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ORIGINAL CONTRIBUTION

Calcium and vitamin D supplementation through fortified dairy products counterbalances seasonal variations of bone metabolism indices: the Postmenopausal Health Study

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Abstract
Purpose To assess the effectiveness of a dietary intervention combined with fortified dairy products on bone metabolism and bone mass indices in postmenopausal women.
Methods Forty postmenopausal women (55–65 years old) were equally randomized into a dietary group (DG), receiving daily and for 30 months, 1,200 mg of calcium and 7.5 μg of vitamin D_3 for the first 12 months that increased to 22.5 μg for the remaining 18 months of intervention through fortified dairy products; and a control group (CG). Differences in the changes of bone metabolism and bone mass indices were examined with repeated measures ANOVA.
Results A significant increase was observed for PTH levels only in the CG during the first six winter months of intervention ($p = 0.049$). After 30 months of intervention, during winter, serum 25(OH)D significantly decreased in the CG while remained in the same high levels as in the summer period in the DG. Serum RANKL levels decreased significantly in the DG compared with the increase in the CG during the 30-month intervention period ($p = 0.005$). Serum CTx decreased significantly in the DG after six (−0.08; −0.12 to −0.03) and 12 (−0.03; −0.08 to −0.02) months of intervention. Finally, the DG had more favorable changes in total body BMD than the CG ($p < 0.001$).
Conclusions Increasing dietary intake of calcium and vitamin D in osteopenic postmenopausal women appears to be effective in producing favorable changes in several bone metabolism and bone mass indices and in counterbalancing seasonal variations in hormonal and biochemical molecules.

Keywords Calcium and vitamin D · RANKL · Bone metabolism · Postmenopausal women · Physiology

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Introduction

The skeleton is a metabolically active organ that undergoes continuous remodeling throughout life. The activities of bone-forming osteoblasts and bone-resorbing osteoclasts are controlled by a variety of hormones, mainly parathyroid hormone (PTH) and calcitriol [1]. During bone resorption, osteoclastic activity leads to the release of breakdown products of type-I collagen, such as C-terminal telopeptides (CTx) [2]. In healthy bone, the resorption cavity created by osteoclasts is completely filled with new osteoid material secreted by active osteoblasts, such as osteocalcin (OC). Besides bone remodeling indices, the identification of the osteoclastogenesis inducer, the receptor activator of nuclear factor-kappaB ligand (RANKL), its cognate receptor RANK, and its decoy receptor osteoprotegerin (OPG), has also contributed enormously to our understanding of the molecular mechanisms involved in osteoclast differentiation and activity. RANKL binds to RANK on the osteoclastic precursors or mature osteoclasts and promotes osteoclastogenesis and bone resorption, while OPG strongly inhibits bone resorption by binding to its ligand RANKL and thereby blocks the interaction between RANKL and RANK [3]. Prevention of pathological bone

loss therefore depends on an appreciation of the mechanisms by which osteoclasts differentiate from their precursors and degrade the skeleton [4].

Disorders of the skeleton, such as osteoporosis, typically represent enhanced osteoclastic bone resorption relative to bone formation. As life expectancy in developed countries is increasing [5], osteoporosis is becoming a major public health problem of great concern, particularly for susceptible population groups, such as postmenopausal women [6]. Women generally lose about 1–2% of their bone per year during and after menopause. Some of the main risk factors for the progression and development of bone loss in these population groups are related to lifestyle and particularly to inadequate dietary intake of certain essential for bone health micronutrients (i.e. calcium and vitamin D) [7]. Even though there is an abundance of clinical trials indicating the beneficial effects of calcium and/or vitamin D supplementation on the prevention of bone loss and on the levels of bone remodeling indices [8–12], there are very few dietary intervention studies examining the effect of increased intake of these micronutrients through fortified foods [13, 14].

The aim of the present study was to examine whether a holistic intervention approach, combining nutrition and lifestyle counseling with consumption of dairy products enriched with calcium and vitamin D for 30 months, would have any potential beneficial effect on bone metabolism and bone mass parameters of Greek postmenopausal women, and how this would be affected by seasonality and supplemented vitamin D dose.

Methods

Sampling

First screening

The study was approved by the Ethical Committee of Harokopio University of Athens and was conducted in accordance with the code of ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans. In July 2004, volunteers were invited to participate by informational brochures and posters distributed in public buildings and community centers in three municipalities within the wider district of Athens, namely Nea Smyrni, Kallithea, and Neo Iraklio. Through this initial screening, conducted in the premises of the aforementioned settings and with the co-operation of the local authorities, a sample of 307 Greek postmenopausal women volunteered to participate. More information on the procedures and the inclusion/exclusion criteria used for recruiting volunteers at this stage is presented in details elsewhere [15].

Second Screening

After the initial screening, 96 women (age 55–65 years) satisfying the inclusion criteria were identified and were invited to participate at the second screening of the study. During the second screening, all volunteers underwent a dual-energy X-ray absorptiometry (DXA; Lunar DPX-MD, Madison, USA) measurement, as well as hematological and biochemical examinations, comprising hematological profile, erythrocyte sedimentation rate and serum calcium, phosphorus, glutamic-oxaloacetic and glutamic-pyruvic transaminases, alkaline phosphatase, and creatinine levels. Those women found to be osteoporotic, according to the data provided by the DXA examination, or having abnormal values on the aforementioned blood indices, were excluded from the study. This second screening yielded 82 eligible women who prior to their entry to the study signed detailed consent forms of participation and proceeded in the intervention study. The intervention component of the study was initiated in October 2004.

Study Groups

From these eligible women, 40 were randomly selected to have a full screening of bone metabolism indices (as described in details below) and were randomized to a dietary intervention group (DG) and a control group (CG), using a table of random digits. The DG consisted of 20 women, who were advised to consume three portions of fortified with calcium and vitamin D_3 low fat dairy products (milk and yogurt) on a daily basis. To ensure compliance to the intervention scheme, lifestyle and nutrition counseling sessions were held biweekly within the settings of the University, and the required quantities of fortified dairy products for the next two weeks were provided at the end of the sessions. The aim of the sessions delivered to the DG was to increase awareness of the subjects on health issues, primarily related to osteoporosis, but also to motivate them to change certain lifestyle and dietary habits in order to improve their health status. The first sessions primarily focused on educating the subjects on the pathophysiology of osteoporosis as well as the risk factors (health related behaviors) related to its development. Gradually, the sessions became more interactive and emphasis was given in guiding and assisting the subjects in changing their dietary habits. In order to avoid excess caloric intake, subjects in the DG were advised to substitute other dairy products in their diet with those provided. The dairy products, produced by Friesland Foods Hellas (Athens, Greece), were milk and yogurt enriched with calcium (33%), which provided a total of 400 mg Ca/portion (one portion equals to 250 mL of milk and to 200 gr of yogurt). Fortification of milk and yoghurt with

calcium was made by adding concentrated milk protein, a natural source of milk calcium, manufactured from fresh skim milk by ultra filtration and spray drying. Regarding the vitamin D content of the dairy products, the quantity used for fortification increased from 2.5 to 10 μg of vitamin D_3 per milk portion, after the first 12 months of intervention, while remained the same in the yogurt products (i.e. 2.5 μg per portion). This led to a total daily supplementation of 22.5 μg of vitamin D_3 to the DG subjects through the consumption of two portions of milk and one portion of yogurt. The vitamin D_3 used for fortification was a white, odorless powder in a carrier of dicalcium phosphate and tricalcium phosphate produced by Watson Foods Co, Inc.

Regarding compliance to the intervention scheme, this was assessed via information obtained at the biweekly sessions, combined with data obtained from the nutritional assessments conducted at baseline and follow-up examinations. These data showed that the compliance to the intervention scheme was reaching a rate of 93% (range 89–100%). Subjects in the CG comprised 20 women, to whom no intervention (i.e. neither fortified dairy products nor nutrition and lifestyle counseling sessions) was delivered as they continued with their usual diet throughout the intervention of 30-month period. The sample sizes in the two groups were adequate since we achieved statistical power greater than 90% for standardized differences for the main outcomes of our study (i.e. BMD) between groups greater than 2.5 (s.e 1.4) at probability of type-I error < 0.05.

Assessment of the effectiveness of the intervention

Nutritional Assessment

At baseline, mid-term and final examinations, the 24-hour recall technique was used to collect dietary intake information for a total of three days, two weekdays and one weekend day, most preferably Sunday. All interviewers were rigorously trained to minimize interviewer effects. Respondents were asked to recall the type and amount of any food and beverage consumed during the previous day in a chronological order, i.e. from the time they woke up in the morning to the same time the following day. To improve the accuracy of food descriptions and portion sizes, standard household measures (cups, tablespoons, etc.) and picture food models (Dairy Food Council, USA) were used during interviews to define amounts when appropriate. Food intake data were analyzed using the Nutritionist V diet analysis software (First Databank, San Bruno, CA), which was extensively amended to include Food Composition Tables for Greek foods and recipes [16, 17] and chemically analyzed commercial food items widely consumed in Greece.

Physical activity assessment

Assessment of physical activity was made by a 3-day activity interview questionnaire. Respondents reported the time spent on various physical activities during two consecutive weekdays and 1 weekend day. The questionnaire classified all work, sport, and leisure activities into four categories, on the basis of their average intensity relative to the impact on the cardiovascular system (low to high), and also by subgrouping activities according to their impact on bone mass (low to high) [18]. The aim of the questionnaire was to determine the frequency and duration (hours per session) subjects devoted weekly in these physical activity categories. The total amount of time devoted weekly on activity categories having intensity higher than four metabolic equivalents was defined as time spent on moderate-to-vigorous physical activities (MVPA).

Biochemical Analyses

Early-morning venous blood samples were obtained from each subject for biochemical screening tests following a 12-h overnight fast. Professional staff performed venipuncture to obtain a maximum of 25 mL blood. The blood was collected in vacutainers with no added anticoagulant, where it was allowed to clot for approximately 2 h as this was designated for serum separation. Centrifugation for serum separation was conducted at 3,000 rpm for 15 min. A part of the collected serum was used for biochemical analyses, while aliquots of 1.5 mL from the remaining serum were pipetted into plastic Eppendorf tubes and stored at −80 °C.

Biochemical analyses included chemiluminescence immunoassay used to assess serum levels of osteocalcin (OC), 25-hydroxy vitamin D (25(OH)D), insulin-like growth factor I (IGF-I) (Nichols Advantage®, Nichols Institute Diagnostics, San Clemente, USA) and intact parathyroid hormone (PTH) (Elecsys® Roche Diagnostics, Mannheim, Germany). Serum type-I collagen cross-linked C-telopeptide (CTx) was measured by an electrochemiluminescence immunoassay (Elecsys® Roche Diagnostics, Mannheim, Germany). The interassay coefficients of variation were 8.6% for osteocalcin, 4.7% for sCTx, 8% for $25(OH)D_3$, 6.7% for IGF-I, and 5.0% for PTH. Furthermore, ELISA method was used for the quantitative determination of human OPG and human ampli-sRANKL, in duplicate in serum samples of the participants by the Biomedica Gruppe immunoassay kits (Biomedica Medizinprodukte GmbH & Co KG, Wien, Austria).

Bone Mineral Density measurements

Total body and lumbar spine (L2-L4) bone mineral density (BMD, g/cm^2) were measured at baseline, at 12 and at

30 months of follow-up examination, respectively, using dual-energy X-ray absorptiometry (DXA; Lunar DPX-MD, Madison, USA) with the analysis software version 4.6. Same geometry at repeated measurements was accounted for by ensuring correct positioning of each study participant, exactly as indicated in the manufacturer's manual. A daily quality assurance check was performed at each time point of follow-up examination, using a calibration standard of known composition, provided by the manufacturer. The scans were performed in the morning by an experienced technician, who was blinded to the therapy.

Statistical analysis

All data are reported as mean (standard deviation: SD) and mean change (95% confidence interval: CI) over baseline. The Kolmogorov–Smirnov test was used to determine normality of distribution of the examined variables. Repeated measures analysis of variance was used to evaluate the significance of the differences between groups at baseline, 6, 12, and 30 months follow-up (treatment effect), the significance of the changes observed within each group (time effect), and the effect of treatment × time interaction. The between-group factor was the study groups (i.e. DG vs. CG); the within-group factor was the time point of measurement (i.e. baseline, 6, 12, and 30 months of intervention). All p values reported were two-tailed. Statistical analysis was conducted with the SPSS (version 13.0). The level of statistical significance was set at $p \leq 0.05$.

Results

Table 1 summarizes the differences observed between groups with respect to the changes in dietary intake of energy, macronutrients, and certain micronutrients that are related to bone metabolism. Regarding energy, fat and carbohydrates dietary intakes, no differences were observed between groups. However, the average dietary intake of protein increased to a higher extent in the DG compared with the CG ($p = 0.020$). Regarding micronutrients, the DG showed higher increases in calcium ($p < 0.001$), phosphorus ($p = 0.014$), magnesium ($p < 0.001$), and vitamin D ($p < 0.001$) intakes, compared with the respective changes observed in the CG. Regarding physical activity, no significant differences were observed between groups in the time spent to MVPA over the intervention period (data not shown).

According to the data presented in Table 2, a significant treatment × time interaction effect was observed for serum PTH ($p = 0.049$) and serum 25(OH)D ($p < 0.001$) levels. Specifically, a significant increase was observed for PTH levels only in the CG during the first six winter months of intervention, resulting to significantly higher PTH levels in the CG compared with the DG ($p = 0.014$). Regarding serum 25(OH)D levels, decreases were observed for both groups during the first six winter months of intervention. However, the decrease was significant only in the CG (−7.2; 95% CI: −10.2 to −4.1) leading to significantly higher mean serum 25(OH)D levels in the DG compared with the CG ($p = 0.007$). After 12 months of intervention (summer period), a significant increase was observed only in the DG (4.3; 95% CI: 1.1–7.5). After 30 months of intervention and during the winter period, serum 25(OH)D significantly decreased only in the CG (−7.5; 95% CI: −11.8 to −3.1) while remained in the same high levels as in the summer period in the DG. These changes resulted in significantly higher mean serum 25(OH)D levels in the DG compared with the CG at the 30-month intervention time point ($p < 0.001$). Furthermore, the decrease observed in the DG for serum RANKL levels was found to differentiate significantly compared with the increase observed in the CG during the 30-month intervention period ($p = 0.005$). Regarding the changes in serum IGF-I and OPG levels and in the OPG-to-RANKL ratio, there were no statistically significant differences between the two groups.

The changes observed in the examined biochemical indices of bone turnover are presented in Fig. 1. A significant treatment × time interaction effect was found for serum CTx levels ($p = 0.001$). Specifically, serum CTx decreased significantly only in the DG after six (−0.08; 95% CI: −0.12 to −0.03) and 12 months (−0.11; 95% CI: −0.16 to −0.06) of intervention while remained unchanged in the CG during the 30-month intervention period. No significant differences were observed between the two groups for serum osteocalcin levels. According to the data presented in Fig. 2 at the end of the intervention period, the DG was found to have more favorable changes in total body BMD ($p < 0.001$) compared with the relative changes observed in the CG. Although lumbar spine BMDs increased in the DG and decreased in the CG, still no statistically significant differences were observed between the two study groups.

Discussion

The objective of the present study was to investigate the influence of a dietary intervention program based on calcium and vitamin D supplementation through fortified dairy products on bone mass and metabolism indices in a sample of 40 apparently healthy postmenopausal women for a period of 30 months. Regarding dietary intake of micronutrients (Table 1) calcium intake was significantly improved in the DG since it remained close to the

Table 1 Differences in dietary intake of energy, macronutrients and micronutrients between women in the intervention ($n = 20$) and control group ($n = 20$) during the 30-month intervention period

	Baseline mean (SD)	6-month follow-up mean (SD)	12-month follow-up mean (SD)	30-month follow-up mean (SD)	p value†
Energy intake (Kcal/day)					0.643
Control group	1720.6 (499.6)	1619.8 (296.9)	1527.8 (354.9)	1744.9 (388.6)	
Intervention group	1687.0 (244.2)	1676.0 (260.4)	1564.7 (196.6)	1946.5 (498.7)	
p value‡	0.798	0.529	0.686	0.162	
Carbohydrates intake (% of Kcal)					0.355
Control group	36.3 (7.3)	34.3 (8.3)	36.3 (6.8)	40.1 (9.7)	
Intervention group	38.5 (8.3)	32.2 (2.8)	37.1 (4.8)	38.8 (8.2)	
p value‡	0.381	0.297	0.673	0.639	
Total fat intake (% of Kcal)					0.696
Control group	33.4 (5.6)	29.5 (7.3)	31.1 (7.6)	30.9 (8.3)	
Intervention group	31.6 (7.0)	27.6 (2.6)	27.6 (3.4)	31.4 (7.1)	
p value‡	0.368	0.276	0.071	0.847	
Protein intake (% of Kcal)					0.020
Control group	12.6 (2.2)	16.5 (5.2)	13.5 (2.2)	12.2 (4.4)	
Intervention group	12.5 (2.3)	19.9 (2.8)	16.8 (3.0)	14.6 (3.9)	
p value‡	0.938	0.015	<0.001	0.077	
Calcium intake (mg/day)					<0.001
Control group	723.4 (250.9)	593.5 (360.8)	634.3 (381.1)	563.6 (309.5)	
Intervention group	675.7 (238.5)	1244.5 (291.3)	1227.5 (295.4)	1336.6 (500.0)	
p value‡	0.552	<0.001	<0.001	<0.001	
Phosphorus intake (mg/day)					0.014
Control group	957.2 (335.3)	955.1 (385.7)	942.5 (327.2)	1132.7 (539.1)	
Intervention group	1014.8 (296.5)	1327.4 (309.9)	1367.0 (289.8)	1561.0 (674.6)	
p value‡	0.568	0.002	<0.001	0.033	
Magnesium intake (mg/day)					<0.001
Control group	214.4 (59.1)	198.3 (65.3)	191.6 (91.4)	247.3 (104.2)	
Intervention group	208.6 (42.1)	292.0 (69.3)	309.1 (64.3)	329.0 (120.4)	
p value‡	0.730	<0.001	<0.001	0.039	
Vitamin D intake (μg/day)					<0.001
Control group	0.55 (0.63)	0.56 (0.91)	0.99 (1.67)	0.77 (1.38)	
Intervention group	0.59 (0.76)	5.76 (2.30)	5.98 (1.74)	18.47 (1.42)	
p value‡	0.867	<0.001	<0.001	<0.001	

† Treatment × Time interaction effect; ‡ Between groups' comparisons at baseline, 6, 12, and 30 months (treatment effect)

recommended level of 1,200 mg per day [19] throughout the intervention period. Furthermore, the increase in the vitamin D content of the fortified dairy products after the first 12 months of intervention led to a total daily supplementation of 22.5 μg per day. This increase was mainly ascribed to the results from the first 12 months of intervention that showed that the daily dose of 7.5 μg of vitamin D provided to the IG was probably not enough to counterbalance the reduction of serum 25(OH)D levels during winter months [15]. In addition, it was based on new emerging scientific evidence suggesting the need of combined supplementation with calcium and vitamin D in the order of 1,000–1,200 mg calcium and 17,5–20 μg of vitamin D daily in osteopenic postmenopausal women [20, 21].

The effect of the intervention, on the examined biochemical indices of calcium homeostasis, should mainly be attributed to the dietary changes, as physical activity levels remained unchanged (data not shown). Specifically, in the first six winter months of intervention, mean serum PTH levels were significantly higher in the CG compared with the DG. This effect could be attributed to the calcium supplementation that improved calcium status and suppressed PTH secretion. Our findings were in consistence with similar studies showing that calcium supplementation or consumption of fortified dietary products suppress serum

Table 2 Changes in serum levels of hormones related to calcium homeostasis (PTH and 25(OH)D), of IGF-I, of RANKL and OPG for women in the dietary ($n = 20$) and control group ($n = 20$) after 6, 12, and 30 months of intervention

	Baseline (summer)	6-month follow-up (winter)	6-month change	12-month follow-up (summer)	12-month change	30-Month follow-up (winter)	30-month change	p value†
	Mean (SD)	Mean (SD)	Change (95% CI)	Mean (SD)	Change (95% CI)	Mean (SD)	Change (95% CI)	
Serum PTH (pg/mL)								
Control group	53.7 (24.6)	64.2 (24.6)	10.4 (1.4. 22.3)	50.4 (15.5)	−3.3 (−10.1, 3.5)	48.8 (20.9)	−4.9 (−11.1, 1.2)	0.049
Intervention group	43.7 (16.2)	45.2 (21.8)	1.5 (−10.3, 13.4)	43.0 (15.8)	−0.7 (−7.5, 6.1)	38.5 (19.8)	−5.1 (−11.3, 1.0)	
p value‡	0.134	0.014		0.140		0.119		
Serum 25(OH) vitamin D (ng/mL)								
Control group	22.8 (7.0)	15.6 (6.0)	−7.2 (−10.2, −4.1)	25.5 (6.6)	2.7 (−0.5, 5.9)	15.3 (6.0)	−7.5 (−11.8, −3.1)	<0.001
Intervention group	23.3 (6.2)	21.0 (6.0)	−2.3 (−5.4, 0.8)	27.6 (5.6)	4.3 (1.1, 7.5)	27.2 (8.4)	3.9 (−0.4, 8.3)	
p value‡	0.818	0.007		0.278		<0.001		
Serum IGF-I (ng/mL)								
Control group	104.7 (35.4)	100.0 (30.0)	−4.6 (−22.1, 12.8)	100.5 (29.1)	−4.2 (−17.3, 8.9)	105.3 (30.1)	0.6 (−16.7, 17.9)	0.382
Intervention group	108.2 (43.6)	114.0 (46.6)	5.9 (−11.6, 23.3)	111.9 (43.3)	3.8 (−9.4, 16.9)	107.3 (33.0)	−0.9 (−18.2, 16.4)	
p value‡	0.782	0.266		0.335		0.842		
Serum RANKL (pg/mL)								
Control group	0.37 (0.11)	0.37 (0.21)	−0.003 (−0.10, 0.09)	0.35 (0.17)	−0.02 (−0.21, 0.17)	0.47 (0.30)	0.01 (−0.07, 0.27)	0.005
Intervention group	0.44 (0.21)	0.39 (0.25)	−0.05 (−0.15, 0.05)	0.53 (0.39)	0.09 (−0.10, 0.28)	0.35 (0.22)	−0.08 (−0.26, 0.08)	
p value‡	0.202	0.766		0.061		0.184		
Serum OPG (pg/mL)								
Control group	4.31 (0.00)	3.89 (0.86)	−0.42 (−0.96, 0.11)	4.02 (0.72)	−0.29 (−0.74, 0.16)	4.10 (1.19)	−0.21 (−0.96, 0.54)	0.063
Intervention group	4.31 (0.00)	4.55 (0.86)	0.24 (−0.29, 0.78)	4.68 (0.72)	0.37 (−0.08, 0.83)	4.68 (1.19)	0.37 (−0.37, 1.12)	
p value‡	–	0.035		0.014		0.179		
OPG-to-RANKL ratio								
Control group	12.1 (0.00)	13.8 (5.1)	1.8 (−1.4, 4.9)	13.2 (8.9)	1.1 (−4.4, 6.7)	12.8 (9.5)	0.8 (−5.1, 6.7)	0.377
Intervention group	12.1 (0.00)	15.1 (5.1)	3.0 (−0.1, 6.2)	14.3 (8.9)	2.3 (−3.3, 7.8)	17.9 (9.5)	5.8 (−0.1, 11.7)	
p value‡	–	0.451		0.699		0.113		

† Treatment × Time interaction effect (in the case of OPG and OPG-to-RANKL ratio, adjustments were also made for baseline values); ‡ Between groups' comparisons at baseline, 6, 12, and 30 months (treatment effect)

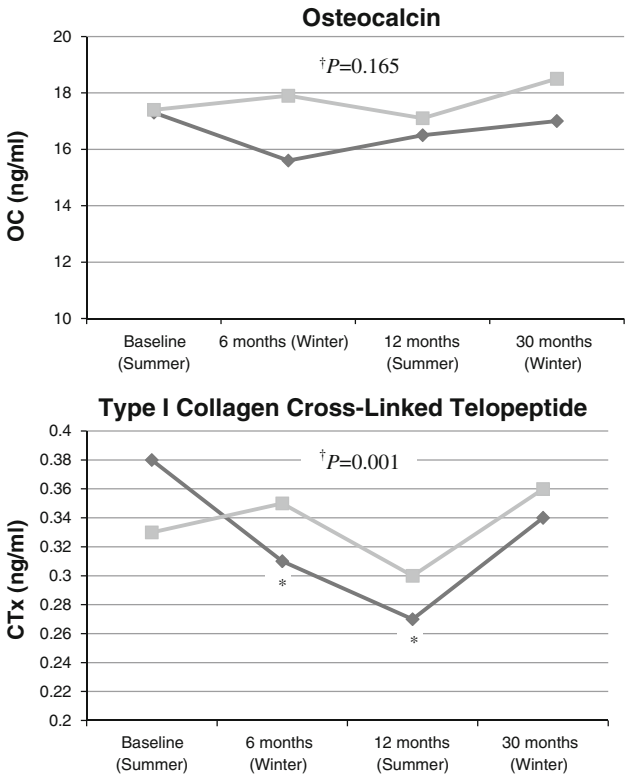

Fig. 1 Changes in serum levels of bone turnover biomarkers for women in the dietary ($n = 20$) and control group ($n = 20$) after 6, 12, and 30 months of intervention. Intervention group; Control group; †Treatment × time interaction effect; *$p < 0.05$ versus baseline within the same group (time effect)

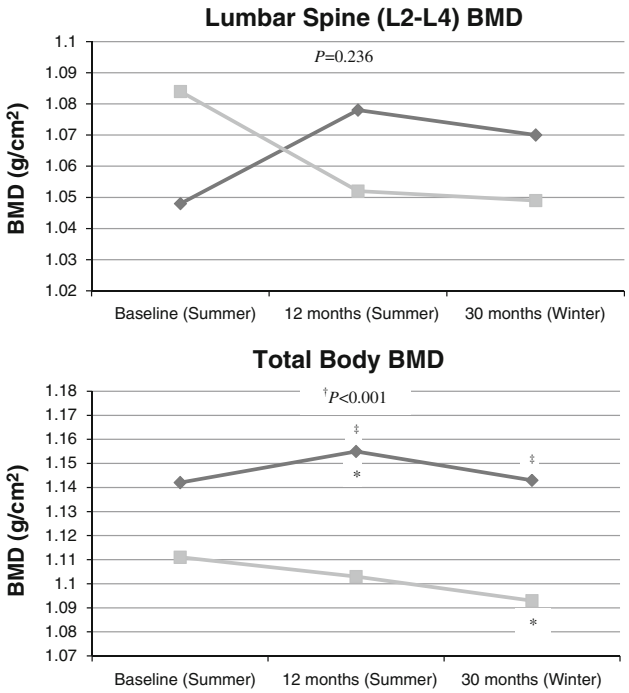

Fig. 2 Changes in lumbar (L2–L4) and total body BMD for women in the dietary ($n = 20$) and control group ($n = 20$) after 6 and 30 months of intervention. Intervention group; Control group; † Treatment × time interaction effect; ‡ $p < 0.05$ Intervention versus control group (Treatment effect); * $p < 0.05$ versus baseline within the same group (time effect)

PTH levels [22]. Furthermore, as dietary protein has been positively associated with increased serum levels of IGF-I, especially among elderly, undernourished, institutionalized osteoporotic patients [23], the significant increase in protein intake in the DG could also be of importance for the interpretation of the current findings. Still, the present study did not show any significant differences with respect to the changes of IGF-I levels between groups, probably because the study participants consisted of apparently healthy, free-living, osteopenic postmenopausal women. Regarding serum 25(OH)D levels, decreases were observed for both groups during the first six winter months of intervention. However, the decrease was significant only in the CG. After 30 months of intervention, during the winter period, serum 25(OH)D significantly decreased in the CG while remained in the same high levels as in the summer period in the DG. Maintenance of 25(OH)D levels during the winter in the same high levels as in the summer could probably indicate that the increase of dietary vitamin D intake from 7.5 μg to 22.5 μg per day was adequate to counterbalance the effect of season on vitamin D status. This observation is consistent with other studies showing that oral supplementation of vitamin D required to sustain serum 25(OH)D at baseline levels should be more than 12.5 μg per day [24]. Furthermore, a dose-dependent increase in serum 25(OH)D levels has also been reported, indicating that for every 2.5 μg of vitamin D ingested the blood levels of 25(OH)D increased by approximately 1 ng/mL [12, 24]. Still, in the present study, the mean serum 25(OH)D levels in the DG remained below what is considered to be a vitamin D sufficiency level (i.e. 30 ng/mL) [21] throughout the intervention period, probably indicating that a higher daily vitamin D dose should be used to increase circulating 25(OH)D above the desirable level of 30 ng/mL. This is in line with earlier studies demonstrating that a daily intake of 100 μg vitamin D is necessary to achieve a 25(OH)D level of 30 ng/mL in almost all individuals with low baseline 25(OH)D levels [25]. Overall, our data revealed that serum 25(OH)D levels in the CG followed a more seasonal pattern compared with the DG, with increases observed in the summer period (i.e. 12 months of follow-up) and decreases in the winter period (i.e. 6 and 30 months of follow-up), when sunlight (UV-B irradiation) exposure, and thus cutaneous 25(OH)D synthesis was decreased.

These hormonal changes could probably have led to the observed changes in serum RANKL and OPG levels. The decrease observed in the DG for serum RANKL levels was found to differentiate significantly compared with the increase observed in the CG during the 30-month

intervention period, indicating that the intervention favorably inhibited secretion of the marker that triggers bone resorption. In contrast to RANKL levels, serum OPG (a marker linked to bone formation) increased in the DG while decreased in the CG. The discovery of the unique role of the RANK/RANKL/OPG signaling has led to the targeting of this pathway as a novel therapeutic approach in the management of osteoporosis [26, 27]. Although very little is known so far about the effect of dietary interventions on bone markers, there have been studies in postmenopausal women where 24 months of genistein administration resulted in higher OPG and lower RANKL levels [28]. On the other hand, our study is, to our knowledge, the first one examining the effect of a dietary intervention on these two osteoclasts' differentiation molecules.

The changes observed in the molecular circuit of OPG/RANKL could have produced the changes in bone remodeling indices, i.e. in serum CTx and OC levels. Serum CTx remained unchanged in the CG during the 30-month intervention period. Nevertheless, a seasonal effect was evident in this group, as mean CTx levels remained higher during winter (6- and 30-month time points) and lower during the summer period (baseline and 12-month time points). Furthermore, serum CTx decreased significantly in the DG after six and 12 months of intervention indicating a strong suppressive treatment effect on this bone resorption marker. Although serum CTx levels increased after 12 months of intervention in the DG, it remained in lower levels compared to baseline. As far as serum OC levels were concerned, there were no significant differences in the observed changes between the two groups. However, similarly to the changes observed for CTx in the CG, serum OC levels in this group followed a seasonal pattern with higher levels in winter months and lower levels in summer months compared to baseline. On the other hand, in the DG serum OC remained at lower levels than at baseline throughout the intervention period. Overall, the changes observed in the CG were indicative of a clear seasonal effect on the rate of bone remodeling, showing increases during winter months and decreases during summer months. On the other hand, the implemented intervention managed to reduce the rate of bone remodeling in the DG. Suppression of this process has been associated with prevention of bone loss from skeletal sites that are susceptible to fracture as well as from the total body [8, 29].

The decreases in serum CTx and OC observed in the DG coincided with the more favorable BMD changes observed in this group. According to the results derived from the DXA measurements, the findings of the present study revealed more favorable changes over the 30-month intervention period in total body BMD in the DG compared with the CG (Fig. 2). In agreement to these findings, Riggs et al. [22] have reported an increase in total body BMD for Caucasian postmenopausal women after a 48-month supplementation of 1,600 mg of calcium per day. However, other dietary intervention studies conducted with Caucasian or Asian have reported either no change [30] or decreases [14, 31, 32] in total body BMD after daily supplementation of 1,000–1,200 mg of calcium and 6–20 μg of vitamin D3 for 24 and 36 months, respectively. Although lumbar (L2-L4) spine BMD increased in the DG during the 30-month intervention period, still this change was not found to differentiate significantly compared with the change in the CG. This finding is consistent to accumulating recent evidence reporting that calcium and vitamin D supplementation may influence only non-vertebral bone mineral density and fracture risk [33, 34]. Still, besides the favorable effect of calcium and vitamin D on fracture risk, their combined supplementation should cautiously be administered to the public, also taking into consideration their association with other clinical conditions, such as cardiovascular disease (CVD). According to recent evidence, supplementation of calcium in doses $\geq$ 1,000 mg per day, without co-administration of vitamin D, was found to be associated with a 30% increased risk of myocardial infarction [35], whereas vitamin D supplementation in doses $\geq$ 25 μg per day was reported to reduce the risk for CVD [36]. On the basis of this new evidence, further research is probably needed in order to establish a safe but efficient threshold of combined calcium and vitamin D supplementation in tackling both osteoporosis and CVD.

In conclusion, increasing dietary intake of calcium and vitamin D through fortified dairy products in osteopenic postmenopausal women appears to be effective in producing favorable changes in several bone metabolism and bone mass indices and in counterbalancing seasonal variations in hormonal and biochemical molecules.

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Conflict of interest YM also works as a science and nutrition consultant for FrieslandCampina Hellas. The study sponsor had no role in the study design; the collection, analysis, or interpretation of the data; the writing of the manuscript; the submission and revision of the paper. None of the other authors had any potential conflict of interest.

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