# Relationship of homocysteine and homocysteine-related vitamins to bone mineral density in Japanese patients with type 2 diabetes

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## ABSTRACT

**Aims/Introduction:** To estimate nutritional risk factors for osteoporosis in patients with type 2 diabetes, bone mineral density, homocysteine level, and intakes and levels of Hcy-related vitamins including folate, vitamin $B_6$ and vitamin $B_{12}$ were analyzed in a cross-sectional study.

**Materials and Methods:** Lumbar spine and femoral neck bone mineral density, serum concentrations of vitamin $B_6$, vitamin $B_{12}$, and folate and plasma homocysteine levels were measured in 125 Japanese patients with type 2 diabetes. Nutrient intake values were evaluated using a food frequency questionnaire.

**Results:** Homocysteine was inversely correlated with bone mineral density, and with both dietary intake and serum concentration of folate. Intake of green vegetables was correlated with intake and level of folate and homocysteine levels. When the population was analyzed across the quartiles, bone mineral density, serum folate concentration, folate intake and intake of green vegetables were lowest in the highest homocysteine group.

**Conclusions:** In patients with type 2 diabetes, the nutritional status of folate might affect the homocysteine level, a putative risk factor for osteoporosis. (J Diabetes Invest, doi: 10.1111/j.2040-1124.2010.00088.x, 2011)

**KEY WORDS:** Osteoporosis, Homocysteine, Folate

## INTRODUCTION

Diabetes is becoming increasingly recognized as a risk factor for osteoporotic fracture. Although fracture risk in patients with type 2 diabetes is increased compared with normal subjects, not only in those with low bone mineral density (BMD) but also in those with normal or high BMD[1–3], decreased BMD is a major determinant of fragility fracture.

Patients with type 2 diabetes often follow a calorie-restricted diet, but few studies have investigated the sufficiency of these nutrients for the maintenance of skeletal health. Generally, nutrient intake increases along with energy intake. *Ad libitum* food intake values obtained from a longitudinal study in institutionalized elderly found that intake values of vitamins increased along with increased energy intake[4]. In contrast, implementation of a low-fat, low-energy diet (1000 or 1500 kcal/day) in patients with overweight and hyperlipidemia has been shown to result in a decrease of the intake of certain nutrients, including B-vitamins[5].

Folate, vitamin $B_6$ and vitamin $B_{12}$ are important enzymatic cofactors in the synthesis of methionine from homocysteine (Hcy), and an elevation of Hcy can be caused by insufficiency of folate, vitamin $B_6$ or vitamin $B_{12}$. Numerous studies have linked high circulating Hcy levels and low concentrations of folate or vitamin $B_{12}$ with increased risk of low BMD in non-diabetic subjects[6–14]. The possibility that elevated Hcy is a risk factor for osteoporosis is suggested by studies of patients with homocystinuria, a rare autosomal recessive disease characterized by markedly elevated levels of plasma Hcy, in which early onset of generalized osteoporosis has occurred[15,16]. The underlying pathophysiological mechanism of osteoporosis in patients with elevated Hcy is not completely understood. Hcy has been reported to interfere with cross-links of newly formed collagen[17,18], and consequently with bone mineralization and strength[19], as well as to stimulate osteoclast formation and activity[20,21]. However, there has been no report on the association of Hcy and Hcy-related vitamins with osteoporosis in patients with diabetes. Furthermore, vitamin insufficiency was evaluated only by serum vitamin concentrations in most of these studies, and there has been no comprehensive investigation of the relationship of dietary intake of nutrients and

serum vitamin concentrations with Hcy and BMD among subjects in the same study.

In the present study, to evaluate nutritional risk factors for osteoporosis in patients with type 2 diabetes, BMD, Hcy level, and intakes and levels of Hcy-related vitamins including folate, vitamin $B_6$ and vitamin $B_{12}$ were analyzed.

## MATERIALS AND METHODS

### Study Population

A total of 125 Japanese patients with type 2 diabetes admitted between December 2008 and June 2009 to Kyoto University Hospital were sequentially enrolled in the study. Lateral lumbar X-ray was carried out to exclude those with scoliosis, compression fractures and ectopic calcifications. Subjects with bilateral hip fractures or prosthesis and other diseases that might influence bone metabolism including liver disease, renal dysfunction (serum creatinine above 2 mg/dL), hyperthyroidism, hyperparathyroidism, hypercorticoidism, and hypogonadism were excluded. All subjects were free of drugs that influence bone and calcium metabolism including glucocorticoids, bisphosphonates, calcitonin injection, estrogens, selective estrogen receptor modulators, vitamin D, vitamin K, thiazide diuretics, heparin and anticonvulsants. The number of patients treated with thiazolidinedione and metformin was 7 and 28, respectively. The present study was cross-sectional in design, and was approved by The Ethical Committee of Kyoto University Hospital and complies with the Helsinki Declaration. Written informed consent was obtained from all participants.

### Measurement of Bone Mineral Density

BMD was measured by dual-energy X-ray absorptiometry (DXA; Discovery; Hologic, Waltham, MA, USA) at the lumbar spine (L1-L4) and femoral neck. The coefficient of variation of the measurements of BMD was 0.39%. BMD ($g/cm^2$) was expressed as $Z$-score calculated on the basis of the normal reference values of the age- and sex-matched Japanese group provided by the DXA system manufacturer. Because male and female patients of different ages were included in the study, comparison of BMD was made based on $Z$-scores. Fat mass and lean body mass (without bone mineral content) were measured by DXA (Hologic Discovery; Hologic) using whole-body absorptiometry software, and each value was expressed in kilograms.

### Biochemical Measurements

Blood samples were obtained after overnight fasting immediately after admission. Glycosylated hemoglobin ($HbA_{1c}$) was measured by high performance liquid chromatography (HPLC). The value for $HbA_{1c}$ (%) is estimated as a National Glycohemoglobin Standardization Program (NGSP) equivalent value (%) calculated by the formula $HbA_{1c}$ (%) = $HbA_{1c}$ [Japan Diabetes Society (JDS); %] +0.4%, considering the relational expression of $HbA_{1c}$ (JDS; %) measured by the previous Japanese standard substance and measurement methods and $HbA_{1c}$ (NGSP)[22]. Fasting serum C-peptide was measured by ELISA (ST AIA-

PACK C-Peptide; Toso Corporation, Tokyo, Japan). Bone-specific alkaline phosphatase (BAP) was measured by enzyme immunoassay (Osteolinks BAP; DS Pharma Biomedical, Suita, Japan), and urine N-terminal cross-linked telopeptide of type-I collagen (uNTx) was measured by ELISA (Osteomark NTx ELISA Urine; Inverness Medical, Waltham, MA, USA). Plasma Hcy levels were determined by HPLC using a thiol-specific fluorogenic reagent, ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfate[23], and the upper limit of Hcy was 13.5 nmol/L. As pyridoxal 5′-phosphate (PLP) is the predominant circulating form of vitamin $B_6$, serum PLP concentrations were measured by HPLC[24,25] for evaluation of vitamin $B_6$ status. For vitamin $B_{12}$ measurement, 0.2 mmol/L acetate buffer (pH 4.8) was added to the serum samples, and the vitamin $B_{12}$ was converted to cyanocobalamin by boiling with 0.0006% potassium cyanide at acidic pH. Cyanocobalamin was determined by the microbioassay method using *Lactobacillus leichmanii*, ATCC 7830[24,25]. Serum folate was determined by the microbioassay method using *Lactobacillus casei* ATCC 2733[24,25].

### Evaluation of Dietary Nutrient Intake

A food frequency questionnaire (FFQ) validated by Takahashi *et al.*[26,27] was used to calculate nutrient intakes. The FFQ used in the present study included questions on the consumption of various food items over the previous 1 or 2 months. Daily nutrient intake was calculated by multiplying the frequency of consumption of each food by the nutrient content of the portion size and summing the products for all food items. The FFQ is validated against 7-day dietary records and the FFQ-estimated nutrient intake values are 72–121% of those of 7-day dietary records[26]. The reproducibility of the FFQ at intervals of 1–2 months is 93–119% for each nutrient[26]. Correlations of dietary folate intake, serum folate concentration, and plasma Hcy level with intakes of various food groups including grain/rice, potato, green vegetables, other vegetables, fruits, seaweeds, beans/soy products, seafood, meats, egg, milk products and oil/fat were evaluated.

### Statistical Analysis

Data were expressed as mean ± SD. SPSS statistical software (version 13.0; SPSS, Chicago, IL, USA) was used for all statistical analyses. Pearson's correlation coefficient was calculated as a measure of association by adjusting for age and sex where appropriate. Stepwise multiple linear regression analyses were carried out to determine independent factors for plasma Hcy levels including (i) dietary vitamin $B_6$, vitamin $B_{12}$ and folate intake values; and (ii) serum PLP, vitamin $B_{12}$ and folate concentrations as independent variables. The relationship between BMD with Hcy and Hcy-related vitamins was further explored using a quartile-based analysis. Statistical differences among the groups were evaluated using analysis of covariance (ANCOVA) adjusted for age and sex, and Dunnett's multiple comparison tests by comparison with the highest Hcy group. $P < 0.05$ was considered significant.

## RESULTS

Clinical characteristics, laboratory data and nutrient intake of subjects are shown in Table 1. The average serum vitamin $B_{12}$ concentration was $1.45 \pm 0.45$ pmol/mL (Table 1) and there was no difference between patients taking metformin ($1.52 \pm 0.49$ pmol/mL, $n = 97$) and those without ($1.43 \pm 0.49$ pmol/mL, $n = 28$). Nutrient intake values were significantly positively correlated with total energy intake (Table 2). Dietary vitamin $B_6$, vitamin $B_{12}$ and folate intake values were positively correlated with serum vitamin $B_6$, vitamin $B_{12}$ and folate levels, respectively (Table 2). Plasma Hcy levels were negatively correlated with both dietary intake and serum concentration of folate (Table 2). Only vitamin $B_6$ intake and not vitamin $B_6$ concentration showed a weak negative correlation with Hcy; the influence of vitamin $B_{12}$ on Hcy elevation was unclear (Table 2). Stepwise multiple linear regression analyses were carried out to

**Table 1** | Background characteristics of the study subjects

| Characteristic | |
| --- | --- |
| No. subjects | 125 |
| Male/female | 79 (63.2%)/46 (36.8%) |
| Age (years) | 61.2 ± 12.4 |
| Duration of diabetes (years) | 11.2 ± 9.4 |
| Diabetes treatment (diet/OHA/Ins/Ins + OHA) | 27 (21.6%)/62 (49.6%)/ 28 (22.4%)/8 (6.4%) |
| BMI (kg/m$^2$) | 24.9 ± 4.9 |
| Fat mass (kg) | 16.5 ± 9.8 |
| Lean body mass (kg) | 45.9 ± 9.3 |
| Fasting plasma glucose (mg/dL) | 160.2 ± 48.6 |
| HbA$_{1c}$ (%) | 9.6 ± 2.2 |
| Fasting serum C-peptide (ng/mL) | 1.71 ± 0.89 |
| Serum BAP (U/L) | 23.5 ± 8.7 |
| uNTx (nMBCE/mmol Cr) | 35.6 ± 19.8 |
| Energy intake (kcal/day) | 2073.2 ± 582.5 |
| Protein/fat/carbohydrate intake (g/day) | 73.6 ± 19.7/64.4 ± 23.7/ 278.7 ± 80.2 |
| Calcium intake (mg/day) | 596.0 ± 213.6 |
| Vitamin D intake (μg/day) | 9.21 ± 4.48 |
| Vitamin B$_6$ intake (mg/day) | 1.22 ± 0.34 |
| Vitamin B$_{12}$ intake (μg/day) | 8.81 ± 4.65 |
| Folate intake (μg/day) | 287.4 ± 100.5 |
| Serum PLP concentration (pmol/mL) | 61.3 ± 29.1 |
| Serum vitamin B$_{12}$ concentration (pmol/mL) | 1.45 ± 0.45 |
| Serum folate concentration (pmol/mL) | 27.5 ± 10.3 |
| Plasma homocysteine concentration (nmol/mL) | 11.2 ± 5.1 |

Data are number of patients (categorized data) or mean ± SD (quantitative data).
BAP, bone-specific alkaline phosphatase; BMI, body mass index; Ins, insulin; OHA, oral hypoglycemic agents; PLP, pyridoxal 5′-phosphate; uNTX, urine N-terminal cross-linked telopeptide of type-I collagen.

**Table 2** | Correlations among dietary nutrient intake values, serum concentrations and plasma homocysteine levels adjusted for age and sex

| | $r$ | $P$ |
| --- | --- | --- |
| Correlations of total energy intake with various nutrient intakes | | |
| Vitamin B$_6$ (mg) | 0.521 | <0.001 |
| Vitamin B$_{12}$ (μg) | 0.253 | 0.005 |
| Folate (μg) | 0.331 | <0.001 |
| Correlations of intake values with serum concentrations | | |
| Vitamin B$_6$ | 0.192 | 0.034 |
| Vitamin B$_{12}$ | 0.336 | <0.001 |
| Folate | 0.400 | <0.001 |
| Correlations of plasma Hcy levels with B vitamins | | |
| Vitamin B$_6$ intake (mg) | −0.207 | 0.022 |
| Vitamin B$_{12}$ intake (μg) | −0.001 | 0.988 |
| Folate intake (μg) | −0.328 | <0.001 |
| Serum PLP concentration (pmol/mL) | 0.002 | 0.982 |
| Serum B$_{12}$ concentration (pmol/mL) | 0.001 | 0.993 |
| Serum folate concentration (pmol/mL) | −0.369 | <0.001 |

Hcy, homocysteine; PLP, pyridoxal 5′-phosphate.

determine independent factors for plasma Hcy levels. Dietary folate intake was a significant predictor of Hcy when dietary vitamin $B_6$, vitamin $B_{12}$ and folate intake values were included as independent variables ($R^2 = 0.088$, β-coefficient = −0.297, $P < 0.001$), and serum folate concentration also was a significant predictor of Hcy when serum PLP, vitamin $B_{12}$ and folate concentrations were included as independent variables ($R^2 = 0.121$, β-coefficient = −0.347, $P < 0.001$). We then evaluated the correlations of folate intake and the concentrations of folate and Hcy with intake of the various food groups determined by FFQ. Dietary folate intake and serum folate concentration were significantly associated with intakes of certain food groups including potato, green vegetables, other vegetables and fruits. Only intake of green vegetables was significantly correlated with the plasma Hcy level (Table 3).

Bone mineral density of lumbar spine (SP-BMD) and femoral neck (FN-BMD) were positively correlated with body mass index (BMI) and fat mass, although no significant correlations were found in diabetes-related parameters including fasting plasma glucose, HbA$_{1c}$ and diabetes duration (Table 4). Both SP-BMD and FN-BMD were positively correlated with fasting serum C-peptide, but these correlations were cancelled when adjusted for BMI. Urinary NTx, a marker of bone resorption, was negatively correlated with FN-BMD. As nutrient intake significantly increases with energy intake, nutrition intakes were also evaluated by adjusting for calories. As a result, calorie-adjusted folate intake was positively correlated with SP-BMD, although the association between calorie-adjusted folate and FN-BMD did not reach statistical significance. There were no significant associations between BMD of both sites and serum concentrations of vitamin $B_6$, vitamin $B_{12}$ and folate. The plasma Hcy concentration was negatively correlated with both

**Table 3** | Correlations of dietary folate intake, serum folate concentration and plasma homocysteine level with various food groups

|  | Dietary folate intake | | Serum folate concentration | | Plasma Hcy level | |
|---|---|---|---|---|---|---|
|  | r | P | r | P | r | P |
| Grain/rice | −0.076 | 0.399 | −0.086 | 0.341 | −0.056 | 0.538 |
| Potato | 0.470 | <0.001 | 0.220 | 0.014 | 0.012 | 0.895 |
| Green vegetables | 0.843 | <0.001 | 0.361 | <0.001 | −0.207 | 0.020 |
| Other vegetables | 0.620 | <0.001 | 0.197 | 0.027 | 0.077 | 0.390 |
| Fruits | 0.338 | <0.001 | 0.206 | 0.021 | 0.018 | 0.839 |
| Seaweeds | 0.322 | <0.001 | 0.072 | 0.426 | 0.071 | 0.435 |
| Beans/soy products | 0.390 | <0.001 | 0.156 | 0.083 | 0.016 | 0.856 |
| Seafood | 0.313 | <0.001 | 0.075 | 0.407 | −0.017 | 0.848 |
| Meats | 0.065 | 0.474 | 0.042 | 0.643 | −0.070 | 0.435 |
| Egg | 0.278 | 0.002 | 0.068 | 0.450 | −0.056 | 0.538 |
| Milk products | 0.108 | 0.230 | 0.113 | 0.208 | −0.035 | 0.698 |
| Oil/fat | 0.145 | 0.107 | 0.161 | 0.073 | −0.112 | 0.214 |

Hcy, homocysteine.

**Table 4** | Correlations of bone mineral density of lumbar spine and femoral neck with diabetes-related parameters, bone turnover markers and B vitamin status

|  | SP-BMD | | FN-BMD | |
|---|---|---|---|---|
|  | r | P | r | P |
| BMI (kg/m$^2$) | 0.288 | 0.001 | 0.463 | <0.001 |
| Fasting plasma glucose (mg/dL) | −0.149 | 0.098 | −0.113 | 0.210 |
| HbA$_{1c}$ (%) | 0.098 | 0.194 | 0.053 | 0.556 |
| Diabetes duration (years) | 0.082 | 0.366 | 0.057 | 0.528 |
| Fasting serum C-peptide (ng/mL) | 0.182 | 0.045 | 0.285 | 0.001 |
| BAP (U/L) | 0.112 | 0.218 | −0.061 | 0.499 |
| uNTx (nMBCE/mmol Cr) | −0.138 | 0.084 | −0.183 | 0.042 |
| Vitamin B$_6$ intake (mg) | −0.032 | 0.727 | −0.053 | 0.559 |
| Vitamin B$_6$ intake (mg/100 kcal) | 0.113 | 0.211 | 0.005 | 0.959 |
| Vitamin B$_{12}$ intake (μg) | 0.012 | 0.899 | 0.166 | 0.065 |
| Vitamin B$_{12}$ intake (μg/1000 kcal) | 0.054 | 0.554 | 0.148 | 0.102 |
| Folate intake (μg) | 0.103 | 0.256 | 0.112 | 0.216 |
| Folate intake (μg/1000 kcal) | 0.198 | 0.027 | 0.153 | 0.090 |
| Serum PLP concentration (pmol/mL) | −0.062 | 0.497 | −0.007 | 0.936 |
| Serum B$_{12}$ concentration (pmol/mL) | 0.023 | 0.799 | 0.058 | 0.524 |
| Serum folate concentration (pmol/mL) | 0.104 | 0.248 | 0.114 | 0.205 |
| Plasma Hcy concentration (nmol/mL) | −0.278 | 0.002 | −0.201 | 0.025 |

BAP, bone-specific alkaline phosphatase; BMI, body mass index; FN-BMD bone mineral density of femoral neck; Hcy, homocysteine; PLP, pyridoxal 5′-phosphate; SP-BMD, bone mineral density of lumbar spine; uNTX, urine N-terminal cross-linked telopeptide of type-I collagen.

SP-BMD and FN-BMD, showing that hyperhomocysteinemia is clearly associated with low BMD in patients with type 2 diabetes (Figure 1).

**(a)** SP-BMD (Z score)  $r = -0.278, P = 0.002$

**(b)** FN-BMD (Z score)  $r = -0.201, P = 0.025$
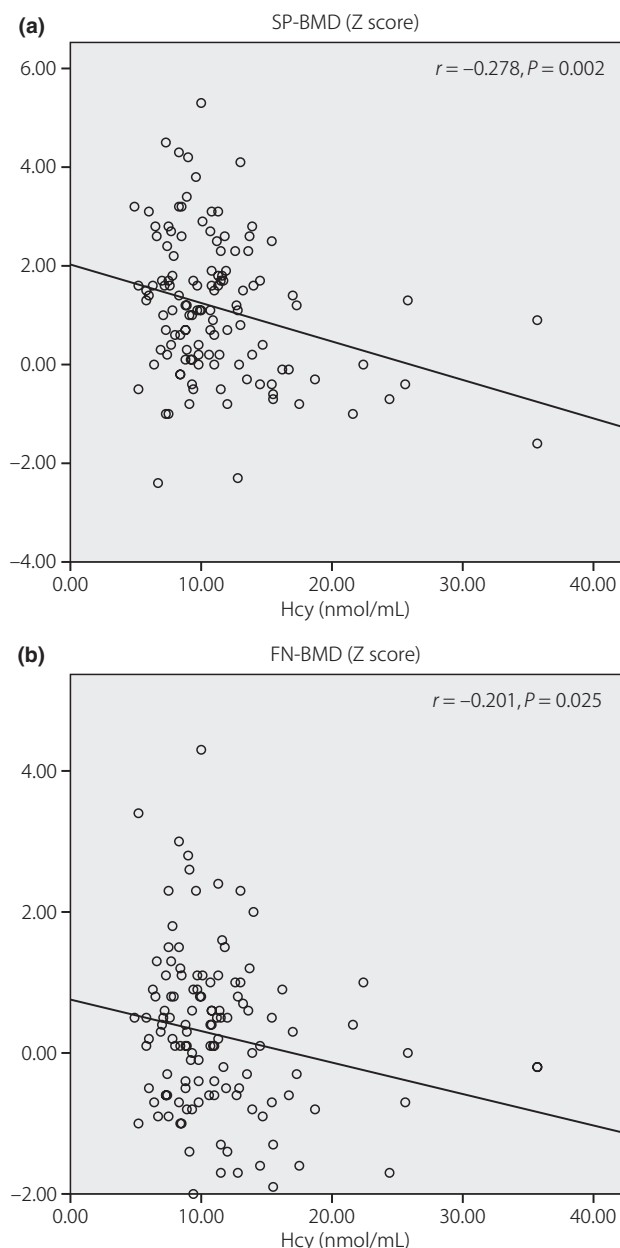
**Figure 1** | The relationship between homocysteine (Hcy) and bone mineral density of lumbar spine (SP-BMD) and femoral neck (FN-BMD).

As hyperhomocysteinemia derived from folate insufficiency has been suggested to be involved in low BMD, we compared clinical characteristics of the study population across the quartiles of Hcy (quartile 1, $n = 31$, Hcy < 8.3 nmol/mL; quartile 2, $n = 32$, Hcy 8.3 to <9.9 nmol/mL; quartile 3, $n = 32$, Hcy 9.9 to <12.8 nmol/mL; quartile 4, $n = 30$, Hcy > 12.8 nmol/mL). There were no significant differences across the quartiles in general clinical characteristics including age, BMI, diabetes-related parameters, energy intake, and vitamin B$_6$ and vitamin B$_{12}$ status (Table 5). However, SP-BMD and FN-BMD were significantly lower in patients in the highest quartile of Hcy than

**Table 5** | Comparison of clinical characteristics according to homocysteine quartiles adjusted for age and sex

| Hcy concentration (nmol/mL) | Quartile 1 (4.9–8.0) | Quartile 2 (8.1–9.9) | Quartile 3 (10.0–12.8) | Quartile 4 (12.8–35.7) | ANCOVA P |
|---|---|---|---|---|---|
| Male/female | 17/14 | 21/11 | 23/9 | 18/12 | |
| Age (years) | 59.3 ± 13.8 | 58.1 ± 12.6 | 63.9 ± 8.7 | 64.0 ± 13.4 | 0.212 |
| BMI (kg/m$^2$) | 25.0 ± 4.4 | 25.8 ± 5.0 | 25.0 ± 5.6 | 23.8 ± 4.5 | 0.461 |
| Fasting plasma glucose (mg/dL) | 158.8 ± 44.8 | 162.0 ± 45.8 | 155.6 ± 50.0 | 164.6 ± 55.4 | 0.721 |
| HbA$_{1c}$ (%) | 10.1 ± 2.3 | 9.9 ± 2.5 | 9.1 ± 1.8 | 9.4 ± 2.1 | 0.378 |
| Diabetes duration (years) | 9.5 ± 8.4 | 10.2 ± 9.7 | 12.6 ± 8.6 | 12.4 ± 9.0 | 0.183 |
| SP-BMD (Z score) | 1.34 ± 1.43* | 1.24 ± 1.38* | 1.39 ± 1.24* | 0.50 ± 1.18 | 0.037 |
| FN-BMD (Z score) | 0.45 ± 0.99** | 0.32 ± 1.23* | 0.26 ± 0.96* | −0.27 ± 1.03 | <0.001 |
| Energy intake (kcal/day) | 2161 ± 543 | 2145 ± 565 | 2069 ± 563 | 1910 ± 650 | 0.260 |
| Vitamin B$_6$ intake (mg) | 1.31 ± 0.35 | 1.26 ± 0.36 | 1.21 ± 0.32 | 1.09 ± 0.29 | 0.136 |
| Vitamin B$_{12}$ intake (μg) | 8.59 ± 3.44 | 8.86 ± 4.64 | 9.49 ± 5.24 | 8.27 ± 5.21 | 0.798 |
| Folate intake (μg) | 323.5 ± 92.2** | 287.2 ± 108.0* | 305.0 ± 91.8** | 231.7 ± 89.1 | 0.001 |
| Intake of green vegetables (g/day) | 101.9 ± 65.3* | 86.1 ± 60.6 | 89.3 ± 47.5 | 68.9 ± 49.2 | 0.043 |
| Serum PLP concentration (pmol/mL) | 65.0 ± 33.1 | 60.0 ± 24.6 | 58.9 ± 32.9 | 61.4 ± 26.2 | 0.943 |
| Serum B$_{12}$ concentration (pmol/mL) | 2.39 ± 0.88 | 2.90 ± 1.61 | 2.50 ± 0.73 | 2.53 ± 0.92 | 0.419 |
| Serum folate concentration (pmol/mL) | 33.6 ± 11.5** | 26.9 ± 7.6* | 26.9 ± 9.0* | 21.7 ± 8.7 | <0.001 |
| Plasma Hcy concentration (nmol/mL) | 6.9 ± 0.9** | 9.1 ± 0.5** | 11.3 ± 0.8** | 17.8 ± 6.1 | <0.001 |

BMI, body mass index; FN-BMD bone mineral density of femoral neck; Hcy, homocysteine; PLP, pyridoxal 5′-phosphate; SP-BMD, bone mineral density of lumbar spine. Mean ± SD, *$P < 0.05$, **$P < 0.01$ relative to the highest homocysteine quartile group.

those in patients in the other quartiles. Furthermore, patients in the highest Hcy quartile showed significantly decreased dietary folate intake, serum folate concentration and intake of green vegetables compared with those in the lower Hcy quartiles. Because the caloric intake was similar across the quartiles, the quality of the diet might be poor in the highest Hcy group. Quartile analysis revealed that the highest Hcy group showed the lowest BMD, the lowest serum folate concentration, the lowest folate intake and the lowest intake of green vegetables.

## DISCUSSION

In the present study, hyperhomocysteinemia was found to be clearly associated with low BMD in type 2 diabetes patients, as it has been reported to be in non-diabetic subjects[6–14]. Furthermore, folate insufficiency might be one of the important factors in hyperhomocysteinemia, as plasma Hcy levels were negatively correlated with both dietary intake and serum concentration of folate.

Osteoporosis is a multifactorial disease, a major health problem characterized by low BMD, deterioration of bone microarchitecture and increased risk of fracture. Elevation of Hcy is one of the important risk factors for osteoporosis[28,29], and can be caused by insufficiency of Hcy-related vitamins, such as folate, vitamin B$_6$ and vitamin B$_{12}$[6–14]. Because dietary risk factors can be improved when recognized, sufficiency of Hcy-related vitamins and its relationship to osteoporosis in patients with type 2 diabetes is of primary concern.

Elevation of Hcy can be caused by insufficiency of folate, vitamin B$_6$ or vitamin B$_{12}$, and the plasma Hcy level is considered to be a fairly sensitive index of folate metabolic status compared

with that of the other factors in non-diabetic subjects. Previous studies reported hyperhomocysteinemia was observed in 86% of subjects with clinically expressed folate deficiency[30]; folate is a major determinant of Hcy levels in healthy people[31,32] and vitamin B$_{12}$ influences Hcy levels less than folate does[33,34]. Folate, vitamin B$_6$ and vitamin B$_{12}$ are water-soluble vitamins, which are in general not readily stored and consistent daily intake is important. Usually, folate and vitamin B$_6$ deficiency develops within a month of insufficient intake. In contrast, it is known that patients with complete loss of intrinsic factor require 3–5 years to become overtly vitamin B$_{12}$ deficient[35]. Vitamin B$_{12}$ is a unique water-soluble vitamin, and because 80% of the 2.5 mg average whole body stock of vitamin B$_{12}$ is reserved in the liver and vitamin B$_{12}$ excreted in the bile and is effectively reabsorbed in the intestine, clinical signs of vitamin B$_{12}$ deficiency take a long time to appear and progress slowly[36]. Some patients in the present study were taking metformin, which is known to inhibit absorption of vitamin B$_{12}$[37], but there was no difference between the patients taking metformin and those not taking metformin. As to vitamin B$_6$, only a weak negative correlation between vitamin B$_6$ intake and Hcy was not enough to conclude that vitamin B$_6$ is a nutritional risk factor for osteoporosis, and there have been no other studies showing the effect of vitamin B$_6$ on BMD.

Leafy green vegetables, such as spinach and broccoli, are rich sources of folate. Folate is also contained in a variety of foods including fruits, beans, seaweeds, liver and egg yolk. To investigate the cause of folate insufficiency, we focused particularly on dietary sources of folate. We evaluated the association of dietary folate intake, serum folate concentration, and plasma Hcy level

with various food groups, and found that intake of green vegetables correlated well with folate status and Hcy levels. Furthermore, it was revealed by the quartile analysis that the highest Hcy group showed the lowest BMD, the lowest serum folate concentration, the lowest folate intake and the lowest intake of green vegetables. This analysis suggests that insufficient intake of green vegetables, but not insufficient caloric intake, causes folate insufficiency in the group with the highest Hcy.

The strength of the present study is that it is the first study to show that nutritional status of folate might affect the homocysteine level, a putative risk factor for osteoporosis, in Japanese patients with type 2 diabetes. The present study is also meaningful in promoting awareness of the importance of diet quality, because patients with diabetes are at high risk of developing osteoporosis. In contrast, the present study has some limitations. First, the sample size was not large enough for conclusions regarding marginal insignificant *P*-values. We estimated sample size using a correlation coefficient obtained from a previous cross-sectional study assessing the relationship between BMD and plasma Hcy[8]. The correlation coefficient of femoral BMD with Hcy was −0.18 and the sample size was estimated to be $n = 153$ (two-sided $\alpha = 0.1$, $\beta = 0.2$), while we analyzed 125 patients. Second, we only analyzed patients with type 2 diabetes and comparison with non-diabetic subjects is necessary. An unanswered question is whether diabetes modulates the effects of nutritional state of folate on Hcy metabolism, and the effects of Hcy levels on BMD. Finally, a longitudinal study is required to examine the effects of Hcy on rate of BMD loss and risk of fracture for a longer duration in patients with type 2 diabetes. It is also necessary to determine whether encouraging patients with higher Hcy levels to eat more green vegetables is useful as a dietary intervention to improve Hcy levels and BMD.

In conclusion, the present study shows that BMD inversely correlates to plasma Hcy levels in Japanese patients with type 2 diabetes, and that dietary intake and the serum concentration of folate are determinant factors of Hcy levels. When our group was analyzed across quartiles, BMD, serum folate concentration, folate intake and intake of green vegetables were lowest in the highest Hcy group. Taken together, in Japanese patients with type 2 diabetes, a diet low in green vegetables rather than a calorie-restricted diet might be the more important factor in the declining nutritional status of folate that increases the Hcy level, a putative risk factor for osteoporosis.

## ACKNOWLEDGEMENTS

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