Impact of energy and casein or whey protein intake on bone status in a rat model of age-related bone loss

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In the elderly, nutritional deficiencies, such as low energy and protein intake, are suggested to increase the risk of osteoporotic fractures. Modulation of the amount and quality of protein intake under energy deficient conditions represents an interesting strategy to prevent aged-related bone loss. We investigated the effect of a 5-month dietary restriction on bone status in 16-month-old male rats. Rats were randomised into six groups (n 10 per group). Control animals were fed a normal diet containing either casein (N-C) or whey protein (N-WP). The other groups received a 40% protein and energy-restricted diet with casein or whey protein (PER-C and PER-WP) or a normal protein and energy-restricted diet (ER-C and ER-WP). Both restrictions (PER and ER) induced a decrease in femoral bone mineral density (BMD), consistent with impaired biomechanical properties and a reduced cortical area at the diaphysis. Plasma osteocalcin and urinary deoxypyridinoline levels suggested a decrease in bone turnover in the PER and ER groups. Interestingly, circulating insulin-like growth factor 1 (IGF-1) levels were also lowered. Overall, normal protein intake did not elicit any bone sparing effect in energy-deficient rats. Regarding protein quality, neither casein nor WP appeared to significantly prevent the BMD decrease. This study confirms that nutritional deficiencies may contribute to osteopenia through decreased IGF-1 levels. Moreover, it seems that impaired bone status could not be significantly prevented by modulating the amount and quality of dietary proteins.

Energy restriction: Protein deficiency: Casein or whey protein: Bone

Age-related osteoporosis mainly affects people older than 70 years of age and results, in most cases, in both vertebral and hip fractures. Given the magnitude of this public health problem and the dramatically increased proportion of older individuals predicted in the next decades, it is necessary to evaluate the potential of every preventive intervention.

In addition to the well-recognised risk factors such as Ca and vitamin D deficiency, other nutritional aspects have been suggested to contribute to the increased incidence of osteoporosis in the elderly. Indeed, ageing has been correlated with a physiological decline in appetite and food intake. This change predisposes the elderly to a poor nutritional status, resulting in a compromised musculoskeletal system characterised by lower muscle mass and a decrease in bone mineral content.

The impact of low energy and protein intake on the skeleton has been investigated in the elderly. Bonjour et al. revisited the concept of Albright who hypothesised that ‘a diet inadequate in protein might lead to a negative nitrogen balance’ and consequently affect bone formation. Several studies have established a positive correlation between bone mineral density (BMD) and both energy and protein intake, and such oral supplementation seemed to improve the clinical outcome in elderly patients with femoral neck fractures.

In animals, energy restriction has been demonstrated to adversely affect bone status. McCay et al. first reported that bones became fragile after long-term energy restriction in rats and that ‘some crumbled with the course of dissection’. In this particular study, the fragility most likely was due to the extreme dietary deprivation, including Ca insufficiency. However, subsequent works have demonstrated altered femoral bone mineral content, BMD and biomechanical properties in old rats under energy restriction.

Protein is a major component of the bone organic matrix and consequently, dietary proteins contribute the essential amino acids necessary for new matrix synthesis. In rats, protein under-nutrition has been associated with lower bone mass and strength, modulated by the growth hormone–insulin-like growth factor 1 (IGF-1) axis, and in animals fed a low protein diet, bone strength was increased by dietary supplementation with essential amino acids. Consequently, modulation of the amount and quality of dietary protein intake represents an interesting approach to preventing bone loss during ageing.

Whey protein (WP) contains a relatively high proportion of essential amino acids and can effectively modulate whole body protein anabolism and prevent body protein loss in elderly subjects. However, few studies have examined the

Abbreviations: BMD, bone mineral density; DPD, deoxypyridinoline; IGF-1, insulin-like growth factor 1; OC, osteocalcin; WP, whey protein; N, ER and PER, treatment with normal, energy-restricted, and protein–energy-restricted diet respectively; -C, and -WP additions to ER, PER and N diets.

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effect of WP intake on bone status. The administration of WP was shown to effectively increase bone strength and enhance bone formation in young animals. On the other hand, casein, which is widely used in experimental diets, has some interesting properties. Casein intake leads to the formation of casein phosphopeptides during digestion and the casein phosphopeptides have been suggested to promote Ca absorption and stimulate bone mineralisation. Therefore, both casein and WP potentially have an impact on bone quality and/or status. However, further studies are required to specifically examine how these proteins modulate skeletal metabolism and status during ageing.

This study was designed to test if an adequate protein intake, provided by casein or WP, could prevent the alteration of bone status induced by energy deficiency in the elderly. Using old male rats, two specific questions were addressed:

(i) What are the long-term effects on bone status of protein and energy restriction (ER) alone, compared to normal protein and energy supply (N)?
(ii) How does the protein source (casein or WP) influence bone status in restricted and unrestricted rats?

### Experimental methods

#### Experimental design

The study was conducted in accordance with the regional Ethics Committee (France).

Male Wistar rats were purchased from Janvier (Le Genest St Isle, France) and housed individually. Male rats were studied in order to eliminate the potential confounding effect of hormonal fluctuations present with an oestrus cycle. Animals were subjected to 12–12 h light–dark cycles and had free access to water.

At the beginning of the study, the rats were 16 months old and the experiment continued for 5 months. During a 2-week adaptation period, the animals were maintained on a standard chow diet to record their daily food intake and body weight. Then, they were randomly assigned to one of six dietary groups (n 10 animals per group). Two control groups received a normal diet (N): a standard semi-purified diet containing 69% carbohydrate, 6% fat and either 17% casein (N-C) or 17% WP (N-WP) as protein source (Table 1). Rats of the N groups were fed a measured amount, corresponding to 90–95% of the average ad libitum intake, to match energy intake and to facilitate the study of healthy, non-obese control animals. The mean energy intake was about 451 kJ/d (108 kcal/d). The protein and energy-restricted groups (PER-C and PER-WP) were limited to 60% of the intake of the N groups (i.e. 10.2% protein and 272 kJ/d (65 kcal/d), respectively). The energy-restricted groups (ER-C and ER-WP) received 60% of the energy of the controls (i.e. 272 kJ/d), but had their protein intake maintained at the level of the N groups. All diet-restricted rats were normalised to the N animals with respect to lipid, fibre, mineral and vitamin intake. Thus, all the animals consumed the same amounts of dietary Ca and P (Ca:P ratio = 1.45). During the experiment, body weight was recorded twice weekly.

At the end of the experiment, rats were fasted for 12 h and sacrificed. Blood as well as prior 24-h urine samples were collected to assay the biochemical parameters. 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 placed in 80% alcohol until BMD was measured.

**Physical measurements**

**Bone mineral density.** BMD was assessed by dual-energy X-ray absorptiometry, using a Hologic QDR-4500 A X-ray densitometer (Hologic, Massy, France). Total femoral BMD, metaphyseal BMD and diaphyseal BMD were determined. For metaphyseal BMD and diaphyseal BMD measurements, scans were cut and analysed as follows: the first cut of the femur was performed at the upper third, and the next cut was made at the lower third. Diaphyseal BMD, which is rich in cortical bone, corresponded to the density of the second third of the femur. Metaphyseal BMD, which mainly contains cancellous bone, was calculated as the mean of the femoral proximal metaphysis density and the femoral distal metaphysis density.

**Femoral mechanical testing.** Femoral length and mean diaphyseal diameter were measured with a precision caliper (Mitutoyo, Shropshire, UK). The femoral failure load was determined using a three-point bending test, with a Universal Testing Machine (Instron 4501, Instron, Canton, MA, USA). The two lower supports were separated by a 20 mm distance and an upper crosshead roller was applied in front of the middle of the bone until failure at a speed of 0.5 mm/min to guarantee that 85–90% of the bone flexure was due to bending.

**Static histomorphometry.** After BMD measurements, distal right femurs were dehydrated in a graded series of ethanol solutions for 5 d prior to embedding in methyl methacrylate (Sigma, L’Isle d’Abeau, France). Blocks were then polished with a grinder (Metaserv 2000, Buchler, Coventry, UK) and 10 μm frontal sections were cut using a RM2165 Leica microtome (Leica Microsystems Nussloch GmbH, Nussloch, Germany). Sections were stained using the Von Kossa silver method (AgNO₃; Sigma). Four sections were analysed per femur. To characterise static cancellous bone, image
acquisition was carried out with an Axioplan EE microscope (Zeiss, Göttingen, Germany) and image analysis performed in the secondary spongiosa of the distal femur metaphysis with the OsteoLab software (Biocom, Paris, France). This allows an evaluation of cancellous bone volume (bone volume:total tissue volume, %), trabecular number, trabecular thickness (μm) and trabecular separation (mm). Cortical bone was assessed at the femoral diaphysis. Cross sections were analysed with the ImageJ 1.34 s software (National Institutes of Health, Bethesda, MD, USA) to measure tissue, marrow and cortical areas (mm²). All histomorphometric parameters were determined according to Parfitt et al.33.

Biochemical analysis

Osteoblastic activity. Plasma osteocalcin (OC) was measured by RIA, using rat 125I-labeled OC, a goat anti-rat OC antibody and a donkey anti-goat secondary antibody (Biochemical Technologies, Stoughton, MA, USA). The sensitivity was 0.01 ng/ml. The intra- and interassay precisions were 6.8 and 8.9%, respectively.

Bone resorption. The urinary deoxypyridinoline (DPD) excretion rate (nmol/24 h) was determined by competitive RIA, using a rat monoclonal anti-DPD antibody adsorbed to the inner surface of a polystyrene tube and 125I-labeled DPD (Pyrilinks-D RIA kit, Metra Biosystems, Mountain View, CA, USA). The sensitivity was 2 nmol/l. The intra- and interassay precisions were 4 and 6%, respectively.

Leptin. Plasma leptin concentrations were assessed by RIA using an anti-rat leptin antibody and a rat leptin as standard (Rat Leptin RIA kit; Linco Research Inc., Missouri, USA). The lowest limit of sensitivity was 0.5 ng/ml, and the intra- and interassay variations were 1.5 and 2.5%, respectively.

Insulin-like growth factor 1. 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. The intra- and interassay variations were 6.8 and 10.7%, respectively.

Urinary calcium excretion. Urinary Ca was determined by atomic absorption spectrophotometry (Perkin Elmer 400, Norwalk, CT, USA). Each sample was diluted appropriately with distilled water and lanthanum chloride (0.1 %) for atomisation. The urinary Ca excretion was calculated using the volume of the 24 h urine samples collected.

Results

Body weight

Changes in body weight are shown in Fig. 1. As expected, animals on dietary restriction exhibited a significant decrease in body weight (P < 0.0001) at the completion of the study, compared to rats fed normal diets. The PER and ER rats weighed about 150 g less than N groups. There was no significant difference between the four restricted groups, whatever the level or the quality of dietary protein. Plasma leptin concentrations are known to correlate with adiposity in mammals33. Here, the leptin levels (ng/ml) were markedly lower in the PER and ER groups than in the N groups (N-C: 11.94 (SEM 1.54); N-WP: 12.31 (SEM 1.45) v. PER-C: 3.46 (SEM 0.39); PER-WP: 4.35 (SEM 0.50); ER-C: 2.28 (SEM 0.31); ER-WP: 3.62 (SEM 0.63)). This suggests a fat-mass reduction in the restricted animals.

Bone mineral density. The BMD was consistently reduced by both types of dietary restriction (PER and ER) in total femur (P = 0.020), as well as at the diaphysial (P = 0.016) and the metaphysial (P = 0.064) sites (Fig. 2(a), (b) and (c) respectively). The casein-fed rats tended to have a higher BMD than those fed the WP diet (P = 0.073).

Biomechanical properties. Femurs from restricted animals (PER and ER) had a lower resistance to fracture compared to those from the N groups (P = 0.013), but resistance to fracture did not differ between the PER and ER groups (Fig. 2(d)). The type of dietary protein had no significant effect. However, femoral biomechanical resistance tended to be higher in the casein groups (P = 0.089) than the WP groups.

Static histomorphometry. Histomorphometric data of the distal femur are shown in Table 2. The trabecular bone volume to total volume ratio (bone volume: total tissue volume; P = 0.043) and trabecular thickness (P = 0.009) were lower in the energy restricted groups (ER), compared to the
protein-energy restricted (PER) groups. Casein intake was associated with an increase in bone volume ($P = 0.045$), as well as an elevated trabecular number ($P = 0.009$) compared to the WP diets, in both restricted and non-restricted rats. Cortical bone parameters were only affected by the dietary restriction factor. Tissue area in the femoral diaphysis decreased in the PER and ER groups compared to the N groups ($P = 0.053$). The same pattern was observed for cortical area ($P = 0.022$).

**Bone biomarkers.** Fig. 3 shows the levels of bone formation (OC) and bone resorption (DPD) markers at the end of the experiment. Plasma OC was reduced in the ER groups ($P = 0.029$) compared to N groups. A similar trend was observed in the PER animals. Moreover, OC levels tended to be higher with casein consumption ($P = 0.072$) than with WP intake. The urinary DPD excretion rate was decreased with both dietary restrictions, compared to the N diets ($P < 0.0001$), and ER animals excreted significantly less DPD than PER rats. Furthermore, using linear regression analysis, a positive correlation ($r = 0.543, P < 0.0001$) was established between formation and resorption markers.

**Plasma Insulin-like growth factor-1.** Plasma IGF-1 concentrations (Fig. 4) were lower in the PER and ER groups compared to the N groups ($P = 0.001$). This decrease in IGF-1 levels was not correlated with the amount of dietary protein. The interaction between dietary restriction and protein effects was significant ($P = 0.014$), with the following relative ranking: (N-WP) = (N-C) = (ER-WP) = (PER-C) > (ER-C) = (PER-WP).

**Calcium excretion.** No statistical change in urinary Ca excretion was recorded in the groups (data not shown).

**Discussion**

Nutritional deficiencies often occur in the elderly and energy and protein undernutrition have been suggested to alter bone health and to increase the risk of osteoporotic fractures. Several studies have assessed the impact of dietary restrictions on bone14–18,32,34–38. However, these studies differ widely in their experimental design, duration, age at onset of restriction and diet composition. Thus, based on these, it is difficult to interpret the effects of dietary restrictions on the skeleton during ageing and to dissociate the respective effects of protein and energy deficiency. To our knowledge, this study is the first to test the effects of protein quality and quantity on bone status during ageing in rats.

Our experimental model was the aged male Wistar rat, which has been established as a relevant model for age-related bone loss in human subjects39. The severity of dietary restriction (40 %) was based on previous rodent studies17,34,35,40. During the experimental period, body weight markedly decreased with both dietary restrictions (a 23 % change compared to the controls; Fig. 1). In the statistical analysis, body weight was included as an independent variable to ensure the assessment of dietary restriction and protein type independently of its variations. Weight loss has been demonstrated to result in decreased BMD as a consequence of reduced mechanical loading, altered hormone levels and dietary factors, and changes in bone composition16,32,34,36,38. Some authors express bone parameters per 100 g body weight16,18,37, which skews the data. Therefore, it remains unclear if there is a direct relationship between body weight and BMD, and whether this link is age-dependent and similar at weight-bearing and non-weight-bearing sites.
Table 2. Effect of dietary restrictions (normal (N)/protein-energy restricted (PER)/energy restricted (ER)) and the type of protein provided in the diet (casein (C)/whey protein (WP)) on histomorphometry of cancellous bone at the distal femoral metaphysis and of cortical bone at the femoral diaphysis

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BV, bone volume; TV, tissue volume; Tb.Th, trabecular thickness; Tb.N, trabecular number; Tb.Sp, trabecular separation; T.Ar, tissue area; Ma.Ar, marrow area; Ct.Ar, cortical area.

Two-way ANCOVA was performed. Comparison between dietary restrictions (N/PER/ER): * significantly different from the N groups; † significantly different from the PER groups. Comparison between protein types (C/WP): ‡ significantly different from the WP groups.
Black et al.\textsuperscript{38} also observed a detrimental effect of dietary restriction on bone in rats and monkeys, but this effect was only attributed to body weight variation. In contrast to these results, our data support the view that BMD variations are not related to body weight reduction, as demonstrated by the ANCOVA analysis. Our results are consistent with those published by LaMothe et al.\textsuperscript{18}, who demonstrated that the impaired tibia structural properties associated with energy restriction were independent of body mass. Therefore, dietary-induced modulation of hormonal factors is likely to contribute to these variations.

BMD changes may be explained by several factors. At the metaphyseal site, the trend recorded for metaphyseal BMD was associated with a decrease in bone volume in the ER animals (Table 2). This decrease in bone volume:total tissue volume seems to be the result of a lower trabecular thickness, whereas trabecular number and separation were unchanged. Surprisingly, no changes were seen in the PER groups. Thus, energy restriction alone seems to have a more pronounced effect than simultaneous protein and energy restriction. In contrast, Bourrin et al.\textsuperscript{41} reported a decrease in trabecular thickness in the tibia proximal metaphysis in response to protein restriction, as well as a decrease in BMD. However, the applied restriction was far more severe (protein level 2.5\%) than in the present study (protein level 10.2\%). In this study, the amount of dietary protein modulated the trabecular volume, but was not correlated with the BMD data. At the diaphyseal site (Table 2), the low BMD recorded in the restricted animals is consistent with a lower cortical tissue area. This indicates a decreased diaphyseal width and a lower diaphyseal cortical area. Both parameters are highly correlated with diaphyseal BMD ($r=0.416, P=0.005$ and $r=0.716, P<0.0001$, respectively). Cortical area changes resulted in altered femoral biomechanical properties in the restricted rats ($r=0.586, P<0.0001$). Nnakwe\textsuperscript{42} and Talbott et al.\textsuperscript{17} similarly identified a decline in ultimate bone strength in rats fed a 40\% restricted diet.

Our results clearly indicate that energy and protein undernutrition affect bone status in aged rats. Nevertheless, adequate protein intake did not prevent the detrimental effects of energy restriction. Indeed, no difference was noted between PER and ER with respect to femoral BMD and the corresponding mechanical data (Fig. 2(a)). Similarly, Bourrin et al.\textsuperscript{41} reported a decrease in BMD and bone strength with protein restriction in aged male rats. Ammann et al.\textsuperscript{39} showed that bone strength was reduced by a low protein diet (only 2.5\% protein in the diet) in adult female ovariectomised rats. However, the consumption of an isoennergic essential amino acid supplement corrected these variations.

The values for the physical bone measurements were associated with a decrease in both bone formation and resorption markers (Fig. 3). The plasma OC levels tended to decrease in the PER groups and reached significant values in the ER groups, indicating a reduced bone formation rate. Similarly, lower urinary DPD excretion rates were recorded in the restricted animals (PER and ER), suggesting a reduced bone resorption. The bone metabolism data were not consistent with the BMD values, and did not explain the decrease in femoral BMD. Indeed, there was no bone remodelling imbalance. This difference might be due to the fact that the DPD and OC assays were carried out on samples collected on the
last day of the experiment. Thus, these reflect the bone status at this specific time point, whereas the BMD variations reflect effects accumulated over the entire duration of the experiment. The exact impact of dietary restrictions on bone biomarkers will require further studies as previous reports have shown conflicting results.\textsuperscript{32,34,43}

Protein restriction (10.2%, compared to the normal level of 17%) did not change the plasma OC levels (Fig. 3), whereas the DPD levels were significantly lower in the PER groups than in the ER groups. It seems that the PER diets induced more resorption than the ER conditions, but this was not correlated with the BMD values. Using different nutritional conditions, Bourrin \textit{et al.}\textsuperscript{41} demonstrated that protein deprivation (2.5% v. 15%) was associated with a decrease in OC levels from the first week of deficiency, while urinary DPD remained unchanged throughout the experiment.

Dietary restrictions (PER and ER) were associated with lower plasma IGF-1 levels (Fig. 4). Nutritional status (especially energy and dietary protein intake) is a critical factor in the regulation of circulating IGF-1 levels\textsuperscript{44}. Considering the bone anabolic effect of IGF-1\textsuperscript{45}, this decrease might explain, at least in part, the changes in femoral BMD and bone biomarkers. This is supported by the positive correlations between IGF-1 levels and BMD (r 0.350, P < 0.025), cortical area (r 0.353, P = 0.032) and biomechanical properties (r 0.317, P = 0.043), respectively.

In this study, the IGF-1 levels did not vary significantly between the two protein intake levels (PER and ER) (Fig. 4). Yet, dietary proteins are known to influence both the production and action of IGF-1\textsuperscript{46}. Plasma IGF-1 levels have been shown to decrease with protein restriction\textsuperscript{41,44,47} and Ammann \textit{et al.}\textsuperscript{15} suggested that an impaired IGF-1 system leads to decreased bone mineral mass and fragility under protein deprivation. However, these conclusions are based on data from animals fed a 2.5% casein diet, which is a drastic deprivation. No significant changes were detected in bone parameters and plasma IGF-1 levels in rats fed diets containing more than 5% protein. This is in agreement with our observations.

The PER-WP and ER-C groups exhibited lower plasma IGF-1 levels. This could be attributed to time-dependent variations between casein and WP digestion\textsuperscript{21,48}. The IGF-1 levels were most likely reduced in the PER-WP group because of the faster absorption rate of WP compared to casein.

Overall, protein quality had little impact on bone status. Nevertheless, rats fed the casein diets exhibited a high number of trabeculae than the WP-fed animals, resulting in an increased bone volume (Table 2). A parallel response was seen in total BMD (P = 0.073) and plasma OC (P = 0.072), even if the trends were not statistically significant. In previous studies of rats, dietary casein was demonstrated to stimulate bone mineralisation by improving Ca deposition in bone and inhibiting bone resorption\textsuperscript{77,49}. In contrast, in mini-pigs casein-derived casein phosphopeptides had only marginal effects on bone mineral content\textsuperscript{28}.

According to our data, WP consumption did not improve bone status more effectively than casein. Paradoxically, Takada \textit{et al.}\textsuperscript{23} found that WP consumption increased the breaking strength and suppressed bone resorption in ovariectomised female rats. Similarly, Kelly \textit{et al.}\textsuperscript{24} demonstrated that WP intake increased alkaline phosphatase activity and IGF-1 mRNA levels in young rats, suggesting enhanced bone formation. In the present study, protein quality had no effect on the OC and plasma IGF-1 levels. These differences can be attributed to the age of our experimental animals (21 months), because age is associated with impaired IGF-1 secretion\textsuperscript{50}, resulting from perturbations to the hypothalamic–adenohypophysial–somatotrope axis\textsuperscript{44,51}.

Urinary Ca excretion was unchanged with the different types of protein. However, casein was previously shown to enhance Ca absorption, due to the bioactive casein phosphopeptides resulting from the digestive breakdown of casein\textsuperscript{28}. In contrast, other studies found no stimulating effect of casein phosphopeptides on intestinal Ca absorption, neither in rats\textsuperscript{52} nor in human subjects\textsuperscript{53}. Zhao \textit{et al.}\textsuperscript{54} reported a Ca absorption-enhancing effect of WP intake, but it was absent during long-term WP-feeding. The lack of variation in Ca absorption in this study could be due to adaptation, which would eliminate the stimulating effect of dietary casein and WP. As suggested in the Zhao study, this effect could be consistent with a down regulation of active Ca absorption, through a suppression of the parathyroid hormone–vitamin D axis, in response to the initial increase in Ca absorption during chronic feeding. In our opinion, the lack of variation in this study may be due to modulation of passive and active Ca transport during ageing. Indeed, it is well established that ageing often is associated with impaired Ca absorption as well as vitamin D deficiency and this can result in secondary hyperparathyroidism\textsuperscript{55}.

To summarise, protein–energy restriction and energy restriction alone induced lower femoral BMD and impaired biomechanical properties, compared to controls, independently of body weight variations. Our study confirms that nutritional deficiencies may contribute to age-related bone loss, since lower BMD and biomechanical resistance are associated with an increased risk of bone fracture. These changes could be attributed to a decrease in IGF-1 levels, but the exact mechanisms need to be identified. No bone-sparing effect has been reported when energy restriction is associated with an adequate protein intake. Under our experimental conditions, neither casein nor WP appear to prevent the detrimental effects of dietary restrictions on bone mass. Nevertheless, diets providing casein seem to preserve bone health more efficiently than those containing WP, as judged by BMD and histomorphometry. In this study, mineral intake was standardised in every group, which is important because energy and protein undernutrition often are associated with Ca deficiency in the elderly. It is conceivable that disruption in dietary Ca intake, in addition to energy and protein restriction, could have a more pronounced effect on bone metabolism.

References
Protein intake and energy deficiency in rat


