# Dietary Protein Supplementation Increases Peak Bone Mass Acquisition in Energy-Restricted Growing Rats

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**ABSTRACT:** Peak bone mass is a major determinant of osteoporosis pathogenesis during aging. Respective influences of energy and protein supplies on skeletal growth remains unclear. We investigated the effect of a 5-mo dietary restriction on bone status in young rats randomized into six groups ($n = 10$ per group). Control animals were fed a diet containing a normal (13%) (C-NP) or a high-protein content (26%) (C-HP). The other groups received a 40% protein energy-restricted diet (PER-NP and PER-HP) or a 40% energy-restricted diet (ER-NP and ER-HP). High-protein intake did not modulate bone acquisition, although a metabolic acidosis was induced and calcium retention impaired. PER and ER diets were associated with a decrease in femoral bone mineral density. The compensation for protein intake in energy-restricted conditions induced a bone sparing effect. Plasma osteocalcin (OC) and urinary deoxypyridinoline (DPD) assays revealed a decreased OC/DPD ratio in restricted rats compared with C animals, which was far more reduced in PER than in ER groups. Circulating IGF-1 levels were lowered by dietary restrictions. In conclusion, both energy and protein deficiencies may contribute to impairment in peak bone mass acquisition, which may affect skeleton strength and potentially render individuals more susceptible to osteoporosis. (*Pediatr Res* 66: 513–518, 2009)

The amount of bone accumulated at the end of skeletal growth is a major determinant of osteoporosis development and fracture risk during aging. Even if genetics seem to account for most of the variance of bone mass, nutritional intakes have been identified as critical factors impacting skeletal modeling and maturation.

In particular, alterations in energy intake have been demonstrated to impair bone quality and strength in rodent models (1–5). Lamothe *et al.* (1) and Mardon *et al.* (2) reported that energy restriction adversely affected bone mineral content (BMC), bone mineral density (BMD), and mechanical properties in old rats, despite a micronutrient compensation. Nevertheless, the impact of energy restriction on bone was suggested to be modulated by the age at onset of restriction (5,6). To date, no clear consensus regarding the effects of energy restriction on peak bone mass acquisition has been established.

Protein intake is widely linked to bone growth, hence to bone strength (7). Indeed, collagen is the major constituent of bone organic matrix, and noncollagenic proteins are involved in the regulation of the mineralization process. Dietary proteins provide the essential amino acids necessary for new matrix synthesis and may modulate circulating IGF-1 levels as well, an osteotrophic growth factor (8). In growing animals, protein deficiency was demonstrated to lead to decreased bone status (9). Ammann *et al.* (10) also reported that a 2.5% casein diet during 16 wk in 5-mo-old rats was associated with bone loss.

Consequently, one would expect high-protein intake to promote bone health. However, other reports suggest that such a diet may alter calcium homeostasis in ways that could lead to bone loss (11,12). The metabolic acidosis, derived from sulfur amino acids catabolism, would also exert a direct stimulatory effect on bone resorption and an inhibitory action on matrix mineralization (13,14). Actually, the role of dietary protein on bone remains controversial.

In the current context of rising incidence of obesity in adolescents, low-energy diets associated with high-protein intakes are widely used in weight management programs. Thus, further investigations would be needed to completely define the role of high- or low- protein intake on both the acid-base metabolism and bone mass acquisition in young individuals and to determine the respective roles of energy and protein intakes on skeletal growth. This study, conducted in young rats, was designed to address two specific questions:

i. Does a high-protein diet (HP, 26%) induce beneficial or detrimental effects on bone acquisition compared with a normal-protein diet (NP, 13%)?
ii. What are the consequences on peak bone mass of a 5-mo period of protein and energy restriction (PER) and energy restriction (ER) alone compared with a control protein and energy supply (C)?

## MATERIALS AND METHODS

*Experimental design.* The study was approved by the Ethics Committee on animal experiments of Auvergne, in France.

Sixty Wistar male rats (10 wk old, average weight 394 g) were purchased from INRA (Institut National de la Recherche Agronomique, Clermont-Ferrand/Theix, France). The animals were housed individually in cages. They were subjected to 12-/12-h light/dark cycles and had free access to water. Their body weight and food intake were measured weekly.

**Abbreviations: BMD,** bone mineral density; **C,** treatment with control diet; **DPD,** deoxypyridinoline; **ER,** treatment with energy-restricted diet; **HP,** treatment with high-protein intake diet; **NP,** treatment with normal protein intake diet; **OC,** osteocalcin; **PER,** treatment with protein energy-restricted diet

**Table 1.** *Daily ration composition*

| Component (g/d) | C-NP | C-HP | PER-NP | PER-HP | ER-NP | ER-HP |
|---|---|---|---|---|---|---|
| Casein | 2.99 | 5.98 | 1.82 | 3.64 | 3.01 | 6.04 |
| Corn starch | 13.27 | 10.83 | 6.97 | 5.50 | 6.02 | 3.55 |
| Sucrose | 3.31 | 2.71 | 1.75 | 1.37 | 1.50 | 0.88 |
| Groundnut and rape oils | 1.39 | 1.39 | 1.40 | 1.40 | 1.40 | 1.40 |
| Cellulose | 0.92 | 0.92 | 0.92 | 0.92 | 0.92 | 0.92 |
| L-cystin | 0.045 | 0.090 | 0.027 | 0.055 | 0.045 | 0.091 |
| Choline bitartrate | 0.058 | 0.058 | 0.059 | 0.059 | 0.059 | 0.059 |
| Mineral mix* | 0.80 | 0.80 | 0.81 | 0.81 | 0.81 | 0.81 |
| Vitamin mix† | 0.23 | 0.23 | 0.23 | 0.23 | 0.23 | 0.23 |
| Total daily intake | 23 | 23 | 14 | 14 | 14 | 14 |

* Expressed in g/kg of mixture: $CaCO_3$, 178.5; $KH_2po_4$, 250; $K_3C_6H_5O_7$, 28.00; NaCl, 74; $K_2SO_4$, 46.60; MgO, 24.00; $FeC_6H_8O_7$, 6.06; $ZnCO_3$, 1.65; $Na_2SiO_3 \cdot 9H_2O$, 1.45; $MnCO_3$, 0.63; $CuCO_3$, 0.30; $CrKO_8S_2 \cdot 12H_2O$, 0.275; $H_3BO_3$, 0.0815; FNa, 0.0635; $NiCO_3$, 0.0318; LiCl, 0.0174; $Na_2SeO_4$, 0.01025; $KIO_3$, 0.010; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.00795; $NH_4VO_3$, 0.0066.

† Expressed in g/kg of mixture: nicotinic acid, 3.000; calcium pantothenate, 1.600; pyridoxine-HCL, 0.700; thiamine-HCL, 0.600; riboflavin, 0.6000; folic acid, 0.200; biotin, 0.020; cyanocobalamin, 2.500; all-rac-α-tocopheryl acetate (500 IU/g), 5.000; all-trans-retinyl palmitate (500000 IU/g), 0.800; cholecalciferol (400000 IU/g), 0.083; phylloquinone, 0.075.

At the beginning of the study, the rats were randomly allocated to one of six dietary groups ($n = 10$ rats/group). Two control groups (C) received a standard semipurified diet containing either a normal protein content (13%) (C-NP) or a high-protein content (26%) (C-HP). The *ad libitum* food intake of C groups was determined during 1 wk of adaptation (C-NP, 24.9 ± 0.8 g *versus* C-HP, 24.1 ± 0.7 g). Then, rats of the C groups were fed a measured amount of diet, corresponding to 90–95% of the average *ad libitum* intake of C-HP group, which had the lowest food intake to match caloric intake and to facilitate the study of healthy, nonobese C animals, as recommended by Pugh *et al.* (15). The mean energy intake of C groups was about 380 kJ/d (91 kcal/d) (Table 1). The protein and energy-restricted groups (PER-NP and PER-HP) were limited to 60% of the intake of the C groups [*i.e.* 7.8 and 15.6% protein, respectively and 228 kJ/d (55 kcal/d)]. The energy-restricted groups (ER-NP and ER-HP) received 40% less energy than the controls (*i.e.* 228 kJ/d), but had their protein intake maintained at the level of the C groups. All diets were purchased from INRA (Jouy en Josas, France). Diet-restricted rats were normalized to the C animals with respect to lipids, fibers, minerals, and vitamins intake. Thus, all the animals consumed the same amounts of calcium (around 58 mg Ca/d) and phosphorus (around 46 mg P/d).

After 5 mo, the rats were killed. One week before sacrifice, the rodents were transferred to individual metabolic cages. After a 3-d adaptation period, food intake was daily controlled, and urine and faeces were collected for 3 consecutive days. At the end of the experiment, the animals were fasted for 12 h and then killed. Blood samples were collected from the abdominal aorta, and plasma was frozen at −20°C until biochemical analysis. Femurs were cleaned from adjacent tissues. Left femurs were harvested in saline solution (9 g NaCl/L) and frozen (−20°C) until mechanical testing. Right femurs were kept in 80% alcohol until BMD measurements.

*Analysis.* Calcium and magnesium concentrations in food, urine, and faecal samples were determined by atomic absorption spectrophotometry (AA800, Perkin Elmer, Norwalk, CT), as described previously (16). Then, calcium retention (μmol/24 h), defined as [calcium intake − (urinary calcium excretion + faecal calcium excretion)] was calculated.

Urines were 400-fold diluted with milli-Q water. Phosphate, sulfate, and citrate concentrations were assayed by ionic chromatography (DX320, Dionex, Sunnydale, CA) (16).

IGF-1 concentrations were measured in serum samples using a two-site immunoenzymometric assay (OCTEIA Rat/Mouse IGF-1 kit, IDS, Paris, France). The sensitivity of the assay was 82 ng/mL. Intra and interassay variations were 5.7 and 10.7%, respectively.

Plasma osteocalcin (OC) concentrations were measured by immunoradiometric assay (Immunotopics, Inc., San Clemente, CA). The sensitivity was 0.01 ng/mL. The intra and interassay precisions were 2.0 and 4.5%, respectively. The urinary deoxypyridinoline (DPD) concentration (nmol/L) was determined by competitive RIA (Pyrilinks-D RIA kit, Metra Biosystems, Mountain View, CA). The sensitivity was 2 nmol/L. The intra and interassay precisions were 4 and 6%, respectively. DPD excretion rate (nmol/24 h) was calculated using the previous 24-h urine volumes.

BMD was assessed by dual-energy x-ray absorptiometry, using a Hologic QDR-4500 A x-ray bone densitometer (Hologic, Massy, France). Total femoral BMD (T-BMD), metaphyseal BMD (M-BMD), and diaphyseal BMD (D-BMD) were determined. Scans were cut and analyzed as follow: the first cut of the femur was performed at the upper quarter, and the next cut at the lower quarter. D-BMD, which contains a high percentage of cortical structure, corresponded to the density of the second and the third quarter of the femur. M-BMD, which mainly contains trabecular structure, was calculated as the mean of the upper and the lower quarters of the femur.

Femoral length and mean diaphyseal diameter were measured with a precision caliper (Mitutoyo, Shropshire, UK). The femoral failure load was then determined using a 3-point bending test, with a Universal Testing Machine (Instron 4501, Instron, Canton, MA). The two lower supports were separated by a 20-mm distance, and an upper crosshead roller (diameter, 6 mm) was applied in front of the middle of the bone at a speed of 0.5 mm/min until failure. Load (Newtons) at rupture was recorded.

*Statistical methods.* Results are expressed as the mean ± SEM and were analyzed with XLSTAT (Addinsoft, Paris, France). The BMD, biomechanical and histomorphometric variables were subjected to a two-way analysis of covariance (ANCOVA) with body weight as the covariate (17). Other parameters were analyzed using a two-way ANOVA, testing for any difference among groups. Thus, the main assessed effects were dietary restriction (C/PER/ER), protein intake (NP/HP), and their interaction (dietary restriction × protein intake). If a result was found significant ($p < 0.05$), the Student-Newman-Keuls multiple comparison test was used to determine specific differences between means. Linear regressions were also performed, to study internal correlations among variables, and the Pearson test was carried out to assess their significance.

## RESULTS

*Body weight.* In C-NP and C-HP groups, body weight increased by 28% during the experimental period (Fig. 1). PER and ER rats on the NP diets exhibited a constant body weight, whereas a significant increase was registered in PER-HP and ER-HP groups (+6 and +11%, respectively). At the end of the study, restricted animals exhibited a lower body weight ($p < 0.0001$) than controls.

*Daily calcium ration.* Calcium concentrations measured by atomic absorption spectrophotometry in the diets confirmed that all groups received an identical calcium ration (mg Ca/ daily ration) (C-NP, 59.0 ± 2.3; C-HP, 58.5 ± 2.7; PER-NP, 57.9 ± 1.9; PER-NP, 58.3; PER-HP, 2.6; ER-NP, 57.8 ± 2.0; ER-HP, 58.6 ± 2.5).

*Urine cation and anion excretion.* After 20 wk of experiment, urinary pH was acidic in all animals (Table 2). Significant differences were recorded among groups ($p < 0.0001$).
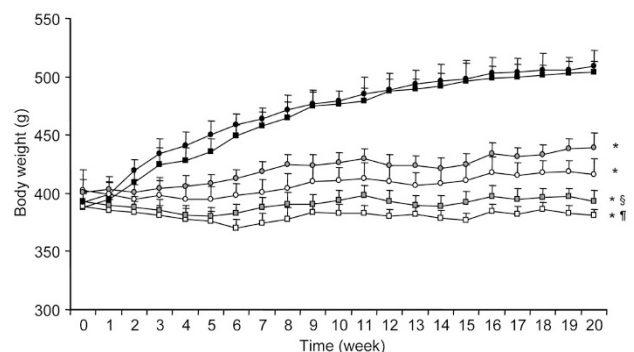


**Figure 1.** Body weight of rats fed control diets (C-NP (■) and C-HP (●)), protein caloric-restricted diets (PER-NP (□), PER-HP (○)), and caloric-restricted diets (ER-NP (■), ER-HP (●)) during the experimental period. *Significantly different from C groups ($p < 0.05$). §Significantly different from ER-HP group ($p < 0.05$). ¶Significantly different from PER-NP and PER-HP groups ($p < 0.05$).

**Table 2.** *Effect of dietary restrictions (C/PER/ER) and the level of protein intake (NP/HP) on urine pH, urinary calcium, magnesium, sulfate, phosphate and citrate excretions*

| | C | | PER | | ER | |
|---|---|---|---|---|---|---|
| | NP | HP | NP | HP | NP | HP |
| Urine pH | | | | | | |
| Mean | 6.05 | 5.59‡ | 6.13 | 5.80*‡ | 5.87*† | 5.58‡† |
| SEM | 0.01 | 0.03 | 0.04 | 0.06 | 0.03 | 0.03 |
| Calcium | | | | | | |
| ($\mu$mol/24 h) | | | | | | |
| Mean | 2.06 | 14.71‡ | 0.95* | 5.93*‡ | 1.50† | 14.93‡† |
| SEM | 0.30 | 2.96 | 0.25 | 1.92 | 0.21 | 3.35 |
| Magnesium | | | | | | |
| ($\mu$mol/24 h) | | | | | | |
| Mean | 84.51 | 82.26 | 85.24 | 82.95 | 80.62 | 82.83 |
| SEM | 8.16 | 7.33 | 7.03 | 5.67 | 7.88 | 3.37 |
| Sulfate | | | | | | |
| (mmol/24 h) | | | | | | |
| Mean | 0.374 | 0.820‡ | 0.285* | 0.601*‡ | 0.528*† | 1.025*‡† |
| SEM | 0.016 | 0.038 | 0.019 | 0.030 | 0.018 | 0.032 |
| Phosphate | | | | | | |
| (mmol/24 h) | | | | | | |
| Mean | 0.798 | 1.190‡ | 0.539* | 0.907‡ | 0.840¶ | 1.281‡† |
| SEM | 0.020 | 0.050 | 0.045 | 0.047 | 0.030 | 0.125 |
| Citrate | | | | | | |
| (mmol/24 h) | | | | | | |
| Mean | 0.052 | 0.023‡ | 0.074* | 0.038‡ | 0.045† | 0.021‡ |
| SEM | 0.007 | 0.001 | 0.007 | 0.005 | 0.007 | 0.001 |

Values are expressed as means $\pm$ SEM.

\* Significantly different from C group with a similar level of protein intake for a given variable ($p < 0.05$).

† Significantly different from PER group with a similar level of protein intake for a given variable ($p < 0.05$).

‡ Significantly different from NP group with a similar dietary restriction for a given variable ($p < 0.05$).



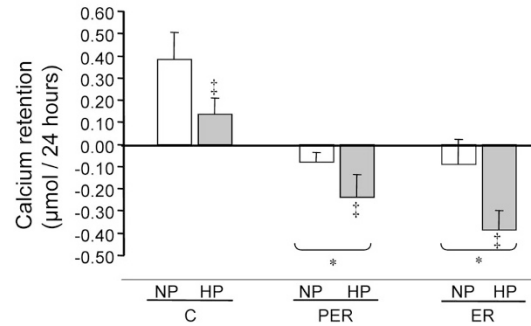**Figure 2.** Effect of dietary restrictions (C/PER/ER) and the level of protein intake (NP/HP) on calcium retention ($\mu$mol/24 h). *Significantly different from C groups. ‡Significantly different from NP groups.

Rats adapted to the HP diets showed lower urinary pH than those receiving the NP diets. Moreover, PER groups exhibited higher urinary pH, whereas ER rats had more acidic urines than controls. Urinary calcium excretion displayed a 6- to 10-fold rise in rats fed the HP diets compared with those fed the NP diets, and calciuria was lower in PER groups than in C and ER rats. Sulfate and phosphate excretions increased with the HP diets compared with the NP diets. They were both lowered in PER groups compared with C rats. Sulfate excretion was increased in all energy restriction animals. In contrast, renal citrate excretion was lower in HP than in NP groups for a given type of dietary restriction. PER groups excreted more citrate compared with C rats. Magnesium excretion did not vary.

*Calcium retention.* At the end of the study, calcium retention was positive in the C groups (Fig. 2). PER and ER groups showed a negative calcium retention compared with C animals ($p < 0.0001$). Moreover, HP diets were associated with even lower calcium retentions compared with NP diets ($p = 0.001$).

*Bone mineral density.* The BMD was lower in both PER and ER rats compared with C rats in total femur ($p = 0.015$) and at metaphysal ($p = 0.009$) and diaphysal ($p = 0.060$) sites (Fig. 3A–C). ER groups exhibited a significantly higher femoral BMD than PER animals. No difference was recorded between NP and HP groups.

*Femoral sizes and biomechanical properties.* PER and ER femur length (mm) was shorter than that of C femurs (C-NP, $39.67 \pm 0.30$; C-HP, $40.10 \pm 0.37$ *versus* PER-NP, $38.06 \pm 0.43$; PER-HP, $38.38 \pm 0.44$; ER-NP, $38.45 \pm 0.46$; ER-HP,

$38.68 \pm 0.33$). These variations accounted for body weight changes because no difference was recorded by ANCOVA analysis. Femur diameter did not vary.

Femoral biomechanical values displayed some similar features to BMD results, which was confirmed by a high positive correlation between fracture load and T-BMD data ($r = 0.618$, $p < 0.0001$). Biomechanical properties tended to be lower in PER and ER groups compared with C groups, even though no significant difference was recorded (Fig. 3D).

*Bone biomarkers.* At the end of the experiment, plasma OC was reduced in PER groups ($p = 0.039$) compared with C groups (Fig. 4A). A similar pattern was observed in ER animals. Urinary DPD excretion rate was lower in both restricted conditions (PER and ER) than in controls ($p < 0.0001$) (Fig. 4B). The OC/DPD ratio (%) (C-NP, $26.21 \pm 2.95$; C-HP, $23.65 \pm 1.75$; PER-NP, $16.24 \pm 3.14$; PER-HP, $16.31 \pm 3.06$; ER-NP, $22.09 \pm 4.15$; ER-HP, $19.54 \pm 2.07$), indicating the level of bone remodelling, was reduced by about 7–10% in PER animals compared with C rats, whereas it was only 4% lower in ER groups. Bone biomarkers were not significantly modulated by the level of protein intake (NP or HP).

*Plasma IGF-1.* Plasma IGF-1 concentrations were lower in the PER and ER groups compared with the C animals ($p < 0.0001$) (Fig. 5). IGF-1 levels increased by 4 to 6% between the NP and the HP groups (non significant).

### DISCUSSION

The respective influences of energy and dietary protein intakes on peak bone mass acquisition are not clearly identified. The present investigation allowed to examine the effect of a high-protein intake (HP, 26%) compared with a normal intake (NP, 13%), on bone acquisition in young male rats, and to differentiate the impact of PER and ER. On the basis previous works (2,3,5,18), our study involved a level of restriction of 40%. Dietary restriction was initiated at 10 wk of age and continued for 5 mo. Peak bone mass is achieved at nearly 9 mo of age in rats (19). Thus, the animals were growing throughout the whole experimental period.

During the experimental period, restricted animals (PER and ER) exhibited little variations in their body weight, whereas groups fed the C diets markedly gained mass. In
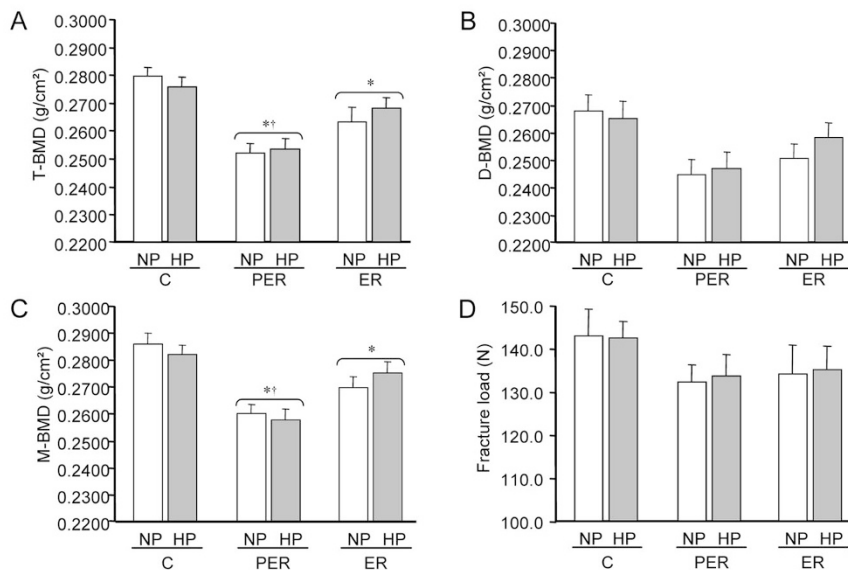
**Figure 3.** Effect of dietary restrictions (C/PER/ER) and the level of protein intake (NP/HP) on total (T-BMD) (*A*), diaphysal (D-BMD) (*B*), metaphysal (M-BMD) (*C*) femoral BMD, and biomechanical resistance (*D*). *Significantly different from C groups. †Significantly different from ER groups.
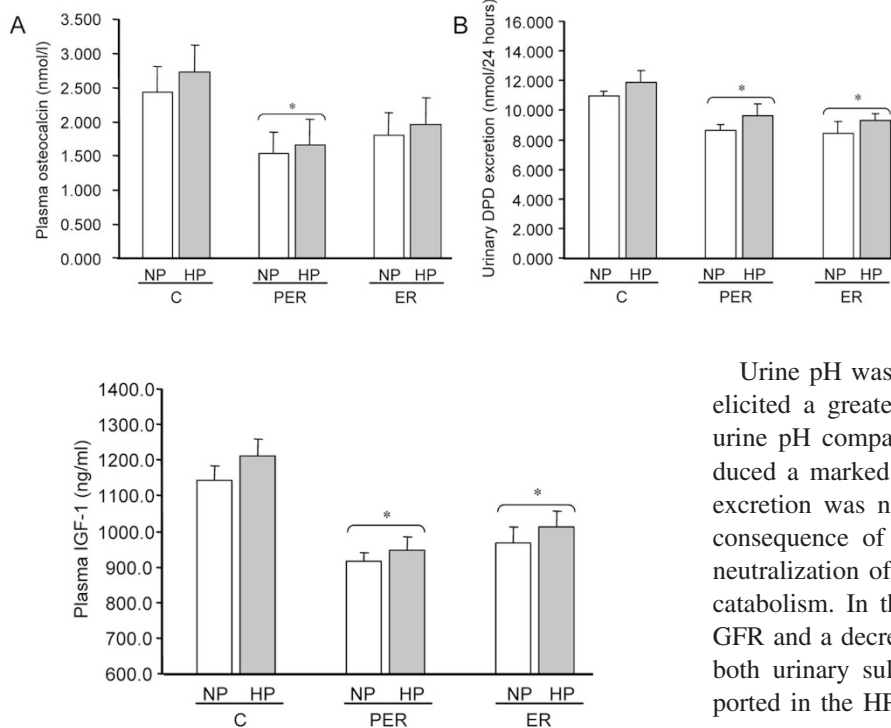


**Figure 4.** Effect of dietary restrictions (C/PER/ER) and the level of protein intake (NP/HP) on plasma OC (*A*) and the urinary DPD excretion rate (*B*). *Significantly different from C groups.



**Figure 5.** Effect of dietary restrictions (C/PER/ER), and the level of protein intake (NP/HP) on plasma IGF-1 concentrations. *Significantly different from C groups.

adequate conditions of energy supply (C groups), the protein content of the diet had no effect on body weight. However, among energy-restricted rats, relative lower body weights were recorded, according to the decreased level of protein intake. Body weight variations are recognized to result in bone mass modulations (4,5). To address the effects of the dietary restrictions and the level of protein intake independently of body weight changes, body weight was included as an independent variable in the statistical analysis of bone parameters (17).

Urine pH was acidic in all groups. Nevertheless, HP diets elicited a greater acid load, characterized by a decrease in urine pH compared with NP diets. Urinary acidification induced a marked rise of calciuria, whereas renal magnesium excretion was not affected. Hypercalciuria is a well-known consequence of high-protein diets (11), resulting from the neutralization of the $SO_4^{2-}$ generated by sulfur amino acids catabolism. In the kidney, it was attributed to an increased GFR and a decreased tubular calcium reabsorption. A rise in both urinary sulfate and phosphate excretion rates was reported in the HP groups. These increases were likely due to greater contents of sulfur amino acids and organic phosphorus, respectively, in the HP diets (20). Hypocitraturia, also associated with high-protein intakes (12), was observed in the HP groups, as well, as a consequence of a decrease in proximal tubule reabsorption of citrate and an impairment of citrate transport in the kidney (20).

The type of restriction (C, PER, or ER) also resulted in urinary parameters changes. Protein deficiency induced higher urinary pH and citrate excretion and lower urinary calcium, sulfate, and phosphate excretions. In the ER diets, there was no change in such parameters compared with the C diets, and sulfate excretion rate was even increased, as a result of a relative high concentration of protein in the mean daily ration.

Calcium retention was decreased significantly by the high-protein (26%) diets. Conversely, some studies in rats found no

effect of high-protein intake on intestinal calcium absorption (21). However, the durations of these studies were shorter. In this study, calcium retention was far more affected by energy restriction.

C rats provided with a higher protein level in the diet (C-HP *versus* C-NP) exhibited no noticeable changes in their femoral BMD, biomechanical properties, or bone remodelling biomarkers. These results are consistent with previous observations in rats (16,21) that reported no change in femoral bone mass, macro-mineral composition, or urinary excretion of pyridinium crosslinks of collagen in response to a dietary protein excess, despite hypercalciuria. Zernicke *et al.* (22) found a greater femoral neck percent ash in 8-wk-old rats fed a 30% protein diet (*versus* 15%), however, without any improvement regarding structural and material properties. Although the reciprocal response of osteoclasts and osteoblasts to metabolic acidosis has been shown to potentially affect bone metabolism (13,14), the dietary acid load generated by higher protein intakes (HP diets *versus* NP diets and C/ER diets *versus* PER diets) had no consequences. No change was recorded in plasma IGF-1 levels between the C-HP and the C-NP groups, even though it is known to be modulated by protein intake (8). Actually, it is likely that the present range of variation in protein intake was not sufficiently wide to detect changes in the stimulating effect it exerts on IGF-1 levels. Thus, all bone parameters concur to conclude that the low-grade metabolic acidosis induced by the hyperproteic diet did not affect the skeleton.

In contrast to the numerous reports of beneficial effects of protein intake on bone (23), some authors support that a high-protein diet would alter bone health (24,25). Amanzadeh *et al.* (12) observed a bone loss, based on histomorphometric analysis, in rats fed a 48% casein diet during 59 d and displaying hypercalciuria and hypocitraturia. This was not relevant with our observations. Actually, it seems that whether dietary protein has a beneficial or detrimental effect on bone also depends on additional nutritional factors, such as calcium intake. An adequate calcium supply, as in this experiment, was suggested to erase the negative effect of protein on bone mass (26). Nevertheless, it remains possible that a diet substantially higher with reduced calcium intake in protein will cause deleterious effects on bone.

Dietary restrictions did not affect bone growth in width. Femurs were longer in C groups compared with restricted rats. However, the normalization for total body weight erased any variations, suggesting that bone growth in length was only influenced by the increased mechanical loading associated with body weight gain, rather than by the diet itself.

Regarding bone status, ultimate femoral BMD was lower in energy (ER) and protein energy-restricted (PER) animals, than in the C groups. At the diaphyseal site, femoral biomechanical properties were highly correlated with BMD values ($r = 0.618$, $p < 0.0001$) and tended to be impaired by the two types of restriction. As previously reported, this observation might also be attributed to decreased diaphyseal cortical area, which contributes to bone fragility (3). Similarly, Talbott *et al.* (18) observed decreased BMD and BMC, respectively, in rats after 9 and 16 wk of 40% energy restriction. Sanderson *et al.* (4)

also reported a detrimental effect of dietary restriction on bone in rats but only attributed to body weight variation. In contrast, the results of our ANCOVA support the view that BMD variations are not related to body weight reduction. This is consistent with the study of LaMothe *et al.* (1), who demonstrated that the impaired tibia structural properties associated with ER were independent of body mass. Therefore, dietary-induced modulation of bone metabolism and hormonal factors are likely to contribute to the BMD variations.

The single restriction in energy was associated with significantly higher femoral BMD than with the PER diets. This suggests that protein and energy deficiencies would have additional detrimental effects on bone status acquisition. The compensation for protein intake in energy-restricted conditions induced a less pronounced bone alteration. Similarly, Bourrin *et al.* (27) reported a decrease in BMD in 6-mo-old rats in response to protein deficiency. Moreover, young rats fed a protein deficient diet for 20 d exhibited lower femoral diaphyseal bending stiffness and breaking strength (9). In agreement with our results, Ammann *et al.* (10) showed that the consumption of an isocaloric essential amino acid supplement was able to correct the bone alterations induced by a low-protein diet in female ovariectomized rats.

BMD changes may be explained by modulations in bone remodelling. Indeed, energy restriction lowered DPD levels in a significant manner, whereas an additional restriction in protein supply decreased both OC and DPD levels. Thus, the OC/DPD ratio was far more reduced in PER than in ER groups, compared with C rats, which was consistent with the BMD data. Nevertheless, the exact impact of dietary restrictions on bone biomarkers remains unclear, given the conflicting results observed in previous reports (5,17,27).

The decrease in plasma IGF-1 levels associated with dietary restrictions (PER and ER) probably played a role on the femoral BMD and bone biomarkers modulations. Nutritional status is a critical factor in the regulation of circulating IGF-1 levels (8). Given its anabolic effect on bone and on epiphyseal cartilage, IGF-1 is determinant for bone growth. Low energy and protein intakes could be detrimental for skeletal integrity by lowering to an inadequate level the production of IGF-1, which could be directly implicated in bone matrix mineralization. It is also possible that circulating levels of IGFBP-1 were modified in response to changes in nutrient supplies, which was suggested to modulate IGF-1 bioavailability by competing for IGF-1 binding with the IGF-1 receptor (28). Despite no significance of difference, IGF-1 levels tended to be higher in ER groups compared with PER animals. This pattern was consistent with the increased femoral BMD induced by the supplementation of protein intake in an energy-restricted context compared with PER conditions. Indeed, elevated protein intake has been shown to be able to prevent the decrease in IGF-1, usually observed in hypocaloric states (29).

The age-sensitive response to energy and protein restriction suspected by Talbott *et al.* (18) was confirmed. Indeed, in contrast with our previous findings in old rats (2,3), dietary restrictions did not induce body weight loss but only erased weight gain, suggesting a slowing down of the growth rate. The supplementation of dietary protein limited the bone loss

due to energy deficiency, whereas it failed to improve bone status in old rats.

As a conclusion, neither beneficial nor detrimental effects associated with a high-protein intake were reported on bone status, despite an induction of metabolic acidosis and an impairment on calcium retention. Regarding dietary restrictions, decreased energy and protein intakes markedly affected bone status in growing rats. Such nutritional deficiencies might contribute to impairment on peak bone mass acquisition and affect skeleton strength throughout later life, potentially rendering the individual more susceptible to osteoporosis. Our study also demonstrated a significant bone sparing effect when ER was associated with an adequate protein intake. Thus, great care should be taken regarding the respective energy and protein supplies during growth, particularly when setting up weight management programs intended for obese adolescents.

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