Consumption of Vitamin D-and Calcium-Fortified Soft White Cheese Lowers the Biochemical Marker of Bone Resorption TRAP 5b in Postmenopausal Women at Moderate Risk of Osteoporosis Fracture

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Abstract

The prevention of increased bone remodeling in postmenopausal women at low 10-y risk of osteoporotic fractures essentially relies on reinforcement of environmental factors known to positively influence bone health, among which nutrition plays an important role. In institutionalized women in their mid-eighties, we previously found that consumption of fortified soft plain cheese increased vitamin D, calcium, and protein intakes, reduced bone resorption biochemical markers, particularly the serum bone specific acid phosphatase tartrate resistant acid phosphatase, isoform 5b (TRAP 5b) that reflects osteoclast activity, and stimulated the serum bone anabolic factor insulin-like growth factor-I (IGF-I). Whether these effects occur in much younger women was tested in a prospective control study. Seventy-one healthy postmenopausal women aged 56.6 ± 3.9 y (mean ± SD) with low spontaneous supply of both Ca and vitamin D were randomized to consume daily (treated, n = 36) or not (controls, n = 35) two servings (2 × 100 g) of skimmed-milk, soft plain cheese for 6 wk. The vitamin D and Ca-fortified dairy product provided daily: 661 kJ, 2.5 μg vitamin D, 400 mg calcium, and 13.8 g protein. At the end of the intervention, the decrease in TRAP 5b and the increase in IGF-I were greater in the treated than in the control group (P, 0.02). The changes in serum carboxy terminal crosslinked telopeptide of type I collagen did not differ significantly between the two groups. In conclusion, like in elderly women, consumption by healthy postmenopausal women of a vitamin D and calcium-fortified dairy product that also increases the protein intake, reduces the serum concentration of the bone resorption biomarker TRAP 5b. This response, combined with the increase in serum IGF-I, is compatible with a nutrition-induced reduction in postmenopausal bone loss rate. J. Nutr. 142: 698–703, 2012.

Introduction

Changes in bone metabolism following the cessation of ovarian function at the time of menopause are characterized by an increased resorption without a commensurate rise in formation (1). This accelerated bone remodeling leads to progressive deterioration of bone micro architecture (1,2), decreased skeletal strength (3,4), and increased risk of fragility fractures with aging (5).

In women, estrogen deficiency represents a major risk of osteoporosis. Therefore, estrogen therapy was considered for many years as the treatment of choice in the prevention of postmenopausal bone loss and occurrence of fragility fractures (6). The results of the Women Health Initiative study fully supported this important notion by demonstrating, in a large, prospective, randomized, placebo-controlled trial, a significant reduction in hip fracture occurrence in response to estrogen treatment (7). In the same study however, this treatment was associated with an increased incidence of both cardiovascular diseases and breast cancer (7). These findings strongly suggested that the risks of hormone replacement therapy outweighed the benefits, particularly for the long-term prevention of postmenopausal bone loss and consecutive osteoporotic fractures. Furthermore, in absence of a severe risk of fragility fractures, it seems inappropriate to use powerful antiosteoporotic drugs within the next 5–10 y following the menopause. Therefore, it appears more appropriate to explore alternative options, particularly by the use of nonpharmacologic means, to attenuate the deleterious increment in bone resorption in early postmenopausal women. One important approach consists of optimizing the consumption of bone-sparing nutrients (8). Among them, vitamin D, calcium, and proteins exert a positive impact on skeletal health throughout life.
by well-characterized physiologic mechanisms (9–12). These three nutrients favor both the acquisition and maintenance of bone mass and its structural integrity, thereby conferring optimal resistance to usual mechanical loading. Furthermore, they can also positively influence the mass and strength of skeletal muscles and reduce the propensity to falling, a preventive measure of growing importance with aging. Thus, associated with moderate but regular physical activity, the adequate dietary supply in vitamin D, calcium, and proteins contribute to reduce the risk of fragility fracture.

In two previous independent studies (13,14), we observed in elderly institutionalized women at high risk of osteoporotic fracture that regular consumption of soft white cheese reduced bone resorption without lowering bone formation markers. In elderly women with mean ages of 85 y (13) and 87 y (14) who daily consumed two servings of soft white cheese that provided ~17–33% of the recommended daily intake of vitamin D, calcium, and proteins, we observed a significant decrease in two circulating biochemical markers of bone resorption: CTX and TRAP 5b (13,14). In the same dietary intervention trials, the circulating level of IGF-I, a bone and skeletal muscle anabolic factor, was significantly increased in response to the consumption of the dairy product (13,14).

The question arises whether a similar favorable response could also be obtained with soft plain cheese consumption in younger women at risk of increased bone resorption as expected after physiological cessation of the ovarian function. We tested this hypothesis in a prospective randomized controlled trial enrolling healthy community-dwelling women with ranging in age from 50 to 65 y who were all postmenopausal for at least 3 y.

### Participants and Methods

Participants were recruited among community-dwelling women via regional newspapers, advertisements, and information letters. Of ~440 volunteers who expressed interest to participate, 71 met all the study design criteria and were enrolled in a prospective trial of 6 wk. Informed consent in conformity with the European Directive and French Code of Public Health was obtained from each woman during the screening visit held not earlier than 15 d before the onset of the 6-wk trial.

Inclusion criteria were as follows: women aged from 50 to 65 y with menopause for at least 3 y; BMI ranging from 18 to 27 kg/m²; calcium intake of <650 mg/d, as assessed by a validated FFQ (15); lower level of vitamin D supply from sun exposure and food intake, according to a dedicated questionnaire (16); no substantive hormone-related therapy for the prevention of postmenopausal disorders during at least the 12 mo preceding the beginning of the trial.

Exclusion criteria were as follows: participation in another clinical trial during the last 3 mo; disorders influencing calcium-phosphate and/or bone metabolism, such as hyperparathyroidism, Paget disease, or chronic conditions requiring cortisone therapy; use of calcium and or vitamin D supplement, taken as pharmaceutical preparation or fortified foods, during the preceding 6 mo; exposure to UV light therapy within the last 3 mo; supplements taken as pharmaceutical preparation or fortified foods, during the preceding 6 mo; exposure to UV light therapy within the last 3 mo; moderate but regular physical activity; chronic gastro-intestinal disorders; or cow milk allergy.

Study design. The study with a 6-wk duration was carried out in one single center. The women who provided informed consent and met all requirements at the screening visit were considered eligible for the clinical trial. Seventy-one eligible participants were enrolled over an 18-wk period from May to September 2009. A prospective, randomized, controlled design was applied. Thirty-six women received the tested food while 35 women served as parallel time controls.

The tested food consisted of skimmed-milk, soft, plain cheese, which was fortified with vitamin D (+1.25 μg/100 g) and calcium, thus achieving a total calcium content of 200 mg compared with 90–120 mg/100 g for standard fresh cheese. Two servings, each weighing 100 g, were consumed every day for 6 wk. They provided daily: 661 kJ (156 kcal), 2.5 μg vitamin D, 400 mg calcium, 233 mg phosphorus, and 13.8 g protein. The control group was advised to maintain their usual diet during the 6-wk randomized trial.

All participants were examined at 4 sequential visits. At the screening visit, held no later than 15 d before the inclusion day, demographic characteristics and inclusion/exclusion criteria were assessed, and calcium and vitamin D questionnaires were completed. At the inclusion visit (d 1), a physical examination was performed and information on medications was collected. At this visit, a FRAX questionnaire (17) for assessing osteoporotic fracture probability was filled in. This assessment, using easily obtained clinical risk factors, enabled us to determine the 10-y probability of sustaining major osteoporotic fractures taken together and hip fractures separately (17). At the inclusion visit (d 1), intermediary (d 21), and end-of-study visits (d 42), a dietary follow-up questionnaire was completed. It was designed to assess whether the recommendation to the participants to not change their food consumption habits during the 6-wk trial were met. Compliance with the product intake was assessed by a self-rating diary, which had to be completed every day. This diary was also aimed at testing the acceptability in terms of portion size suitability and whether or not there was, with time, feelings of constraint and tiredness with the product consumption.

Blood samples for various laboratory tests were collected at the inclusion (d 1) and end-of-study visits (d 42). Both compliance and acceptability for the consumed dairy product were checked at d 21 and 42.

The main endpoints were the changes in the two serum markers of bone resorption, CTX and TRAP 5b (18,19). Secondary endpoints included serum changes of IGF-I, 25OHD, PTH, and bone formation markers.

Biochemical measurements. Serum calcium was measured by colorimetry, sodium and potassium by indirect potentiometry with the use of specific electrodes, and creatinine by the Jaffé reaction (Roche Diagnostics) (20). Serum PTH, osteocalcin, P1NP, and CTX (Cross-laps) were measured by automated immunochromoluminoscence on the Elecsys platform (Roche Diagnostics) as previously described (20). For these 4 biochemical analyses, the within- and between-run CV were <5%, whatever the concentration tested. For PTH, the reference range was established in a population sample with serum 25OHD concentration >50 nmol/L (21). The upper limit of the PTH reference range was 46 ng/L (21), somewhat lower than that commonly used as provided with the commercial kit (20). The reference ranges of the three other assays were established in a group of 59 premenopausal healthy women aged 35–48 y. All of them had regular menses associated with plasma FSH concentration <12 mIU/L. They received a single dose of 2 mg of cholecalciferol 1 wk before the blood sampling. The reference ranges were 10–46 ng/L, 13–32 μg/L, 19–50 μg/L, and 0.7–3.0 nmol/L for PTH, osteocalcin, P1NP, and CTX, respectively. BAP was measured by automated immunochromoluminoscence on the Access II platform (Beckman-Coulter). BAP within-run CV was 6.7% at a mean concentration of 13.2 μg/L and <5% at a concentration >25 μg/L. The reference range established in healthy premenopausal women was 4–15 μg/L.

TRAP 5b, a surrogate marker of osteocalcist number (19), was determined by immunoassay. The method uses a monoclonal antibody raised against TRAP 5b purified from human osteoclasts and recombinant human TRAP as a standard (Bone TRAP Enzyme Assay kit, SBA Sciences). Intra- and inter-assay CV were <4.8 and 5.2%, respectively.

The serum concentration of 25OHD was measured by RIA (DiaSorin) as previously reported (22). Vitamin D insufficiency was defined as a serum concentration of 25OHD <75 nmol/L. Serum IGF-I was measured by an immunoradiometric assay (IGF-I RIA-CT, Schering-Cis Bio) based on the use of two monoclonal antibodies directed toward different IGF-I...
epitopes. In this method, bound IGF-I is displaced from IGFBP by acidification. A large excess of IGF-II is then added to the acid-treated serum to prevent reassociation of IGF-I with its carrier proteins when buffer is added. The analytical properties of this assay were recently reported (20). The normal range of serum IGF-I in women aged 36–85 y \( (n = 197) \) was 68–247 μg/L (personal data).

**Statistical analysis.** Determination of the sample size was estimated from the effects on changes in two bone resorption markers, CTX and TRAP5b (13,14), observed in previous nutritional interventions with dairy products. The intra- and inter-individual variability was found to be less with TRAP5b compared to CTX. To achieve a power of 80% and a 2-sided \( \alpha \) of 0.05, the sample size was estimated to be 96 (48/group) and 62 (31/group) participants with CTX and TRAP5b, respectively. An unexpected difficulty limited the number of enrolled women to 71 (36 and 35 in the treated and control groups, respectively) instead of 96 as initially foreseen.

Changes after the 6-wk randomized controlled trial. Daily calcium consumption increased from baseline in the two groups but, as expected, much more in the treated than in the control group (\( P < 0.0001 \)) (Table 3). Protein consumption increased from baseline in the treated but not in the control group, resulting in a difference in changes of 10.5 g/d (\( P = 0.02 \)) (Table 3). At baseline, the daily intakes of lipids, carbohydrates, and energy did not differ between the two groups (Table 1). From the dietary follow-up questionnaire completed on d 21 and 42, the consumption of these did not change in either group (Table 3). In agreement with these FFQ data, the dietary follow-up monitoring variables significantly differed between the two groups (Table 2). In agreement with the inclusion selection, 25OHD was low, at a concentration considered to indicate vitamin D insufficiency (25). The low circulating concentration of 25OHD was not associated with a mean serum PTH concentration greater than the upper limit of the reference range (Table 2). As expected in postmenopausal women, the biochemical expression of increased bone turnover was observed, with CTX and PINP concentrations higher than the reference range established in premenopausal women aged 35–48 y (Table 2). Other markers of bone formation (BAP and osteocalcin) were still within the premenopausal reference ranges. Serum TRAP5b was not above a reference range established in premenopausal women aged 22–54 y (Table 2). Serum concentrations of albumin, prealbumin, and IGF-I were within the corresponding reference ranges (Table 2). We expected these results, because the women had a daily protein intake of 1.25 g/kg body weight (i.e., 74.3 g/d for women with mean body weight of 59.6 kg) compared to the recommended allowance of 0.8–1.0 g/kg body weight for the French population (24) (Table 1).

**Results**

**Baseline characteristics.** The demographic characteristics recorded at the inclusion did not significantly differ between the control and treated groups (Table 1). The mean time elapsed since menopause was \( \sim 7.5 \text{ y} \), indicating that the clinical expression of ovarian function cessation was very close to 50 y of age. BMI was within the reference range for the French female population (23). According to the inclusion criteria, the mean daily calcium consumption, 560 mg/d, was relatively low, corresponding to 47% of the French RDA, which is set at 1200 mg/d for women aged >55 y (24). It amounted to 70% of the two-thirds of French RDA (24).

Using the French version of the FRAX algorithm (17), the 10-y risk of major osteoporotic fractures and hip fracture alone was <4.0% (range 2.2–13.0%) and 1.0% (range 0.2–5.0%), respectively (Table 1). Note that 11 of the 71 (15.5%) enrolled women, with a median age of 57 y, reported having experienced a fracture before the last 12 mo (14.3 and 16.7% in the control and treated groups, respectively).

At the inclusion visit, none of the measured biochemical variables significantly differed between the two groups (Table 2). In agreement with the inclusion selection, 25OHD was low, at a concentration considered to indicate vitamin D insufficiency (25). The low circulating concentration of 25OHD was not associated with a mean serum PTH concentration greater than the upper limit of the reference range (Table 2). As expected in postmenopausal women, the biochemical expression of increased bone turnover was observed, with CTX and PINP concentrations higher than the reference range established in premenopausal women aged 35–48 y (Table 2). Other markers of bone formation (BAP and osteocalcin) were still within the premenopausal reference ranges. Serum TRAP5b was not above a reference range established in premenopausal women aged 22–54 y (Table 2). Serum concentrations of albumin, prealbumin, and IGF-I were within the corresponding reference ranges (Table 2). We expected these results, because the women had a daily protein intake of 1.25 g/kg body weight (i.e., 74.3 g/d for women with mean body weight of 59.6 kg) compared to the recommended allowance of 0.8–1.0 g/kg body weight for the French population (24) (Table 1).

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did not reveal any significant changes in participant food habits or food substitution.

From d 1 to 42, the serum bone resorption marker CTX did not change (+4.0%; P = 0.38) in the control or treated (−4.7%; P = 0.19) group (Fig. 1; Table 4) and the changes did not differ between the groups (P = 0.23). The other serum bone resorption marker, TRAP 5b, decreased in both the control (P = 0.008) and treated (P < 0.0001) groups and the decline was greater (P = 0.011) in the treated group than in the control group (Fig. 1; Table 4).

In the treated group, a negative correlation (r = −0.64; P < 0.0001; n = 36) was computed between the initial concentration and the magnitude of the decrease of TRAP 5b after 6 wk of intervention.

By the end of the intervention, besides the absolute (Table 4) and relative (Fig. 1) changes in bone resorption markers, there was also a significant increase in serum IGF-I in the treated group.

The variations in the concentration of serum 25OHDL were similar in the two groups (Table 4), but the absolute values remained lower by 8–10 nmol/L than the inferior limit (75 nmol/L) of the reference range (21) (Table 2). Between d 1 and 42, serum PTH did not significantly change in either group (Table 4).

From self-rating questionnaires, compliance was high throughout the 42 d of dairy product consumption. The acceptability in terms of taste and portion size was satisfactory, as also corroborated by the absence of tiredness in consuming the tested dairy product.

Discussion

In two previous studies carried out in institutionalized elderly women with mean ages of 84.8 and 86.9 y, we reported (13,14) that the regular consumption of soft white cheese significantly reduced both serum CTX and TRAP 5b, two biochemical markers of bone resorption. In the present trial carried out in healthy postmenopausal women with mean ages of 56.6 y, thus younger than the two previously institutionalized cohorts, we confirmed the effect on TRAP 5b, but not on CTX. As indicated in “Methods,” a 26% reduction in the eventually enrolled sample (Table 4) and end (d 42) of the intervention. A different from baseline, P < 0.05; *different from control, P < 0.05. BAP: bone alkaline phosphatase; CTX, carboxy terminal crosslinked telopeptide of type I collagen; IGF-I, insulin-like growth factor-I; 25OHD, 25-

Hydroxyvitamin D; P1NP, amino-terminal propeptide of type I procollagen; PTH, parathyroid hormone; TRAP 5b, tartrate resistant acid phosphatase, isoform 5b.


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In our two aforementioned studies (13,14), similar significant negative relationships were observed between the initial concen-
tation and the magnitude of the absolute reduction of either serum CTX or TRAP 5b as recorded 4 or 6 wk after the beginning of the dairy product consumption. The initial concentration of serum CTX, which was lower in healthy postmenopausal women in their mid-fifties than in institutionalized women in their mid-eighties, might account for the difference in the magnitude of the CTX response to the same dietary intervention.

The two serum markers, CTX and TRAP 5b, used in this study should theoretically reflect different steps in the process of bone resorption (18,19,27). The former is a collagen degradation product that reflects bone matrix resorption, whereas the second is an acid phosphatase enzyme secreted by the osteoclast (18,19,27). The measured TRAP 5b isomer is a circulating acid phosphatase specific for bone (27). The absolute decline in the antiresorptive response is less pronounced than with other markers of bone resorption, including serum CTX (27). However, the signal:noise ratio is better with TRAP 5b than with CTX (27). Furthermore, it has the advantage of not being influenced by food intake and its diurnal variation is minimal (27). Last but not least, the within-subject variability of TRAP 5b is lower than that of many other urinary and serum telopeptide markers (27). It has the advantage of reflecting the activity of the osteoclasts, the cells directly responsible for resoring bone, rather than collagen degradation like CTX, which is secondary to this cellular activity (19,27). These biological and analytical favorable characteristics may be particularly advantageous when the statistical power of a study is not optimal and/or the inhibitory effect on bone resorption is relatively moderate, as in the foregoing study. TRAP 5b could be the resorption marker of choice when food intervention studies are carried out in populations with mild bone remodeling elevation as in healthy women <10–15 y after the beginning of menopause. Furthermore, TRAP 5b is produced all along the osteoclastogenesis process (19), whereas CTX is released only when bone matrix is degraded by mature, treated osteoclasts. Therefore, it is possible that in nutritional interventions leading to an inhibition of bone resorption, the number of osteoclastic cells secreting TRAP 5b might decrease earlier than the consecutive functional consequence of reduced bone resorption, as expressed by the decline in the circulating concentration of CTX.

Our study can be considered as part of the nutritional approach to the prevention of postmenopausal osteoporosis, with the consumption of a nutrient-rich food according to the formal ranking scoring system (28). The combined increase in intakes of both calcium and protein, as well as other nutrients present in the dairy products (8,29), can explain the reduction in the bone resorption marker, TRAP 5b, associated with the significant increase in the serum IGF-I concentration. These dual biochemical changes have already been observed with dairy product supplementation in postmenopausal women over a wide range of age (13,14,30–33). That the increased IGF-I was mostly due to the additional protein supply rather than the possible associated increase in energy was demonstrated in a 6-mo, randomized, controlled intervention trial in which an isocaloric supplement was used as a placebo against a 20-g/d casein supplementation (34). There is some evidence indicating that protein and calcium intakes act synergistically on bone health when supplied in adequate amounts (35,36).

In the present study, a significant fall in serum PTH in response to the increased calcium intake was not found; hence, it is unlikely that this hormone was involved in the observed reduction of bone resorption. There is evidence that calcium, through its specific cation-sensing receptor, could directly inhibit osteoclast-mediated bone resorption [for review, see (37)]. Furthermore, besides vitamin D, calcium, and proteins, other food constituents, such as magnesium, potassium, several B vitamins (8), and certain fatty acids (29), can contribute to the nutritional prevention of osteoporosis. The tested dairy product also contains some of these nutrients, which thereby might have contributed to the changes in bone metabolism observed in our intervention study.

As observed in many regions of the world, vitamin D insufficiency is not uncommon (38) among women with no particularly unhealthy habits. In the present study, this was the case for a substantial number of the French women who were first screened by a questionnaire that inquired about vitamin D supply (16) and then by measuring the serum concentration of 25OHD.

According to the French FRAX assessment, the 10-y fracture probability appears to be relatively low for postmenopausal women with ammean age of 56.6 y (39,40). Indeed, two independent French population studies clearly reported a higher recorded incidence rate of fractures than predicted from the French FRAX algorithm (39,40). Therefore, it remains possible that in the present cohort of postmenopausal women, the actual incidence of 10-y fracture risk may also be higher than the relatively low predicted rate. As expected from the FRAX algorithm (41) in our cohort, the risk increased with both age and reduction in BMI.

Our study has limitations and strengths. A first limitation was to enroll only 74% of the initial sample size estimate, thus reducing the power of the study to detect a significant decrease in serum CTX. Nevertheless, the enrolled number of women was larger than the estimate needed to detect a difference in TRAP 5b, the other marker of bone resorption. Another limitation was that the 6-wk duration may have been too short for assessing significant decreases in both serum PTH and CTX along with that measured for TRAP 5b. However, a longer study may have impaired the notable adherence of all participants to the protocol. Whereas the tested dairy food provided a substantial daily supplement of calcium (+300 mg/d) and proteins (+10.5 g/d), the additional vitamin D supply (+2.5 μg/d) was not sufficient to raise the serum 25OHD concentration of the treated group to the normal range. The positive results obtained with the dairy product might be limited to a very small fraction of the general population of healthy postmenopausal women with low calcium intake. However, during the screening phase, only 1 of 4 women consumed >650 mg/d of calcium. This observation indicates that a few years after menopause, a large sample of the general healthy French population has a calcium intake of about one-half the recommended allowance and therefore could benefit from an additional supply.

The main strength of the study is its randomized, controlled design with very similar demographic, nutritional, and biochemical characteristics, as determined in the two groups at baseline. Another strength is the high degree of compliance with no enrolled participants dropping out, thus allowing the data evaluation of all participants by intention-to-treat analysis. Finally, the maintenance of the food habits during the 6-wk intervention, as documented by the dietary follow-up indicating no significant difference in the intake of lipids, carbohydrates, and energy between the two groups, can be placed on the positive side in a trial testing the influence of a specific dairy product.

In conclusion, increased bone resorption after the cessation of the ovarian function is a main detrimental factor enhancing the risk of fragility fracture in later life. In this 6-wk, randomized, controlled study, we showed that the regular consumption of fortified soft white cheese by healthy, community-dwelling women at relatively low risk of osteoporotic fracture significantly reduced the serum concentration of TRAP 5b, a marker of osteoclastic bone resorption. TRAP 5b appears to be a sensitive biochemical factor that can be used in nutritional interventions aimed at attenuating the osteoporotic process from early post-
menopausal years in healthy women to later life in institutionalized patients at high risk of fracture.

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Literature Cited