Usual Dietary Intake of Fermented Soybeans (Natto) Is Associated with Bone Mineral Density in Premenopausal Women

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Summary Fermented soybeans (Natto), a traditional Japanese food, contain more than 100 times as much vitamin K2 as various cheeses and are considered to promote γ-carboxylation. Thus it is conceivable that Natto may play a preventive role in the development of osteoporosis. In this study, the relationships between the bone stiffness index measured by ultrasound, bone turnover markers, and lifestyle factors, including Natto intake, were examined in relation to vitamin D receptor (VDR) polymorphism. Among 117 premenopausal volunteers, approximately 75% were bb homozygotes, 20% were Bb heterozygotes, and only 5% were BB homozygotes. The B allele group and the bb group were subdivided according to Natto intake. In a monovariate analysis, no significant differences in indices for dietary intake, including Ca and vitamin D intake, were observed. The stiffness index in the B allele group, however, was slightly lower than in the bb groups when there was no Natto intake. There were no significant differences in serum ALP and Gla-osteocalcin, bone formation markers, or NTx and Ca in urine, bone resorption markers. A logistic regression test, including the interactional effect of Natto intake and VDR RFLP, indicated that the B allele group was a risk factor of bone mineral loss and that Natto was effective in maintaining bone stiffness in this group. Although the present study was cross sectional and requires longitudinal investigation, Natto may improve the bone health of people who have a low affinity receptor for vitamin D.

Key Words Natto, bone mineral density, vitamin D receptor polymorphism, stiffness, bone turnover markers

With the rapidly increasing proportion of elderly in industrialized countries, including Japan, osteoporosis has become a major public health problem affecting quality of life and increasing costs to health care providers (1). Although osteoporosis results in inadequate skeletal strength predisposing its sufferers to fracture, most osteoporotic patients are diagnosed after the occurrence of fractures, since osteoporosis itself lacks specific symptoms. Even if osteoporosis is diagnosed, it is difficult to avoid the risk of fracture because it takes significant time to recover bone mineral density (BMD). In females, BMD reaches its maximum in adolescence and gradually decreases until menopause, after which it decreases dramatically (2). Since reduction caused by menopause is unavoidable, it seems important to make a maximum level of BMD as high as possible in adolescence and to prevent reducing BMD before menopause.

Development of quantitative ultrasound methods (QUS) (3) has made it possible to measure BMD not only for clinical use, but also for screening purposes because QUS systems are portable and inexpensive (4). QUS is also recognized as a means of reflecting information on bone structure and density that is not measured by ionizing radiation methods (5). The rate of bone formation or degradation can also be assessed either by measuring the enzymatic activity of osteoblastic or osteoclastic cells, such as alkaline phosphatase activity, or by measuring components of the bone matrix released into circulation during formation or resorption, such as osteocalcin and type I collagen breakdown products (6). These bone turnover markers may also be useful not only to predict the occurrence of osteoporosis, but also to assess the efficiency of antosteoporotic treatment.

Vitamin D3 regulates calcium (Ca) homeostasis that is recognized to be essential to prevent osteoporosis (7) and the synthesis of osteocalcin, which is the most plentiful protein in bone (8). Vitamin K2 (VK2) is essential to activate osteocalcin through carboxylation of glutamate to γ-carboxyglutamic acid (9). On the other hand, because of allelic variants caused by restriction fragment length polymorphisms (RFLP), the vitamin D receptor (VDR) consists of b and B subtypes with respectively high affinity and low affinity to vitamin D (10). Compared to women with the bb variants, women with the B allelic variants have reduced calcium absorption efficiently on low Ca intake (11), which is reportedly less in Japanese than in Caucasians (11). Therefore it is
attractive to consider that VK$_2$ supplementation may activate osteocalcin and prevent bone mineral loss for the B variants, especially in women with low Ca intake. Although its deficiency in humans is less likely because it is synthesized by intestinal flora (12), the administration of menaquinone-4 (MK-4), a VK$_2$ component to patients with established osteoporosis for 48 wk increased metacarpal BMD and serum osteocalcin and reduced urinary Ca excretion (13). However, whether a dietary intake of VK$_2$ is useful in maintaining bone turnover and in preventing osteoporosis remains unclear (12). Soybeans fermented by Bacillus Natto (Natto), a traditional Japanese food, contain in excess of 100 times more VK$_2$ (mainly MK-7) than various cheeses (14) and are recognized to be a healthy food.

To assess whether dietary intake of VK$_2$ plays a preventive role in the development of osteoporosis, we examined the levels of bone biochemical markers and stiffness measured by QUS among premenopausal women whose Natto intake differed, in relation to VDR polymorphism.

SUBJECTS AND METHODS

Subjects. All 140 female workers at a local hospital in Japan were recruited with informed consent. Twenty-three were excluded because of menopause, cardiovascular diseases, bilateral ovariectomy, use of anticoagulant therapy, use of drugs known to interfere with bone metabolism, and the rest, 117 premenopausal workers (mean age 33.2±9.8 y of age), were included in the present study.

This study was performed under the guidelines for Medical Ethics of Kawasaki Medical School.

Lifestyle factors. The usual dietary intake was assessed by the use of a food frequency questionnaire according to an 11-food-group classification (15). This questionnaire had been designed and validated previously against a 7-d frequent food intake, and daily dietary intakes were calculated from this 7-d weighed-food intake. Besides each intake being compared among the groups divided according to Natto intake, all dietary intakes, including lipids, carbohydrates, dietary fiber, and vitamins A, B, and C, were estimated by making a score according to the method by Takahashi et al. (15).

The Natto intake of each subject was requested by separately. Moreover, since the subjects noted their fondness of Japanese foods instead of Western foods, VK$_2$ intake through cheese, butter, and similar products was negligible in this study.

The physical activity level was assessed by the number of steps taken per day as measured by a pedometer. Wearing lightweight clothes, the subjects had their heights and weights measured, and their body mass indexes (BMI) were calculated.

Bone stiffness. Regarding bone stiffness, the speed of sound (SOS; m/s) and the broadband ultrasound attenuation (BUA; dB/MHz) in the calcaneus were measured with an Achilles (A-1000) ultrasound bone densitometer (Lunar Corporation, Madison, WI). The SOS was recognized to reflect the density and elastic properties of bone, and BUA was thought to relate to the structure and density of bone (5). The mathematical index, designated as stiffness index, was automatically calculated with this formula: stiffness index=0.67×BUA+0.28×SOS−420 (2, 16). Since the index was suitable for Caucasians, “% young adult” was further calculated by dividing the stiffness index of each subject by the peak stiffness of normal Japanese females, which was 91.6 (2), to estimate bone mineral loss associated with aging.

Biochemical markers for bone turnover. Second-morning urine specimens were collected and blood samples were obtained at QUS measurement. The serum and the urine specimens were both stored at −20°C until measurement. Serum bone specific alkaline phosphatase (ALP-s) was measured as a bone formation marker. Serum γ-carboxylated osteocalcin (Gla-osteocalcin) was measured with a Gla-type osteocalcin EIA kit (Takara, Osaka, Japan). Osteocalcin correlates well with bone formation, but is readily subjected to rapid degradation in serum (17). Not only Gla-osteocalcin and undecarboxylated osteocalcin (Glu-osteocalcin) but also their fragments of various sizes coexist in the circulation, different immunoassays have yielded varying results (18). In the present study, a kit utilizing an antibody specific to 17Gla and its adjacent region was used to isolate Gla-osteocalcin. Although γ-carboxylation preferentially occurred at residues 21 and 24 (19), 17Gla was essential for a Ca-dependent conformational transition (20), indicating that the 17Gla-specific antibody could recognize osteocalcin fully γ-carboxylated to be active. Ca (Ca/Cr) and type I collagen cross-linked N-telopeptides (NTx/Cr) in urine were measured as bone resorption markers. Both were corrected by creatinine in urine. The menstrual cycle of each subject was not considered because the effects of menstrual cycle on bone turnovers were thought to be small (21).

VDR polymorphism. Genomic DNA was extracted from leukocytes, and intron 8 of the VDR gene, where the BsmI digestion site was located, was amplified by the polymerase chain reaction (PCR) method (22). The polymorphisms were signified as b or B, respectively, in the presence or absence of the BsmI digestion site. Allele b corresponded to VDR with high affinity to vitamin D and allele B to VDR with low affinity.

Statistical analysis. A one-way analysis of variance (ANOVA) with Fisher’s PLSD as a post hoc test was performed to compare differences in lifestyle factors, stiffness, and biochemical markers of bone turnover for each Natto intake group. A two-way ANOVA and a logistic regression test were performed to examine interaction between Natto intake and VDR RFLP. When significant differences were detected, contrast analyses were performed with Sheffe’s multiple comparison tests. Odds ratios as estimated risk with 95% confidential intervals were calculated by logistic regression by the use of SPSS (Medical Pack 6.1).
RESULTS

Lifestyle factors and VDR RFLP of subjects

The subjects were divided into three groups according to their Natto intake, namely, high intake, more than once a week; low intake, about once a month; and no intake. Since 30–50 g of Natto, containing approximately 9 μg/g of VK₂ (14), was usually taken at a time, it seemed that the high-intake group might ingest more than 1 mg of VK₂ per week. A previous report showed that serum menaquinone-7 concentration of 31 British women taking Natto at least twice a week in approximately 9 μg/g of VK₂ (14), was usually taken at a time, it seemed that the high-intake group might ingest more than 1 mg of VK₂ per week. A previous report showed that serum menaquinone-7 concentration of 31 British women taking Natto at least twice a week it was 7.915±6.914 ng/mL; of 13 Japanese women taking Natto once a week it was 2.814±3.137 ng/mL (23). Although serum menaquinone-7 concentration was not measured in this study, it is indicated that serum menaquinone-7 concentration of the low-intake group seems to be twice as high as that of the no-intake group, and that of the high-intake group also seems to be 10 times higher than that of the no-intake group. When VDR RFLP were considered, in good accordance with a previous report (22), approximately 75% were bb homozygotes, 20% were Bb heterozygotes, and only 5% were BB homozygotes. Thus the VDR RFLP groups were subdivided into bb and B allele groups. There were no significant differences in mean age or BMI among these six groups (Table 1). The p value of mean age between bb and B allele in the low-intake group was 0.19. Although the physical activities of the B allele groups were significantly lower than those of the bb groups, interaction between Natto intake and VDR RFLP was not observed. Moreover, no significant differences in the indices of dietary intakes, i.e., the intakes of energy, protein, Ca, vitamin D, and soy diet, were observed. However, Ca intake was insufficient in all groups. On the other hand, a nutritional survey using this food-frequency questionnaire was performed on 1,621 female residents (ranging from their 20s to 40s) in Tokushima Prefecture (24). It is indicated that the mean score of these subjects was 63.5±4.0. The mean score in this study is thought to be not different from the one in a large epidemiological study. No statistical differences in the scores of all dietary intakes were observed. Among the subjects, 20% and 50% had smoking and drinking habits, respectively. However, those who had a drinking habit drank as little as drink I (25) per day on the average. Drink I was defined as being equivalent to 1.5 ounces (44 mL) of liquor, 4 ounces (118 mL) of wine, or 12 ounces (355 mL) of beer. Thus the effects of smoking and drinking were negligible in the present study.

Monovariate comparison of bone stiffness in each group

The average of the stiffness index of all subjects was 86.9±13.5; therefore the average of % young adult was calculated to be 94.9±14.7% (Table 2). No statistical differences in the stiffness index were observed among either group divided by Natto intake or those by VDR RFLP. Furthermore, no interactional effect between Natto intake and VDR RFLP was observed. The stiffness index in the B allele groups, however, was slightly lower than in the bb groups when there was no Natto intake, though no statistical difference was observed. It is conceivable that Natto intake may affect bone turnover in the B allele groups, but not in the bb groups. To assess which step of bone turnover was preferentially affected, the biochemical markers for bone turnover were examined in each group.

Monovariate comparison of biochemical markers for bone turnover in each group

ALP-s differed significantly among the Natto intake groups (Table 3). This was because the B allele with the low-intake group included a subject having a very low ALP level. Neither the difference between the VDR RFLP groups nor the interactional effect was significant. There were no significant differences in Glα-osteocalcin among the groups. Bone resorption markers, NTx/Cr and Ca/Cr, displayed no significant differences among groups divided by Natto intake and VDR RFLP, as well as in interaction.

Logistic regression test and risk determinants for bone mineral loss

The monovariate analyses seemed to be insufficient to exclude confounding factors and to estimate which parameter was associated with the bone mineral density. Since an average of the stiffness index of normal 30 to 40-y-old Japanese females has been reported to be 86 to 88 (2), a logistic regression test for possible risk factors was performed to determine whether the stiffness index was lower than 86.

Logistic regression for all subjects indicated that none of the determinants was a potential risk for lowering the stiffness index when the interactional effect of Natto intake and VDR RFLP was not recognized as a determinant (Table 4). When the interactional effect was included as a determinant, the B allele group could be considered a risk factor, and its odds ratio was as high as 5.77. An addition of the interactional effect seemed to expose a concealed effect of difference in VDR RFLP, and the interactional effect itself reduced risk with an odds ratio of 0.22. Therefore a logistic regression test was further performed on groups divided by VDR RFLP (Table 5). None of the determinants had a significant effect in the bb group, but age showed a slightly increased risk. In the B allele group, Natto intake more than once a week based on no intake displayed a low odds ratio of 0.03, the p value of which was 0.04.

DISCUSSION

Whether VDR polymorphism has an influence on BMD is still controversial because several investigations have shown that the BB genotype of VDR is associated with low BMD (22, 26, 27); the absence of such an association in Korean (28) and Danish (29) populations has also been reported. BB genotype is also reported to be associated with reduced Ca absorption when Ca intake is low (11). Moreover, the B allele displays low affinity to vitamin D, which plays an important role in synthesizing osteocalcin in osteoblasts. However, an association of the B allele with a high level of circulating osteocalcin is also reported (30). Such bidirectional roles of the B allele on BMD seem to make the interpre-
Table 1. Comparison of lifestyle factors between Natto intake and VDR RFLP.

|                  | n   | bb          | B allele |
|------------------|-----|-------------|----------|
|                  | 117 | 85          | 32       |
| No intake        | 42  | 27          | 15       |
| Low intake       | 28  | 21          | 7        |
| High intake      | 47  | 37          | 10       |
| Age              |     | 32.9±10.0   | 33.8±9.3 |
| 33.2±9.8         |     | 31.2±9.8    | 31.7±8.5 |
| No intake        | 31.4±9.2 | 32.0±9.3    | 37.7±11.5|
| Low intake       | 33.4±10.0 | 34.7±10.5   | 34.4±8.7 |
| High intake      | 34.6±10.0 | 34.2±2.4    | 21.7±2.9 |

|                  |     | 21.2±2.5   | 21.4±2.3 |
| BMI              |     | 21.3±2.6   | 21.4±2.3 |
| 21.3±2.6         |     | 21.5±3.1   | 21.4±2.3 |
| No intake        | 21.4±2.9 | 20.6±1.7   | 22.2±4.0 |
| Low intake       | 21.0±2.5 | 21.3±2.4   | 21.7±2.9 |
| High intake      | 21.4±2.5 | 21.3±2.4   | 21.7±2.9 |
| Physical activity (steps/d) |     | 9146.3±2950.8 | 7454.8±2089.6** |
| 8780.3±2843.8    |     | 9039.4±3414.5 | 6879.5±2261.1|
| No intake        | 8337.4±3224.2 | 8999.4±2509.6 | 7439.7±2042.4|
| Low intake       | 8595.1±2459.7 | 9308.1±2876.1 | 8297.6±1774.0|
| High intake      | 9106.0±2705.4 | 9039.4±3414.5 | 6879.5±2261.1|
| Energy (kcal/d)  |     | 1812.9±368.9 | 1805.1±422.0 |
| 1810.9±381.2     |     | 1806.4±413.8 | 1781.4±310.3 |
| No intake        | 1783.4±388.3 | 1765.2±317.1 | 1878.1±359.0 |
| Low intake       | 1793.4±325.0 | 1844.6±368.4 | 1848.7±582.2 |
| High intake      | 1845.4±410.9 | 1844.6±368.4 | 1848.7±582.2 |
| Protein (g/d)    |     | 63.9±17.6   | 65.2±22.8 |
| 64.3±18.9        |     | 60.2±15.7   | 62.2±18.4 |
| No intake        | 60.8±16.4 | 65.2±19.2   | 67.8±29.3 |
| Low intake       | 65.8±18.5 | 65.9±19.2   | 67.8±29.3 |
| High intake      | 66.3±21.1 | 65.9±19.2   | 67.8±29.3 |
| Ca Intake (mg/d) |     | 507.7±234.8 | 494.2±200.5 |
| 504.3±225.8      |     | 446.1±181.5 | 499.0±230.7 |
| No intake        | 456.0±185.2 | 499.0±230.5 | 523.7±250.5 |
| Low intake       | 505.2±213.4 | 557.6±264.1 | 496.6±182.6 |
| High intake      | 545.7±249.7 | 557.6±264.1 | 496.6±182.6 |
| Vitamin D (IU/d)|     | 178.2±94.3  | 181.0±103.4 |
| 178.9±96.2       |     | 152.6±67.3  | 160.6±85.1 |
| No intake        | 155.2±72.5 | 152.6±67.3  | 160.6±85.1 |
| Low intake       | 200.5±101.3 | 202.4±106.9 | 194.6±89.4 |
| High intake      | 186.5±108.1 | 183.2±101.3 | 199.9±139.2 |
The subjects were divided into three groups according to their Natto intake: no intake, low intake; around once a month, high intake: more than once a week.

Statistical difference in physical activity was observed between bb and B allele (** p<0.01).

Table 2. Comparison of stiffness index between Natto intake and VDR RFLP.

| Natto Intake | bb     | B allele |
|--------------|--------|----------|
| No intake    | 59.4±9.9| 59.6±11.0|
| Low intake   | 57.0±9.5| 58.7±12.1|
| High intake  | 60.0±9.7| 58.4±8.0 |

( ): % young adult of stiffness index.

No statistical difference was observed among groups.

ANOVAs with post hoc tests allow a comparison between pairs or a combination of groups and can analyze the differences among dependent variables. That is, they are omnibus tests of differences in the data, testing various combinations of means to determine individual group differences (33). Thus they are useful for comparing data obtained from patient-control groups and groups with different levels of treatment, for example. However, the present study aimed to analyze risks not yet apparent in premenopausal women who are considered to be healthy. The data consisted of multiple dependent variables with varying confounding factors; e.g., bone mineral density is known to be dependent on age (5), but it is also altered by BMI, physical activity,
Table 3. Comparison of bone turnover markers between *Natto* intake and VDR RFLP.

| Bone formation markers | 
|------------------------|
| **ALP-s (U/L)**        |
| 57.7±21.2              |
| **bb**                 |
| 56.7±19.3              |
| **B allele**            |
| 60.2±25.7              |
| No intake              |
| 63.4±19.2              |
| Low intake             |
| 51.3±17.7*             |
| High intake            |
| 56.3±23.7              |
| **Gla osteocalcin (ng/mL)** |
| 1.86±1.45              |
| **bb**                 |
| 1.79±1.43              |
| **B allele**            |
| 2.05±1.52              |
| No intake              |
| 1.99±1.49              |
| Low intake             |
| 1.50±1.20              |
| High intake            |
| 1.96±1.54              |

| Bone resorption markers | 
|-------------------------|
| **NTx/Cr (nm BCE/mm Cr)** |
| 41.1±18.8               |
| **bb**                  |
| 41.9±19.2               |
| **B allele**             |
| 39.2±18.1               |
| No intake               |
| 41.7±22.6               |
| Low intake              |
| 40.2±16.8               |
| High intake             |
| 41.1±16.6               |
| **Ca/Cr (g/g Cr)**      |
| 0.14±0.10               |
| **bb**                  |
| 0.15±0.10               |
| **B allele**             |
| 0.12±0.09               |
| No intake               |
| 0.13±0.08               |
| Low intake              |
| 0.15±0.11               |
| High intake             |
| 0.16±0.10               |

A statistical difference was observed in ALP-s between no intake and low intake (two-way ANOVA, *p<0.05*).

Table 4. Risk determinants in the reduction of stiffness without or with interaction.

| Without interaction | With interaction |
|----------------------|------------------|
|                      | Odds ratio | 95% CI  | Odds ratio | 95% CI  |
| Age                  | 1.03       | 0.99–1.07 | 1.03       | 0.99–1.08 |
| BMI                  | 0.94       | 0.79–1.11 | 0.95       | 0.80–1.13 |
| Score                | 0.99       | 0.94–1.03 | 0.98       | 0.94–1.02 |
| Physical activity    | 1.00       | 0.99–1.00 | 1.00       | 0.99–1.00 |
| ALP-s                | 1.02       | 0.99–1.04 | 1.02       | 0.99–1.05 |
| Gla osteocalcin      | 0.98       | 0.73–1.32 | 1.03       | 0.75–1.40 |
| Ca/Cr                | 1.22       | 0.12–121.6| 0.77       | 0.01–88.4 |
| NTx/Cr               | 1.01       | 0.99–1.04 | 1.01       | 0.99–1.04 |
| *Natto* intake       |            |          |            |          |
| 1/mo based on none   | 1.50       | 0.50–4.49 | 2.44       | 0.74–8.00 |
| 1/wk based on none   | 0.91       | 0.35–2.36 | 1.88       | 0.62–5.77 |
| B allele based on bb | 1.30       | 0.49–3.44 | 5.77*      | 1.13–29.38 |
| *Natto* intake×VDR RFLP |        |          | 0.22**     | 0.06–0.73 |

Odds ratios indicating that stiffness was lower than 86 to higher than 86 were calculated according to potential risk factors. *p<0.05, **p<0.01*.
nutrient intakes, and so on (5, 7, 34). Therefore multivariate analysis such as logistic regression was applied to exclude the effects of these confounding factors.

Nutrient intakes, excluding Natto, had no significant difference between groups in the monovariate analysis. Since the Ca intake of Japanese is less than 600mg/d (35), which is less than that of Caucasians (11), possibly all Japanese may suffer from low Ca absorption. In fact, the subjects of the present study took only about 500mg/d, which is less than the Japanese average. However, nutrient intakes, including Ca, were neither potential risks nor preventive factors in the logistic analysis. The interactional effect of Natto intake and VDR RFLP was recognized as a preventive factor and the B allele group itself as a risk factor, indicating that Natto was effective in maintaining bone stiffness, especially in the B allele group. Aside from a monovariate analysis, logistic regression analyses successfully excluded confounding factors and made it possible to explore the risk of bone mineral loss.

Among 14 isomers of VK2 (menaquinone; MK), MK-4 most potently enhanced mineralization in vitro (36). A small amount of MK-4, around ng/g, is found, for example, in margarine, sesame, cheese, and butter, as well as in Natto (14). Cheese also contains a variety, but a small amount, of VK2s, but Natto has an extremely high content of MK-7, about 10µg/g, which is a precursor of MK-4 (37). When experimental diets containing only MK-7 were fed to ovariectomized rats, both MK-4 and MK-7 accumulated in femoral bone and significantly increased the Ca content of femur (37). Natto intake increases MK-4 in bone and possibly promotes Ca accumulation even in premenopausal women. Furthermore, Natto contains about 1 mg/g Ca and genistein, since it is made from soybeans (38). One dish of Natto is equivalent to about 10% of the daily intake of Ca in Japanese. Isoflavones, including genistein, have structural similarities to estrogen and may have an influence as phytoestrogens (39). In ovariectomized mice, genistein showed no reversal of uterine atrophy, which was completely restored by 17 β-estradiol, but it recovered the trabecular bone volume loss of the femoral distal metaphysis (39). Genistein is thought to be useful for preventing bone loss caused by estrogen deficiency in females. The daily intake of isoflavones in Asians is estimated at 25–200 mg, and 10–100 nM of active isoflavones exist in their serum regardless of Natto consumption (40). Premenopausal women consuming a soy diet displayed higher serum estradiol level in their follicular phase than those with a control diet (41). Aside from VK2, Natto intake could play a protective role by supplying phytoestrogens and Ca. Although the effect of VK2 on the prevention of bone mineral loss is still unclear, Natto intake seems to be useful for maintaining bone mineral content, especially for women with B allele. Because all subjects in the present study were premenopausal, it will be important to study the effect of Natto on the postmenopausal population.

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