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Two-Year Randomized Controlled Trial of Vitamin K_1 (Phylloquinone) and Vitamin D_3 Plus Calcium on the Bone Health of Older Women

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ABSTRACT: Dietary supplementation with vitamin K_1, with vitamin D_3 and calcium or their combination, was examined in healthy older women during a 2-year, double-blind, placebo-controlled trial. Combined vitamin K with vitamin D plus calcium was associated with a modest but significant increase in BMC at the ultradistal radius but not at other sites in the hip or radius.

Introduction: The putative beneficial role of high dietary vitamin K_1 (phylloquinone) on BMD and the possibility of interactive benefits with vitamin D were studied in a 2-year double-blind, placebo-controlled trial in healthy Scottish women ≥60 years of age.
Materials and Methods: Healthy, nonosteoporotic women ($n = 244$) were randomized to receive either (1) placebo, (2) 200 μg/day vitamin K_1, (3) 10 μg (400 IU) vitamin D_3 plus 1000 mg calcium/day, or (4) combined vitamins K_1 and D_3 plus calcium. Baseline and 6-month measurements included DXA bone mineral scans of the hip and wrist, markers of bone turnover, and vitamin status. Supplementation effects were tested using multivariate general linear modeling, with full adjustment for baseline and potential confounding variables.
Results: Significant bone mineral loss was seen only at the mid-distal radius but with no significant difference between groups. However, women who took combined vitamin K and vitamin D plus calcium showed a significant and sustained increase in both BMD and BMC at the site of the ultradistal radius. Serum status indicators responded significantly to respective supplementation with vitamins K and D. Over 2 years, serum vitamin K_1 increased by 157% ($p < 0.001$), the percentage of undercarboxylated osteocalcin (%GluOC) decreased by 51% ($p < 0.001$), serum 25-hydroxyvitamin D [25(OH)D] increased by 17% ($p < 0.001$), and PTH decreased by 11% ($p = 0.049$).
Conclusions: These results provide evidence of a modest synergy in healthy older women from nutritionally relevant intakes of vitamin K_1 together with supplements of calcium plus moderate vitamin D_3 to enhance BMC at the ultradistal radius, a site consisting of principally trabecular bone. The substantial increase in γ-carboxylation of osteocalcin by vitamin K may have long-term benefits and is potentially achievable by increased dietary intakes of vitamin K rather than by supplementation.
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Key words: vitamin K, vitamin D, osteoporosis, bone densitometry, bone turnover markers, osteocalcin, undercarboxylated osteocalcin

INTRODUCTION

POPULATION-BASED PREVENTATIVE MEASURES that reduce the rate of bone mineral loss and therefore extend years of active, good-quality life offer a potential means of improving bone health. Dietary change is one potentially achievable and sustainable approach. Undernutrition is often observed in older people and is likely to be a significant contributing risk factor to the pathogenesis and outcome of hip fracture in the elderly.[1,2] Dietary intervention strategies have focused mainly on calcium and vitamin D; supplementation with either or both of these nutrients having been shown in some studies to be effective in maintaining BMD or in reducing the incidence of fractures.[3–6] Not all studies have shown these benefits,[7] including three recent fracture prevention trials carried out in the United Kingdom[8,9] and the United States.[10] Another nutrient that is being increasingly linked to bone health and osteoporosis is vitamin K.[2,11–13]

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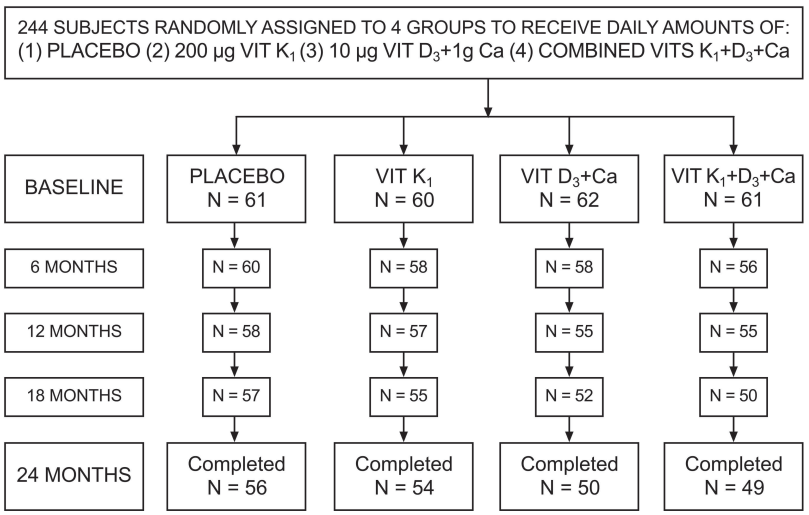

FIG. 1. Study design showing the four treatment arms and subject numbers recruited and retained.

Vitamin K is needed as a co-factor to convert certain peptide-bound glutamate (Glu) residues to γ-carboxyglutamate (Gla) residues in target proteins (Gla proteins) that are synthesized by a variety of tissues. Bone matrix contains several Gla proteins, of which osteocalcin is the most abundant.[11,12] The synthesis of Gla residues imbues vitamin K–dependent proteins with structural integrity, metal binding characteristics, and functionality; in the case of osteocalcin, the Gla residues confer high affinity binding to bone hydroxyapatite.[14] Although its precise function is unclear, osteocalcin seems to act as a regulator of bone remodeling and/or bone mineral maturation.[15] A clear rationale for ensuring optimal dietary intakes of vitamin K is the evidence that a high circulating level of undercarboxylated osteocalcin (GluOC) is an independent risk predictor of bone fractures[16–19] and of low BMD.[20,21] In addition, short-term studies show that the γ-carboxylation status of osteocalcin responds to increased vitamin K intakes in the usual dietary range.[22,23] The possibility that vitamin D itself may also influence the γ-carboxylation status of osteocalcin has been raised by evidence that the free Gla content of animal bones[24] and circulating GluOC in elderly women[16] both reflect vitamin D status. This unsubstantiated effect of vitamin D on posttranslational γ-carboxylation is independent of the well-established role of 1,25-dihydroxyvitamin D as a potent enhancer of osteocalcin expression.[25]

To date, the only published trials that have assessed the effects of vitamin K on postmenopausal bone loss have used supradietary daily doses of either 1 mg of vitamin K_1[26] or 45 mg of vitamin K_2 (menaquinone-4; menatetranone).[27–29] Our placebo-controlled study was designed to establish the influence of an additional daily intake of 200 μg vitamin K_1 on the bone health of older women. This extra vitamin K_1 intake was equivalent to the 95th percentile of younger adult intake in Scotland[30] and equivalent to that contained in a 40- to 60-g portion of leafy green vegetables such as collards and spinach.[31] This intake could reasonably be considered to be attainable by dietary modification rather than from supplementation. Supplementation at a high dietary level is justified by evidence from population studies in the United States that low consumption of vitamin K in some groups is associated with elevated fracture risk[32,33] or lower bone mass[34] and that an impaired vitamin K status is associated with low bone mass[35] or increased bone turnover.[36] Our trial was also designed to study the effects, and possible interaction with vitamin K, of vitamin D and calcium at additional daily intakes of 10 μg (400 IU) and 1000 mg, respectively. The vitamin D intake was as recommended for elderly people in the United Kingdom at the time this study was initiated.[37]

MATERIALS AND METHODS

Participants and intervention protocol

Healthy women ≥60 years of age were recruited from the general population to provide approximately equal numbers in the age groups 60–64, 65–69, 70–74, and ≥75 years. Exclusion criteria were clinical osteoporosis or chronic disease (e.g., diabetes mellitus, cardiovascular disease, cancer, fat malabsorption syndromes), routine medication that interferes with vitamin K, vitamin D, or bone metabolism (notably warfarin and steroids), and consumption of nutrient supplements that provided in excess of 30 μg vitamin K, 10 μg (400 IU) vitamin D, or 500 mg calcium daily. Hoffmann-La Roche (Basel, Switzerland) provided the supplementation tablets. One set of these tablets was in a chewable form and contained either 1000 mg of elemental calcium as calcium carbonate plus 10 μg of vitamin D_3 or placebo ingredients in a matching form. A second set of smaller, oval-shaped tablets contained 200 μg of vitamin K_1 (or placebo); these were swallowed whole with a drink. The 244 participants were randomly assigned to one of the four study groups: (1) placebo, (2) vitamin K_1 200 μg/day, (3) calcium 1000 mg plus vitamin D_3 10 μg (400 IU)/day, or (4) combined supplementation with vitamin K_1 and vitamin D_3 plus calcium at the levels in groups 2 and 3 (Fig. 1). To ensure double-blinding, an independent statistician at Hoffmann-La Roche, who had no other connection to the study,

provided a computer-generated randomization list to the researchers. Each participant was asked to take two tablets every day with their breakfast. The Tayside Committee on Medical Research Ethics approved the study, and all participants gave written informed consent.

Baseline measurements were performed in the winter, and subsequent measurements were performed every 6 months for 2 years. On each occasion, participants visited between 8:30 and 9:30 a.m. after an overnight fast, and a venous blood sample was taken, spun, aliquoted, and frozen for storage at –70°C within 1 h of collection. Weight and height were measured. Smoking habits, sunshine exposure, adherence to protocol by pill counting, and medication changes were noted. Dietary intake was assessed using a validated food frequency questionnaire (FFQ) designed for studies of osteoporosis in Scottish women,[38] and nutrient intakes from supplements were added to those derived from diet alone to produce a total energy and nutrient intake profile for participants. Subjects provided a urine sample between 10:00 and 11:00 a.m. after an early morning void.

Bone densitometry

DXA of the femur was carried out every 6 months using the same Lunar (DPXα) machine with software version 1.11 and 1.15 (Lunar Corp., Madison, WI, USA). Changes in software did not affect earlier analyses. The recommended Lunar scanning procedure guidelines were followed. Medium scan mode was used for all subjects, and the current was either 750 or 3000 μA, depending on the body mass index (BMI) of the subject. Previous scans were always examined before and during follow-up scans. The Osteoscan pDXA was used for the scanning of the radius, with software version 2.1x (NIM, Verona, Italy). In the standard analysis, the Osteoscan automatically marks the bone edges and positions two region of interest (ROI) boxes, of fixed size: one at the ultradistal site (just distal to the ulnar styloid) and one at the mid-distal site (starting at one third of the distance between the ulna styloid and the olecranon). The nondominant wrist was measured unless there had been a fracture, and on repeat scans, the analysis ROI boxes were matched to the baseline scan. Bone scan results were expressed as BMC (mg) and BMD (BMC divided by bone area; mg/cm^2). The CVs for BMD and BMC measurements, respectively, were as follows: femoral neck, 1.7% and 2.1%; Ward's, 5.1% and 8.7%; trochanter, 2.2% and 7.1%; ultradistal radius, 1.8% and 2.6%; mid-distal radius, 1.9% and 2.1%.

Biochemical analyses

Serum vitamin K_1 was measured by HPLC as previously described.[39] Serum 25-hydroxyvitamin D [25(OH)D] was measured by radioimmunoassay using a ^{125}I radioimmunoassay (RIA) kit (68100E; DiaSorin, Wokingham, UK). PTH was measured with the Immulite intact PTH assay (EURO/DPC, Llanberis, Wales, UK). Serum bone-specific alkaline phosphatase (BALP) analyses were carried out using the Alkphase-B immunoassay kit (Metra Biosystems, Great Haseley, UK). Undercarboxylated and carboxylated serum osteocalcin (GluOC and GlaOC) were determined by separate immunoassays using the respective ELISA kits from Takara Shuzo (Otsu, Shiga, Japan). The sum of GluOC and GlaOC was used as a measure of total OC. As previously recommended,[40] the vitamin K status of bone was also evaluated by expressing the GluOC fraction as a percentage of total OC. Midmorning urine samples were analyzed for creatinine with a COBAS Bio multichannel analyser using the Boehringer alkaline picrate colorimetric method (124192; Boehringer Mannheim, Mannheim, Germany). Urinary cross-linked N-teleopeptides of type I collagen (NTX) was measured using the NTX ELISA kit Osteomark (Ostex, Seattle, WA, USA).

The above biochemical indices were monitored at baseline and 6-month intervals (BALP, GluOC, GlaOC, NTX) or at baseline and yearly intervals [25(OH)D, vitamin K_1, PTH]. Measurements of each analyte were carried out on samples batched by visit except for BALP and PTH, which were performed after the study completion, with samples for each subject batched and analyzed in the same assay run. Assay performance was monitored against external QA schemes for 25(OH)D, vitamin K_1, and PTH or, when no scheme was available, against in-house quality assurance (QA) schemes.

Statistical methods

Statistical procedures were carried out in SPSS (version 10) and SAS. Descriptive statistics were performed for all variables at baseline and on each 6-month visit. Data were transformed toward normality and parametric tests used, with statistical significance taken as $p < 0.05$. Correlation and linear regression procedures were used to study relationships between variables. ANOVA and χ^2 tests were used to detect differences between groups and distributions.

Separate analyses of the bone mineral variables (BMC and BMD) were carried out to address the possibility that BMD does not sufficiently correct for influences of body size, because its derivation as BMC/bone area indicates an areal rather than a volumetric density. Use of BMC with separate adjustment for bone area has been advocated by Prentice et al.[41] to be more statistically rigorous (because it allows the relationship between these two measures to be unforced) and to provide a better adjustment for body size. In line with this approach, we used BMC as the dependent variable and included bone area, weight, and height as independent variables in all multiple regression models.[41] To study the influence of baseline values on response to supplementation, changes in bone mineral and biochemical markers [e.g., 25(OH)D, GluOC, GlaOC] were expressed as a percentage of baseline values and compared across fourths of the baseline distribution using ANOVA.

Cross-sectional multiple regression analysis was used to reveal factors contributing to variance in BMD and BMC at baseline and other time-points. Repeated-measures ANOVA and general linear model (GLM) procedures (SPSS) were used initially to study the significance of changes in BMC and BMD within and between the intervention groups, with appropriate adjustment for covariates (including baseline age, bone area, weight, height, physical

Table 1. Baseline Characteristics and Basal Nutrient Intakes of the 209 Women Who Completed the Study

	Intervention group			
	Placebo	*Vitamin K_1*	*Vitamin D + calcium*	*Vitamins K and D + calcium*
Final *n* (*n* at recruitment)	56 (61)	54 (60)	50 (62)	49 (61)
Baseline characteristics				
Age (years)	67.8 ± 6.0	67.7 ± 4.9	69.4 ± 6.4	67.8 ± 5.4
Age at menopause (years)	47.8 ± 4.8	49.8 ± 4.3	48.3 ± 8.4	49.5 ± 4.1
Weight (kg)	66.0 ± 8.5	67.5 ± 9.3	64.9 ± 8.4	64.5 ± 9.0
BMI (kg/m^2)	26.2 ± 3.3	26.4 ± 3.7	25.8 ± 3.4	26.1 ± 3.7
Basal nutrient intakes				
Energy (kJ/day)	7594 ± 1935	7790 ± 1955	8082 ± 1916	7762 ± 1852
Protein (% energy)	16.5 ± 1.9	15.8 ± 1.9	16.2 ± 1.8	16.4 ± 1.9
Fat (%energy)	30.1 ± 4.6	30.7 ± 3.0	31.6 ± 3.6	30.6 ± 4.0
Carbohydrate (% energy)	48.5 ± 4.9	49.1 ± 2.9	47.9 ± 3.9	48.4 ± 4.8
Vitamin D (μg/day)	5.0 ± 2.3	5.4 ± 2.6	5.6 ± 3.2	5.9 ± 3.1
Vitamin K_1 (μg/day)	83.0 ± 24.5	86.9 ± 26.6	82.0 ± 26.8	87.1 ± 28.8
Calcium (mg/day)	1068 ± 280	1046 ± 244	1078 ± 248	1026 ± 219

Values are mean ± SD. Basal nutrient intakes are from foods plus approved supplements and were derived from five 6-month FFQ assessments for each subject over the 2-year intervention. There were no significant differences between groups for any variable.

activity, total energy intake, calcium and other nutrient intakes, serum markers of vitamin D and vitamin K status, and markers of bone turnover). Separate GLMs, with pairwise comparisons against baseline values, were carried out by a statistician, unrelated to the study, who was blind to the groups (Dr Mishra), using the SAS package.

RESULTS

Subject history, compliance, and dietary intakes

A flow chart with the numbers of subjects randomly assigned and retained in each treatment arm at successive 6-month visits is shown in Fig. 1. Of the 244 eligible women randomized into the study, 209 (85.6%) completed the 2-year study, with good supplement adherence based on pill count (median, 99; interquartile range [IQR], 97.3–99.8%). Reasons for withdrawal were illness unrelated to the study ($n = 17$); volunteer preference, noncompliance, or other violations of the inclusion criteria ($n = 14$); and low BMD necessitating further medical intervention ($n = 4$). Of six subjects who specifically reported dislike of the tablets as a reason for dropping out, the distribution between groups was vitamin K ($n = 2$), vitamin D plus calcium ($n = 2$), and combined vitamins K and D plus calcium ($n = 2$).

There were no between-group differences in age, age at menopause, history of oophorectomy, smoking habit, past use of oral contraceptives, or hormone replacement therapy. Table 1 shows the baseline characteristics of the participants and their basal dietary intakes of relevant nutrients. Energy and macronutrient intakes at baseline did not differ significantly between groups. The mean calcium and phylloquinone intakes were adequate, according to UK guidelines,[37] but vitamin D intakes in all but three subjects were below the 10 μg/day recommended in this age group. There were no significant group differences for intakes of the trace elements copper, magnesium, manganese, potassium, selenium, and zinc (data not shown). Regular supplement taking was permitted as part of usual dietary intake if daily intakes were within the inclusion criteria of <10 μg vitamin D, <500 mg calcium, and <30 μg vitamin K_1. Fifty-one percent of the study population reported consuming at least one supplement on at least one of the five 6-month visits. Food supplement use did not differ by group and had a substantial effect on the recorded intakes of vitamin C (~20%) and of vitamin D (~40%) but not on vitamin K_1 or calcium.

Changes in BMD and BMC

The unadjusted mean changes (95% CI) in BMD and BMC over the 2-year study are shown in Table 2. There were no significant differences between intervention groups at any bone site. However, significant changes from baseline were found at some sites. At the mid-distal radius, BMD fell significantly from baseline in all groups ($p < 0.001$), indicating bone loss of between 1% and 2% per year, irrespective of intervention. At the ultradistal radius, whereas there were no significant differences from baseline in the placebo, vitamin K, or vitamin D plus calcium groups, there was a significant increase in BMD of 0.8% per year ($p < 0.01$) in the combined vitamin K and vitamin D plus calcium group. Unadjusted BMC at the ultradistal radius showed the same significant gain in bone mineral ($p < 0.01$) in women who had received combined supplementation with vitamin K and D. Although there was a slight 2-year rise in both BMD and BMC at the ultradistal radius in the placebo group, this did not achieve significance. At the femoral trochanter, there were no significant differences from baseline in BMD, but there were significant 2-year rises in BMC in the placebo group, vitamin D group, and combined vitamin groups (Table 2).

The DXA data were also analyzed by GLM procedures, with appropriate adjustment of covariates as described in the Materials and Methods section. An essentially similar picture was obtained for both BMD and BMC results at all bone sites except for the femoral trochanter, where there were unexplained significant gains in BMC but not BMD at

TABLE 2. UNADJUSTED TWO-YEAR MEAN CHANGES (95% CI) IN BMD AND BMC IN THE FOUR INTERVENTION GROUPS

Bone site	*Interval (months)*	*Intervention group*				
		Placebo	*Vitamin K_1*	*Vitamin D + calcium*	*Vitamins K and D + calcium*	*ANOVA from baseline*
Femoral neck BMD (mg/cm^2)	0–24	0.7 (−10.2, 11.6)	−4.2 (−15.6, 7.2)	1.9 (−6.5, 10.3)	1.0 (−8.0, 10.1)	NS
Femoral neck BMC (mg)	0–24	−32.1 (−127, 0.6)	−49.6 (−141, 42.0)	25.8 (−59.7, 111)	9.9 (−86.6, 66.8)	NS
Femoral trochanter BMD (mg/cm^2)	0–24	8.9 (−3.9, 21.6)	4.3 (−9.1, 17.8)	7.8 (−2.3, 17.8)	6.9 (−3.9, 17.7)	NS
Femoral trochanter BMC (mg)	0–24	630† (218, 1040)	346 (−100, 793)	460† (158, 763)	549† (207, 890)	$p < 0.01$
Femoral Ward's BMD (mg/cm^2)	0–24	0.1 (−19.6, 19.4)	2.2 (−20.1, 16.2)	11.4 (−6.3, 29.2)	6.0 (−21.8, 9.9)	NS
Femoral Ward's BMC (mg)	0–24	−36.7 (−143, 70.3)	−30.9 (−126, 64.5)	39.9 (−60.3, 140)	25.6 (−106, 54.5)	NS
Mid-distal radius BMD (mg/cm^2)	0–24	−20.4* (−26.8, −13.9)	−18.6* (−26.7, −10.5)	−16.6* (−23.3, −9.9)	−16.9* (−23.7, −10.1)	$p < 0.001$
Mid-distal radius BMC (mg)	0–24	−20.4* (−25.4, −15.3)	−16.3* (−22.1, −10.6)	−14.9* (−20.8, −9.0)	−14.1* (−20.7, −7.6)	$p < 0.001$
Ultradistal radius BMD (mg/cm^2)	0–24	3.6 (−0.2, 7.4)	−1.9 (−7.5, 3.8)	1.7 (−3.1, 6.5)	6.2† (2.6, 9.8)	$p < 0.01$
Ultradistal radius BMC (mg)	0–24	7.2 (−4.9, 19.3)	−6.3 (−20.8, 8.3)	−1.5 (−17.4, 14.4)	30.7† (15.2, 46.2)	$p < 0.01$

Values are the mean changes (95% CI) in BMD and BMC from baseline over the 2-year intervention period for each group. Significant differences between intervention groups and/or from baseline were investigated by GLM, repeated measures with pairwise comparisons. There were no significant differences between intervention groups at any bone site.

* Significant bone loss from baseline.

† Significant bone gain from baseline.

NS, not significant ($p > 0.05$).

some visits in all groups. There were no significant differences for adjusted BMD or BMC between the four treatment groups, but changes from baseline were observed. For the most part, only adjusted BMC results are presented here, in line with previous recommendations for the reporting of bone mineral measurements in population-based research.[41] The 6-month changes from baseline in covariate-adjusted BMC at the three sites of the femur and for the mid-distal radius are shown in Fig. 2. As found for the unadjusted results, there were significant increases from baseline in adjusted BMC at the femoral trochanter after 1 year and beyond in both the vitamin D group and in the combined vitamins K and D group. On the other hand, there was a significant increase in trochanter BMC in the placebo group after 2 years. Adjusted values for BMC at the mid-distal radius confirmed that there was a progressive loss of bone mineral at this site over 24 months.

The only consistent intervention effect on BMD and BMC was seen at the ultradistal radius. GLM-adjusted changes (95% CI) in BMD and BMC are shown in Fig. 3. This rigorous analysis confirmed the findings from the unadjusted data that bone mineral had increased from baseline at the ultradistal radius in the combined intervention group, with significant increases in BMD at 18 and 24 months and in BMC at all visits from 6 months onward. A significant increase in BMD in the placebo group was only evident at the 6-month time-point.

Changes in indicators of vitamin K and vitamin D status

The effect of intervention on tissue and functional markers of vitamin K and vitamin D status during the study are shown in Table 3. With the exception of %GluOC and 25(OH)D, the status indicators showed a skewed distribution; for these indicators, geometric means are given in Table 3 because they represent better average measures for such non-normally distributed data. Fasting vitamin K_1 serum concentrations rose significantly in response to vitamin K such that the geometric mean for all supplemented subjects had risen by 157% (95% CI, 101, 212) after 2 years. Vitamin K supplementation induced a significant 40% decrease in the absolute concentration of GluOC and a significant 105% increase in GlaOC over 2 years (Table 3). The apparent lower influence of vitamin K on the GluOC fraction compared with GlaOC is explained by the finding that both fractions rose with time independently of vitamin K. This non–vitamin K–dependent rise was slightly greater for GluOC than for GlaOC. Over 2 years, the geometric means for GluOC increased in the nonsupplemented groups by 49% (placebo) and 69% (vitamin D plus calcium), respectively, whereas for GlaOC, the equivalent increases were 30% and 24%, respectively. The bone vitamin K status indicator, %GluOC,[40] responded substantially to vitamin K supplementation, with 2-year decreases of 48% (vitamin K) and 54% (vitamins K and D) and an overall decrease of 51% (95% CI, −47.5, −54.0). These changes in the γ-carboxylation status of osteocalcin were evident, and consistent, from 6 months onward. In contrast, there was a trend for %GluOC to rise over 2 years in the non–vitamin

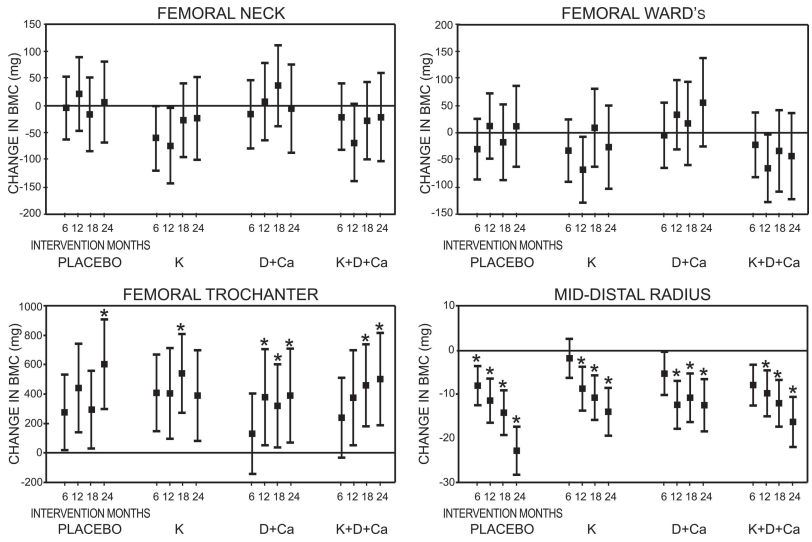

FIG. 2. GLM-adjusted mean changes (95% CI) in BMC from baseline at the femoral neck, femoral Ward's, femoral trochanter, and mid-distal radius after successive 6-month intervals of supplementation. *Significant change from baseline.

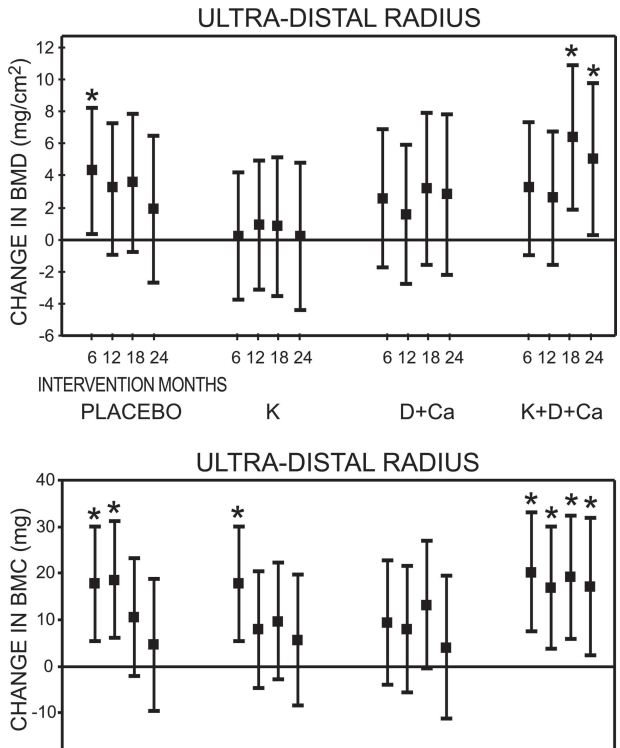

FIG. 3. GLM-adjusted mean changes (95% CI) in BMD and BMC from baseline at the ultradistal radius after successive 6-month intervals of supplementation. *Significant increase from baseline.

K–supplemented groups, but the increases of 7.1% (placebo) and 15.5% (vitamin D plus calcium) only reached significance in the latter. Quartile analysis showed that the greatest decreases in GluOC and %GluOC in the vitamin K supplementation groups occurred in those women with the highest values at baseline (results not shown).

Serum 25(OH)D rose significantly ($p < 0.001$) in response to vitamin D supplementation, with a mean increase of 16.7% (95% CI, 9.0, 24.4) over 2 years. This rise in 25(OH)D was accompanied by a significant fall ($p = 0.002$) in PTH of 10.9% (95% CI, –20.6, –1.2). In contrast, 25(OH)D fell significantly ($p < 0.001$) in the non–vitamin D–supplemented subjects, with a mean decrease of 17.5% (95% CI, –13.5, –21.4) over 2 years, but PTH showed no significant change. Quartile analysis showed that the women showing higher percentage increases in 25(OH)D had lower concentrations at baseline (results not shown)

For every status marker measured, there was no evidence of an additive effect in women who took combined vitamin K and vitamin D plus calcium.

Relationship between serum 25(OH)D and PTH

Baseline 25(OH)D was negatively correlated to PTH before ($r = –0.23$, $p < 0.01$) and after age adjustment ($r = –0.26$, $p < 0.001$), and this relationship was unaffected by vitamin D supplementation. Further analyses using a repeated-measure, multivariate linear regression model were carried out to study the ability of serum 25(OH)D to predict change in serum PTH concentrations over time. To avoid seasonal influence, the chosen time intervals were baseline, 12 months, and 24 months, representing subject visits during October to January. This data has been previously reported in abstract form.[42] In brief, change in serum 25(OH)D was found to be a strong independent negative predictor of the change in PTH, with regression coefficient × 100 (±SE) = –0.9 (0.2), $p < 0.0001$. Previous PTH also positively predicted the change in PTH, with coefficient × 100 (±SE) in the regression model = 94 (3.7), $p < 0.0001$. When "current" 25(OH)D was substituted for "change" in 25(OH)D in the model, it was not a significant independent predictor of change in PTH. Neither age nor calcium intake was a significant predictor of change in PTH.

Changes in indicators of bone turnover

No significant differences were found between groups for the markers of bone formation and resorption represented

TABLE 3. SERUM INDICATORS OF VITAMIN K AND VITAMIN D STATUS IN THE FOUR INTERVENTION GROUPS

		Intervention group				
	Study month	*Placebo*	*Vitamin K_1*	*Vitamin D + calcium*	*Vitamins K and D + calcium*	*ANOVA by intervention group*
Serum vitamin K_1 (μg/liter)	0	0.25 (0.20, 0.31)	0.22 (0.17, 0.27)	0.21 (0.16, 0.29)	0.21 (0.16, 0.27)	NS
	12	0.23 (0.17, 0.32)	0.66 (0.53, 0.82)	0.22 (0.16, 0.30)	0.55 (0.45, 0.68)	$p < 0.001$
	24	0.29 (0.24, 0.34)	0.55 (0.46, 0.67)	0.23 (0.21, 0.32)	0.53 (0.44, 0.64)	$p < 0.001$
ANOVA* (within-group)		NS	$p < 0.001$	NS	$p < 0.001$	
Serum GluOC (μg/liter)	0	5.03 (4.15, 6.10)	5.52 (4.66, 6.53)	4.81 (3.95, 5.87)	5.77 (4.67, 7.14)	NS
	12	7.02 (6.19, 7.95)	4.04 (3.45, 4.73)	6.77 (5.98, 7.66)	3.67 (3.02, 4.45)	$p < 0.001$
	24	7.48 (6.51, 8.58)	3.77 (3.19, 4.46)	8.13 (7.04, 9.38)	3.15 (2.55, 3.88)	$p < 0.001$
ANOVA* (within-group)		$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	
Serum GlaOC (μg/liter)	0	5.80 (5.04, 6.68)	6.04 (5.34, 6.82)	5.81 (4.89, 6.90)	5.66 (4.89, 6.54)	NS
	12	6.99 (6.2, 7.98)	10.61 (9.92, 11.36)	6.80 (5.99, 7.71)	9.90 (9.08, 10.79)	$p < 0.001$
	24	7.52 (6.86, 8.25)	12.11 (11.24, 13.05)	7.20 (6.40, 8.08)	11.88 (11.12, 12.69)	$p < 0.001$
ANOVA* (within-group)		$p < 0.001$	$p < 0.001$	$p = 0.001$	$p < 0.001$	
Percent GluOC	0	46.4 ± 18.0	48.0 ± 15.3	45.8 ± 17.7	50.0 ± 17.8	NS
	12	49.9 ± 14.3	28.8 ± 10.8	50.1 ± 12.2	28.6 ± 13.5	$p < 0.001$
	24	49.7 ± 13.8	25.2 ± 10.9	52.9 ± 14.4	23.0 ± 13.8	$p < 0.001$
ANOVA (within-group)		NS	$p < 0.001$	$p = 0.001$	$p < 0.0001$	
Serum 25(OH)D (μg/liter)	0	22.8 ± 6.1	23.1 ± 6.7	25.0 ± 6.2	24.7 ± 7.0	NS
	12	19.1 ± 6.9	18.4 ± 6.8	29.7 ± 6.1	29.2 ± 6.5	$p < 0.001$
	24	19.5 ± 5.3	18.3 ± 6.4	29.8 ± 6.0	28.3 ± 6.6	$p < 0.001$
ANOVA (within-group)		$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	
Serum PTH (pM)	0	4.38 (3.99, 4.81)	4.69 (4.25, 5.17)	4.86 (4.37, 5.40)	4.30 (3.84, 4.81)	NS
	12	4.75 (4.28, 5.28)	4.91 (4.41, 5.47)	4.31 (3.85, 4.83)	4.00 (3.58, 4.48)	$p = 0.037$
	24	4.42 (3.98, 4.90)	4.64 (4.16, 5.18)	4.47 (4.03, 4.96)	3.71 (3.22, 4.27)	$p = 0.036$
ANOVA* (within-group)		NS	NS	$p = 0.003$	$p < 0.001$	

Values are geometric means (95% CI) for (log-transformed) skewed distributions and arithmetic means ± SD for normal distributions. Significance differences within each intervention group with time were investigated by ANOVA for repeat measures using GLM. Between-group significances at each subject visit were investigated by ANOVA.

* ANOVA on log-transformed data.

NS, not significant ($p > 0.05$).

by serum BALP and urinary NTX, respectively. Total OC represented by the sum of the GluOC and GlaOC fractions also showed no significant group differences. Significant increases with time were seen for total OC in all groups ($p < 0.01$) and for urinary NTX in all but the placebo group ($p < 0.05$).

DISCUSSION

This was a novel study, designed to address the specific question of whether taking regular, high-dietary amounts of vitamin K_1, either alone, or in combination with moderate supplements of vitamin D_3, could reduce the rate of bone mineral loss in healthy, nonosteoporotic older women. Calcium was included in the vitamin D treatment group to ensure adequacy but was not the nutrient of primary interest. The only comparable vitamin K_1 supplementation trial published thus far is the Maastricht study, which differed in design and had a 5-fold higher vitamin K_1 dose.[26]

The power of our study to detect changes in bone mineral accretion was diminished both by the wide interindividual variation in BMD at baseline and by the lower than expected rate of bone loss in the placebo group. However, it is recognized that the rate of bone loss in women of this age varies greatly, depending on the bone site, nutritional status, and other factors.[43,44] For example, in the recent Women's Health Initiative (WHI) trial in women 50–79 years of age,[10] the placebo group showed a steady 9-year increase in BMD for the whole body and total spine, whereas BMD for the total hip declined by only 1% over the same period. Lesser[45] suggested that this may have been attributable to the "therapeutic" background intakes of calcium (1154 mg/day) and vitamin D (9.2 μg/day) in that trial. In our study, the mean calcium intake at baseline of 1055 mg/day was similar to that in the WHI trial but considerably higher than the 690 mg/day found in the British National Diet and Nutrition Survey (NDNS) of 1994/5 in women 65–84 years of age.[46] The mean background vitamin D intake in our study of 5.4 μg/day was less than that of the WIH trial but greater than the nationally representative intake of 3.5 μg/day in British women 65–84 years of age.[46] In our study, the only bone site showing a consistently significant bone loss was the mid-distal radius, but the loss was not significantly different between placebo and supplementation groups. On the other hand, at the site of the ultradistal radius, we did observe a significant increase in BMC and BMD from baseline in those women who had received combined calcium, vitamin D, and vitamin K. This increase was evident for unadjusted BMD and BMC and after adjustment for covariates. With its high content of

trabecular bone, the ultradistal forearm has a higher metabolic turnover rate than predominantly cortical bone and may be more responsive to intervention effects. A study of healthy, postmenopausal women in Massachusetts suggested that vitamin D supplementation at 10 μg/day was more effective in preventing bone loss of trabecular bone than of cortical bone.[47] The ultradistal forearm (but not the mid-distal forearm or lumbar spine) was previously shown by some of us to be similarly responsive to a 2-year combined exercise plus calcium intervention in older women.[48] The possible greater sensitivity of the ultradistal radius to a vitamin K effect is supported by the findings from a meta-analysis of nine studies in which BMD changes had been measured after exposure to oral anticoagulants.[49] Although this meta-analysis showed a trend to bone loss at most sites, a significant decrease in BMD was only observed at the ultradistal radius.[49] Thus, the effect of vitamin K antagonists seems to mirror our findings of vitamin K (with vitamin D) supplementation but in reverse.

We did not measure vertebral BMD because of the confounding effect of vascular calcification in older people and because matrix Gla protein is a vitamin K–dependent inhibitor of vascular calcification.[13] This decision seems justified in view of our previous exercise study[48] and the results of a 3-year intervention study from Maastricht in which a supplementation effect of combined vitamin K, vitamin D (with calcium), and additional minerals was found at the femoral neck but not at the lumbar spine.[26] In the Maastricht study, the rate of bone loss at the femoral neck was significantly slowed over 3 years in this combined vitamin K group but not in subjects receiving vitamin D plus minerals alone.[26] However, at 1 mg/day, the supplementary vitamin K_1 dose in the Maastricht study was 5-fold higher than in our study and would be difficult to achieve from dietary sources alone.

The lack of a significant effect on BMD in the vitamin D plus calcium-supplemented group suggests that the improvement in vitamin D status alone provided no additional benefit in these healthy older women who, on the basis of calcium intake, were already calcium replete. The efficacy of vitamin D given alone or with calcium in the prevention of bone loss or fractures is controversial, with some trials showing significant benefit[3,5,6] and some not.[7–10] A variety of factors could account for these differences, but important variables include vitamin D status at baseline[50] and habitual calcium intake of the population under study. Our participants were younger, less frail, and were less likely to have vitamin D insufficiency and secondary hyperparathyroidism than participants in some trials where a vitamin D/calcium intervention effect was seen; this may have limited any effect on bone mass. Evidence of a supposedly satisfactory vitamin D status in most of our subjects was supported by their mean baseline 25(OH)D concentration of 24 μg/liter (60 nM) and by the fact that none fell below the level considered adequate (10 μg/liter; 25 nM) by a UK working group.[46] Despite this, supplementation with 10 μg/day vitamin D_3 not only significantly raised 25(OH)D levels but also significantly lowered PTH levels. The suppression of PTH, in particular, suggests that suboptimal vitamin D status was common in our participants. As in a large, global study,[50] we found that the increase in 25(OH)D after vitamin D supplementation is strongly dependent on the baseline value, with maximal increases being seen in those women with an initially low serum 25(OH)D. Our analyses also revealed that the change in 25(OH)D is predictive of the inverse change in PTH. These data support the concept that pre-existing vitamin D status is a major factor in determining the change in 25(OH)D in response to vitamin D supplementation and therefore of the degree of lowering in PTH levels.[50] Although methodological differences usually preclude comparisons of absolute values, the overall 2-year changes of 17% for 25(OH)D and 11% for PTH were slightly lower than in other studies for an equivalent vitamin D dose.[6,50,51] This is again likely to reflect the better initial vitamin D status of our subjects. This is supported by comparing our results with a recent intervention study in free-living elderly in Randers, Denmark.[6] Comparison with this Danish study is especially valid because the doses of vitamin D (and calcium), the 2-year sampling period, and the methodology for 25(OH)D and PTH measurements were exactly the same in both studies. In a small subset of the Danish participants, the equivalent 2-year changes for 25(OH)D and PTH were 27% and 15%, respectively. These greater changes probably reflected the lower baseline 25(OH)D of 15 μg/liter in the Danish subjects compared with 24 μg/liter in our Scottish subjects. Coincidentally, Randers and Dundee share exactly the same latitude (56°28′).

The basal intakes of vitamin K_1 (mean, 85 μg/day) are representative of intakes found in a UK National Survey of people ≥65 years of age,[52] except that a lower proportion of our study population (27% versus 59%) failed to meet the current UK guideline of 1 μg/kg body weight/day.[37] However, nearly two thirds of the women in our study failed to meet the more recent U.S. adequate intake of 90 μg/day.[53]

Our study supports the concept[12,22,54,55] that much higher dietary intakes of vitamin K are needed for γ-carboxylation of bone OC compared with the hepatic coagulation proteins. This is the first study to show that regular, long-term supplementation with vitamin K_1 at a high dietary level can substantially improve and maintain vitamin K status as evidenced by increases in both tissue stores (serum vitamin K_1) and a functional marker (OC carboxylation). Although absolute values for %GluOC are assay dependent,[40] the reduction of ~50% is consistent with that seen in short-term repletion studies after supplementation with ~300 μg of vitamin K_1[22,23,55] but smaller than that after a supradietary dose of 1000 μg/day.[55] In our study, maximal OC carboxylation was attained by 6 months and did not change significantly thereafter. It is noteworthy that those women with the most impaired vitamin K status at baseline (as represented by the highest quartiles of GluOC and %GluOC and lowest quartile of GlaOC, respectively) showed the greatest changes in OC carboxylation after vitamin K supplementation.

We chose vitamin K_1 for this study because this molecular form accounts for 90% of dietary vitamin K intakes.[56] A proprietary preparation of menaquinone-4 (menatetranone) is used as an anti-osteoporotic agent in Japan, and a

recent meta-analysis of Japanese trials showed an association of menatetranone supplementation with reduced fracture incidence.[57] These menatetranone trials differed from our study in that the administered doses were extremely large (generally 45 mg/day) and the target groups already had pre-existing involutional[27–29] or secondary[58,59] osteoporosis or osteopenia. Enhanced effects on BMD have been found when menatetranone is used in combination with 1α-hydroxycholecalciferol.[28,29] There is also evidence that the effectiveness of menatetranone in inhibiting bone loss is related to the specific geranyl-geranyl side chain of this form.[60,61] The existence of an unusual metabolic pathway that converts a fraction of dietary vitamin K_1 to menaquinone-4[62] does not exclude the possibility that any benefit of supplementary vitamin K_1 might be attributable to the menaquinone-4 thus formed.

Our study provided no evidence that vitamin K_1 taken alone at high dietary intakes had any influence on maintaining bone mass despite the improvement in OC carboxylation. However, the Japanese experience suggests that the effectiveness of vitamin K_2 in preventing fractures is greater than that predicted from its ability to maintain bone mass.[27] This is supported by the finding in older American women that serum GluOC is more strongly related to ultrasonic transmitted velocity than to femoral neck density[63]; this relationship prompted the authors to suggest that vitamin K insufficiency might have a greater effect on bone quality than on BMD. Our findings showing no demonstrable effect of supplementation on markers of bone formation (BALP) and resorption (NTX) are in agreement with the lack of consistent effect on equivalent bone markers after long-term supplementation with 1 mg vitamin K_1[26] or 45 mg menatetranone.[27] The opposite findings of a rise[27] or fall[26] in OC may be artefactual (e.g., a consequence of the varying affinities of different OC antibodies to changing carboxylation status); this variability in OC response after vitamin K supplementation has been noted previously.[54,64] In this study, OC, as represented by the sum of the GluOC and GlaOC fractions, showed no differences between groups. Subsequent work using a standard RIA for the intact OC (and 1–43 fragment) also showed no differences in OC between groups (MJ Shearer and CM Gundberg, unpublished observations).

Although several large population studies have revealed significant associations of dietary intakes of vitamin K with fracture rates and/or BMD,[32–34] the results are not always consistent or uniform across the sexes or age groups. The same is true of associations with indicators of vitamin K status.[35] Clearly it is difficult to distinguish vitamin K status from all the other factors influencing changes in BMD, and one consistent criticism of these associations has been the difficulty in excluding the confounding effect of overall poor nutrition.[11,33] Nevertheless, in the Framingham study,[33] it is noteworthy that the top quartile of vitamin K_1 intake (median, 250 μg/day), which was associated with reduced fracture risk, was comparable with the mean total dietary intake achieved in this study of 285 μg/day.

This study lends support to the concept that vitamin K may act in concert with vitamin D to reduce bone loss. Three other studies have shown an enhanced effect of combined vitamin K and vitamin D supplementation,[26,28,29] but ours is the first in which the extra vitamin K intake can be considered to be within the dietary range. The mechanism(s) whereby parallel improvements in the status of both vitamins D and K might have an enhanced effect over either vitamin alone is unclear. It is feasible that any synergy between vitamins D and K derives from separate effects exerted independently or alternatively from their concerted action through common proteins or pathways. It is known that 1,25-dihydroxyvitamin D enhances the transcription of vitamin K–dependent bone proteins (e.g., osteocalcin, matrix Gla protein, and Gas6). Other evidence has led to the suggestion that vitamin D might directly influence the γ-carboxylation reaction of bone Gla proteins.[16,24] This hypothesis was not supported by our findings because we showed that γ-carboxylation of osteocalcin was neither enhanced by vitamin D (relative to the placebo group) nor by its combination with vitamin K (relative to the vitamin K group).

In summary, dietary supplementation with a combination of nutritionally relevant amounts of vitamin K with vitamin D and calcium in healthy older women was associated with a modest but significant increase in BMC at one site, consisting predominantly of trabecular bone. Similar changes were not observed in the either the vitamin K group alone or in the calcium plus vitamin D group, suggesting a synergistic role of the combination as suggested by previous reports.

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