, 1999), its role in osteoporosis is poorly understood, probably because of the lack of well-controlled clinical trials. The epidemiological study from the Framingham cohort in elderly men and women, with cross-sectional and longitudinal components, showed positive effects of Mg and K, as part of a diet rich in fruit and vegetables on BMD (Tucker *et al*, 1999). Similarly, New *et al* were the first to show in their studies that nutrients rich in fruit and vegetables have beneficial effects on bone, probably due to the alkaline ash these foods create, confirming the hypothesis of the role of bone in acid–base balance regulation (New *et al*, 1997, 2000). Our study, although much smaller and of a cross-sectional nature, is pointing in the same direction.

Vitamin C is required for collagen crosslinking (Combs, 1998) and along with other antioxidant vitamins may serve to protect the skeleton from oxidative stress, especially from smoking (Melhus *et al*, 1999). Two recent population-based studies showed that use of long-term vitamin C supplements was beneficial for BMD in various skeletal sites (Leveille *et al*, 1997) and the effect was augmented by estrogen and Ca supplements use (Morton *et al*, 2001) in postmenopausal women. In our study, the stronger influence was seen with the vitamin C from food only than total (food and supplement), probably because the supplemental intake is not regular and/or of longer duration. This study, showing clearly the relationship between vitamin C and BMD of several skeletal sites indicates its importance in bone health, previously just speculated about and proven mostly in animals.

In our evaluation, Zn, Cu and Fe were associated mostly with trabecular bone (spine, trochanter, ultradistal forearm), indicating that their effect is regulated by osteotropic cytokines (Mundy, 1999), and therefore probably even harder to detect. The significant association was found mostly in one skeletal site (except for Zn), and therefore considered not clinically important. It is worth noting, however, that both Zn and Cu play a role in connective tissue metabolism, acting as cofactors for several enzymes necessary for bone mineralization and formation of collagenous structure of bone (Beattie & Avenell, 1992). Iron may play an important role in bone formation acting as a cofactor for enzymes involved in collagen synthesis (Prockop, 1971). A study by Medeiros *et al*, showed that bone-breaking strength was lower in Fe deficient rats, suggesting that inadequate Fe may induce bone fragility (Medeiros *et al*, 1997). We recently examined the relationship between bone mass and ferritin (as an indicator of Fe status) in a 4y clinical trial of Ca supplementation in adolescent girls (Ilich-Ernst *et al*, 1998). There was a trend for a positive association between serum ferritin and forearm BMD at baseline and the total body BMD in year 4 in the placebo group of the study. The positive association between bone mass and dietary iron in the present study adds to the above trends. However, further

studies are necessary, particularly in people who are Fe-deficient, to bring more insight into this relationship.

In summary, this study in elderly women, although of a cross-sectional nature and taking into account powerful biological factors like age, body composition and calcitropic hormones, as well as physical activity, was able to discern the individual relationship between energy, protein, Ca and few other micronutrients with bones. Based on these results, we could speculate that diet rich in protein and Ca, as well as Mg, Zn and vitamin C, is crucial for bones and that low intakes of these (typically present in elderly), particularly if chronic and co-existing, may predispose to lower bone mass. However, the exact involvement of these nutrients, their interaction and co-linearity, and their clinical significance in bone health in elderly need to be further elucidated. Understanding relationships among nutrients, not limited just to calcium and vitamin D, but others that have not been investigated to such extent, is an important step toward identifying preventive methods for age-related bone loss.

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