The results of the stepwise multiple regression analysis are shown in . BMI was the predominant independent variable, followed by age and serum OC concentration for both BMDs of the lumbar spine and femoral neck. The serum 25(OH)D concentration was independently associated with BMD of the femoral neck, although its R^2 was smaller than those of BMI, age and serum OC concentration.

shows ORs for "low BMD (t score ≤ -2.5 SD)" by level of serum 25(OH)D. After adjustment for model covariates, prevalence of low BMD for the lumbar spine was significantly higher in the 40- to 50-nmol/L group compared to the reference group (≥ 70 nmol/L). Similarly, a significantly higher prevalence of low BMD of the femoral neck was observed in the 30- to 40-nmol/L and 40- to 50-nmol/L groups compared to the reference group (≥ 70 nmol/L). The serum 25(OH)D concentration was not significantly associated with the serum OC concentration ($P=0.1715$) or the serum NTX concentration ($P=0.2355$). The lack of these associations remained after subjects were restricted to those with serum 25(OH)D concentrations <50 nmol/L ($P=0.4839$ for serum OC and $P=0.9574$ for serum NTX).

The serum 25(OH)D concentration is generally believed to be associated with physical strength. However, the serum 25(OH)D concentration was significantly associated with neither thigh muscle strength ($P=0.1144$), grip strength ($P=0.3131$), nor the TUG test ($P=0.6140$). Even when comparing in these three physical variables between lower and higher subgroups by using any thresholds, there were no significant differences in any variables between them.

Discussion

This is the first large-scale epidemiologic study exploring a possible association between vitamin D status and bone mass, bone metabolism, or physical strength in postmenopausal Asian women. The mean serum 25(OH)D concentration (55.6 nmol/L) and prevalence of vitamin D insufficiency observed in this population were similar to those of other populations of ambulant Japanese elderly women [11,12]. The vitamin D status of ambulant elderly Japanese, including this study population, is well maintained even in winter, due in part to high dietary intake of vitamin D from fish [11,13]. This study demonstrated that the serum 25(OH)D concentration was linearly associated with BMD of the femoral neck in subjects with a serum 25(OH)D concentration of 30 nmol/L or higher. This finding is in accordance with the result of a large epidemiologic study recently conducted [6] and supports a rationale that the serum 25(OH)D levels should be maintained 75–80 nmol/L or higher [3,14]. By contrast, an association between the serum 25(OH)D concentration and BMD of the lumbar spine was not significant. This discrepancy has not been frequently reported in the literature, but may be explained by the fact that vitamin D status affects cortical bone more than spongy bone. This hypothesis is supported by Stone et al.'s [15] finding that lower 25(OH)D levels are associated with hip but not calcaneal bone loss. Regarding the association between the serum 25(OH)D concentration and BMD of the lumbar spine, 50 nmol/L appears to be an inflection point ( ). This study may have failed to detect a true association due to the relatively small number of subjects at high 25(OH)D levels. Further studies should address this issue.

The present study showed that the serum 25(OH)D concentration of 50 nmol/L or lower was associated with low BMD (t score ≤ -2.5 SD) of both the lumbar spine and femoral neck (no significant increase in the prevalence of low BMD was observed in the <30 nmol/L group due to limited sample size). Study findings also suggest that vitamin D insufficiency is more strongly associated with low BMD in the femoral neck than in the lumbar spine.

Despite the significant associations observed between serum 25(OH)D concentration and BMD, the low R^2 values associated with vitamin D status in multivariate analysis indicate that it accounted for only a small proportion of the variance in BMD in the study population. Results of the present study are in line with the findings of two recent population-based investigations targeting postmenopausal women. The Rancho Bernardo Study [16] showed a slight but significant association between serum 25(OH)D and femoral BMD, and the OFELY Study [17] showed serum 25(OH)D not to be a significant determinant of bone loss. On the other hand, there have been two clinic-based studies in which the serum 25(OH)D concentration was correlated moderately with both spinal and femoral BMDs in postmenopausal women [18,19]. As such, the strength of the association between vitamin D status and BMD seems to depend on which population is targeted.

Numerous studies have shown an inverse association between the serum 25(OH)D and intact PTH serum concentrations [20–22]. The present study confirmed such an association with a threshold of 50 nmol/L of the serum 25(OH)D concentration for elevated serum intact PTH concentrations. This finding suggest that maintenance of serum 25(OH)D concentrations of at least 50 nmol/L is essential for maintaining bone health in postmenopausal Japanese women.

This study failed to confirm an association between serum 25(OH)D concentration and markers of bone turnover. Gallagher et al. [23] also reported no or only a slight association between the serum 25(OH)D concentration and markers on bone turnover in a healthy elderly population. On the other hand, Jesudanson et al. [24] showed a negative association between serum 25(OH)D concentration and serum bone resorption markers and alkaline phosphatase levels in postmenopausal women attending an osteoporosis clinic. Furthermore, an inverse relationship between serum 25(OH)D and markers of bone turnover was found in postmenopausal women with established osteoporosis [25]. Taken together, these studies suggest an association between the serum 25(OH)D and markers of bone turnover may be observed in frail populations, such as osteoporotic women, but not in the general population of postmenopausal women.

Our study also demonstrated that serum intact PTH is associated with BMD of the femoral neck, but not with BMD of the lumbar spine. The lack of the association with the lumbar spine may be due to the fact that PTH affects cortical bone mass [26] to a greater extent than spongy bone mass or because PTH does not have as great of an effect on bone mass in elderly Asian

populations compared to their European counterparts [29]. Moreover, BMD of the femoral neck was independently associated with serum PTH and 25(OH)D, which suggests that each plays an independent role in bone metabolism and bone mass. PTH may affect BMD partly via increased bone turnover because high serum PTH was associated with both serum OC and NTX in this study. On the other hand, serum 25(OH)D may affect BMD not via increased bone turnover, as serum 25(OH)D did not link to bone turnover markers in this study but probably via increased calcium absorption in the intestine. The cross-sectional nature of this study has limitations in its ability to make causal relationships, and this hypothesis should be confirmed by a longitudinal study.

Low levels of vitamin D have been reported to be associated with impaired physical functions [27,28]. To the contrary, the present study failed to demonstrate such an association between vitamin D status and muscle strength or the TUG test. The lack of the associations in this study may be due to relatively good vitamin D status (mean serum 25[OH]D concentration, 55.6 nmol/L), the study population being relatively young (mean age, 64.5 years), or ethnicity [29].

The elderly Japanese population has some characteristics in terms of diet and bone health that make them different from other general populations. They have lower calcium intake and higher vitamin D intake than elderly whites [11]. In the present population, 95% of the subjects had total calcium intake of less than 800 mg/day, a daily calcium requirement in Japan [30]. Their low calcium intake (527 mg/day) might diminish an effect of vitamin D on bone, and increase of calcium intake is hypothesized to alter strength of the association between vitamin D status and bone mass.

This study had some limitations. This study employed a cross-sectional design, which is limited in its ability to detect causal relationships. An intervention trial is needed in order to establish causality. In addition, subjects' participation rate of this study was approximately 50%, and thus selection bias may have occurred. For example, it is likely that healthier or more active women tended to participate in this study. Generalizations of our results to other populations should thus be made with caution.

In summary, the present study was the largest study to date to examine the relationship between vitamin D levels and bone health among Asian postmenopausal women. Our results suggest that higher serum 25(OH)D concentrations are associated with increased BMD of the femoral neck, and that a serum 25(OH)D concentration of at least 70 nmol/L is needed to obtain high BMD of the femoral neck, and that of at least 50 nmol/L is needed to achieve normal PTH levels and prevent low BMD. While significant associations were observed between vitamin D status and BMD of the femoral neck, the contribution of vitamin D status to BMD is relatively small, suggesting a role for other factors in low bone mass.

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ORIGINAL ARTICLE

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Low plasma phylloquinone concentration is associated with high incidence of vertebral fracture in Japanese women

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Abstract It has been reported that vitamin K supplementation effectively prevents fractures and sustains bone mineral density in osteoporosis. However, there are only limited reported data concerning the association between vitamin K nutritional status and bone mineral density (BMD) or fractures in Japan. The objectives were to evaluate the association between plasma phylloquinone (K_1) or menaquinone (MK-4 and MK-7) concentration and BMD or fracture in Japanese women prospectively. A total of 379 healthy women aged 30–88 years (mean age, 63.0 years) were consecutively enrolled. Plasma K_1, MK-4, MK-7, and serum undercarboxylated osteocalcin (ucOC) concentrations, BMD, and incidence of vertebral fractures were evaluated. In stepwise multiple linear regression analyses, L_{2-4} BMD and a bone turnover marker, log K_1, concentrations were independently correlated with vertebral fracture incidence. When subjects were divided into low and high K_1 groups by plasma K_1 concentration, the incidence of vertebral fracture in the low K_1 group (14.4%) was significantly higher than that in the high K_1 group (4.2%), and its age-adjusted RR was 3.58 (95% CI, 3.26–3.93). L_{2-4} BMD was not different between the two groups. These results suggest that subjects with vitamin K_1 insufficiency in bone have increased susceptibility for vertebral fracture independently from BMD.

Key words vitamin K · undercarboxylated osteocalcin · vertebral fracture · bone mineral density (BMD) · Japanese women · phylloquinone

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Introduction

Vitamin K is well known for its role in the synthesis of a number of blood coagulation factors. Vitamin K is also an important factor for bone metabolism via γ-carboxylation of vitamin K-dependent proteins such as osteocalcin (OC), matrix Gla protein, and protein S [1,2]. Low dietary phylloquinone (K_1) intake has been shown to be associated with increased hip fracture risk, notably among postmenopausal women [3,4]. Low dietary K_1 intake is also associated with low bone mineral density (BMD) at the hip and spine in pre- and postmenopausal women [5,6], and circulating levels of vitamin K_1 or K_2 were reported to be decreased in patients with hip fracture [7–10]. Those studies were mainly performed in Caucasians. There is only a limited amount of data concerning the association between vitamin K nutritional status and BMD or fractures in Japan. It has been reported that the intake of *natto*, which contains a high concentration of menaquinone-7 (MK-7), prevents hip fractures in Japanese [11] or promotes bone formation in premenopausal women [12]. However, another report showed that no differences in plasma K_1, menaquinone-4 (MK-4), and MK-7 were observed between patients with vertebral or hip fracture and normal subjects [13]. In animal models of osteoporosis, the effects of vitamin K_2 supplementation on bone mass, strength, and structure has been reported to be effective [14–17], or to be negative in ovariectomized rats [18–20], and the evidence is still equivocal. Although a relationship between vitamin K status and fracture risk has been reported, the relationship between BMD or fracture and vitamin K status is still controversial. Recently, it has been reported that vitamin K stimulates the differentiation of osteoblasts via not only γ-carboxylation but also steroid or xenobiotics receptors (SXR) [21].

Therefore, in the present study, we evaluated the association between plasma vitamin K (K_1, MK-4, and MK-7) concentrations and incidence of fracture or BMD in Japanese women prospectively, and assessed the importance of vitamin K status or γ-carboxylation of OC in reduction of fracture risk and increase of BMD.

Subjects and methods

Subjects

Japanese women in their thirties to eighties were consecutively enrolled in this study (2002–2003), and followed up by 2006. Women with metabolic bone diseases other than primary osteoporosis and women who were taking medicine related to bone metabolism such as active vitamin D, vitamin K, vitamin K antagonists, estrogen, bisphosphonates, or steroids were excluded. Women who had extremely low body mass index (BMI) (lower than 16) were also excluded. A total of 379 women (mean age, 63.0 ± 10.8 years; range, from 30 to 88 years) met the selection criteria for this study. The subjects consisted of 48 women aged 30–49 years, 202 women aged 50–69 years, and 129 women aged 70 years or older (70– years). Subjects were living in a rural area of Nagano. Most subjects have a backyard with their house, and they had the habit of frequently eating vegetables that they cultivated in their backyard.

Measurements

Plasma, serum and urine samples were collected from the subjects in the morning and stored immediately at –30°C until measurement. Plasma vitamin K (K_1, MK-4, and MK-7) was determined by the high-performance liquid chromatography-tandem mass spectrometry (LC-APCI-MS/MS) method [22]. ucOC as a sensitive marker for vitamin K insufficiency was measured by electrochemiluminesence immunoassay (ECLIA) (Sanko Junyaku, Japan). The antibody used in this ECLIA method is the same antibody used in the "Takara assay." However, the ucOC concentrations measured using this novel method were higher than those obtained using other methods, including the Takara assay. Intact OC was determined by immunoradiometric assay (IRMA) (Mitsubishi Kagaku Bio-Clinical Laboratories, Japan).

Serum concentrations of 25-hydroxyvitamin D [25-OH-D; radioimmunoassay (RIA); DiaSorin, Stillwater, MN, USA], and intact (1-84, 7-84) parathyroid hormone [intact PTH, immunoradiometric assay (IRMA); Scantibodies Laboratory, Santee, CA, USA] were determined. A bone resorption marker, urinary excretion of *N*-telopeptide (NTX; as measured by enzyme-linked immunosorbent assay (ELISA; Osteomark, Ostex International Seattle, WA, USA), and a bone formation marker, bone-derived alkaline phosphatase (BAP; EIA; DS Pharma Biomedical, Japan), were measured. For the evaluation of calcium metabolism, serum concentrations of calcium (Ca) and phosphorus (P) were measured. Body mass index (BMI) was calculated as the weight in kilograms divided by the square of the height in meters.

Lumbar spine (L_{2-4}) and femoral neck (FN) BMD was measured by dual-energy X-ray absorptiometry (DXA) using a Lunar DPX-IQ (Lunar, Radisson, WI, USA). The interassay variance of this method in our laboratory was 0.5% ± 0.5% [coefficient of variation (CV) ± SD] [23]. Incident vertebral fracture was first defined by the semiquantitative method reported by Genant et al. [24]. When a marginal fracture was obtained, we performed quantitative measurements of vertebral body heights at the posterior, central, and anterior margins in both baseline and follow-up vertebral films. We then redefined the presence or absence of incident vertebral fractures in accordance with the criteria proposed by Fukunaga et al. [25]. Fractures were evaluated by one of the coauthors who had contributed to development of the method of Fukunaga et al. [25]. Incident fractures with apparent major trauma were excluded from the present study because we wanted to examine the relationship between vitamin K nutrition and fragility fracture occurrence.

Statistical analysis

All statistical analyses were performed by using statistical software JMP 6.0 J (SAS Institute, Cary, NC, USA). Logistic regression analysis was used to test univariable associations between the incidence of vertebral fracture and anthropometric parameters, bone metabolic parameters, or plasma vitamin K concentrations. Stepwise multiple linear regression analyses were performed to explore determinants of incident vertebral fractures. The following plausible predictors were included in the original model: (1) age, BAP, and K_1 concentration, and (2) L_{2-4} BMD, BAP, and K_1 concentration. Variables that correlated strongly with each other, such as age and L_{2-4} BMD, were not entered simultaneously in the original model. Forward stepwise regression was performed, and $P < 0.25$ was used to enter variables. Values of vitamin K concentrations were logarithmically transformed to improve normality in this analysis because plasma vitamin K concentrations were not normally distributed. A Cox proportional hazards model was used to assess the relationship between plasma K_1 concentration and vertebral fracture. Hazard ratios and 95% confidence intervals are evaluated by no adjusted model or adjusted model for BMD or BMI.

In the second analysis, subjects were divided into low and high K_1 groups by median K_1 concentration (2.67 nmol/l). Parametric comparisons used Student's *t* test. The incidence of vertebral fracture in the two groups was evaluated by the chi square test and crude or age-adjusted relative risks (RRs). Moreover, the age and L_{2-4} BMD values at which 25% of subjects would suffer fractures in the four groups were inversely predicted by logistic regression analysis.

Ethical considerations

The comprehensive study protocol including nutritional evaluation was reviewed by the ethics committee of Research Institute and Practice for Involutional Diseases (RIPID), and comprehensive written informed consent was obtained from all participants.

Table 1. Subject characteristics

n	379
Age (years)	63.0 (10.8)
Body weight (kg)	52.1 (7.3)
Body height (cm)	151.6 (6.0)
BMI (kg/m^2)	22.6 (2.8)
K_1 (nmol/l)	3.51 (2.70)
MK-4 (nmol/l)	0.20 (0.31)
MK-7 (nmol/l)	10.0 (15.1)
ucOC (ng/ml)	4.68 (3.15)
iOC (ng/ml)	8.69 (7.13)
25-OH-D (nmol/l)	51.8 (16.3)
iPTH (pmol/l)	4.9 (1.8)
Ca (mmol/l)	2.30 (0.10)
P (mmol/l)	1.12 (0.15)
BAP (U/l)	31.4 (11.2)
NTX (pmol BCE/μmol Cr)	57.3 (25.5)
L_{2-4} BMD (g/cm^2)	0.970 (0.186)
L_{2-4} Z-score	0.178 (1.405)
FN BMD (g/cm^2)[a]	0.750 (0.128)
FN BMD Z-score[a]	0.398 (0.857)

All values are mean (SD)
K_1, phylloquinone; MK, menaquinone; ucOC, undercarboxylated osteocalcin; iOC, intact osteocalcin; 25-OH-D, 25-hydroxyvitamin D; iPTH, intact parathyroid hormone, BAP, bone-derived alkaline phosphatase; NTX, N-terminal telopeptide; BCE, bone collagen equivalent; BMD, bone mineral density; L_{2-4}, lumbar spine$_{2-4}$; FN, femoral neck
[a]FN BMD and FN BMD Z-score were measured in 176 subjects
Plasma and urinary biochemical parameters were within the normal range

Results

Subject characteristics

The subject characteristics are summarized in Table 1. The plasma K_1, MK-4, and MK-7 concentrations (mean ± SD) of the 379 Japanese women were 3.51 ± 2.70, 0.20 ± 0.32, and 10.0 ± 15.1 nmol/l, respectively. Other plasma and urinary biochemical parameters were within the normal range. The location and number of incident fracture were as follows: vertebrae, 35 (9.2%); forearm, 8 (2.1%); femoral neck, 1 (0.3%); and others, 5 (1.3%). Because there were few cases of forearm and femoral neck fractures, the incidence of vertebral fracture was used to evaluate the association between vitamin K status and bone fracture.

Association between plasma vitamin K concentration and incidence of vertebral fracture

Table 2 shows the association between the incidence of vertebral fracture and age, anthropometric parameters, bone metabolic parameters, and plasma vitamin K concentrations. Age ($P < 0.001$) and BAP ($P = 0.011$) were associated positively, and L_{2-4} BMD ($P < 0.001$), K_1 ($P = 0.007$), and log K_1 ($P < 0.001$) were associated negatively with the incidence of vertebral fracture. MK-4 and MK-7 concentrations were not associated with the incidence of vertebral fracture. NTX and log ucOC showed a tendency to be positively associated with the incidence of vertebral fracture, and their P values were almost equal (NTX, $P = 0.089$; log ucOC, $P = 0.088$).

Table 2. Association between incidence of vertebral fracture and age, anthropometric parameters, bone metabolic parameters, and plasma vitamin K concentrations

	β-Coefficient	*P*
Age (years)	0.064	<0.001
BW (kg)	0.028	0.240
BH (cm)	−0.032	0.274
L_{2-4} BMD (g/cm^2)	−3.956	<0.001
NTX (pmol BCE/μmol Cr)	0.012	0.089
BAP (U/l)	0.042	0.011
ucOC (ng/ml)	0.057	0.271
ucOC/iOC	0.145	0.698
Log ucOC	0.487	0.088
Log ucOC/iOC	0.213	0.518
K_1 (nmol/l)	−0.244	0.007
MK-4 (nmol/l)	−0.345	0.602
MK-7 (nmol/l)	−0.005	0.672
Log K_1 (nmol/l)	−0.899	<0.001
Log MK-7 (nmol/l)	−0.057	0.672

Logistic regression analysis was used to test univariate associations of anthropometric or bone metabolic parameters and plasma vitamin K concentrations with incidence of vertebral fracture
Age and BAP were associated positively, and L2–4 BMD, K1, and log K1 were associated negatively with vertebral fracture incidence

Table 3. Relationship between vertebral fracture incidence and age, L_{2-4} BMD, BAP, or plasma vitamin K_1 concentration evaluated by stepwise multiple regression analysis

a. Plausible predictors (age, BAP and log K_1)

	Estimate	r^2	*P*
Age	0.050	0.055	0.017
Log K_1	−0.783	0.033	0.014
BAP	0.040	0.029	0.017

b. Plausible predictors (L_{2-4} BMD, BAP, and log K_1)

	Estimate	r^2	*P*
L_{2-4} BMD	−4.125	0.096	0.001
Log K_1	−0.760	0.033	0.017
BAP	0.036	0.022	0.039

Stepwise multiple linear regression analyses were performed to identify determinants of vertebral fracture incidence
The following plausible predictors were included in the original model: (1) age, BAP, and vitamin K_1 concentration (log K_1), (2) L_{2-4} BMD, BAP, and vitamin K_1 concentration (log K_1)
Variables that correlated strongly with each other, such as age and L_{2-4} BMD, were not entered simultaneously into the original model
Age, L_{2-4} BMD, BAP, and log K_1 concentration were independently associated with vertebral fracture incidence

Stepwise multiple linear regression analyses were performed to explore the determinants of vertebral fracture incidence. In both models, (1) age, BAP, and log K_1 and (2) L_{2-4} BMD, BAP, and log K_1were included in the original model, and age, L_{2-4}, BMD, BAP, and log K_1 concentration were independently associated with the incidence of vertebral fracture (Table 3). Moreover, a Cox proportional hazards model was used to assess the relationship between plasma K_1 concentration and vertebral fracture (Table 4). Hazard ratios and 95% confidence intervals are evaluated by no adjusted model or adjusted model for BMD or BMI. Both plasma K_1 concentration and log K_1 concentration

significantly decreased hazard ratio of vertebral fracture in the no adjusted model and adjusted model for BMD or BMI. Significant association between vitamin K_1 concentration and vertebral fracture was not observed in the age-adjusted model, because age and vitamin K_1 concentration became a strong confounding factor in the Cox proportional hazards model including a time course factor

Vertebral fracture incidence in low and high K_1 groups

Comparison of the incidence of vertebral fracture between the low and high K_1 groups was divided by the median plasma K_1 concentration (2.67 nmol/l) (Table 5). The incidence of vertebral fracture in the low K_1 group (n = 27, 14.4%) was significantly higher than that in the high K_1 group (n = 8, 4.2%), $P < 0.001$. The age of the low K_1 group was significantly higher than that of the high K_1 group. However, no significant difference was observed in L_{2-4} BMD between the two groups. The unadjusted RR for vertebral fractures in the low K_1 group was 3.43 [95% confidence interval (CI), 1.60–7.35) and the age-adjusted RR was 3.58 (95% CI, 3.26–3.93). No significant differences of plasma 25-OH-D (low K_1, 52.8 ± 17.3; high K_1, 51.0 ± 15.3 nmol/l) or PTH (low K_1, 3.3 ± 1.4; high K_1, 3.3 ± 1.2 pmol/l) concentrations were observed between the two groups. Moreover, the inverse prediction values of L_{2-4} BMD at which 25% of subjects would suffer fractures were estimated from logistic regression analysis in the two groups. The predicted L_{2-4} BMD in the low K_1 group was 0.707 (95% CI, 0.053–0.847, P = 0.007), and that in the high K_1 group was 0.578 (95% CI, 0.004–0.711, P = 0.003). These results suggest that subjects with low vitamin K status would suffer fractures at a higher BMD than those with high vitamin K status.

Table 4. Hazard ratio (HR) of vertebral fracture evaluated by Cox proportional hazards model

Variables	HR	95% CI	P	Adjustment
K_1	0.628	0.404–0.899	0.008	No
	0.691	0.453–0.982	0.038	BMD
	0.656	0.415–0.940	0.018	BMI
Log K_1	0.561	0.363–0.867	0.009	No
	0.612	0.397–0.948	0.028	BMD
	0.517	0.332–0.808	0.004	BMI

A Cox proportional hazards model was used to assess the relationship between plasma K_1 concentration and vertebral fracture; hazard ratios (HR) and 95% confidence intervals (CI) are evaluated by no adjusted model or adjusted model for BMD or body mass index (BMI)

Discussion

The associations between dietary vitamin K intake, biochemical indicators of vitamin K status such as plasma K_1 or ucOC concentration, and bone loss and risk of hip fracture were evaluated in several studies [3–10,25]. Low dietary K_1 intake has been reported to be associated with increased hip fracture risk, most notably in postmenopausal women [3,4]. In the Framingham Heart Study, low dietary K_1 intake was not associated with low BMD at either the hip or spine, even though low intake was associated with increased hip fracture risk [3]. However, in the Framingham Heart Study (1996–2000) [6], low plasma K_1 concentration after adjustment for plasma triglyceride concentration was associated with low BMD at the femoral neck among the men and low plasma K_1 concentration was associated with low spine BMD in postmenopausal women. In other studies, low dietary K_1 intake was associated with low BMD in women aged 29–86 years [5], and low plasma K_1 concentration was shown to be associated with low BMD at the spine [26]. The vitamin K concentration in elderly women with hip fractures was reported to be low [7–10]. Although an apparent relationship between vitamin K status and fracture risk has been reported, the relationship between BMD and vitamin K status is still controversial. Therefore, the mechanism(s) responsible for reducing fracture risk with high vitamin K intake or high serum level of vitamin K are not fully understood.

In the present study, the associations between plasma K_1, MK-4, and MK-7 concentrations and incidence of fracture were evaluated in Japanese women. The results showed a significant association between plasma K_1 concentration and incidence of vertebral fracture. Moreover, we could demonstrate that K_1 concentration was associated with vertebral fracture incidence independently of age, L_{2-4} BMD, and BAP. However, vitamin K status and femoral neck or other fractures could not be evaluated in the present population because of the lack of statistical power of these long

Table 5. Relative risk of vertebral fracture incidence in two groups divided by plasma vitamin K_1 concentration

Groups	n	Age	BMD	BAP	Incidence of vertebral fracture	RR (95% CI)	Age-adjusted RR (95% CI)
Low K_1	188	65.3 (12.1)	0.966 (0.195)	31.0 (11.7)	14.4%	3.43 (1.60–7.35)	3.58 (3.26–3.93)
High K_1	191	62.7 (10.1)	0.973 (0.177)	31.8 (10.7)	4.2%	1	1
P		0.020	0.708	0.478	<0.001		

Mean (SD)
Subjects were divided into two groups according to the median of plasma K_1 concentration (2.67 nmol/l)
Student's t test was used to compare the age of the two groups
Crude and age-adjusted relative risks (RRs) for the vertebral fracture incidence are presented with 95% confidence intervals
Crude and age-adjusted RRs for vertebral fracture incidence of the low K_1 group were significantly higher than those of the high K_1 group

bone fractures. In the present study, the numbers of incident femoral neck and forearm fractures were 1 and 8, respectively. A lower prevalence of hip fracture in the Japanese population than in Caucasians was reported [27]. Thus, evaluation of the role of vitamin K in long bone fracture in the Japanese population will require a larger sample size.

In a previous study, it was shown that high serum MK-7 concentration resulting from eating *natto*, which is a high-MK-7-content food, may contribute to the relatively low hip fracture risk in Japanese women [11]. However, in the present study, we did not find that plasma MK-7 concentration was associated with vertebral fracture incidence. It has been reported that MK-7 has equivalent potency regarding γ-carboxylation of OC to K_1 [28–30] and that *natto* intake promotes bone formation in premenopausal woman [12]. The reason why the association between MK-7 concentration and vertebral fracture was weaker than the associations between K_1 concentration and vertebral fracture is not clear. In a previous study [12], the association between the prevalence of femoral neck fracture and the consumption of *natto* was evaluated by comparison of the rate of the fracture between areas with and without the custom of eating *natto*. However, almost all subjects were *natto* eaters in the present study, which may be one of the reasons why no significant association between plasma MK-7 concentration and vertebral fracture incidence was observed. Moreover, a survey of the period or interval of MK-7-rich food intake seems more important than the measurement of serum MK-7 concentration for evaluating the relationship between bone metabolism and MK-7 in Japan. However, unfortunately, a food questionnaire was not employed in the present study, and this will be necessary in future.

Not only the circulating K_1 concentration but also the serum ucOC concentration has been reported to be associated with hip fracture [31–34]. We have reported that circulating K_1 and MK-7 concentrations were negatively correlated with the serum ucOC concentration; however, the level of vitamin K_1 or MK-7 required to reduce the serum ucOC concentration increased with advanced age [35]. In the present study, ucOC concentration or the ratio of ucOC/intactOC did not show a significant association with incident vertebral fracture. Recent studies revealed that vitamin K may play two important roles in bone metabolism, one of which is regulating posttranslational modification of Gla-containing proteins, and the other is regulating the SXR-mediated cellular regulatory system. Recently, Ichikawa et al. [36] reported that collagen accumulation in osteoblastic MG63 cells was enhanced by vitamin K_2 treatment, and the transcription of the extracellular matrix-related gene "*tsukushi*," which is involved in collagen assembly, was regulated by vitamin K_2 via steroid and xenobiotic receptor (SXR). Therefore, vitamin K plays a significant role in bone homeostasis, not only by affecting γ-carboxylation but also by affecting transcriptional regulation of the collagen gene, which may be one of the reasons why the association between ucOC and fracture incidence was weak as compared with that between K_1 and fracture.

In the second analysis, subjects were divided into low and high K_1 groups according to median K_1 concentration (2.67 nmol/l). The low K_1 group showed a higher incidence of vertebral fracture (Table 5). The age of the low K_1 group was also higher than that of the high K_1 group (Table 5). However, both the unadjusted and age-adjusted RRs demonstrated that risk of vertebral fracture was greater in the low K_1 status group. Moreover, L_{2-4} BMD was not different between the two groups, suggesting that K_1 status may be associated with vertebral bone strength, not with L_{2-4} BMD. The inverse predicted value of L_{2-4} BMD at which 25% of the subjects would suffer fractures was significantly higher in the low K_1 group. This finding suggests that subjects with low vitamin K_1 status would easily suffer fractures even with higher L_{2-4} BMD.

In the present study, the average of K_1 concentration was 3.51 nmol/l, and it was two or three times higher than previous reports. Averages of circulating K_1 concentrations in European or U.S. subjects have been reported approximately within the range of 0.7 to 1.7 nmol/l [6,10,37–42]. In other reports of Japanese subjects, 1.58 [26], 1.07 [13], 1.86 [43], and 2.66 [44] nmol/l K_1 concentrations were reported. Average of K_1 concentration in our other epidemiological study of Japanese elderly subjects was 1.71 nmol/l (data have not been published). Precision and accuracy of LC-APCI-MS/MS method used in present study to measure the vitamin K concentration had been confirmed by the HPLC fluorescence determination method [45]. Correlation coefficient and the corresponding P value for K_1 concentration determined by LC-APCI-MS/MS and HPLC fluorescence determination methods were $r = 0.989$ and $P < 0.001$ ($y = 0.841\ x + 0.035$ ng/ml; y, HPLC fluorescence determination method; x, LC-APCI-MS/MS method). From these results, the circulating K_1 concentration of Japanese subjects is considered to be higher than that of European or U.S. subjects, and dietary K_1 intake of Japanese people suggests that the K_1 intake in Japanese may be higher than that in Europe countries of the United States. The reason why the average K_1 concentration in the present study was particularly higher than other studies not only in Europe and the United States but also in Japan was not clear. Subjects were living in a rural area of Nagano. Most subjects have a backyard at their house, and they have the habit of frequently eating the vegetables that they cultivate in their backyard. Thus, although a food questionnaire was not employed in the present study, it is predicted that the dietary K_1 intake of present subjects may be relatively high.

There were some limitations of the present study. The design was a prospective study, but the participants were recruited from a hospital in a rural area of Japan (refer to the paper by Shiraki et al. [46] for the characteristics of this population). Thus, a nationwide prospective survey is required to assess the role of vitamin K in bone fractures conclusively in the near future. Although there were some limitations of the present study, it can be concluded that the incidence of vertebral fractures was associated with the plasma K_1 concentration. Because subjects with low vitamin K_1 status showed increased risk of vertebral fractures

regardless of their L_{2-4} BMD, low vitamin K_1 status may be an indicator of low bone quality.

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Low calcium intake is associated with increased bone resorption in postmenopausal Japanese women: Yokogoshi Study

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Abstract

Objective: Low Ca intake is common among Japanese women, but its effect on bone metabolism has not been fully elucidated. The aim of the present study was to determine the relationship between Ca intake and serum markers of bone turnover in postmenopausal Japanese women.

Design: A cross-sectional study.

Setting: A community setting.

Subjects: Subjects were 595 home-dwelling postmenopausal Japanese women. Ca intake was assessed by a validated FFQ. Serum type I collagen cross-linked N-telopeptides (NTX) and osteocalcin were measured as markers of bone turnover. The relationships between demographic characteristics, lifestyles, serum Ca, vitamin D and intact serum parathyroid hormone and bone turnover were also assessed.

Results: The average age of the subjects was 64·5 (SD 5·8) years and the mean Ca intake was 527 (SD 160) mg/d. Ca intake was significantly associated with serum NTX ($P = 0{\cdot}0104$), but not with serum osteocalcin. Mean serum NTX concentration in the lowest quartile of Ca intake (<417 mg/d) was significantly higher than in the fourth, referent quartile. Among these Japanese postmenopausal women, very low Ca intake (less than ~400 mg/d) was associated with increased bone resorption but not bone formation.

Conclusions: Increased bone resorption may be one mechanism by which this Ca-depleted population normalizes bone metabolism and prevents osteoporosis.

Keywords
Bone metabolism
Bone resorption
Calcium intake
Postmenopause

Low Ca intake is recognized as a risk factor for osteoporosis and osteoporotic fractures in postmenopausal women. Daily Ca intake of at least 1200 mg is recommended for postmenopausal women[1,2]. However, Ca intake among Japanese women and other East Asian populations is typically well below present recommendations. The National Nutrition Survey of Japan (2003) showed that the average daily Ca intake of peri- and postmenopausal Japanese women was only 562 mg[3]. This is lower than present clinical guidelines and also lower than that of people in many European and North American countries. Nevertheless, the incidence of hip fractures in Japan has been estimated to be less than half that in the USA[4], suggesting that the prevalence of osteoporosis in Japan is also lower. This paradox may partly be explained by the fact that populations consuming Ca-depleted diets exhibit physiological adaptations that maintain normal bone metabolism. However, the relationship between Ca intake and bone

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metabolism in populations with low Ca intake has not been well studied.

Previous studies have demonstrated that a higher rate of bone turnover leads to bone loss, disruption of trabecular networks and reduced connectivity, and that bone turnover markers indicating bone resorption predict subsequent osteoporotic fractures independent of bone mineral density[5]. In fact, high bone remodelling may be a primary cause of osteoporotic bone fragility[6]. Markers of bone turnover can thus be used to assess the relationship between Ca intake and clinically significant aspects of bone metabolism. The aim of the present study was to determine the relationship between Ca intake and two serum markers of bone turnover – serum type I collagen cross-linked N-telopeptides (NTX)[7,8] and osteocalcin[9] – among postmenopausal Japanese women with low Ca intake.

Subjects and methods

Subjects

On 31 March 2006, we targeted all 1310 women aged 55–74 years who lived in the town of Yokogoshi, Japan. Of these women, 667 women agreed to participate and underwent measurement of serum bone turnover markers in the baseline investigation of the Yokogoshi Study, a community-based, epidemiological study on bone health for postmenopausal women[10]. The medical examination was conducted in November 2005. All subjects were non-institutionalized, ambulatory and independent. The following women who had medical histories that may have affected bone metabolism were excluded from analysis: (i) twelve women with a history of bilateral oophorectomy; (ii) seven women who had undergone corticosteroid therapy; and (iii) fifty-three women treated with bisphosphonates, selective oestrogen receptor modulators, active vitamin D analogues, vitamin K (menatetrenone), oestrogen or calcitonin for suspected osteoporosis. Ultimately, 595 of 667 women were analysed. Written informed consent was received from all subjects. The study protocol was approved by the Ethics Committee of Niigata University School of Medicine. Further details of the Yokogoshi Study have been published elsewhere[10].

Biochemical measurements

A fasting blood specimen was drawn during the daytime, at least 6 h following the last ingestion of any food or drink. Each specimen was immediately maintained at 4°C and the serum was obtained within the day of collection by centrifugation at 1613*g* for 10 min.The specimen was subsequently stored at −80°C prior to biochemical analysis. Serum NTX concentration, a marker of bone resorption, was determined by ELISA (Osteomark NTX Serum; Ostex International, Inc., Seattle, WA, USA; reference value: 10·7–24·0 nmol BCE/l), which had an inter-assay CV of 2·8%. Serum osteocalcin concentration, a marker of bone formation, was determined by an immunoradiometric assay (Mitsubishi Kagaku Medical, Inc., Tokyo, Japan; reference value: 3·1–12·7 ng/ml) with an inter-assay CV of 6·6%. Serum vitamin D concentration, measured as 25-hydroxyvitamin D (25(OH)D), was determined by RIA (DiaSorin, Stillwater, MN, USA) with an inter-assay CV of 9·9%. Serum intact parathyroid hormone (PTH) concentration was measured with a two-site immunoradiometric assay (Nichols Institute Diagnostics, San Clemente, CA, USA), which has an inter-assay CV of 1·5%.

Other measurements

Age, medical history, reproductive history, current medication list and lifestyle information were obtained from all patients. Current Ca intake was assessed with a previously validated FFQ for the Japanese diet[11], with the correlation coefficient between values measured by this method and the conventional 3 d diet record being 0·668. Physical activity levels were assessed based on whether subjects engaged in the following activities at least once weekly: (i) light exercise, such as gate ball (or croquet), taking walks, etc., as light activity; and (ii) moderate exercise, such as farm work, gardening, etc., as moderate activity. Body height and weight of the subjects in light underwear were measured to the nearest 1 mm and 100 g, respectively. BMI was calculated by dividing body weight (kg) by the square of body height (m^2).

Statistical analysis

All continuous variables were assessed for normality. Serum NTX and intact PTH concentrations were skewed to higher values, and thus they were transformed logarithmically when conducting statistical tests. Categorical variables, such as 'light exercise' and 'moderate exercise', were coded as 0 for 'no' and 1 for 'yes'. Pearson's product moment correlation coefficients were calculated to evaluate an association between two continuous variables. Student's *t* test was used to test associations between physical activity measures and the two serum bone turnover markers. A stepwise method of multiple regression analysis was used to explore independent variables associated with outcome variables. Candidate predictor variables were significant variables obtained by the bivariate analyses. Analysis of covariance with Dunnett's multiple comparisons was used to compare one reference mean value with other mean values. Computations were performed using the SAS statistical software package release 8·02 (SAS Institute Inc., Cary, NC, USA). A *P* value less than 0·05 was considered statistically significant.

Results

Characteristics of the subjects are shown in Table 1. Regarding physical activity, 569 (95·6%) subjects engaged in light activity and 293 (49·2%) engaged in moderate activity. Serum markers of bone turnover may have an intra-day fluctuation. Nevertheless, there was no

significant difference in mean log-transformed serum NTX concentration between subjects who underwent blood collection in the morning and in the afternoon ($P = 0{\cdot}3234$). The mean serum osteocalcin concentration among samples collected in the afternoon (10·3 mg/ml) was significantly higher ($P = 0{\cdot}0486$) than among those collected in the morning (9·6 mg/ml).

Correlation coefficients between predictor variables and log-transformed serum NTX or osteocalcin concentration are shown in Table 2. Age, weight, BMI, Ca intake and log-transformed serum intact PTH concentration were significantly correlated with log-transformed serum NTX concentration. Years since menopause, weight, BMI and log-transformed serum intact PTH concentration were significantly correlated with serum osteocalcin concentration. None of the physical activity measures was associated with the log-transformed serum NTX or osteocalcin concentration. Additionally, a correlation coefficient between Ca intake and log-transformed serum intact PTH concentration was of borderline significance ($r = -0{\cdot}072$, $P = 0{\cdot}0776$).

Results of the stepwise multiple regression analysis are shown in Table 3. Age, weight, Ca intake and log-transformed serum intact PTH concentration were independently associated with log-transformed serum NTX concentration. BMI and log-transformed serum intact PTH concentration were independently associated with serum osteocalcin concentration.

Table 4 shows mean values of serum NTX and osteocalcin concentration according to quartile relative to the highest quartile of Ca intake. The lowest quartile of Ca intake had significantly lower log-transformed serum NTX concentrations. There was no significant association, however, between Ca intake and serum osteocalcin concentration.

Table 1 Demographic and laboratory characteristics of the study subjects: home-dwelling postmenopausal Japanese women (*n* 595), Yokogoshi Study, 2006

	n	Mean	SD
Age (years)	595	64·5	5·8
Years since menopause	578	14·5	6·9
Height (cm)	595	150·7	5·5
Weight (kg)	595	53·1	8·4
BMI (kg/m^2)	595	23·4	3·5
Total Ca intake* (mg/d)	595	527	160
Serum 25(OH)D (nmol/l)	595	55·5	14·6
Serum intact PTH (pmol/l)	595	4·25	1·40
Serum NTX (nmol BCE/l)	595	21·0	6·5
Serum osteocalcin (ng/ml)	595	9·94	3·95

25(OH)D, 25-hydroxyvitamin D; PTH, parathyroid hormone; NTX, type I collagen cross-linked N-telopeptides.
*Ca intake from dietary sources was 518 (SD 146) mg/d.

Discussion

Previous research has shown that increased bone resorption markers are associated with increased fracture risk

Table 2 Correlation coefficients (*r*) between selected variables and serum NTX* and osteocalcin concentrations: home-dwelling postmenopausal Japanese women (*n* 595), Yokogoshi Study, 2006

	Serum NTX*		Serum osteocalcin	
Variable	*r*	*P* value	*r*	*P* value
Age (years)	0·081	0·0482	−0·019	0·6376
Years since menopause	0·004	0·9318	−0·084	0·0442
Height (cm)	−0·023	0·5703	−0·018	0·6574
Weight (kg)	−0·113	0·0060	−0·115	0·0049
BMI (kg/m^2)	−0·105	0·0102	−0·115	0·0051
Ca intake (mg/d)	−0·096	0·0194	−0·020	0·6200
Serum 25(OH)D (nmol/)	0·049	0·2355	−0·052	0·2038
Serum intact PTH* (pmol/l)	0·089	0·0302	0·100	0·0149

NTX, type I collagen cross-linked N-telopeptides; 25(OH)D, 25-hydroxyvitamin D; PTH, parathyroid hormone.
*Logarithmically transformed.

Table 3 Results of a stepwise multiple linear regression analysis with log-transformed serum NTX and osteocalcin concentrations as outcomes: home-dwelling postmenopausal Japanese women (*n* 595), Yokogoshi Study, 2006

Independent variable	Regression coefficient (β)	SE	*P* value
Serum NTX*			
Age (years)	0·00407	0·00193	0·0353
Weight (kg)	−0·00374	0·00132	0·0048
Ca intake (mg/d)	−0·000179	0·0000695	0·0104
Serum intact PTH* (pmol/l)	0·07806	0·0342	0·0230
Serum osteocalcin			
BMI (kg/m^2)	−0·148	0·047	0·0017
Serum intact PTH* (pmol/l)	1·03	0·51	0·0457
Time of blood collection (0, morning; 1, afternoon)	0·726	0·327	0·0269

NTX, type I collagen cross-linked N-telopeptides; PTH, parathyroid hormone.
*Logarithmically transformed.

Table 4 Mean values of serum NTX concentration at each quartile of calcium intake: home-dwelling postmenopausal Japanese women (n 595), Yokogoshi Study, 2006

	Serum NTX* (nmol BCE/l)	
	Mean	95 % CI
Ca intake (mg/d)		
Q1 (<417)	22·6†	21·2, 24·0
Q2 (≥417, <525)	20·0	19·1, 20·9
Q3 (≥525, <619)	21·3	20·3, 22·3
Q4 (≥619)	20·2	19·4, 21·1 (reference)
	Adjusted P for trend = 0·0135	

NTX, type I collagen cross-linked N-telopeptides; Q1, first quartile; Q2, second quartile; Q3, third quartile; Q4, fourth quartile.
*Logarithmically transformed.
†Mean value was significantly different from that of reference group (analysis of covariance and Dunnett's multiple comparisons, with age, weight and log-transformed serum parathyroid hormone as covariates): $P<0·05$.

independent of bone mineral density[5]. Therefore, the correlates of bone resorption are relevant to assessing the aetiology of osteoporotic fractures. In the present study, we measured serum NTX concentrations as a bone resorption marker. This approach is more robust because serum-based markers of bone turnover show less variability than urine-based markers[12] and serum NTX measurements can assess bone resorption with decreased intra-subject variability[7].

The present study demonstrated that Ca intake was inversely associated with serum NTX concentration. This finding has been reported previously by a small study[13] and is consistent with previous work showing that Ca supplementation reduces bone resorption[14–16]. Increased bone resorption by low Ca intake is hypothesized to be mediated through hyperparathyroidism induced by low Ca intake[16,17]. Our data, however, failed to demonstrate a significant association between Ca intake and intact PTH concentration, but rather showed that serum NTX concentration was associated with Ca intake independent of PTH. This suggests that a different pathophysiological mechanism may be responsible for the effects of low Ca intake on bone metabolism.

An additional key finding of the present study was that mean NTX concentration in the lowest quartile of Ca intake (<417 mg/d) was significantly lower than that in the highest, reference quartile (≥619 mg/d). Interestingly, this finding is in accordance with an epidemiological study demonstrating that the lowest quartile of Ca intake in peri- and postmenopausal Japanese women has a significantly increased risk of vertebral fracture than the highest, reference quartile[18]. Taken together, these data suggest that the lowest levels of Ca intake may be a particularly serious problem in this population.

Average daily Ca intake of most subjects in the present study was only 527 mg, lower than that recommended for peri- and postmenopausal women by the Ministry of Health and Welfare of Japan[19]. This is consistent with the National Nutrition Survey[3], which reported that Ca intake of Japanese persons aged 50–69 years was 568 mg/d on average, much lower than that of people in many European and North American countries. Nevertheless, only a quarter of women with very low Ca intake exhibited increased bone resorption in the present study. Japanese people historically have had a low-Ca diet[20] and therefore they may be physiologically adapted to low Ca through increased Ca absorption.

Although serum osteocalcin concentration is a major predictor of bone mineral density of the elderly[10,21], the strength of the association between serum osteocalcin concentration and fracture occurrence shows considerable variation[5]. In the present study, Ca intake was not associated with serum osteocalcin concentration.

The present study also showed that low body weight or BMI is associated with high bone resorption and formation markers. The inverse association between body weight (or BMI) and bone turnover markers has been reported by others[22–24]. A number of studies have also shown that low body weight is a major predictor of bone mineral density and bone loss of the elderly[21,25,26] and that this association may be mediated by increased bone turnover.

Exercise is known to affect bone turnover markers[27]. However, the present study did not find physical activity to be associated with bone turnover markers. One major reason for this may be that we obtained qualitative, rather than quantitative, data for physical activity, because assessment of physical activity in the elderly is difficult. Physical activity is one of the important determinants of bone health, and future studies should clarify this association in elderly populations.

One limitation of our study is that we measured only serum NTX and osteocalcin concentrations, which reflect only certain aspects of bone turnover or bone quality. Another limitation is that an FFQ is not an ideal method to evaluate Ca intake, although the FFQ used in our study was validated with improved accuracy over other FFQ. These limitations weaken the association of Ca intake with serum NTX concentration seen here.

In summary, the present study showed that very low Ca intake (less than ~400 mg/d) is associated with increased bone resorption in peri- and postmenopausal Japanese women. These results suggest that Ca supplementation programmes should focus on those women at highest risk. On the other hand, a population approach is also of interest because Ca intake in most adults in Japan is lower than current recommendations. Future studies are needed to develop interventions that effectively address low Ca intake among postmenopausal women.

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