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Enhancement of Calcium/Vitamin D Supplement Efficacy by Administering Concomitantly Three Key Nutrients Essential to Bone Collagen Matrix for the Treatment of Osteopenia in Middle-Aged Women: A One-Year Follow-Up

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Summary **Two vitamins and proline (CB_6Pro), three nutrients essential for bone collagen, were used in combination to a 1000 mg calcium/250 IU vitamin D (Ca/D) daily supplement to treat osteopenia as a preventive measure against osteoporosis later in life. Middle-aged women not using estrogen were screened for osteopenia using the WHO criteria and divided into three groups (n = 20 each): 1) placebo healthy controls with normal bone mineral density (BMD); 2) control Ca/D-treated osteopenic patients; and 3) Ca/D + CB_6Pro-treated osteopenic patients. The three groups were comparable at baseline except for BMD. After one-year treatment, cortical diaphyseal BMD remained constant in each group, but trabecular bone loss persisted (at 5 lumbar sites) in osteopenic group 2. No further bone loss was detected in osteopenic group 3. A loss of 2% was evidenced in the placebo group at one lumbar site. Markers of bone formation (which increase in coupling to resorption) decreased significantly in both osteopenic groups. Although biomarkers of resorption did not change, hormone (PTH and 1,25$(OH)_2D_3$)-induced osteoclastic activity was significantly reduced. No decline in BMD occurred at any bone site in osteopenic group 3, highlighting the importance of improving the quality of bone matrix concomitantly to mineral replacement.**

Key Words: **osteopenia, vitamins C & B_6 and proline, calcium/vitamin D, collagen matrix, bone metabolism and density**

Introduction

A slow decline begins to occur after peak bone mass has been reached. Baran [*1*] suggested that the loss of bone mass starts at about age 35 in women. According to Garton *et al.* [*2*], ovulatory changes that amplify as women progress through perimenopause are associated with bone loss despite normal serum estradiol levels and regular menstrual cycles. A relatively modest but significant amount of cancellous bone, especially in the vertebral bodies and femur Ward's triangle, is lost within 10 years preceding menopause, whereas cortical bone mass is maintained until menopause [*3*].

Women account for more than two out of three cases of osteoporosis. They are particularly at risk due to high nutritional demand for calcium during pregnancy and lactation [*4*]. To our knowledge, no nutrient supplemental intervention attempts have been made to prevent further bone loss in middle-aged eumenorrheic women, at risk of fracture

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due to diagnosed low bone mass (but otherwise healthy). This is despite the fact that it is now firmly established that low bone mass (osteopenia) is a significant risk factor for osteoporotic fractures later in life [*5*]. Reduced bone mass results from an imbalance in the bone-remodeling unit as a function of enhanced bone turnover without a commensurate increase in bone formation [*6*].

Calcium supplementation is the leading nutritional intervention for osteoporosis treatment and prevention. Other nutrients have received far less attention. Controversy prevails in regard to calcium although several studies have addressed the effects of this mineral on bone density and turnover, particularly in postmenopausal women [*7–10*]. According to Fardellone *et al.* [*7*], two months of calcium supplementation was efficient in reducing biomarkers of bone turnover in postmenopausal women, with a greater effect in women with a low dietary intake. It would take a much longer time to observe a change in bone mineral density (BMD) because the bone-remodeling cycle ranges from 30 to 80 weeks according to Heaney's simulation model [*6*]. This investigator referred to a reference mineralization period of 40 weeks. On the basis of highly reliable static and tetracycline-based (dynamic) histomorphometric data on humans, the average length of time required to complete the remodeling cycle is approximately 6 months [*11*].

Bone matrix is a complex, highly mineralized tissue with a structural framework composed primarily of collagen [*12*]. Several nutritional factors other than calcium are involved, but their influence on BMD and biomarkers of bone turnover, particularly the new one (type I collagen helical peptide), is still largely unknown. An early correlation study showed that several nutrients were related to bone loss in women (aged between 35–65 years) [*13*]. Vitamins C (ascorbic acid) and B_6 are vital components in the biology of bone cells and resultant bone mass. Vitamin C is the required coenzyme in the hydroxylation of proline and lysine during collagen synthesis in osteoblasts (bone cells) whereas vitamin B_6 is involved as coenzyme in the assembly process of collagen (aldehyde cross-link formation) in the extracellular matrix. Masse *et al.*'s biomechanical study demonstrated the importance of this extracellular process for the strength of bone using a vitamin B_6-deficient animal model [*14*]. The only clinical study on this vitamin in relation to bone was reported by Reynolds *et al.* [*15*] who showed that half of their hip fracture patients were vitamin B_6-deficient. Administering to healthy postmenopausal women, either calcium alone (1450 mg) or with a multivitamin/mineral preparation containing small doses of vitamins C and B_6 (120 mg and 2 mg, respectively), Jensen *et al.* [*16*] obtained significant reductions of parathyroid hormone (PTH) and pyridinium collagen cross-links after one-year supplementation. This multivitamin preparation succeeded in reducing bone loss in contrast to calcium taken alone. Morton *et al.*'s [*17*] postmenopausal women taking vitamin C in pharmacological dose (745 mg in average) had significantly higher BMD at all sites, but they were on hormone (estrogen) replacement therapy. According to Leveille *et al.* [*18*], long term use of vitamin C supplement was associated with a higher BMD in the early postmenopausal years (when bone loss is the most rapid) and among never users of estrogen. Studies on vitamin C in relation to bone are limited and showed inconsistent results. Results from the PEPI study indicated that the positive association between vitamin C and BMD is modified by the level of dietary calcium intake, with diets high in calcium enhancing the association [*19*]. To our knowledge, there are no well-controlled studies on the effects of pharmacological doses of vitamin B_6, and proline as well, in relation to bone health status. Proline is a very specific amino acid in the sequence of the three collagen polypeptide chains and is also essential for the molecular stability of this triple helical molecular structure.

The present follow-up study on middle-aged osteopenic patients was aimed to treat their bone loss using a nutritive preparation containing three nutrients essential in collagen formation, administered in pharmacologic doses for one-year, in conjunction with a conventional calcium/vitamin D supplement, as an early preventive measure against osteoporosis. This new approach is based on the hypothesis that improvement and stabilization of the collagenous bone matrix structure will enhance the efficacy of calcium/vitamin D supplementation conventionally prescribed to counteract bone mineral loss.

Materials and Methods

Subjects

Female volunteers, aged between 35 and 55 year-old and non-estrogen users, were recruited during a mid-spring/summer to enrol in a one-year nutritional intervention project on osteopenia in middle age. Over 150 women from a homogenous Caucasian population living in the same Eastern Canada urban area (latitude 46° North) responded to various public advertisements in which all other selection criteria were clearly specified: non-smoker, eating a normal mixed (non-vegetarian) diet, non obese (BMI<30 kg/m^2) and in good health (as self-assessed) without history of bone fracture [*20*], hysterectomy and no regular use of medication or vitamin/calcium supplements. These specific criteria were aimed to recruit a biologically homogeneous population sample and to avoid factors susceptible to modify bone metabolism and density. An initial screening of all respondents was conducted over the phone to exclude non-eligible subjects.

Women who met the inclusion criteria attended the initial information meeting and signed a consent form. They were assigned a number to keep their anonymity and filled out a

general questionnaire, which included items on socioeconomic background, reproductive characteristics, personal and family medical history. The research protocol was approved by the Ethics Committee on Human Research of the Université de Moncton and by the Review Board of Dumont Hospital, Moncton, NB, Canada.

Dual energy X-ray absorptiometry (DEXA, Lunar Corp., Madison, WI) and the well-established WHO criteria [*21*] were used for the screening of osteopenia. It is defined as a value of BMD between 1.0 and 2.5 SD below the average value (T-score) of the peak bone mass of healthy 30 year-old adults. Women with normal BMD served as healthy placebo controls ($n = 20$). Osteopenic patients were treated either with a conventional calcium supplement containing vitamin D (OsCalD®, Wyeth-Ayerst Canada, Montréal, Canada) or with OsCalD® plus CB_6Pro, a nutritive capsule containing 500 mg ascorbic acid (vitamin C), 75 mg pyridoxine hydrochloride (vitamin B_6) and 500 mg proline. This capsule, prepared by Peter Ford Apothecary Laboratory (Moncton, Canada), was taken orally once a day with breakfast. The OsCalD® tablets were taken twice daily, at breakfast and bedtime. Each tablet contained 500 mg calcium carbonate and 125 IU vitamin D. A full-year supply of placebo, calcium/vitamin D tablets, and CB_6Pro capsules were double-blind (to main investigator and subjects) provided to each subject by the pharmacist.

Evaluation of bone mineral density (BMD)

BMD of the lumbar spine (L1 to L4 and L*i*-L*j* [$i = 1$ to 3; $j = i + 1$ to 4]) and femoral sites (neck, Ward's triangle, trochanter and diaphysis) were evaluated at the beginning and at the end of the study. All scans were performed and analysed by the same experienced technician. The bone densitometer was calibrated on a daily basis according to the manufacturer's instructions. Reproducibility was calculated as a coefficient of variation (CV) obtained by weekly measurements of a standard aluminium bar phantom on the instrument and *in vivo* by repeated measurements (5 times) obtained in three patients of different ages. The CV of the instrument was 0.5% with the standard phantom; *in vivo*, the CV was calculated to be 1.1% for the lumbar spine, 1.5% for the neck of femur, 3.2% for Ward's triangle, 1.8% for trochanter and 1.0% for diaphysis. For the diaphyseal measurements, the upper shaft of the proximal femur was scanned below the intertrochanteric line.

Anthropometric measurements

Subjects were weighed and measured for height. Their body mass index (BMI) was calculated from measured weight (kg) and height (m) as weight/(height)^2. Body frame size was assessed by the elbow width method (cm) according to Frisancho and Flegel [*22*]. Waist and hip circumferences (cm) where used to calculate the waist-to-hip ratio, an indicator of body fat distribution [*23*]. Adipose tissue mass (%) was estimated from skinfold thickness using the prediction equation of Durnin and Womersley [*24*]. Skinfold thickness was measured in triplicate to the nearest 0.5 mm with a constant-pressure Harpenden skinfold caliper, at three different sites of the left side of the body: 1) triceps, halfway between the acromion and the olecranon; 2) subscapular, about 20 mm below the tip of the scapula, at an angle of 45° to the lateral side of the body; and 3) supra-iliac, about 20 mm above the iliac crest, in the axillary line.

Evaluation of self-selected diets

Subjects were asked at the beginning of the study to record their food intakes for 3 non-consecutive days (including one week-end day) preceding the first blood collection. Written instructions were given to all of them and explained by a registered dietitian to maximize completeness and accuracy of recording. Subjects were asked not to modify their regular food intakes while recording and to record all foods and beverages immediately after consumption. Dietary records were checked for completeness by the same dietitian in the presence of the subjects when they met individually for a second time. Daily energy and nutrient intakes, including proline and lysine, were determined using Food Processor® (Version 8.2, 2000, Esha Research, Salem, OR). Energy and nutrient intakes were averaged ($n = 3$ days) to compare the three subject groups prior to the intervention and to assess the nutritional adequacy of their diets with respect to current nutrition standards [*25*]. Ca: P and P: protein ratios were calculated.

Biochemical analyses

In the morning, after an overnight fast, antecubital venous blood and urine samples were collected twice, the same day as bone densitometry, before and after the nutritional intervention. All samples were kept frozen at −70°C until the end of the study to analyze pre- and post-treatment samples in parallel. Ten-mL Vacutainer® tubes were used for collecting blood sample to assay the concentrations of serum estradiol, calcium, inorganic phosphorus and intact (i) PTH. Blood was allowed to clot for 1h at 37°C and centrifuged at 2000 × g for 10 min at 4°C. An enzyme immunoassay (Abbott IMx®, Abbott Laboratories, Chicago, IL) was used for the analytical determination of serum estradiol. Serum iPTH, calcium and inorganic phosphorus were determined by automated routine procedures.

Two other blood samples, withdrawn in a 5 mL tube containing either heparin or EDTA (depending on the analysis), were centrifuged within 15 min at low speed (2000 × g for 10 min) to obtain the plasma. Plasma bone turnover markers were analysed in a laboratory of the Palo Alto VA Medical Center, CA, by methods that were validated and reported previously [*26*, *27*]. Markers of bone resorption were mea-

sured on nonhydrolyzed urine samples using a competitive enzyme immunoassay for free deoxypyridinoline (fDpd) (Metra DPD, Quidel Corporation, Mountain View, CA) and the most recent marker, type I collagen helical peptide (Metra Helical, Quidel Corporation). Both measurements were corrected for the urinary concentration of creatinine (Cr), which was determined using a colorimetric technique based on Metra assay kit (Quidel Corporation). Plasma osteocalcin (OC) and bone specific alkaline phosphatase (bALP) were measured with a 2-site immunoradiometric assay kit (Diagnostic Systems Laboratories, Webster, TX) and with a monoclonal antibody that demonstrates specificity for bALP using Tandem R Ostase Beckman Coulter kit (Fullertown, CA), respectively. Plasma calcitriol [1,25(OH)$_2$D$_3$] concentration was measured using a radioimmunoassay from Nichols Institute Diagnostics (San Juan Capistrano, CA).

Plasma pyridoxal'5-phosphate (PLP), active coenzyme form of vitamin B$_6$, and its end metabolite in urine (4-pyridoxic acid, 4-PA), were determined using cation exchange HPLC [*28*]. These two metabolites were used to assess the vitamin B$_6$ nutritional status and the compliance with the CB$_6$Pro prescription, respectively. Urinary 4-PA was expressed as mmol per mmol creatinine. Plasma concentration of calcidiol [25(OH)D$_3$], hepatic precursor of 1,25(OH)$_2$D$_3$, which was used as an indicator of vitamin D nutritional status, was determined following extraction of serum in reagent alcohol (90% ethanol, 5% methanol, 5% isopropanol) with a competitive protein-binding assay kit (Nichols Institute Diagnostics). The intra-assay CV for all assays was less than 8%.

Statistical analyses

GraphPadInstat® (Version 2.0, 1998, GraphPad Software, San Diego, CA) was used for statistical analysis. Data are reported as means ± standard deviation (SD), except where indicated. Data were first analysed to verify for Gaussian distribution and equality of variance. One-way analysis of variance (ANOVA) followed by Bonferroni *post-hoc* test was used to test the significance of differences between mean values obtained from the evaluation of the three subject groups at baseline. The effect of each treatment (placebo, calcium/vitamin D and calcium/vitamin D + CB$_6$Pro) was analysed by a two-tailed paired Student's *t* test. Values of $p \leq 0.05$ were considered significant.

Results

The three groups were comparable at baseline in several aspects, namely, socio-economic background (not shown), age, body weight and frame, height, BMI, serum estradiol, biochemical markers of calcium and bone metabolism, and vitamins B$_6$ and D nutritional status as assessed by plasma PLP and 25(OH)D$_3$, respectively (Table 1). They were also comparable in terms of dietary intakes, including proline and lysine, amino acids essential for collagen synthesis and its structural stability in bone matrix (Table 2). Body weight was normal in each group. Dietary intakes of proteins, vitamins and minerals involved in bone metabolism, were adequate, except for vitamin D, magnesium, copper and zinc for the three groups. Their calcium intakes almost reached the newly established high requirement of 1000 mg. Vitamins C and B$_6$ were above the current nutritional recommendations. Subjects were not B$_6$- or D-deficient as judged by plasma PLP and 25(OH)D$_3$ levels within normal ranges, respectively (Table 1). The calcium/vitamin D + CB$_6$Pro-treated osteopenic group complied with the CB$_6$Pro prescription as judged by the significant ($p<0.01$) 12-fold elevation of urinary 4-PA excretion at the end of the one-year treatment (Fig. 1). No side effects were observed throughout

Table 1. Baseline basic characteristics of each subject group (n = 20)

		Normal BMD control group	*Osteopenic groups*	
		Placebo	Calcium/Vit. D	Calcium/Vit. D + CB$_6$Pro
Age	(years)	44.6 ± 6.9	49.0 ± 5.6	47.9 ± 7.7
Weight	(kg)	69.8 ± 11.7	68.3 ± 9.3	63.8 ± 9.1
Height	(m)	1.60 ± 0.05	1.59 ± 0.06	1.60 ± 0.08
BMI	(kg/m^2)	27.2 ± 3.9	26.9 ± 3.2	24.9 ± 3.5
Elbow width	(cm)	6.45 ± 0.61	6.47 ± 0.53	6.28 ± 0.4
Waist hip ratio	(cm/cm)	0.78 ± 0.08	0.78 ± 0.06	0.78 ± 0.07
Body fat	(%)	35.8 ± 5.2	38.4 ± 5.3	37.5 ± 6.5
Serum estradiol	(pmol/L)	236 – 767[§]	164 – 556[§]	178 – 503[§]
Plasma PLP	(nmol/L)	31.01 – 41.77[§]	27.77 – 69.12[§]	30.02 – 59.41[§]
Plasma 25(OH)D$_3$	(nmol/L)	81.35 ± 46.30	72.72 ± 18.65	72.25 ± 33.82

Means ± SD, except where indicated. BMD, bone mineral density; BMI, body mass index; PLP, pyridoxal'5-phosphate. None of the differences between groups were significant. [§] 95% confidence interval.

the study period in any of the groups and no subjects dropped out.

Table 3 summarizes the effect of the calcium/vitamin D + CB_6Pro treatment on bone metabolism and turnover in comparison to control osteopenic group and healthy normal controls having received a calcium/vitamin D supplement alone and a placebo, respectively. No biochemical change was observed in the healthy controls on placebo. Both markers of bone formation (bALP and OC, which increase in osteopenia secondarily to resorption) were significantly lowered in both osteopenic groups (Table 3). Although no reduction of resorption was evidenced biochemically (fDpd

Table 2. Daily pertinent nutrient intakes for each subject group (n = 20)

			Normal BMD control group	*Osteopenic groups*	
		DRI[a]	Placebo	Calcium/Vit. D	Calcium/Vit. D + CB_6Pro
Energy	(kcal)	—	2002 ± 740	1880 ± 611	1815 ± 428
Proteins	(g)	50	84.0 ± 20.6	80.4 ± 31.4	68.5 ± 16.7
Proline	(g)	—	3.9 ± 0.9	3.3 ± 1.1	3.1 ± 1.0
Lysine	(g)	—	5.0 ± 1.4	4.2 ± 1.4	4.0 ± 1.2
Ca	(mg)	1000	952 ± 357	920 ± 220	914 ± 223
P	(mg)	700	1409 ± 348	1463 ± 681	1338 ± 267
Ca:P	(mg/mg)	—	0.70 ± 0.14	0.70 ± 0.27	0.70 ± 0.14
P:proteins	(mg/g)	—	16.90 ± 2.28	17.20 ± 3.48	18.60 ± 3.03
Mn	(mg)	2	2.3 ± 0.8	2.3 ± 1.0	2.3 ± 1.0
Mg	(mg)	320	269 ± 81	260 ± 93	260 ± 90
Cu	(μg)	2	1.3 ± 0.4	1.2 ± 0.3	1.3 ± 0.5
Zn	(mg)	12	9.7 ± 3.2	9.3 ± 4.5	9.1 ± 1.5
Vit C	(mg)	60	105 ± 50	104 ± 33	102 ± 32
Vit A	(μg RAE)	800	1192 ± 841	1167 ± 457	1188 ± 323
Vit B_6	(mg)	1.3	1.9 ± 0.4	1.7 ± 0.6	1.6 ± 0.6
Vit D	(μg)	5	3.6 ± 1.2	2.4 ± 1.6	2.1 ± 1.3

Means ± SD. BMD, bone mineral density; DRI, dietary reference intakes; RAE, retinol activity equivalents. None of the differences between groups were significant. [a] From the Institute of Medicine (2000) [*25*].

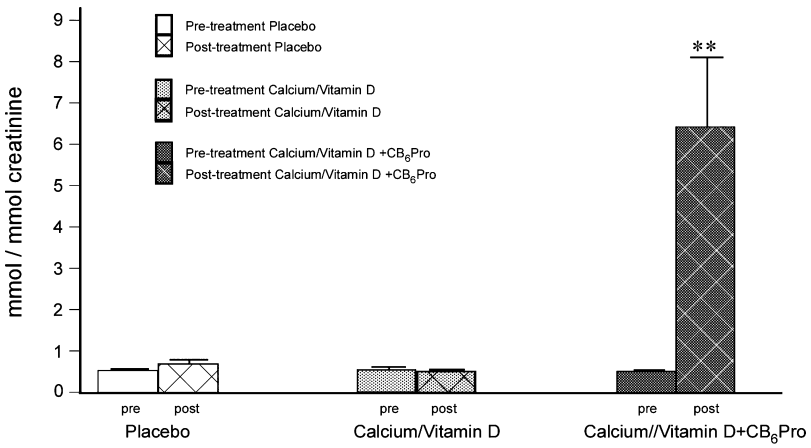

Fig. 1. Compliance of the calcium/vitamin D + CB_6Pro-treated osteopenic group (n = 20) to the nutritive prescription (CB_6Pro), as assessed by urinary excretion of pyridoxic acid (4-PA), vitamin B_6 end-metabolite. Plain and hatched dark grey bars, respectively, represent pre- and post-treatment values. Pre- and post-treatment values of 4-PA are also shown for the normal controls receiving a placebo and the control osteopenic group treated with calcium/vitamin D (white and light grey bars, respectively) (n = 20 per group). Data are means ± SD. Asterisks indicate the significance level for pre- and post-treatment comparison for the experimental osteopenic group: $^{**}p<0.01$. There was no significant difference for the normal and osteopenic control groups not receiving vitamin B_6 (CB_6Pro) as expected.

Table 3. Pre- and post-treatment biochemical indices of bone metabolism and turnover in each subject group (n = 20)

		Normal BMD control group		*Calcium/Vit. D osteopenic group*		*Calcium/Vit. D + CB_6Pro osteopenic group*	
		Pre	Post	Pre	Post	Pre	Post
Ca	(mmol/L)	2.32 ± 0.11	2.33 ± 0.09	2.29 ± 0.08	2.30 ± 0.08	2.37 ± 0.13	2.33 ± 0.08
Pi	(mmol/L)	1.05 ± 0.15	1.06 ± 0.12	1.06 ± 0.14	1.06 ± 0.14	1.05 ± 0.13	1.08 ± 0.13
bALP	(μg/L)	6.13 ± 3.56	6.39 ± 3.18	7.49 ± 3.09	5.73 ± 2.63***	6.95 ± 2.58	5.11 ± 2.01***
Osteocalcin	(ng/mL)	37.14 ± 8.39	36.18 ± 11.72	39.34 ± 8.25	33.65 ± 11.67***	44.36 ± 5.60	40.44 ± 5.92**
Helical peptide	(μg/mmol Cr)	44.68 ± 28.98	40.05 ± 25.82	46.74 ± 21.16	43.24 ± 32.86	53.49 ± 23.42	48.65 ± 22.92
fDpd	(nmol/mmol Cr)	4.42 ± 1.39	4.62 ± 1.24	4.97 ± 2.11	4.43 ± 1.82	4.69 ± 1.54	4.36 ± 1.29
iPTH	(pmol/L)	2.99 ± 1.51	3.12 ± 1.20	3.83 ± 1.47	2.85 ± 1.22***	2.95 ± 0.92	2.56 ± 0.73*
25(OH)D_3	(nmol/L)	81.35 ± 46.30	90.52 ± 54.52	72.72 ± 18.65	79.00 ± 36.00	75.25 ± 33.82	81.25 ± 31.67
1,25$(OH)_2D_3$	(pmol/L)	0.63 ± 0.33	0.69 ± 0.24	0.83 ± 0.35	0.66 ± 0.32*	0.71 ± 0.25	0.59 ± 0.20

Means ± SD. bALP, bone alkaline phosphatase; BMD, bone mineral density; Cr, creatinine; fDpd, free deoxypyridinoline; iPTH, intact parathyroid hormone; Pi, inorganic phosphorus. *p<0.05 Pre vs Post. **p<0.01 Pre vs Post. ***p<0.001 Pre vs Post.

and helical peptide) at the end of the one year-treatment, osteoclastic activity, as assessed by two major calciotropic hormones, was significantly reduced in both osteopenic groups. The 13 and 25% reductions of iPTH were significant in both groups. Like PTH, 1,25$(OH)_2D_3$ seemed to be more sensitive to calcium/vitamin D administered alone (control osteopenic group). 1,25$(OH)_2D_3$ decreased by 20% (p<0.05) in this group. The 17% reduction in the calcium/vitamin D + CB_6Pro-treated osteopenic group almost reached a significant level (p = 0.06). The concentration of 25(OH)D_3 did not change regardless of group.

BMD in the healthy control group having received a placebo did not change after one year, except that a significant 2% loss (p<0.05) at L1-L2 site occurred (Fig. 2B). Osteopenic patients treated with the conventional calcium/vitamin D supplement continued to lose bone minerals, to a much greater extent than the normal BMD controls, as shown by significant reductions in BMD at five lumbar sites (L3, L1-L2, L1-L3, L1-L4, L2-L3; significance levels indicated on Fig. 2A and B) at the end of the study. However, the femur neck BMD was significantly improved (p<0.05) (Fig. 2C). The combination of calcium/vitamin D with the three key collagen-related nutrients (vitamins C and B_6 + proline) administered to osteopenic group 3 deterred further bone loss at all bone sites, at least during the one-year treatment period, compared to the results obtained in the two other groups, especially the other osteopenic group treated with calcium/vitamin D alone. Cortical diaphyseal bone remained constant in each group during the study.

Discussion

The occurrence of bone loss in mid-age when serum estradiol levels are within normal range can indicate either that maximum peak bone mass was not achieved, or the beginning of a decline in new bone formation not matching the normal resorption rate. The first event is very difficult to demonstrate without a long-term longitudinal study starting during adolescence. The second event may be influenced by the quality of the collagenous matrix, as suggested by fundamental research data from our vitaminB_6-deficient animal model [*14*]. The present study showed the effect of a nutritional intervention targeting both major bone components (collagen matrix and minerals) for treating osteopenia in middle-aged menstruating women. It provided evidence that treating bone loss as a resorptive problem that commands calcium restoration with no attempt to improve bone matrix is not optimal.

Nutritional vitamin B_6 status of the three subject groups was considered adequate based on the cut-off of 20 nmol/L [*29*]. Nutritional vitamin C status could not be assessed biochemically due to the unavailability of the technique in our laboratory at the time of the study. However, by extrapolating from the dietary intake values which were almost 2-fold the recommendation, nutritional status inadequacy seemed unlikely. Low plasma 25(OH)D_3 concentration can lead to osteopenia [*30*]. Our three subject groups had adequate vitamin D status as assessed by this biochemical indicator (cut-off level established at 50 nmol/L [*31*]) despite insufficient dietary intakes. Dietetic evaluation, which only provides an estimate of total vitamin D intake from natural sources and fortified foods, does not take into consideration endogenous photosynthesis of vitamin D in the skin under influence of sunlight (or artificial ultraviolet radiation). Accurate dietary estimates of vitamin D intakes are not available, in part because the vitamin D composition of fortified foods is highly variable [*32*]. Using food composition data from the second National Health and Nutrition Examination Survey (NHANES II), a median vitamin D intake of 2.3 μg (90 IU) per day, was estimated for adult women. The level proposed as adequate plasma 25(OH)D_3 concentration to prevent compensatory hypersecretion of

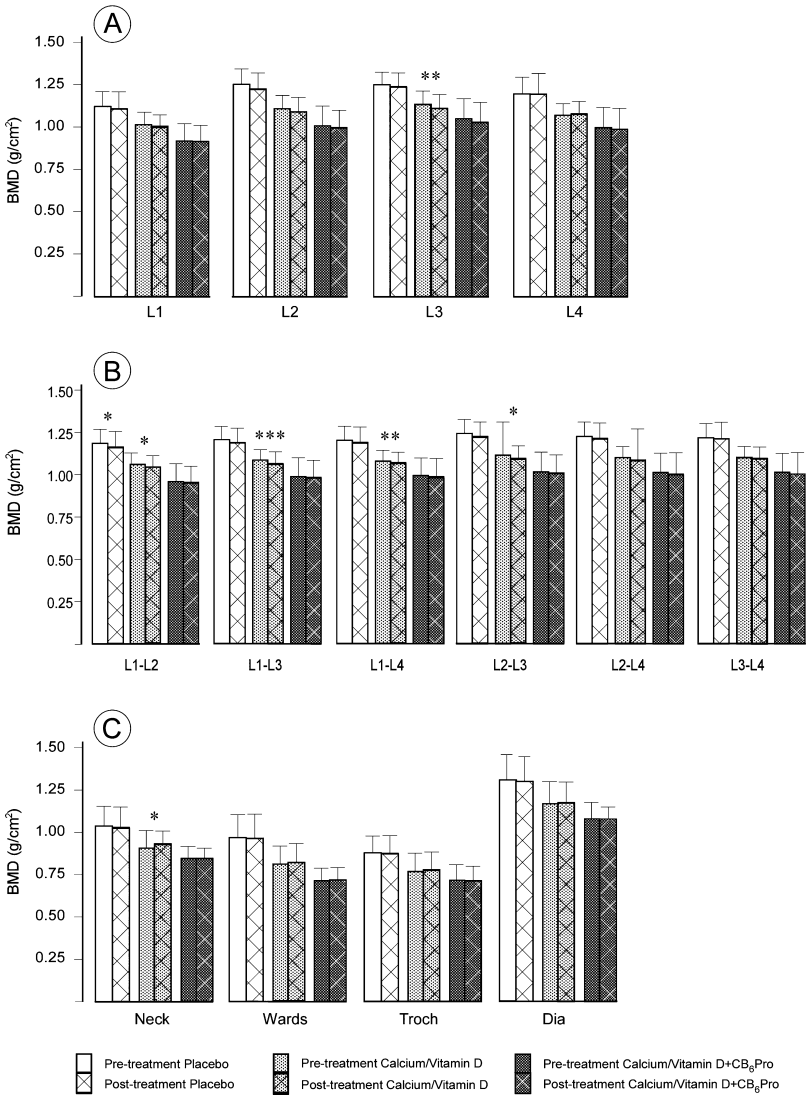

Fig. 2. Pre- and post-treatment bone mineral density (BMD, g/cm^2) at lumbar (A and B) and femoral (C) sites in each subject group ($n = 20$) for all sites under investigation. Pre- and post-treatment are indicated by plain and hatched bars, respectively. White, light grey and darker grey bars represent, respectively, the normal controls, the control osteopenic group treated with calcium/vitamin D, and the experimental osteopenic group treated with calcium/vitamin D combined with three other bone-related nutrients relevant to collagen matrix. Data are means ± SD. Neck: femur neck; Wards: femur Ward's triangle; Troch: femur trochanter; Dia: femur diaphysis. Asterisks indicate significance levels for pre- and post-treatment comparisons for each group: *p<0.05; **p<0.01; ***p<0.001.

PTH varies from 62 to 100 nmol/L [*16*, *27*]. The mean plasma concentration of this vitamin was within this range for the three subject groups in the present study. The total daily dose of 250 IU provided by two tablets of OsCalD to osteopenic patients was insufficient to increase their plasma 25(OH)D_3 concentration. According to Dawson-Hugues *et al.* [*33*], an increase from 5.0 µg (200 IU) to 20 µg (800 IU) caused an increment of only 8 nmol/L in postmenopausal women. It is a different case in older women because they are likely to be vitamin D-deficient. For instance, Ooms *et al.* [*34*] obtained an elevation of 35 nmol/L for elderly women when using 400 IU.

In the present study, both bALP and OC (which increase in coupling to bone resorption) were significantly reduced in both treated osteopenic groups, probably as a consequence of the inhibition of bone resorption. bALP is known to be the most sensitive biomarker to treatment [*35*]. In effect, in the present study, it responded the most to both calcium/vitamin D and calcium/vitamin D + CB_6Pro treatments, with reductions of 23 and 26%, respectively. The expression of bALP starts after cessation of cell proliferation of osteoblasts and reaches a maximum during matrix maturation,

but declines during matrix mineralization [*36*]. OC is the most abundant non-collagenous protein in bone produced by osteoblasts. In women taking estrogen, Hannon *et al.* [*37*] found that OC and bALP start to decrease after 6–8 weeks of treatment, which is consistent with the theory of coupling of bone formation to bone resorption. In the present study, the halt in bone loss evidenced by densitometry and biochemistry (PTH reduction) in osteopenic women whose treatment aimed at modifying both components of bone (matrix and minerals) was coupled with a reduction in both markers of bone formation. Biochemical markers of bone resorption (fDpd and type I collagen helical peptide) were weak predictors of bone changes in this study, probably because the calcium/vitamin D supplement reduced bone turnover rate and truncated the ranges of these two markers. The 9% reduction in type I collagen helical peptide observed after one-year calcium/vitamin D + CB_6Pro treatment was noteworthy, although not significant. First, this assay has been shown to be one of the most sensitive indicators of resorption to show the antiresorptive effect of estrogens and bisphosphonates [*38*]. Furthermore, helical peptide has a morphological significance. Constituting the major structural part of the collagen molecule, a greater urinary excretion of this peptide, as occurs when there is osteoporosis, can only result from a degradation of collagen. This protein degenerative process, in line with bone resorption, outlines the contribution of bone matrix.

No bone loss was detectable at the spine and femur bone sites in the calcium/vitamin D + CB_6Pro-treated osteopenic group as compared to the other two groups who did exhibit some bone loss. This finding is especially noteworthy considering that osteopenia was more severe in this group, non only based on T-score (WHO criteria), but also on more recent absolute reference values used by bone researchers, *i.e.* <0.9 g/cm^2 for lumbar spine and <0.795 g/cm^2 for femur neck, respectively [*39–40*]. It appears that the CB_6Pro preparation enhanced the calcium/vitamin D supplement efficacy in building new bone and this could explain the success of their combination in halting bone loss. Reduced osteoclastic activity was confirmed biochemically by the reduction of PTH. The observed decrease in $1,25(OH)_2D_3$ obtained with the calcium/vitamin D supplement, together with an unchanged serum phosphate level, as found in the present study in agreement with Elders *et al.*'s study [*41*], could be interpreted to mean that PTH is the most important mediator. The bone-sparing effect observed with the calcium/vitamin D-CB_6Pro treatment is interesting because it was achieved not so much through a reduction in osteoclastic activity, suggesting that a beneficial effect at the level of bone matrix also took place. This improvement in architecture and structural stability of the bone tissue, attributed to the role of vitamins C, B_6 and proline in collagen, could never have been obtained with a mineral supplement. A bone matrix of better quality would be more efficient at retaining minerals and preventing further bone loss. Several recent animal studies have highlighted this newly recognized issue of bone matrix quality.

The ability of the calcium/vitamin D + CB_6Pro treatment as compared to calcium/vitamin D supplement alone to stabilize bone mass and prevent further bone loss was the most worthy finding of this study. BMD was then maintained in this group, at all bone sites of interest, after one year when three major nutrients essential to bone matrix structure were prescribed in pharmacological doses, together with a 1000 mg calcium supplement. BMD at the femur neck in the control osteopenic group, receiving the calcium/vitamin D supplement alone, significantly increased, but decreased at five lumbar sites. Morton *et al.* [*17*] also showed improvement at the femur neck when using calcium but only when it was combined to vitamin C supplement (745 mg in average). According to Riis *et al.* [*42*], calcium supplement may have a minor effect on the loss of cortical bone, but it had no effect on trabecular bone. The placebo group in the present study lost 2% bone minerals after one year at one lumbar site. According to Alekel *et al.* [*43*], the loss at the vertebral sites in perimenopausal bone is 2–3% per year.

Other studies, mostly carried out on postmenopausal women, have also shown the partial effect of calcium supplement in preventing bone loss. In Agnusdei *et al.*'s [10] double-blind, placebo-controlled study for prevention of postmenopausal spinal bone loss, only patients treated with ipriflavone, a new nonsteroidal agent, a synthetic derivative of naturally occurring isoflavones (a group of phytoestrogens), had a significant increase in skeletal mineral content compared with women who received oral calcium supplement or placebo. In a meta-analysis, it was concluded that the published literature until 1990 shows a consistent positive effect of calcium supplement in postmenopausal women at all sites, except the lumbar spine [*44*]. Few years later, Riggs *et al.* [*8*] and Storm *et al.* [*9*] found significant BMD improvement in postmenopausal women at both lumbar and appendicular sites using 1600 mg or 1000 mg, respectively. The effect of calcium on BMD is still a controversial subject, varying according to duration of study, anatomic site, age, baseline dietary level and BMD values, but independently of estrogen status [*41*]. The literature on osteopenic women of middle-age is scarce.

In conclusion, the question of the efficacy of calcium supplementation to treat lumbar bone loss should be considered still open. In the present one-year follow-up study, lumbar bone loss persisted in osteopenic patients treated with conventional calcium/vitamin D supplement. In contrast, further bone loss was prevented at all lumbar and femoral sites in the osteopenic group of women treated with three nutrients involved in the cell-mediated remodeling of

bone through its collagenous matrix, in addition to calcium/vitamin D. The good safety and tolerability profile of both calcium/vitamin D and CB_6Pro makes their combination a promising option for the treatment of mid-age osteopenia as an early preventive measure against osteoporosis. Large well-controlled investigations are needed to confirm the efficacy of this new approach. Its long-term effect should also be tested to verify if the positive results observed in the present placebo-control study with CB_6Pro supplementation in conjunction with a calcium/vitamin D supplement, after one-year treatment, can be sustained over a longer period of time.

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Abbreviations

bALP, bone alkaline phosphatase; BMD, bone mineral density; CB_6Pro, vitamins C and B_6 + proline; Cr, creatinine; DRI, dietary reference intakes; fDpd, free deoxypyridinoline; iPTH, intact parathyroid hormone; OC, osteocalcin; 4-PA, 4-pyridoxic acid; Pi, inorganic phosphorus; PLP, pyridoxal'5-phosphate.

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