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ORIGINAL ARTICLE

Association between vitamin K intake from fermented soybeans, *natto*, and bone mineral density in elderly Japanese men: the Fujiwara-kyo Osteoporosis Risk in Men (FORMEN) study

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Abstract
Summary A cross-sectional analysis of 1,662 community dwelling elderly Japanese men suggested that habitual *natto* intake was significantly associated with higher bone mineral density (BMD). When adjustment was made for undercarboxylated osteocalcin levels, this association was insignificant, showing the *natto*–bone association to be primarily mediated by vitamin K.
Introduction Low vitamin K intake is associated with an increased risk of hip fracture, but reports have been inconsistent on its effect on BMD. Our first aim was to examine the association between BMD and intake of fermented soybeans, *natto*, which contain vitamin K1 (20 μg/pack) and K2 (380 μg/pack). Our second aim was to examine the association between undercarboxylated osteocalcin (ucOC), a biomarker of vitamin K intake, and BMD to evaluate the role of vitamin K in this association.
Methods Of the Japanese men aged ≥65 years who participated in the baseline survey of the Fujiwara-kyo Osteoporosis Risk in Men study, 1,662 men without diseases or medications known to affect bone metabolism were examined for associations between self-reported *natto* intake or serum ucOC levels with lumbar spine or hip BMD.
Results The subjects with greater intake of *natto* showed significantly lower level of serum ucOC. Analysis after adjustment for confounding variables showed an association of greater intake of *natto* with both significantly higher BMD and lower risk of low BMD (T-score<−1 SD) at the total hip and femoral neck. This association became insignificant after further adjustment for ucOC level.
Conclusion Habitual intake of *natto* was associated with a beneficial effect on bone health in elderly men, and this association is primarily due to vitamin K content of *natto*, although the lack of information on dietary nutrient intake, including vitamin K1 and K2, prevented us from further examining the association.

Keywords Bone density · Men · Osteocalcin · Soy foods · Undercarboxylated osteocalcin · Vitamin K

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Introduction

Vitamin K plays an important role in bone metabolism and is expected to have beneficial impact on bone health [1].

Vitamin K naturally exists in two major forms: vitamins K1 and K2. Vitamin K1 is widely distributed in green and leafy vegetables, while vitamin K2 is produced by bacteria during fermentation or is contained in animal-derived foods. The predominant dietary form of vitamin K in the USA, Europe, and most parts of the world is vitamin K1. However, the major form is vitamin K2 in Japan, especially menaquinone-7 (MK-7), which is a component of the fermented soybean product referred to as "*natto*."

Many epidemiologic studies have been conducted to evaluate the association between vitamin K intake and bone health, mostly in subjects from the USA or Europe. Feskanich et al. first reported that low intake of vitamin K1 was associated with increased risk of hip fracture in the Nurses' Health Study [2]. This finding was supported by the Framingham Osteoporosis Study where a protective effect of dietary vitamin K1 for hip fracture was observed in men as well as in women, but unexpectedly no effect was seen on bone mineral density (BMD) [3]. Booth et al. further examined the data from the Framingham Offspring Study and reported that low dietary vitamin K1 intake was associated with low BMD at the spine and hip only in women [4], while low plasma vitamin K1 level was related to low BMD at the femoral neck only in men in a subgroup of the same cohort [5]. The reason for these inconsistencies remains unclear, but they could be due to inaccurate estimation of the intake of green vegetables (the primary source of vitamin K1) obtained from food-frequency questionnaire data.

On the other hand, most studies that have evaluated the association between vitamin K2 and bone health have been conducted in Japan. This is because *natto*, a major source of vitamin K2 in Japan, is still consumed widely and frequently almost exclusively in Japan [6]. *Natto* is sold in a plastic pack that usually contains about 40 g of *natto*, i.e., the quantity considered to be suitable for a meal in Japan. One pack of *natto* contains about 20 μg of vitamin K1 and about 380 μg of vitamin K2 [6]. Kaneki et al. suggested a possible beneficial effect of *natto* intake on bone health by showing an inverse association between *natto* consumption and incidence rate of hip fracture in a prefecture-level correlation study in Japan [7]. This ecological finding led to exploration of the issue in epidemiologic studies. Using data from the Japanese Population-based Osteoporosis (JPOS) Cohort Study, Ikeda et al. found a significant positive association between *natto* intake and the rate of change in BMD at the hip in postmenopausal women [8]. *Natto* is usually sold throughout Japan in a plastic package containing approximately 40 g. This facilitates the accurate assessment of *natto* intake from self-reports in contrast to that of green vegetable intake and may account for the significant association obtained using JPOS study data.

Thus, proper evaluation of the association between vitamin K intake and BMD requires an accurate estimate of vitamin K intake for each individual. Objective biomarkers of vitamin K intake may satisfy this requirement. However, very few studies have used biomarkers for this purpose especially in men. Since vitamin K is a cofactor of γ-carboxylase (which converts glutamate residues to γ-carboxyglutamyl (Gla) residues in osteocalcin (OC), matrix Gla protein, and protein S) [9, 10], the amount of OC with uncarboxylated glutamate residues, i.e., under-γ-carboxylated OC (ucOC), is considered to be a sensitive marker of vitamin K status in the human body [11]. Validity of ucOC level as an indicator of vitamin K intake has been supported by several studies that showed significant inverse correlation between plasma vitamin K level and plasma ucOC level [12–14]. ucOC level may therefore be used to evaluate the association between vitamin K intake and BMD. The primary objective of the present study was to examine the association between *natto* intake and spine and hip BMD in healthy elderly Japanese men. The secondary objective was to examine the association between ucOC levels and spine and hip BMD to evaluate the role of vitamin K in this association.

Methods

Subjects

The Fujiwara-kyo Osteoporosis Risk in Men (FORMEN) study was conducted as part of a larger cohort study, the Fujiwara-kyo study (primary investigator: Norio Kurumatani, MD, PhD, Professor and Chairman, Department of Community Health and Epidemiology, Nara Medical University School of Medicine). Its aim was to provide a scientific basis for strategies used to prevent frailty, prolong healthy life expectancy, and maintain quality of life of elderly men and women in Japan. Details of the FORMEN study and Fujiwara-kyo study are described elsewhere [15]. The FORMEN study examined 2,174 male volunteers asked to participate in a baseline survey of the Fujiwara-kyo study conducted in 2007 and 2008. Participants were aged 65 years or older at baseline, living in their homes in the cities of Kashihara, Nara, Yamato-Koriyama, and Kashiba, and could walk without the assistance of another person. Of the 2,174 participants, 2,012 completed the study items for the FORMEN study. We excluded 350 men according to the exclusion criteria, including missing *natto* intake information (five men) and a history of illness or medication usage known to affect bone metabolism (parathyroid disease in 1 participant, connective tissue disease in 20, asthma in 24, ossification of posterior longitudinal ligament in 2, steroid therapy in 19, thyroid diseases with thyroid hormone therapy in 19, surgery for stomach cancer or ulcers in 83, diabetes

mellitus with insulin therapy or HbA1c ≥6.5% in 170, prostate cancer with anti-androgen therapy in 37, and no information in 1; 29 men had multiple reasons for exclusion). The final sample consisted of 1,662 men. The study protocol was approved by the Medical Ethics Committee of Nara Medical University and the Ethics Committee of Kinki University School of Medicine. The participants provided written informed consent before enrolment in the study.

Bone mass measurement

BMD was measured by dual-energy X-ray absorptiometry (DXA) at the lumbar spine (L2–4) and the right hip in a posteroanterior projection (QDR4500A, Hologic Inc., Bedford, MA, USA). When subjects had a history of fractures or bone disease in the right hip, the subjects were scanned on the left side. The short-term precision as measured by the coefficients variation (CV) of the BMD measurements in vivo was 1.2%, 1.2%, and 1.6% for the lumbar spine, total hip, and femoral neck, respectively. BMD values at the spine with osteophytes of stage 4 (Nathan's classification [16]) or excessive calcification due to osteoarthrosis were considered missing. According to the World Health Organization, osteopenia is defined as a T-score higher than −2.5 and lower than −1.0 [17]. Thus, we defined low BMD as ≥1 SD below the young adult mean (i.e., T-score<−1).

Bone turnover markers

Blood samples were collected following an overnight fast, and serum was obtained for several kinds of conventional biochemical tests planned in the Fujiwara-kyo study. The remaining serum was stored at −80°C until measurement of bone turnover markers. We measured levels of undercarboxylated osteocalcin (OC; ucOC) as a biomarker of vitamin K intake, OC as a marker of bone formation, and tartrate-resistant acid phosphatase isoenzyme 5b (TRACP-5b) as a marker of bone resorption [15]. Serum ucOC was measured by an electrochemiluminescence immunoassay. Serum OC was measured by a two-site immunoradiometric assay. Serum TRACP-5b was measured by a fragments absorbed immunocapture enzyme assay. The intraassay CV, interassay CV, and overall CV in the measurements for ucOC were 4.1%, 3.5%, and 5.4%, respectively, 4.9%, 3.7%, and 6.1% for OC, and 4.9%, 7.3%, and 8.8% for TRACP-5b.

Explanatory variables

Height (centimeters) and weight (kilograms) were measured using an automatic scale (Tanita TBF-215, Tanita Inc., Japan). Body mass index (BMI, kilograms per square meter) was calculated from these measurements.

Detailed interviews were conducted to confirm the information given on a questionnaire, including 250 items covering past medical history, medication history, smoking and drinking habits, intake of dairy products, intake of *natto*, and marital status. *Natto* is sold in a plastic pack that usually contains about 40 g of *natto*, i.e., the quantity considered to be suitable for a meal in Japan. One pack of *natto* contains about 20 μg of vitamin K1 and about 380 μg of vitamin K2 [6]. Participants were asked about the number of packs of *natto* consumed over a 1-week period and were classified into four groups (less than one pack/week, one pack/week, several packs/week, one pack/day and more). Energy expenditure index by daily physical activities was estimated using International Physical Activity Questionnaire [18] validated for the Japanese elderly [19]. These interviews were conducted by trained public health nurses or medical doctors.

Statistical analysis

SAS statistical software (version 9.1; SAS Institute, Cary, NC, USA) was used for all statistical analysis. We transformed the data of OC level into ranks for analysis since the OC data were not normally distributed, and the values are presented as medians. The geometric mean and SD are used for TRACP-5b and ucOC levels because they followed a logarithmic normal distribution. Analysis of variance (ANOVA) was used to evaluate the significance of the difference in mean BMD and other continuous variables among groups categorized according to *natto* intake. The chi-square statistic was used to compare the prevalence of lifestyle factors, such as smoking, drinking, milk intake, and history of illness. Analysis of covariance (ANCOVA) was used to evaluate the significance of the difference in mean BMD among groups based on *natto* intake or quartiles of serum ucOC concentration with adjustments for confounding factors. Adjusted mean BMD was obtained as the least square mean from the ANCOVA model with Tukey–Kramer adjustment for multiple comparisons. Multivariate logistic regression analysis was performed to assess the effect of *natto* intake on the risk of low BMD after adjustment for potential confounding factors. Also, to evaluate the association of serum ucOC concentration with low BMD, we used the multivariate logistic regression model. Next, we used R^2 as a generalized linear model and Akaike's Information Criterion (AIC) for the logistic regression model to assess how well the adjusted model fit the data.

Results

Table 1 shows demographic, lifestyle, and clinical characteristics of participants classified by *natto* intake. There was no difference in age or BMI among the groups based

Table 1 Characteristics of elderly Japanese male participants classified by *natto* intake, The FORMEN Study

	Total (*n*)	*Natto* intake (*n*)				*p* value[a]
		Less than 1 pack/week	1 pack/week	Several packs/week	1 pack/day and more	
Age, year	73.1±5.2 (1,662)	73.3±5.4 (952)	72.8±5.1 (291)	72.7±4.8 (265)	73.4±5.3 (154)	0.158
Height, cm	162.8±5.7 (1,662)	162.5±5.8 (952)	163.2±5.4 (291)	163.2±5.5 (265)	163.7±5.4 (154)	0.036
Weight, kg	61.2±8.5 (1,662)	60.8±8.6 (952)	61.3±8.3 (291)	62.2±8.4 (265)	61.9±8.3 (154)	0.097
BMI, kg/m^2	23.1±2.7 (1,662)	23.0±2.8 (952)	23.0±2.7 (291)	23.3±2.7 (265)	23.1±2.5 (154)	0.422
Physical activity, METs·min/day	254.3±258.2 (1,598)	252.9±268.4 (911)	244.8±247.4 (280)	266.2±246.9 (258)	260.8±234.2 (149)	0.788
Smoking habit,%						
Current smoker	17.5 (289)	20.5 (194)	15.1 (44)	15.8 (42)	5.8 (9)	<0.001
Ex-smoker	59.6 (987)	56.8 (537)	61.2 (178)	61.9 (164)	70.1 (108)	
Never smoker	22.9 (379)	22.6 (214)	23.7 (69)	22.3 (59)	24.0 (37)	
Drinking habit,%						
6 times/week or more	24.2 (399)	27.1 (256)	20.3 (59)	16.2 (43)	26.6 (41)	<0.001
3–5 times/week	12.7 (209)	14.1 (133)	12.1 (35)	8.3 (22)	12.3 (19)	
1–2 times/week	5.4 (90)	4.2 (40)	6.9 (20)	7.5 (20)	6.5 (10)	
Occasionally	9.4 (155)	8.5 (80)	12.8 (37)	8.3 (22)	10.4 (16)	
Never	48.4 (799)	46.0 (434)	47.9 (139)	59.6 (158)	44.2 (68)	
Milk intake,%						
2 cups/day or more	7.8 (129)	6.8 (65)	6.2 (18)	7.9 (21)	16.2 (25)	<0.001
1 cup/day	43.0 (715)	41.1 (391)	46.4 (135)	43.8 (116)	47.4 (73)	
1 cup/2–3 days	15.5 (257)	13.3 (127)	17.2 (50)	21.9 (58)	14.3 (22)	
1 cup/week	7.2 (120)	7.2 (69)	8.6 (25)	7.5 (20)	3.9 (6)	
Never	26.5 (441)	31.5 (300)	21.6 (63)	18.9 (50)	18.2 (28)	
Marital status,%						
Married	91.5 (1,513)	91.1 (860)	93.5 (272)	92.8 (246)	87.7 (135)	0.161
Not married	8.5 (141)	8.9 (84)	6.5 (19)	7.2 (19)	12.3 (19)	
History of diseases,%						
Diabetes mellitus	5.4 (90)	4.3 (41)	4.8 (14)	8.7 (23)	7.8 (12)	0.021
Coronary heart disease	0.4 (6)	0.5 (5)	0.3 (1)	0.0 (0)	0.0 (0)	0.525
Hypertension	34.0 (565)	32.3 (307)	37.5 (109)	36.2 (96)	34.4 (53)	0.327
Stroke	3.9 (64)	4.3 (41)	3.8 (11)	3.0 (8)	2.6 (4)	0.640

Values represent mean ± SD or percentage with number in parentheses. One pack of *natto* contains about 20 μg of vitamin K1 and about 380 μg of vitamin K2

METs metabolic equivalent tasks

[a] Chi-square test or ANOVA was performed over the category of *natto* intake

on *natto* intake. Smoking, drinking, and milk intake were significantly associated with *natto* intake. Higher prevalence of diabetes mellitus was associated with greater *natto* intake.

The relationship of levels of biochemical markers of bone turnover and BMD with *natto* intake is shown in Table 2. With increasing *natto* intake, a significant dose-dependent decrease in ucOC level and increase in total hip and femoral neck BMD were observed.

Table 3 shows mean values of BMD in the *natto* intake groups adjusted for age, BMI, milk intake, smoking, alcohol drinking, physical activity, and diabetes mellitus. There was a significant positive association between *natto* intake and mean values of total hip and femoral neck BMD. The significance of this association did not change when an additional adjustment for OC or TRACP-5b level was made, but it disappeared when the ucOC level was entered into the model for adjustment.

Table 4 shows the results of multiple logistic regression analysis. After adjustment for the same confounding factors used in the analysis shown in Table 3, a statistically significant odds ratio (OR) of low total hip or femoral neck BMD was observed. The OR remained significant when additional adjustments for OC or TRACP-5b levels were made, but it became insignificant when ucOC level was entered into the model for adjustment.

Table 5 shows the adjusted mean BMD and adjusted OR of low BMD in every quartile of serum ucOC concentration. The ucOC level showed a significant negative association with mean BMD and a significant positive association with OR of low BMD even after adjustment for covariates including *natto* intake.

Discussion

Role of vitamin K in *natto*'s effect on bone health

In this large-scale community-based single-center study of elderly Japanese men, subjects with a greater intake of *natto* had significantly higher BMD and a lower prevalence of low BMD at the total hip and femoral neck than subjects with lower intakes of *natto*, and this association was significantly attenuated when an additional adjustment was made for ucOC levels. Thus, the present study suggests that the *natto*–BMD association is primarily mediated by vitamin K contained in *natto*. To our knowledge, this is the first report to identify an association between *natto* intake and BMD in elderly men, and to examine the association through a biomarker of vitamin K status.

Although only a few studies have been published on the association between *natto* intake and BMD, there have been clinical trials on the effects of vitamin K2 supplementation

Table 2 Biochemical markers of bone turnover and BMD in elderly Japanese male participants classified by *natto* intake, The FORMEN Study

	Total		*Natto* intake frequency								*p* value[a]
			Less than 1 pack/week		1 pack/week		Several packs/week		1 pack/day or more		
	N	Mean ± SD	*N*	Mean ± SD	*N*	Mean ± SD	*N*	Mean ± SD	*N*	Mean ± SD	
Biochemical marker of bone turnover											
OC (ng/ml)	1,626	4.9 (3.2, 6.4)	931	4.9 (3.1, 6.6)	285	4.8 (3.0, 6.1)	260	4.8 (3.2, 6.6)	150	5.0 (3.9, 6.6)	0.184
TRACP-5b (mU/dl)	1,656	209.3 (203.8, 214.9)	948	211.3 (121.1, 368.5)	290	199.3 (114.7, 346.5)	264	211.2 (125.0, 356.9)	154	213.0 (124.5, 364.4)	0.426
ucOC (ng/ml)	1,626	2.9 (2.9, 3.0)	931	3.4 (1.9, 6.0)	285	2.7 (1.6, 4.7)	260	2.4 (1.4, 4.2)	150	2.1 (1.2, 3.7)	<0.001
BMD, g/cm^2											
Lumbar spine	1,580	1.029±0.188	893	1.020±0.192	284	1.036±0.182	255	1.045±0.182	148	1.044±0.190	0.169
Total hip	1,658	0.882±0.123	950	0.873±0.124	289	0.886±0.123	265	0.905±0.122	154	0.897±0.117	<0.001
Femoral neck	1,658	0.743±0.114	950	0.732±0.112	289	0.746±0.113	265	0.770±0.117	154	0.759±0.109	<0.001

OC values represent median with interquartile limits in parentheses. ucOC and TRACP-5b values represent geometric mean with values for M–SD and M+SD in parentheses. One pack of *natto* contains about 20 μg of vitamin K1 and about 380 μg of vitamin K2

BMD bone mineral density, *OC* osteocalcin, *ucOC* undercarboxylated osteocalcin, *TRACP-5b* tartrate-resistant acid phosphatase isoenzyme 5b

[a] ANOVA was performed for all groups divided on the basis of *natto* intake

Table 3 Multivariate adjusted mean BMD in elderly Japanese male participants classified by *natto* intake, The FORMEN Study

Natto intake	Adjusted mean BMD[a], g/cm^2			Adjusted mean BMD[b], g/cm^2		
	Lumbar spine Mean ± SE	Total hip Mean ± SE	Femoral neck Mean ± SE	Lumbar spine Mean ± SE	Total hip Mean ± SE	Femoral neck Mean ± SE
Less than 1 pack/week	1.030±0.013	0.881±0.007	0.740±0.007	1.033±0.013	0.882±0.007	0.741±0.007
1 pack/week	1.044±0.015	0.888±0.009	0.749±0.009	1.040±0.016	0.882±0.009	0.744±0.009
Several packs/week	1.037±0.015	0.900±0.009*	0.767±0.009**	1.029±0.016	0.889±0.009	0.758±0.009
1 pack/day or more	1.050±0.019	0.903±0.011*	0.764±0.010**	1.035±0.019	0.886±0.011	0.751±0.011
p value[c]	0.541	0.030	<0.001	0.912	0.852	0.132

One pack of *natto* contains about 20 μg of vitamin K1 and about 380 μg of vitamin K2

BMD bone mineral density

[a] Adjusted for age, BMI, milk intake, smoking, alcohol drinking, physical activity, and diabetes mellitus

[b] Adjusted for age, BMI, milk intake, smoking, alcohol drinking, physical activity, diabetes mellitus, and undercarboxylated osteocalcin level

[c] ANCOVA was performed for all groups divided on the basis of *natto* intake

**$p<0.01$ and *$p<0.05$ vs. "less than one pack/week"

on fracture risk or BMD. A systematic review and meta-analysis of randomized controlled trials (RCTs) by Cockayne et al. reported that vitamin K2 supplementation reduced the incidence of vertebral fracture by 60%, hip fracture by 77%, and non-vertebral fractures by 81% and reduced BMD loss by 0.27 SD [20]. However, the trials included in the meta-analysis were all conducted in postmenopausal Japanese women. More recent trials examining Caucasian

Table 4 Odds ratio of low BMD in elderly Japanese male participants classified by *natto* intake, The FORMEN Study

Natto intake	Number		Adjusted OR[b] (95%CI)	Number		Adjusted OR[c] (95%CI)
	Low BMD[a]	Normal		Low BMD[a]	Normal	
Lumbar spine						
Less than 1 pack/week	150	704	1	148	689	1
1 pack/week	32	240	0.64 (0.42, 0.98)	31	235	0.71 (0.46, 1.10)
Several packs/week	32	217	0.83 (0.54, 1.28)	32	212	0.98 (0.63, 1.54)
1 pack/day or more	17	126	0.66 (0.38, 1.16)	17	122	0.85 (0.48, 1.52)
p value for trend			0.389			0.838
Total hip						
Less than 1 pack/week	159	749	1	159	728	1
1 pack/week	42	235	0.94 (0.62, 1.41)	41	230	1.05 (0.69, 1.58)
Several packs/week	28	230	0.72 (0.45, 1.14)	28	225	0.85 (0.53, 1.37)
1 pack/day or more	12	137	0.44 (0.23, 0.84)	12	133	0.56 (0.29, 1.10)
p value for trend			0.025			0.106
Femoral neck						
Less than 1 pack/week	159	750	1	158	730	1
1 pack/week	39	239	0.85 (0.57, 1.28)	38	234	0.94 (0.62, 1.43)
Several packs/week	26	232	0.65 (0.41, 1.04)	26	227	0.76 (0.47, 1.23)
1 pack/day or more	12	137	0.42 (0.22, 0.81)	12	133	0.53 (0.28, 1.04)
p value for trend			0.001			0.029

One pack of *natto* contains about 20 μg of vitamin K1 and about 380 μg of vitamin K2

BMD bone mineral density, *95% CI* 95% confidence interval

[a] Low BMD; T-score<−1 SD

[b] Adjusted for age, BMI, milk intake, smoking, alcohol drinking, physical activity, and diabetes mellitus

[c] Adjusted for age, BMI, milk intake, smoking, alcohol drinking, physical activity, diabetes mellitus, and undercarboxylated osteocalcin level

Table 5 Multivariate adjusted BMD values and odds ratio of low BMD in elderly Japanese male participants classified by quartile ofucOC level, The FORMEN Study

Serum concentration of ucOC	BMD[a], g/cm^2	Number		OR[a] (95% CI)
	Mean ± SE	Low BMD[b]	Normal	
Lumbar spine				
1st quartile (≤2.04 ng/ml)	1.058±0.014	40	337	1
2nd quartile (2.04 ng/ml < and ≤ 2.94 ng/ml)	1.046±0.015	50	318	1.34 (0.84, 2.14)
3rd quartile (2.94 ng/ml < and ≤4.36 ng/ml)	1.035±0.015	48	333	1.25 (0.78, 2.01)
4th quartile (4.36 ng/ml<)	0.999±0.016* *** ******	90	270	2.59 (1.65, 4.07)
p value for trend	0.045			0.151
Total hip				
1st quartile (≤2.04 ng/ml)	0.911±0.008	41	357	1
2nd quartile (2.04 ng/ml < and ≤2.94 ng/ml)	0.903±0.009	44	341	1.02 (0.63, 1.66)
3rd quartile (2.94 ng/ml < and ≤4.36 ng/ml)	0.884±0.009* ****	49	344	1.13 (0.70, 1.83)
4th quartile (4.36 ng/ml<)	0.844±0.009* *** *****	106	274	2.53 (1.62, 3.94)
p value for trend	0.048			0.183
Femoral neck				
1st quartile (≤2.04 ng/ml or less)	0.768±0.008	44	356	1
2nd quartile (2.04 ng/ml < and ≤2.94 ng/ml)	0.760±0.008	42	338	0.92 (0.57, 1.49)
3rd quartile (2.94 ng/ml < and ≤4.36 ng/ml)	0.752±0.008**	48	350	1.02 (0.64, 1.63)
4th quartile (4.36 ng/ml<)	0.717±0.009* *** *****	100	280	2.14 (1.38, 3.31)
p value for trend	0.069			0.219

BMD bone mineral density, *ucOC* undercarboxylated osteocalcin

[a] Adjusted for age, BMI, *natto* intake, milk intake, smoking, alcohol drinking, physical activity and diabetes mellitus

[b] Low BMD; T-score<−1 SD

*p<0.01 and **p<0.05 vs. 1st quartile; ***p<0.01 and ****p<0.05 vs. 2nd quartile; *****p<0.01 and ******p<0.05 vs. 3rd quartile

women reported different results. An RCT examining early menopausal Norwegian women showed that MK-7, taken in the form of *natto* capsules, over a 1-year period, reduced serum levels of ucOC but did not influence bone loss rates [21]. Another study in postmenopausal American women also failed to find a significant beneficial effect of MK-4 on BMD or proximal femur geometric parameters [22].

For vitamin K1 supplementation, a double-blind controlled trial in Caucasians, Blacks, Hispanics, and Asians showed that the supplemented group had a significantly greater decrease in the proportion of non-carboxylated OC in the total OC than the control group, although there was no difference in BMD change between the groups [23]. Six months of vitamin K1 supplementation did not improve either the spine or femoral neck BMD in an RCT of healthy white women [24]. Observational studies on the association between self-reported dietary vitamin K1 intake and BMD have shown conflicting results. But the Framingham Offspring Study which used an objective measure of vitamin K intake as an exposure variable showed that vitamin K was associated with increased BMD in elderly men [5].

Most studies that reported a beneficial effect of vitamin K on BMD or fracture risk were conducted in Japan on Japanese women and assessed the effect of vitamin K2. In contrast, most studies conducted in Caucasians reported insignificant effects of vitamin K1 intake on BMD, and Gundberg commented that vitamin K supplementation was unlikely to prevent fracture [25]. Thus, ethnic differences between Caucasians and Japanese, including dietary culture and environmental and genetic factors, may exist with regard to the effects of vitamin K intake on BMD or fracture risk [26]. Currently, we have no convincing explanation for the apparent ethnic differences in the effectiveness of vitamin K on bone health, which should be studied further.

A review of RCTs reported that vitamin K1 or K2 supplementation reduced serum ucOC levels, regardless of dose and despite the absence of a significant change or only a modest increase in BMD, and that vitamins K1 and K2 supplementation improved bone strength in the femoral neck, improving femoral neck width and maintaining indices of compression, bending, and impact strength, and reducing the incidence of clinical fractures [12]. The risk reduction for fractures reported for MK-4 supplementation

in Japanese subjects was much larger than expected from the change in BMD, indicating that vitamin K may have improved so-called bone quality, including bone geometric properties and/or material properties, rather than simply bone mass [12]. MK-4 supplementation improved hip bone geometry indices in postmenopausal women [27], and age-related changes in the section modulus and buckling ratio in the HSA indices in elderly Japanese women reportedly differ from those in Caucasian women [28]. However, further studies are necessary to explore and explain the apparent ethnic differences as well as to provide a mechanism for the fracture risk reduction by vitamin K.

In most of RCTs conducted in Japan, 45 mg/day of MK-4 was administered and resulted in relatively modest increase in BMD [12, 20]. *Natto* contains only a small amount of MK-4 (2 μg/100 g) but an extremely large amount of menaquinone-7 (MK-7) (939 μg/100 g) or 100 times the MK-7 content of various kinds of cheese [6, 29]. Nevertheless, the vitamin K content in a pack of *natto* is only a few percent of the pharmaceutical dose. MK-7 had a much longer half-life in serum than other forms of vitamin K (3 days vs. 1–2 h for vitamin K1) [29, 30]. Thus, people who habitually consume *natto* may maintain higher serum levels of MK-7 leading to reduction in bone loss over time.

Other mechanisms that may account for the effects of *natto* intake on bone metabolism

In addition to the above, *natto* may work via other mechanisms to regulate bone metabolism. As shown in Table 4, the OR of low BMD in the group with the greatest *natto* intake became statistically insignificant when adjusted for the ucOC level. However, the OR remained below 1. This may indicate that *natto* also protects bone via pathways independent of vitamin K. *Natto* contains large amounts of isoflavones in addition to vitamin K. Some studies have shown that soy isoflavone can effectively decrease bone resorption [31], and a high isoflavone-containing product may reduce spinal bone loss in postmenopausal women [32]. *Natto* is also relatively rich in calcium (90 mg per 100 g of *natto*). Calcium supplementation, alone or in combination with vitamin D, has been reported to reduce bone loss in a meta-analysis [33]. Thus, habitual *natto* intake may prevent bone loss by ensuring an adequate supply of isoflavones and calcium as well as vitamin K.

Association between *natto* intake and diabetes mellitus

In the present study, subjects with greater intakes of *natto* had significantly higher prevalences of diabetes mellitus than subjects with lower intakes. We also showed that *natto* intake was inversely associated with plasma ucOC level. Recently, it was reported that ucOC increased pancreatic β-cell proliferation and secretion of insulin, while enhancing insulin sensitivity and reducing the development of obesity and glucose intolerance in mice [34]. In previous reports, ucOC was inversely associated with fasting plasma glucose levels and fat mass in diabetic patients [35] and in mostly diabetic subjects [36]. However, a beneficial effect of vitamin K1 on glucose tolerance was reported in a subgroup analysis comprising male participants from a randomized controlled trial [37]. Provided that the hormonal functions of ucOC in mice are conserved in humans, *natto* intake may have an adverse effect on glucose metabolism.

Strengths and limitations of the study

The present study has some advantages over previous studies. The scale of this study was large enough to afford sufficient statistical power. Self-reports of *natto* intake from participants should be quite reliable because *natto* intake level was strongly correlated with ucOC levels, a biomarker for vitamin K intake.

However, the present study also has several limitations. First, participants were not randomly selected from the population, and patients with severe or symptomatic diseases may not have participated in the study. This sampling method may have biased the sample towards healthier individuals. Second, the present study was a baseline survey of a cohort, and cross-sectional analyses of these data cannot establish causality between *natto* or vitamin K intake and BMD. The effects of *natto* or vitamin K intake on change in BMD and on risk of osteoporotic fracture should be investigated in follow-up studies. Third, *natto* intake was significantly correlated with smoking, drinking, and milk intake, which were adjusted for in the multivariate analyses. R^2 values of the generalized linear model for BMD increased 10–30 times in all models, and AIC of the logistic regression model for low BMD decreased by approximately 50 by the adjustment, indicating that the adjusted models achieved a substantially better fit of the data. However, these adjustments may not eliminate the confounding effects, and other confounding factors that we did not consider or a healthier lifestyle related to *natto* intake that we did not assess may exist. Fourth, we obtained a significant *natto*–BMD association at the hip or femoral neck but not in the spine. Measurement of lumbar spine BMD using DXA cannot exclude aortic calcification and is affected by spondylosis deformans and osteoarthrosis [38]. We could not eliminate the effects of these extra-skeletal calcifications or vertebral deformities from the real association. Finally, information on dietary intake of energy, macro- or micronutrients, and vegetables was not available. We understand that this is a serious

limitation of our study. If differences in these intakes among the *natto* intake groups existed, they may have confounded the association of interest. Using ucOC levels as a biomarker for vitamin K intake may have reduced any possible confounding effects due to energy or micro/macronutrients in the assessment of the *natto*–BMD association. However, ucOC levels do not represent intakes of vitamins K1 and K2 separately. Intake of vegetables that provide vitamin K1 may have confounded the association. However, a previous study found no difference in vegetable intakes between groups of Japanese with differing *natto* intakes [7]. Nevertheless, we accept that differences in vegetable intakes among the *natto* intake groups may have affected our results.

Conclusion

The FORMEN study showed that high *natto* intake was associated with lower ucOC and higher BMD. Habitual intake of *natto* was associated with a beneficial effect on bone health in elderly men, and this association is primarily due to the vitamin K content of *natto*.

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Conflicts of interest None.

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