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ORIGINAL CONTRIBUTION

Serum level of under-carboxylated osteocalcin and bone mineral density in early menopausal Norwegian women

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Abstract
Purpose Serum level of under-carboxylated osteocalcin (ucOC) is considered a sensitive measure of vitamin K status, and ucOC levels are associated with bone mineral density (BMD) and fracture risk in elderly persons. The aim of this study was to assess the relationship between ucOC and BMD in early menopausal women.
Methods The data reported here come from the enrolment in a double-blinded placebo-controlled randomized trial comprising 334 healthy Norwegian women between 50 and 60 years, 1–5 years after menopause, not using warfarin or medication known to affect bone metabolism. Total hip, femoral neck, lumbar spine, and total body BMD and serum level of ucOC and total osteocalcin were measured, and information of lifestyle was collected through questionnaires. The association between ucOC and BMD at all measurement sites was assessed by multiple regression analyses adjusting for possible confounding variables.
Results The absolute serum level of ucOC was significantly and negatively associated with BMD at all measurements sites, both in univariate analyses ($p < 0.01$) and in multivariate analyses adjusting for years since menopause, smoking status and weight ($p < 0.01$). However, serum ucOC, expressed as percentage of the total osteocalcin level, was not associated with BMD at any site.
Conclusions Achievement of adequate vitamin K nutritional intake is important, but ucOC expressed as percentage of total osteocalcin levels as reflection of vitamin K status does not seem to play a central role in determining BMD levels in early menopausal women.

Keywords Vitamin K · Under-carboxylated osteocalcin · Bone mineral density · Menopause

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Introduction

Vitamin K is a family of different molecular forms: vitamin K1 (phylloquinone or phytonadione) is a single form synthesized in green plants and vitamin K2 contains a spectrum of multiple forms called menaquinone-*n* (MK-*n*). Both vitamin K1 and K2 may equally contribute to human vitamin K status [1], and MK-4 and MK-7 are probably the molecular species most closely associated with bone mass

and fracture risk [2–4]. Vitamin K1 is found in green leafy vegetables and vegetable oils, MK-4 in animal products, and one source rich of MK-7 is the Japanese food NATTO, soybeans fermented with the bacteria Bacillus subtilis natto (1,103 μg/100 g) [1, 5–7].

Osteocalcin (OC) is a bone protein synthesized by osteoblasts during bone matrix formation [8]. It contains three glutamate residues that are γ-carboxylated in a vitamin K-dependent process. These γ-carboxyglutamate residues are responsible for its specific affinity to the hydroxyapatite molecule. Only a small proportion of OC is not bound to bone, and thus detectable in serum as a marker of bone formation [9]. In vitamin K deficiency, due to decreased γ-carboxylation, a larger fraction of OC does not undergo the complete carboxylation process and is referred to as under-carboxylated osteocalcin (ucOC) [10]. Serum ucOC levels expressed as fraction of total OC are considered a sensitive measure of vitamin K status [11] and associated with low dietary intakes of vitamin K [12]. A negative association between serum levels of ucOC and bone mineral density (BMD) at the hip has been reported [13], and it has been argued that a high ucOC level may be a marker of hip fracture risk in elderly women [14, 15].

Supplementation with phytonadione or MK-4 reduces bone loss and fracture risk in Japanese women [2]. However, in three recently published double-blind studies, high doses of vitamin K1 and K2 (MK-4 and MK-7) significantly lowered ucOC levels in the treatment groups without any apparent effect on BMD [16, 18]. Thus, the relationship between vitamin K, serum ucOC levels and bone mass remains controversial [19, 20]. In the present study, we have used data from the baseline measurements in a double-blind placebo-controlled randomized trial comprising 334 healthy postmenopausal women between 50 and 60 years [18] to assess the association between ucOC, as a surrogate measure for all vitamin K intake and BMD.

Materials and methods

Inclusion of study participants

Through newspaper advertisements and media coverage from January to October 2006, healthy women between 50 and 60 years of age from the Norwegian cities of Tromsø and Bergen were invited to participate in a double-blind placebo-controlled randomized controlled trial [18]. In short, 455 women were assessed for eligibility by telephone interview and were included in the study if they were 50–61 years of age, were between 1 and 5 years since last menstruation, were not using warfarin, were not under any form of hormonal therapy (HT) or other medications known to influence bone remodelling. Altogether 334 women were included in the study that was conducted by the National Research Center in Complementary and Alternative Medicine (NAFKAM), in collaboration with the University Hospital of North Norway (UNN) and Haukeland University Hospital in Bergen. The participants from the two centres did not differ significantly in any important aspects of the study.

Questionnaire

Entering the study, all participants filled in a questionnaire concerning general health, smoking habits, alcohol intake, physical activity level and use of dietary supplementations, such as vitamin D or cod liver oil (high content of vitamin D). From the questionnaires, smoking status was categorized into either smoking or not smoking, and alcohol intake into minimal (less than few times last year), modest (once a month to once a week) and moderate (more than once a week). Physical activity level was derived from two identical questions on light and heavy physical activity with four alternatives on hours per week. These alternatives were combined into a common score with three alternatives; low, moderate or high physical activity level. Use of vitamin D and/or cod liver oil was combined into one variable of vitamin D intake (yes or no).

Height, weight, BMD and biochemical measurements

Height and weight were measured to the nearest centimetre/half kilogram with the participants wearing light clothing and no shoes. BMD was measured as g/cm^2 at both centres by Dual X-ray Absorptiometry (Prodigy, GE-LUNAR, Madison, WI, USA) at the total body, at the lumbar spine (L2–L4) and at the total hip, including the femoral neck. The densitometers were calibrated in vitro as well as in vivo at the study start, and no differences between the two densitometers were detected. The coefficient of variation (CV %) for total hip measurements was 1.14% in Tromsø and 0.82% in Bergen. The measurements were performed according to the same protocol, and one trained technician reviewed all the scans.

Two non-fasting blood samples were drawn from each participant. The blood samples were centrifuged at 4° and frozen until analysis at the Hormone Laboratory, Haukeland University Hospital. The assays used were enzyme-linked immunosorbent assays (ELISA). Total serum osteocalcin (N-mid OC) was measured by assays from Nordic Bioscience Diagnostics, Herlev, Denmark. The cOC and ucOC assays were obtained from TaKaRa Bio. Inc., Japan. The mean sample pair variation was 4.1% for N-mid OC, 4.1% for cOC and 8.4% for ucOC. Inter-assay CVs were 9.2% (mean value 17.0 ng/L) and 5.5% (mean value 43.9 ng/L) for N-mid OC, 20% (mean value 1.33 ng/mL)

and 5.67% (mean value 6.47 ng/mL, manufacturer's data) for ucOC, and 23% (mean value 2.91 ng/mL) and 1.0% (mean value 12.1 ng/mL, manufacturer's data) for cOC.

Valid serum N-mid OC measurements were obtained in 308 participants, and among these, ucOC measurements were obtained from 288 participants (blood donation was refused by 26 participants, and 20 samples were unsatisfactory for full analysis). Altogether 285 participants had both N-mid OC and ucOC measurements. Participants with measurements were 0.5 years more after menopause ($p = 0.023$) and had a total body BMD that was −0.027 g/cm^2 lower ($p = 0.031$) than participants without; otherwise, there were no significant differences in characteristics between them.

Ethics, informed consent and quality control

The regional Committee of Research Ethics and the Norwegian Data Inspectorate approved the study. The Norwegian Directorate of Health and Social Services approved the establishment of the biobank for serum specimens. Written informed consent was obtained from all participants at inclusion. After the examination, the participants were informed about their BMD status, and all participants with total hip or lumbar spine T-scores at or below −2.0 were offered appropriate clinical follow-up.

Statistical analyses

The normal distribution of parameters was evaluated with visual inspection of histograms, and the dependent variables, the BMD values of the different sites, were considered normally distributed. The univariate association between BMD at the total hip, femoral neck, lumbar spine, total body and absolute ucOC levels was first assessed in univariate analyses. The association between the dependent variables (BMD at the different sites), the independent variable and the possible confounding variables: age, years since menopause, height, weight, BMI, physical activity levels, smoking status, alcohol and vitamin D intake [21–27] was assessed using either Spearman's or Pearson's correlation. Variables that significantly correlated with either BMD at any site or with ucOC were included as covariates in an initial regression model. Following this, the association between ucOC and BMD at the different sites was examined in multiple regression models adjusting for (a) years since menopause, weight and smoking status, (b) years since menopause and weight. Then, ucOC levels were calculated as percentage of total osteocalin levels (ucOC/N-mid OC), and the association was examined using the same procedure as for absolute ucOC. All statistical analyses were performed using the Statistical Package for Social Sciences version 15.0 and 19.0 (SPSS Inc., Chicago, Ill, USA), and two sided p values < 0.05 were considered statistically significant.

Table 1 Central characteristics of the study participants, values are means (±SD) or *n* (%)

Characteristics	All participants*
Age, years	54.4 (2.5)
Height, cm	166 (567)
Weight, kg	67.5 (9.4)
BMI, kg/m^2	24.5 (3.1)
Age at menopause, years	51.0 (2.7)
Self-perceived health	
Poor	59 (18)
Good	267 (82)
Smoking status	
Non-smokers	284 (85)
Smokers	50 (15)
Alcohol intake	
Minimal	51 (16)
Low	189 (58)
Moderate	86 (26)
Physical activity level	
Low	43 (13)
Moderate	248 (76)
High	35 (11)
Present vitamin D intake	
Yes	19 (6)
No	315 (94)
Femoral neck bone mineral density, g/cm^2	0.875 (0.11)
Total hip bone mineral density BMD, g/cm^2	0.919 (0.12)
Lumbar spine (L2–L4) bone mineral density, g/cm^2	1.095 (0.15)
Total body bone mineral density, g/cm^2	1.117 (0.08)
Under-carboxylated osteocalcin, ng/mL (ucOC)	4.12 (2.59)
Carboxylated osteocalcin, ng/mL (cOC)	13.35 (6.12)
N-mid osteocalcin, ng/mL (N-mid OC)	22.12 (9.67)
ucOC/N-mid OC, %	18.77 (10.60)

* N, $N = 334$, except self-perceived health, alcohol intake, physical activity level: $N = 326$, cOC and ucOC: $N = 288$, N-mid OC: $N = 308$, ucOC/N-mid OC: $N = 285$

Results

Participant's characteristics are shown in Table 1, and the distribution of ucOC, N-mid OC and ucOC/Nmid OC is displayed in Fig. 1. In univariate analyses, there was a significant and negative association between absolute ucOC levels and BMD at all measurement sites ($p < 0.006$) (Table 2). There was furthermore a significant correlation between smoking status and ucOC levels ($p < 0.01$), between years since menopause, weight,

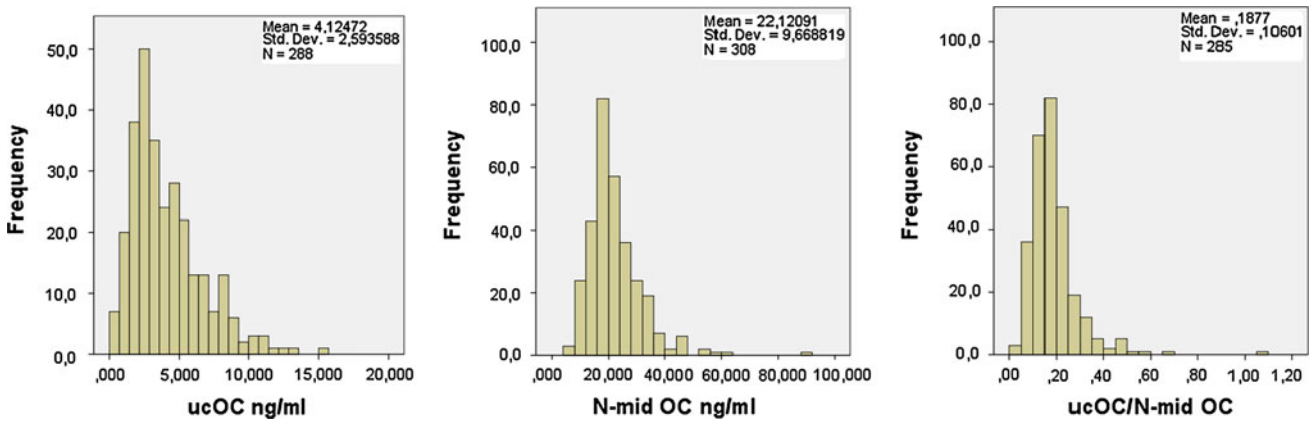

Fig. 1 The distribution of serum levels of absolute under-carboxylated osteocalcin (ucOC) ng/mL, total osteocalcin (N-mid OC) ng/mL and ucOC/N-mid OC in early postmenopausal women

Table 2 Association between bone mineral density (BMD) g/cm^2 and under-carboxylated osteocalcin (ucOC), ng/mL, in univariate analyses, both as an absolute measure (ucOC) and as fraction of total osteocalcin levels (ucOC/N-mid OC)

	Univariate analyses	
	Beta value (SE)	*p* value
Association ucOC–BMD		
ucOC–Femoral neck BMD	−0.007 (0.002)	0.004
ucOC–Total hip BMD	−0.007 (0.003)	0.006
ucOC–Lumbar spine BMD	−0.010 (0.003)	0.003
ucOC–Total body BMD	−0.006 (0.002)	0.001
Association ucOC/N-mid OC–BMD		
ucOC/N-mid OC–femoral neck BMD	−0.44 (0.062)	0.47
ucOC/N-mid OC–total hip BMD	0.011 (0.065)	0.86
ucOC/N-mid OC–lumbar spine BMD	−0.019 (0.083)	0.82
ucOC/N-mid OC–total body BMD	0.00 (0.044)	0.99

Table 3 Association between bone mineral density (BMD) g/cm^2 and under-carboxylated osteocalcin (ucOC), ng/mL, in multiple regression analyses, both as an absolute measure (ucOC) and as fraction of total osteocalcin levels (ucOC/N-mid OC)

Model: adjusted for weight and years since menopause at each site

	Multiple regression model	
	Beta value (SE)	*p* value
Association ucOC–BMD		
ucOC–Femoral neck BMD	−0.007 (0.002)	0.002
ucOC–Total hip BMD	−0.007 (0.002)	0.008
ucOC–Lumbar spine BMD	−0.008 (0.003)	0.008
ucOC–Total body BMD	−0.005 (0.002)	0.003
Association ucOC/N-mid OC–BMD	*Model 2*	
ucOC/N-mid OC–femoral neck BMD	−0.056 (0.059)	0.34
ucOC/N-mid OC–total hip BMD	−0.002 (0.061)	0.97
ucOC/N-mid OC–lumbar spine BMD	−0.030 (0.077)	0.70
ucOC/N-mid OC–total body BMD	−0.007 (0.039)	0.86

height, BMI and BMD levels at the total body, lumbar spine, femoral neck and total hip ($p < 0.04$) (data not shown). In an initial multiple regression model, including absolute ucOC, years since menopause, height, weight and smoking status, all variables were significantly associated with BMD at each site ($p < 0.02$), except height, and smoking was only associated with BMD at total hip ($p = 0.049$) and femoral neck ($p = 0.048$). In a final model adjusting for years since menopause and weight, absolute ucOC level was a significant and negative predictor of BMD at each measurement site (Table 3). However, ucOC expressed as percentage of total OC levels

(ucOC/N-mid OC) was no longer a predictor of BMD at any site or in univariate (Table 2) or multivariate analyses (Table 3).

Discussion

In this study, absolute serum ucOC levels were a negative predictor of BMD at the femoral neck, total hip, lumbar spine and total body, also after adjustments for years since menopause, weight and smoking. However, ucOC expressed as percentage of total OC levels (ucOC/N-mid OC) was no longer a predictor of BMD at any site.

In previous studies, higher serum ucOC levels were observed in elderly institutionalized women compared to young premenopausal women, and ucOC serum level was reported to be an independent determinant of femoral BMD [13] and a marker of hip fracture risk [14, 28]; but in all these studies, the correlation decreased when ucOc was expressed as percentage of the total OC [11]. In the EPIDOS study, elevated levels of ucOC were found in 29% of elderly women in the general population, predicting hip fracture risk independently of femoral neck BMD with an odds ratio of 1.9 [15]. In contrast, total OC was not associated with hip fracture risk, and ucOC was considered an independent risk factor for hip fracture [15]. In women above 60 years, increasing serum ucOC levels were negatively associated with femoral neck BMD, with a stronger association on bone quality measured by ultrasonic transmitted velocity (UTV) at os calcis than on BMD [29]. This was also indicated in another study including early menopausal women, where serum levels of ucOC were moderately associated with bone quality, but not with femoral BMD [30]. However, in all these studies, ucOC levels may rather be a reflection of accelerated bone metabolism than a marker of vitamin K status [11].

In the Nurses Health Study, women with the lowest vitamin K intake (below 109 μg per day) had an increased risk of hip fracture compared to women where the estimated daily intake were between 109 and 242 μg (median intake 163 μg/day) [31]. No linear dose-dependent trend ($p = 0.32$) was observed, indicating a threshold below which the risk of hip fracture increased [31]. In the Offspring cohort of the Framingham Heart Study, the estimated mean daily intake of vitamin K was 153 and 171 μg/day in men and women, respectively [32]. In women, low dietary vitamin K intake (<101 μg/day) was associated with low BMD at the hip and spine, after adjustments for covariates [32]. The cross-sectional differences in BMD across Vitamin K intake—quartiles were, however, modest [32]. Plasma levels of vitamin K and percentage ucOC (%ucOC) were measured in the same cohort [22]. After adjustments, low plasma vitamin K level and high serum %ucOC were associated with low BMD at the hip in men, but not in premenopausal women and postmenopausal women [22], comparable to the findings from the present study.

There are several trials assessing the relationship between serum ucOC levels and BMD changes with conflicting results. A positive effect of treatment with vitamin K (daily doses of 45 mg of MK-4) on BMD is seen in studies of patients with different chronic diseases [33–38], with established osteoporosis [39–44] or in conjunction vitamin D3 [40]. In trials including healthy premenopausal women taking NATTO (MK-7) for a period of 1 year [45], or postmenopausal women taking 45 mg MK-4 for a year [46, 47], ucOC levels decreased without influencing bone loss. In a study where healthy Dutch postmenopausal women received 45 mg MK-4 for 3 years, bone mineral content (BMC) and femoral neck width (FNW), but not BMD, increased relative to the placebo group [48]. In a double-blind controlled trial in elderly US men and women [17] and in postmenopausal US women [16], neither phylloquinone nor MK-4 supplementation had any effect on BMD changes, although serum ucOC declined significantly in the treatment group. These findings were similar to the prospective data from our study where MK-7 supplementation over 12 months did not influence BMD changes at any measured site, despite significantly declined serum ucOC and increased tCO in the treatment group [18]. Taken together, these results might reflect a situation where an increased intake of vitamin K above recommended levels does not add any extra benefits to bone, despite the influence of vitamin K intakes on absolute serum ucOC levels as indicated in a recently published Japanese study [49].

In summary, absolute serum ucOC was associated with BMD, whereas ucOC expressed as a fraction of total OC [11] was not, in a homogenous group of healthy Norwegian women in a narrow age span with a wide range of total hip BMD levels. The two densitometers used in the study were cross-calibrated and followed the same quality control routines. A potential limitation of the study is the lack of information on participant's vitamin D status in view of a possible interaction between vitamin K and vitamin D3 [40, 50–53]. Variation in ucOC levels related to dietary vitamin K intake may be a marker for an overall healthy diet [23]. However, the biological role of ucOC levels in serum remains unknown [54], and its possible role may reflect other functions [54, 55].

In conclusion, in prevention of osteoporosis and later fracture risk, adequate vitamin K should be included in overall healthy diets. However, as reflection of vitamin K status, ucOC calculated as percentage of total OC levels in serum does not seem to play a central role for BMD levels in early menopausal women.

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Conflict of interest None.

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