Original Article

Hypovitaminosis D and K are highly prevalent and independent of overall malnutrition in the institutionalized elderly

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There have been methodological problems for studying hypovitaminosis D and K in the elderly. First, studies were done either by evaluating food intake or measuring their circulating levels, but rarely by both in Japan. In this paper, vitamin D and K intakes and their circulating levels were simultaneously determined. Second issue is whether hypovitaminosis D and K are independent of general malnutrition, prevalent in the elderly. We tried to statistically discriminate them by principal component analysis (PCA). Fifty institutionalized elderly were evaluated for their circulating 25 hydroxy-vitamin D (25OH-D), intact parathyroid hormone (PTH), phylloquinone (PK), menaquinone-7 (MK-7) levels, and their food intake. Although average vitamin D intake (7.0 μg/day) exceeded the Japanese Adequate Intake (AI) of 5.0 μg/day, average serum 25OH-D concentration was in the hypovitaminosis D range (11.1 ng/mL). Median vitamin K intake was 168 μg/day, approximately 2.5 times as high as AI for vitamin K. Nevertheless, plasma PK and MK-7 concentrations were far lower than those of healthy Japanese elderly over 70 years old. PCA yielded four components; each representing overall nutritional, vitamin K_2, vitamin D, and vitamin K_1 status, respectively. Since these components are independent of each other, vitamin D- and K-deficiency in these subjects could not be explained by overall malnutrition alone. In summary, institutionalized elderly had a high prevalence of hypovitaminosis D and K, and the simultaneous determination of their circulating level and dietary intake is mandatory in such studies. PCA would yield fruitful results for eliminating the interference by confounders in a cross-sectional study.

Key Words: hypovitaminosis D, hypovitaminosis K, principal component analysis, adequate intake, institutionalized elderly

INTRODUCTION

Vitamin D is of utmost importance in enhancing the intestinal absorption of calcium and phosphorus,[1,2] with its deficiency causing skeletal mineralization defect; rickets and osteomalacia. Recently, it has come to the general attention that inadequate supply of vitamin D, even in its milder form (vitamin D insufficiency), is associated with increased risk of fracture through negative calcium balance, hence secondary hyperparathyroidism.[1,2] Vitamin D insufficiency is also reported to be associated with muscle weakness. Recent clinical studies have indicated that intervention with vitamin D supplementation reduced the incidence of falling in elderly subjects.[3] Clinically important non-vertebral fractures, such as hip and wrist fractures are triggered by falling. Thus, vitamin D insufficiency would render the elderly subjects more prone to fracture through its effects both on the skeleton and muscle. Recently, lower serum level of 25 hydroxy-vitamin D (25OH-D) was reported to be a significant risk factor even for mortality.[4]

Vitamin D insufficiency is quite common in the elderly population,[5,6] and institutionalized elderly are at even higher risk for vitamin D insufficiency.[7-10] Factors hitherto postulated to be responsible include low dietary vitamin D intake,[7,9] reduced dermal capacity to produce vitamin D with aging and minimal sun exposure.[11,12]

In contrast to vitamin D, the skeletal action of vitamin K has called our attention only quite recently. The only biological action of vitamin K has been considered to be its role as the coenzyme of γ-glutamyl carboxylase (GGCX) in the liver, by which additional carboxyl group is introduced into the glutamic acid residue in four of the

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Table 1. Background profiles and results from blood tests of the study subjects.

	Total	Male	Female	*p* value
n	50	15	35	-
Age (y)	87.6±8.0 (88.5)	84.9±7.9 (83.0)	88.7±7.8 (90.0)	0.133
Level of care needed	3.6±1.1 (4.0)	3.3±1.0 (3.0)	3.7±1.2 (4.0)	0.228
Body height (cm)	144.0±11.6 (142.0)	157.0±7.8 (159.0)	138.4±7.8 (139.0)	< 0.01
Body weight (kg)	43.6±9.3 (43.2)	50.3±7.9 (49.9)	40.7±8.3 (38.1)	0.001
Body mass index (kg/m2)	21.0±3.8 (20.1)	20.5±3.4 (19.6)	21.3±4.0 (20.2)	0.476
Serum albumin (g/dL)	3.7±0.4 (3.7)	3.8±0.4 (3.9)	3.6±0.4 (3.6)	0.136
Serum total cholesterol (mg/dL)	184±37 (184)	186±26 (195)	183±41 (183)	0.828
Serum triglyceride (mg/dL)	98±41 (92)	96±47 (75)	98±39 (93)	0.403
Serum aminotransferase (U/L)	22±11 (19)	20±7 (17)	22±12 (19)	0.603
Serum alanine aminotransferase (U/L)	16±10 (13)	16±7 (13)	16±12 (12)	0.235
eGFR (mL/min./1.73m2)	61±20 (60)	67±19 (67)	59±21 (57)	0.208
Serum 25-hydroxyvitamin D (ng/mL)	11.1±3.1 (11.2)	10.3±3.5 (9.3)	11.5±3.0 (11.6)	0.274
Serum parathyroid hormone (pg/mL)	30.8±11.8 (30.0)	29.9±11.1 (31.0)	31.3±12.2 (30.0)	0.736
Plasma phylloquinone (ng/mL)	0.73±0.70 (0.58)	0.62±0.29 (0.60)	0.77±0.82 (0.53)	0.992
Plasma menaquinone-7 (ng/mL)	0.53±0.37 (0.45)	0.59±0.47 (0.47)	0.51±0.32 (0.44)	0.849

Data are expressed as mean±SD with the values in parentheses showing the median.
Comparison of indices between males and females were done by unpaired t test or Mann-Whitney test depending on normality.
eGFR; estimated Glomerular Filtration Rate.

Table 2. Daily dietary intakes of the study subjects.

	Total	Male	Female	*p* value
Energy (kcal)	1322±159 (1387)	1374±96 (1416)	1300±175 (1386)	0.160
Protein (g)	51.0±5.8 (53.3)	53.1±3.6 (54.6)	50.2±6.3 (53.5)	0.091
Fat (g)	32.8±3.9 (34.6)	34.2±2.4 (35.3)	32.2±4.3 (34.5)	0.095
Carbohydrates (g)	178±20 (186)	185±12 (189.7)	175±21 (186)	0.093
Calcium (mg)	494±53 (504)	503±50 (506)	490±54 (502)	0.157
Vitamin D (μg)	7.0±1.4 (7.7)	7.4±0.9 (7.8)	6.9±1.5 (7.6)	0.107
Vitamin K (μg)	155±30 (168)	164±19 (172)	151±33 (168)	0.107

Data are expressed as mean±SD with the values in parentheses showing the median. Comparison of indices between male and women were done by unpaired t test or Mann-Whitney test depending on normality.

blood coagulation factors (II, VII, IX, X) to yield γ-glutamic carboxyl (Gla) residue.[13] Other extrahepatic proteins are also γ-carboxylated by GGCX, such as osteocalcin (bone Gla protein; BGP) and matrix gla protein (MGP).[14] Recent evidences suggest that vitamin K deficiency is associated with increased risk of fracture. When subjects were categorized into quartiles according to their vitamin K intake, fracture risk in the lowest quartile was twice as high as that in the highest quartile.[15] The age-adjusted incidence of vertebral fracture was significantly higher in subjects with low plasma phylloquinone levels than those with high plasma levels in Japanese women.[16] In addition, the association of circulating vitamin K level and bone mineral density (BMD) has also been reported. For example, low plasma phylloquinone concentration was associated with low BMD at the femoral neck in men, and lower spine BMD in postmenopausal women without estrogen replacements.[17] High serum concentration of undercarboxyled osteocalcin (ucOC), which is a sensitive indicator of skeletal vitamin K insufficiency, was a significant risk factor of hip fracture independent of BMD.[18,19]

Plasma phylloquinone level is subject to alteration by aging,[20,21] and elderly subjects have been reported to have low plasma phylloquinone concentrations.[22] Of note is the report that elderly nursing home residents generally had a poor dietary vitamin K intake compared to the ambulatory elderly.[23]

Studies on the role of hypovitaminosis D and K in the elderly, especially the institutionalized ones are greatly hampered by the fact that they are also generally malnourished. Arguments against the significance of these vitamins have been made that decreased serum concentrations of these vitamins is merely a reflection of overall malnutrition. In this paper, we have tried to statistically discriminate hypovitaminosis D and K from general malnutrition by using principal component analysis (PCA), which has been employed in clinical nutrition for the analyses of dietary pattern.[24,25]

MATERIALS AND METHODS

Subjects

The study subjects were 50 institutionalized elderly (male 15, female 35) in a nursing home, Kayu-Shirakawa. Exclusion criteria were routine medication that has potential interference with vitamin D or vitamin K status. Detailed information about this study was given and written consent was obtained from the subject or the proxy. The study protocol was approved by the ethical committee in Kyoto Women's University.

Laboratory Data

Blood was obtained after overnight fasting. After centrifugation, serum was kept frozen at –30°C until analysis. Serum concentration of 25OH-D was measured by radioimmunoassay (RIA) (DiaSorin, Stillwater, MN, USA). Circulating level of intact parathyroid hormone (PTH) was measured by electro chemiluminescent immunoassay (ECLIA) (Roche Diagnostics, Mannheim, Germany). Plasma vitamin K_1 (phylloquinone; PK), and menaquinone-7 (MK-7) levels were determined by high-performance liquid chromatography-tandem mass-mass spectrometry with atmospheric pressure chemical ionization (LC-APCI-MS/MS) using a HPLC system (Shimadzu, Kyoto, Japan) and API3000 LC-MS/MS System (Applied Biosystems, Foster City, CA) with ^{18}O-labeled vitamin K as the internal standard.[26]

Nutrition Intake Study

Since the subjects were institutionalized and their diet was supplied from the institution, their nutrients and energy intake were calculated by multiplying the supplied nutrients on the basis of the Standard Tables of Food Composition in Japan, 5th ed. with the average percentage intake in a preceding month by the staff.[27] Percentage intake was assessed for each subject at every meal, and the monthly average percentage intake was calculated. Based on these records, their intake of energy and nutrients was calculated using software (Healthy Maker Pro 501, Mushroom Software Corp, Okayama, Japan).

Statistical Analyses

Statistical analyses were performed with SPSS 15.0J (SPSS Japan Inc., Tokyo, Japan). Comparison of two independent groups was made with Student's t-test or Mann-Whitney test depending on normality. Multiple regression analyses by stepwise method were performed to determine independent factors for circulating levels of vitamin D and K levels. The relationship between various nutritional indices and circulating vitamin D- and K- levels was analyzed with principal component analysis (PCA), which is a statistical method to summarize the various parameters into a small number of summary factors (components). These components are obtained in such a way that the first component is extracted from the initial raw data with the maximal amount of information (eigenvalue), and the second one is extracted from the remaining information. Therefore, each component is mutually independent. Components with the eigenvalue greater than 1 were adopted, as in usual practice.

RESULTS

Biochemical markers and Circulating Concentrations of Vitamin D and K

Baseline characteristics and data from blood examination are shown in Table 1. There was no gender difference in the age and level of care needed, which is a 5-grade score in the long-term care insurance in Japan with a higher number indicating the need for more intensive care. The level of care needed was higher than grade 3 in 78% of subjects. Most of the present subjects required wheelchair for transportation. Body height and body weight were significantly higher in males than in females. Body mass index (BMI), or serum albumin, total cholesterol and triglyceride concentrations did not significantly differ between the two groups. Generally, serum albumin level less than 3.5 g/dL is considered to indicate malnutrition. Serum albumin level was below this value in 26% of subjects. Inasmuch as the advanced age and high level of care needed, nutritional parameters remained within the reference range in most of the subjects. None of the study subjects had severe hepatic or renal dysfunction. There is a general consensus that a serum 25OH-D concentration less than 20 ng/mL indicates hypovitaminosis D.[2] Serum 25OH-D concentration was <10 ng/mL in 40% of subjects, 10-20 ng/mL in 58%, and ≥20 ng/mL in only one subject. None of the subjects had a serum PTH level above the cut-off value (65 pg/mL). Plasma PK and MK-7 concentrations in all of the subjects were 0.73±0.70 ng/mL and 0.53±0.37 ng/mL, respectively. In the present study, serum PK was less than 1 ng/ml and serum MK-7 was less than 1 ng/ml, in 85% and 90% of the subjects, respectively. The interpretation for these values will be given in the "Discussion" section. There were no gender differences in plasma vitamin K levels, serum 25OH-D or PTH.

Nutritional intake in the study subjects

The nutrients intake in the males and females were not statistically different as shown in Table 2. During the preparation of this paper, Dietary Reference Intake (DRI) for Japanese 2010 (DRI 2010) was released on May 29, 2009.[28] Since this work was done in 2006, however, consideration is made basically according to DRI 2005.[29] The intake of macronutrients such as protein, fat and carbohydrates appeared appropriate for their age and sex. The adequate intakes (AI) for calcium in Japan are 750 mg for men and 650 mg for women over 70 years. The AI for vitamin D is 5 μg/day, and that for vitamin K is 75 μg/day for men and 65 μg/day for women respectively. Although average calcium intakes in both groups were lower than the AI in DRI 2005, the average daily vitamin D intake was 7.0 μg, which is 140% of the AI in DRI 2005. The average daily intake of vitamin K in whole subjects was 155 μg, which is more than twice the AI for each gender. Thus, apparently these subjects had sufficient intakes of vitamin D and K based on AI in DRI 2005.

Multiple regression analyses for the determination of independent factor for circulating vitamin D, K concentrations.

In multiple regression analyses, vitamin D intake was a significant determinant of serum 25OH-D level, although the R^2 was low. Serum triglyceride level was the only significant predictor for plasma MK-7 concentration, and vitamin K intake and serum triglyceride concentrations significantly contributed to plasma PK level (Table 3).

Principal Component Analysis (PCA)

Since institutionalized elderly are generally malnourished, it is quite important to determine whether the low vitamin D - and K -status is independent of overall malnutrition or not. Then PCA was performed with the parameters included for analysis being serum albumin, triglyceride, cholesterol, 25OH-D, PTH levels and plasma PK, MK-7

Table 3. Multiple regression analyses for the determination of independent factors for circulating vitamin D, K concentrations.

	R^2	*p* value	Variable	β	*p* value
Serum 25OH-D	0.095	0.033	Vitamin D intake	0.309	0.033
Plasma PK	0.181	0.011	Vitamin K intake	0.290	0.042
			Triglyceride	0.380	0.009
Plasma MK-7	0.255	<0.001	Triglyceride	0.505	<0.001

Only significant predictors are shown. The abbreviations are β for β coefficient, and *p* for *p* value. Independent predictor for serum 25OH-D or plasma PK, MK-7 concentrations was analyzed by multivariate analysis with stepwise regression. Age, level of care needed and serum triglyceride and total cholesterol concentrations were included in all analyses. Vitamin D intake was additionally included in the analysis for plasma 25OH-D concentration. For plasma PK and MK-7, vitamin K intake was additionally included.

Table 4. Principal component analysis of nutrition indices

	Component 1	Component 2	Component 3	Component 4
Serum Albumin	0.880	0.004	0.047	0.059
Serum triglyceride	0.229	0.734	0.119	0.380
Serum total cholesterol	0.800	0.320	-0.046	-0.060
Serum 25OH-D	0.434	-0.457	-0.658	-0.033
Serum PTH	0.156	-0.273	0.877	-0.090
Plasma PK	-0.014	0.030	-0.071	0.986
Plasma MK-7	0.117	0.832	-0.238	-0.152

Factor loadings to four components after varimax rotation are shown. Loadings greater than 0.35 are shown in bold
Four components thus obtained were considered to represent the following nutritional status; component 1: overall nutritional status, component 2: vitamin K_2 status, component 3: vitamin D status, and component 4: vitamin K_1 status.

concentrations. Four components were obtained and explained 82% of the variance. The first component was composite of high albumin, total cholesterol and 25OH-D, and second component consisted of high triglyceride, low 25OH-D, and high MK-7. The third component was composite of low 25OH-D and high PTH, and the fourth component was composed of high triglyceride and high PK. The interpretation of each component was made as follows; the first component representing overall nutritional status, the second component, vitamin K_2 status, the third component, vitamin D status, and the fourth component representing vitamin K_1 status (Table 4).

DISCUSSION

Nutritional status would be adequately assessed by both evaluating the subjects' food intake and measuring their circulating or urinary markers. This principle would hold true especially in the elderly, since they are at high risk for malabsorption or utilization defects of nutrients. Unfortunately in Japan, vitamin D and K status in the elderly has been studied either by evaluating their food intake, as in the annual National Nutrition Survey Japan (NNS-J) or by measuring circulating level of these vitamins,[21,30-33] but rarely by both.[12,34]

Institutionalized elderly have been our special concern, since they are much more susceptible to hypovitaminosis D and K deficiency than the healthy elderly. The NNS-J in 2006 showed that subjects over 70 years of age, including both genders, had the following daily nutrients intakes: energy 1761 kcal, calcium 551 mg, vitamin D 9.0 μg, vitamin K 273 μg,[35] which were higher than those of the subjects in the present study. Gastrointestinal absorption of nutrients in the present study subjects would be impaired also. These considerations led us to simultaneously evaluate both vitamin D and K intakes and its circulating levels in the present study.

Before the interpretation of our data, determination procedure for vitamin K deserves some discussion. There have been discrepancies on the plasma concentration of vitamin K in the previous literature, which is at least partly due to the different determination procedure employed. Recently we have developed a novel procedure for the determination of vitamin K analogs with high sensitivity and specificity, based on high-performance liquid chromatography-tandem mass-mass spectrometry with atmospheric pressure chemical ionization (LC-APCI-MS/MS).[26] With this procedure, plasma concentrations of PK and MK-7 were 0.73±0.70 ng/mL (median 0.58 ng/mL) and 0.53±0.37 ng/mL (median 0.45 ng/mL), respectively in the current study. In our recent study, plasma concentrations for PK and MK-7 were 1.29±1.09 ng/mL (median 0.94 ng/mL) and 4.21±6.81 ng/mL (median 2.14ng/mL), respectively in the healthy Japanese elderly over 70 years old using the same assay procedure.[21] In the same study, lowest concentration of plasma vitamin K level to avoid the elevation of serum ucOC concentration was 2.5 ng/ml for PK and 6.4 ng/ml for MK-7.[21] Since serum ucOC level is a sensitive indicator of skeletal vitamin K insufficiency, these figures can yield a rough estimate of circulating vitamin K levels needed by the skeleton.

The median intake of vitamin K in the current subjects was 168 μg, which was more than twice the AI in DRI 2005. The AI for vitamin K was not altered in DRI 2010. Dietary vitamin K intake has been identified as an important determinant of plasma phylloquinone concentration

in previous studies.[36,37] In the present study, vitamin K intake was also significantly associated with plasma PK, but not with plasma MK-7. Since they were not supplied with fermented soybean; natto, which contains exraordinary amount of MK-7,[38] phylloquinone from green vegetables is likely to be the major contributors to the total vitamin K intake in our subjects. Thus plasma PK alone correlated with total vitamin K intake, adjusted by serum triglyceride. These data strongly suggest that these subjects are vitamin K-deficient in spite of the fact that their dietary intake is far above the AI in according to DRI 2005, and increased vitamin K intake would be effective in improving plasma PK levels in institutionalized elderly in present study.

As in the case of vitamin K, average dietary intake of vitamin D was around 7 μg/day, which is approximately 140% of the AI in subjects in the present study. Nevertheless, the average serum 25OH-D concentration was only 11.1 ng/mL. Thus, most subjects in the present study had hypovitaminosis D in spite of apparently sufficient vitamin D intake.

Although the multiple regression analysis has identified vitamin D intake as the significant contributor to serum 25OH-D concentration, the R^2 value was low, which indicates that the current model could explain only a small portion of variation. Several factors could be responsible for the above results. First, because of walking disability and other physical dysfunction, the chance of sun exposure was minimal in most of the current study subjects, but it was not null. Thus, sun exposure may also partly explain the above results. Unfortunately, however, detailed information about sun exposure was unavailable. Furthermore, ADL itself has been reported to be related to serum 25OH-D levels,[39] on which detailed information is not available in the current study. Secondly, the intestinal absorption of vitamin D is likely to decrease due to factors such as compromised intestinal ability for nutrients absorption and limited fat intake.[40] Nevertheless, oral vitamin D intake seems to be of value in the institutionalized elderly for improving their vitamin D status. Cashman *et al.* reported dose-dependent increase in serum 25OH-D concentration after incremental supplementation with vitamin D_3 in free-living adults over 64 years of age.[41] Although AI for vitamin D slightly increased to 5.5 μg/day in recently issued DRI 2010, the elderly subjects are likely to require much more vitamin D intake to avoid hypovitaminosis D considering the various problems to interfere with absorption and utilization as discussed above. A second issue with regard to the above discussion; disturbed intestinal absorption and limited fat intake, will also apply to the discrepant intake and circulating level of vitamin K.

Although serum 25OH-D level was extremely low, average serum PTH level was within the reference range. Circulating 25OH-D concentrations showed significant negative correlation with serum PTH levels ($r = -0.293$, $p = 0.041$; data not shown), which suggests that the negative feedback regulation of PTH secretion by vitamin D is not impaired in the current population. Kuchuk *et al.* reported that the elevation of serum PTH concentration by vitamin D deficiency is moderate in its magnitude, and usually fell into the reference range.[42] Thus they stressed the importance of serum 25OH-D level, and argued that for bone health maintenance and physical performance in the elderly, serum 25OH-D concentration above 50-60 nmol/L (20-24 ng/mL) was required.

Although the institutionalized elderly are considered to be generally malnourished,[43-45] nutritional status appeared rather satisfactory in the present study subjects in face of hypovitaminosis D and K. Then we analyzed the relationship between the overall nutrition and circulating levels of vitamin D and K by PCA. The PCA have yielded four components representing: overall nutritional status, vitamin D status, vitamin K_2 status, and vitamin K_1 status respectively. Serum 25OH-D also exhibited some association with the first component, representing the overall nutritional status. One of the reasons for the above results would be that 25OH-D is bound to vitamin D-binding protein (DBP) and albumin during its transport in circulation.[46] Since these components are independent of each other by their definition, these results suggest that hypovitaminosis D and K in the institutionalized elderly do not merely reflect general malnutrition, and have their own role. Confounders are serious challenge in the clinical studies. In the intervention studies, randomization would eliminate the interference by the confounders. It would be less problematic in the case of cohort studies. Adjustment for confounders is quite difficult in the cross-sectional studies like the current one. Multivariate analyses such as PCA would be of help in eliminating the interference by confounders in this type of studies.

In conclusion, institutionalized elderly had high prevalence of hypovitaminosis D and K in spite of their dietary intake exceeding the AI in DRI 2005 in Japan, which suggests that the requirement for these vitamins would be higher in these subjects. Additionally, hypovitaminosis D and K were shown to be independent of general malnutrition by PCA, which would be a useful analytical procedure for eliminating the interference by confounders in cross sectional studies.

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AUTHOR DISCLOSURES

None of the authors have any conflicts of interest.

REFERENCES

1. Bilezikian JP, Silverberg SJ. The role of parathyroid hormone and vitamin D in the pathogenesis of osteoporosis. In: Marcus R, Feldman D, Kelsey J, editors. Osteoporosis 2nd ed. San Diego CA: Academic Press; 2001. pp.71-84.
2. Lips P. How to define normal values for serum concentrations of 25-hydroxyvitamin D? An overview. In: Feldman D, Pike JW, Glorieux FG, editors. Vitamin D 2nd ed. San Diego CA: Academic Press; 2005. pp. 1019-28.
3. Bischoff-Ferrari HA, Dawson-Hughes B, Willett WC, Staehelin HB, Bazemore MG, Zee RY, et al. Effect of Vitamin D on falls: a meta-analysis. JAMA. 2004;291:1999-2006.
4. Kuroda T, Shiraki M, Tanaka S, Ohta H. Contributions of 25-hydroxyvitamin D, co-morbidities and bone mass to mortality in Japanese postmenopausal women. Bone. 2009; 44:168-72.

5. Eastell R, Lawrence Riggs B. Vitamin D and osteoporosis. In: Feldman D, Pike JW, Glorieux FG, editors. Vitamin D 2nd ed. San Diego CA: Academic Press; 2005. pp.1101-20.
6. Nakamura K, Nishiwaki T, Ueno K, Yamamoto M. Age-related decrease in serum 25-hydroxyvitamin D concentrations in the frail elderly: a longitudinal study. J Bone Miner Metab. 2007;25:232-6.
7. Lips P, van Ginkel FC, Jongen MJ, Rubertus F, van der Vijgh WJ, Netelenbos JC. Determinants of vitamin D status in patients with hip fracture and in elderly control subjects. Am J Clin Nutr. 1987;46:1005-10.
8. Lips P, Wiersinga A, van Ginkel FC, Jongen MJ, Netelenbos JC, Hackeng WH, et al. The effect of vitamin D supplementation on vitamin D status and parathyroid function in elderly subjects. J Clin Endocrinol Metab. 1988;67:644-50.
9. Suominem M, Laine T, Routasalo P, Pitkala KH, Rasanen L. Nutrient content of served food, nutrient intake and nutritional status of residents with dementia in a finnish nursing home. J Nutr Health Aging. 2004;8:234-8.
10. Przybelski R, Agrawal S, Krueger D, Engelke JA, Walbrun F, Binkley N. Rapid correction of low vitamin D status in nursing home residents. Osteoporos Int. 2008;19:1621-8.
11. Holick MF, Matsuoka LY, Wortsman J. Age, vitamin D, and solar ultraviolet. Lancet. 1989;2:1104-5.
12. Nashimoto M, Nakamura K, Matsuyama S, Hatakeyama M, Yamamoto M. Hypovitaminosis D and hyperparathyroidism in physically inactive elderly Japanese living in nursing homes: relationship with age, sunlight exposure and activities of daily living. Aging Clin Exp Res. 2002;14:5-12.
13. Vermeer C. Gamma-carboxyglutamate-containing proteins and the vitamin K-dependent carboxylase. Biochem J. 1990; 266:625-36.
14. Booth SL. Skeletal functions of vitamin K-dependent proteins: not just for clotting anymore. Nutr Rev. 1997;55:282-4.
15. Booth SL, Tucker KL, Chen H, Hannan MT, Gagnon DR, Cupples LA, et al. Dietary vitamin K intakes are associated with hip fracture but not with bone mineral density in elderly men and women. Am J Clin Nutr. 2000;71:1201-8.
16. Tsugawa N, Shiraki M, Suhara Y, Kamao M, Ozaki R, Tanaka K, et al. Low plasma phylloquinone concentration is associated with high incidence of vertebral fracture in Japanese women. J Bone Miner Metab. 2008;26:79-85.
17. Booth SL, Broe KE, Peterson JW, Cheng DM, Dawson-Hughes B, Gundberg CM, et al. Associations between vitamin K biochemical measures and bone mineral density in men and women. J Clin Endocrinol Metab. 2004;89:4904-9.
18. Seibel MJ, Robins SP, Bilezikian JP. Serum undercarboxylated osteocalcin and the risk of hip fracture. J Clin Endocrinol Metab. 1997;82:717-8.
19. Vergnaud P, Garnero P, Meunier PJ, Bréart G, Kamihagi K, Delmas PD. Undercarboxylated osteocalcin measured with a specific immunoassay predicts hip fracture in elderly women: the EPIDOS Study. J Clin Endocrinol Metab. 1997; 82:719-24.
20. Sokoll LJ, Sadowski JA. Comparison of biochemical indexes for assessing vitamin K nutritional status in a healthy adult population. Am J Clin Nutr. 1996;63:566-73.
21. Tsugawa N, Shiraki M, Suhara Y, Kamao M, Tanaka K, Okano T. Vitamin K status of healthy Japanese women: age-related vitamin K requirement for gamma-carboxylation of osteocalcin. Am J Clin Nutr. 2006;83:380-6.
22. Sadowski JA, Hood SJ, Dallal GE, Garry PJ. Phylloquinone in plasma from elderly and young adults: factors influencing its concentration. Am J Clin Nutr. 1989;50:100-8.
23. Tse SL, Chan TY, Wu DM, Cheung AY, Kwok TC. Deficient dietary vitamin K intake among elderly nursing home residents in Hong Kong. Asia Pac J Clin Nutr. 2002;11:62-5.
24. Northstone K, Ness AR, Emmett PM, Rogers IS. Adjusting for energy intake in dietary pattern investigations using principal components analysis. Eur J Clin Nutr. 2008;62: 931-8.
25. Kesse-Guyot E, Bertrais S, Péneau S, Estaquio C, Dauchet L, Vergnaud AC, et al. Dietary patterns and their sociodemographic and behavioural correlates in French middle-aged adults from the SU.VI.MAX cohort. Eur J Clin Nutr. 2009;63:521-9.
26. Suhara Y, Kamao M, Tsugawa N, Okano T. Suhara Y, Kamao M, et al. Method for the determination of vitamin K homologues in human plasma using high-performance liquid chromatography-tandem mass spectrometry. Anal Chem. 2005;77:757-63.
27. Resources Council, Science and Technology Agency, Japan. Standard Tables of Food Composition in Japan, 5th ed. Tokyo: Printing Bureau, Ministry of Finance; 2000. 2009/11/4 [cited 2000/11/22]; Available from: http://www.mext.go.jp/english/news/2000/11/001150.htm#top. (in Japanese).
28. Ministry of Health, Labour, and Welfare, Japan. Dietary reference intakes for Japanese 2010. Tokyo: Daiichi-Shuppan; 2010. 2009/11/4 [cited 2009/5/29]; Available from: http://www-bm.mhlw.go.jp/shingi/2009/05/s0529-4.html. (in Japanese).
29. Ministry of Health, Labour, and Welfare, Japan. Dietary reference intakes for Japanese 2005. Tokyo: Daiichi-Shuppan; 2005. 2009/11/4 [cited 2004/11/22]; Available from: http://www.mhlw.go.jp/houdou/2004/11/h1122-2.html. (in Japanese).
30. Nakamura K, Nashimoto M, Yamamoto M. Summer/winter differences in the serum 25-hydroxyvitamin D3 and parathyroid hormone levels of Japanese women. Int J Biometeorol. 2000;44:186-9.
31. Nakamura K, Nashimoto M, Hori Y, Yamamoto M. Serum 25-hydroxyvitamin D concentrations and related dietary factors in peri- and postmenopausal Japanese women. Am J Clin Nutr. 2000;71:1161-5.
32. Nakamura K, Nashimoto M, Yamamoto M. Are the serum 25-hydroxyvitamin D concentrations in winter associated with forearm bone mineral density in healthy elderly Japanese women? Int J Vit Nutr Res. 2001;71:25-9.
33. Kaneki M, Hodges SJ, Hosoi T, Fujiwara S, Lyons A, Crean SJ, et al. Japanese fermented soybean food as the major determinant of the large geographic difference in circulating levels of vitamin K2: possible implications for hip-fracture risk. Nutrition. 2001;17:315-21.
34. Nakamura K, Tsugawa N, Saito T, Ishikawa M, Tsuchiya Y, Hyodo K, et al. Vitamin D status, bone mass, and bone metabolism in home-dwelling postmenopausal Japanese women: Yokogoshi Study. Bone.2008;42:271-7.
35. Ministry of Health, Labour, and Welfare, Japan. The National Nutrition Survey 2006. Tokyo: Daiichi-Shuppan; 2009. 2009/11/4 [cited 2008/04]; Available from: http://www.mhlw.go.jp/houdou/2008/04/h0430-2.html. (in Japanese).
36. McKeown NM, Jacques PF, Gundberg CM, Peterson JW, Tucker KL, Kiel DP, et al. Dietary and nondietary determinants of vitamin K biochemical measures in men and women. J Nutr. 2002;132:1329-34.
37. Shea MK, Benjamin EJ, Dupuis J, Massaro JM, Jacques PF, D'Agostino RB Sr, et al. Genetic and non-genetic correlates of vitamins K and D. Eur J Clin Nutr. 2009;63:458-64.
38. Ikeda H, Doi Y. A vitamin-K2-binding factor secreted from Bacillus subtilis. Eur J Biochem. 1990;28:219-24.
39. Nakamura K, Nishiwaki T, Ueno K, Yamamoto M. Serum 25-hydroxyvitamin D levels and activities of daily living in

noninstitutionalized elderly Japanese requiring care. J Bone Miner Metab.2005;23:488-94.
40. Kuwabara A, Tanaka K, Tsugawa N, Nakase H, Tsuji H, Shide K, et al. High prevalence of vitamin K and D deficiency and decreased BMD in inflammatory bowel disease. Osteoporos Int. 2009;20:935-42.
41. Cashman KD, Wallace JM, Horigan G, Hill TR, Barnes MS, Lucey AJ, et al. Estimation of the dietary requirement for vitamin D in free-living adults >=64 y of age. Am J Clin Nutr. 2009;89:1366-74.
42. Kuchuk NO, Pluijm SM, van Schoor NM, Looman CW, Smit JH, Lips P. Relationships of serum 25-hydroxyvitamin D to bone mineral density and serum parathyroid hormone and markers of bone turnover in older persons. J Clin Endocrinol Metab.2009;94:1244-50.
43. Visvanathan R. Under-nutrition in older people: a serious and growing global problem!Visvanathan R. J Postgrad Med. 2003;49:352-60.
44. Kagansky N, Berner Y, Koren-Morag N, Perelman L, Knobler H, Levy S. Poor nutritional habits are predictors of poor outcome in very old hospitalized patients. Am J Clin Nutr. 2005;82:784-91.
45. Vellas B, Lauque S, Andrieu S, Nourhashemi F, Rolland Y, Baumgartner R, et al. Nutrition assessment in the elderly. Curr Opin Clin Nutr Metab Care. 2001;4:5-8.
46. Bikle DD, Gee E, Halloran B, Kowalski MA, Ryzen E, Haddad JG. Assessment of the free fraction of 25-hydroxyvitamin D in serum and its regulation by albumin and the vitamin D-binding protein. J Clin Endocrinol Metab. 1986;63:954-9.

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Depressed expression of Klotho and FGF receptor 1 in hyperplastic parathyroid glands from uremic patients

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Fibroblast growth factor 23 (FGF23) exerts its effect by binding to its cognate FGF receptor 1 (FGFR1) in the presence of its co-receptor Klotho. Parathyroid glands express both FGFR1 and Klotho, and FGF23 decreases parathyroid hormone gene expression and hormone secretion directly. In uremic patients with secondary hyperparathyroidism (SHPT), however, parathyroid hormone secretion remains elevated despite extremely high FGF23 levels. To determine the mechanism of this resistance, we measured the expression of Klotho, FGFR1, and the proliferative marker Ki67 in 7 normal and 80 hyperplastic parathyroid glands from uremic patients by immunohistochemistry. All uremic patients had severe SHPT along with markedly high FGF23 levels. Quantitative real-time reverse transcription PCR showed that the mRNA levels for Klotho and FGFR1correlated significantly with their semi-quantitative immunohistochemical intensity. Compared with normal tissue, the immunohistochemical expression of Klotho and FGFR1 decreased, but Ki67 expression increased significantly in hyperplastic parathyroid glands, particularly in glands with nodular hyperplasia. These results suggest that the depressed expression of the Klotho–FGFR1 complex in hyperplastic glands underlies the pathogenesis of SHPT and its resistance to extremely high FGF23 levels in uremic patients.

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KEYWORDS: chronic kidney disease; FGF23; FGFR1; Klotho; parathyroid; secondary hyperparathyroidism

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Secondary hyperparathyroidism (SHPT) is a common complication of chronic kidney disease. Phosphate retention, hypocalcemia, and calcitriol deficiency have long been considered to contribute to the pathogenesis of SHPT.[1–3] In addition, recent data suggest that fibroblast growth factor 23 (FGF23), a novel phosphaturic hormone, has a central role in the progressive decline of calcitriol levels and in the concomitant parathyroid hormone (PTH) hypersecretion in chronic kidney disease.[4–6]

FGF23 is a 32 kD protein that is mainly produced by osteocytes,[7] and it exerts its hormonal effects by binding to its cognate fibroblast growth factor receptor 1 (FGFR1) in the presence of its obligatory co-receptor, Klotho.[8,9] Klotho is a transmembrane protein that determines the tissue specificity of FGF23. In the kidney, FGF23 interacts with the Klotho–FGFR1 complex present in the distal tubule,[10] and thereby presumably inhibits sodium-dependent transporter and 1α-hydroxylase (CYP27B1) activities in the proximal tubule, leading to phosphaturia and reduced synthesis of calcitriol.[11,12] In patients with chronic kidney disease, FGF23 levels increase as kidney function declines to help maintain normal serum phosphate levels, but this results in the aggravation of SHPT because of decreased feedback inhibition by calcitriol.[4–6] Once on dialysis, serum FGF23 levels markedly increase in response to hyperphosphatemia and calcitriol therapy.[13–15]

Importantly, the parathyroid gland also expresses both FGFR1 and Klotho and is a target organ for FGF23.[16,17] Recent studies have shown that FGF23 directly decreases PTH gene expression and secretion. These data suggest that the direct action of FGF23 on PTH secretion is in contrast to its indirect action by inhibition of renal calcitriol production. It is, however, noteworthy that in uremic patients undergoing dialysis, such an indirect effect of FGF23 may be less evident, as renal production of calcitriol is substantially impaired and active vitamin D sterols are frequently used to control SHPT.

Thus, extremely elevated FGF23 levels would be expected to decrease serum PTH levels in uremic patients. However, in these patients, PTH secretion remains elevated despite extremely high FGF23 levels.[14,15] A similar paradox has been observed in refractory SHPT, in which parathyroid glands do not respond to calcium supplementation and calcitriol therapy, which should decrease PTH secretion. In the past, such a resistance to medical treatment has been explained by a decrease in the expression of calcium-sensing receptor and vitamin D receptor, particularly in glands with nodular hyperplasia, which is a more severe form of parathyroid hyperplasia.[18–22]

In the present study, we examined the expression of Klotho and FGFR1 in surgically excised parathyroid glands of uremic patients and compared it with the expression in normal human parathyroid tissue. The recognition of abnormal Klotho–FGFR1 complex expression would provide a new insight into the mechanisms involved in dysregulated PTH secretion and parathyroid cell proliferation in uremic patients with extremely high FGF23 levels.

RESULTS

Patient characteristics

Parathyroid tissue specimens were obtained from 5 patients with normal kidney function and 23 dialysis patients with SHPT. Clinical characteristics of the patients included in the study are shown in Table 1. Control patients had normal levels of serum whole PTH and FGF23, whereas all uremic patients had severe SHPT requiring parathyroidectomy and showed extremely high levels of serum FGF23, as reported previously.[23] Serum calcium, phosphate, and alkaline phosphatase levels were also significantly higher in dialysis patients than in control patients. Nearly all patients with SHPT (21 of 23) were treated with vitamin D sterols at the time of parathyroidectomy, and 43 percent (10 of 23) had a history of treatment with cinacalcet hydrochloride.

Histology and weight of removed parathyroid glands

A total of 7 normal parathyroid glands and 80 hyperplastic parathyroid glands were obtained from the participating patients. All normal parathyroid glands showed clusters of parenchymal cells mixed with a considerable amount of adipose tissue. Hyperplastic parathyroid glands were divided into two types: diffuse hyperplasia ($n=19$) and nodular hyperplasia ($n=61$). Diffuse hyperplasia was defined as an increased number of parenchymal cells with normal lobular structures, and nodular hyperplasia was defined as at least one well-circumscribed, encapsulated, and virtually fat cell-free accumulation of parenchymal cells.[24] All uremic patients with SHPT had at least one hyperplastic gland with nodular hyperplasia. The weight of glands with nodular hyperplasia (672 ± 80 mg) was significantly higher than those with diffuse hyperplasia (172 ± 41 mg, $P<0.001$).

Table 1 | Baseline characteristics of the study population

Variable	Normal (N=5)	SHPT (N=23)	P-value
Age (years)	67 ± 2	54 ± 3	0.002
Sex (male/female)	3/2	9/14	0.36
Duration of dialysis (months)	NA	135 ± 12	—
BUN (mg/dl)	15.3 ± 2.2	60.0 ± 3.7	<0.001
Creatinine (mg/dl)	0.86 ± 0.08	11.19 ± 0.56	<0.001
Calcium (mg/dl)	9.45 ± 0.15	9.98 ± 0.10	0.019
Phosphate (mg/dl)	3.90 ± 0.32	6.08 ± 0.30	<0.001
Alkaline phosphatase (U/l)	196 ± 19	704 ± 189	0.014
PTH(1–84) (pg/ml)	19 ± 3	583 ± 155	0.001
FGF23 (pg/ml)	37 ± 13	12,629 ± 2697	<0.001

BUN, blood urea nitrogen; FGF23, fibroblast growth factor 23; PTH, parathyroid hormone; SHPT, secondary hyperparathyroidism.

Immunohistochemical expression of Klotho, FGFR1, and Ki67

Representative immunohistochemical staining of Klotho, FGFR1, and Ki67 is shown in Figure 1. Ki67 is a proliferation marker expressed in all phases of the cell cycle. In normal glands, expression of Klotho and FGFR1 revealed distinct staining along the parathyroid cell surface (Figure 1a and f), suggesting that the parathyroid is a target organ for FGF23 in humans also. In contrast, the expression of these proteins was substantially reduced in hyperplastic parathyroid glands from uremic patients, particularly in glands with nodular hyperplasia (Figure 1b–d, g–i). Ki67 expression showed mainly nuclear localization, and its positive cells were evidently increased in hyperplastic parathyroid glands compared with normal glands (Figure 1k–n). Interestingly, careful evaluation of serial sections revealed that expression of Klotho and FGFR1 were virtually negative in parathyroid cells of nodular lesions, whereas cells outside such lesions showed weak but definitive positive staining (Figure 2a, b, d–g). A serial section of the gland showed significantly increased Ki67-positive cells in the nodular lesion compared with that in the outside area (Figure 2c, h and i).

We scored the immunohistochemical signals of Klotho, FGFR1, and Ki67 as described previously to perform a semi-quantitative analysis.[21] To confirm the validity of the semi-quantification, we measured Klotho and FGFR1 mRNA levels by quantitative real-time reverse transcription PCR in aliquots from 41 different parathyroid glands. In these patients, half of the surgically removed parathyroid glands were used for immunohistochemistry and the other half were used for real-time reverse transcription PCR. Linear regression analysis showed that immunohistochemical expression of Klotho and FGFR1 correlated significantly with the respective mRNA levels ($r=0.34$, $P=0.028$; and $r=0.36$, $P=0.021$, respectively).

Next, we compared the semi-quantitative immunohistochemical expression of Klotho, FGFR1, and Ki67 in hyperplastic parathyroid glands obtained from uremic patients with that in normal parathyroid tissue. We found that both Klotho and FGFR1 scores decreased significantly in uremic hyperplastic glands compared with normal tissue, and that these trends were more pronounced in glands with nodular hyperplasia (Figure 3a and b). Ki67 scores increased significantly in glands with nodular hyperplasia compared with either normal tissue or glands with diffuse hyperplasia

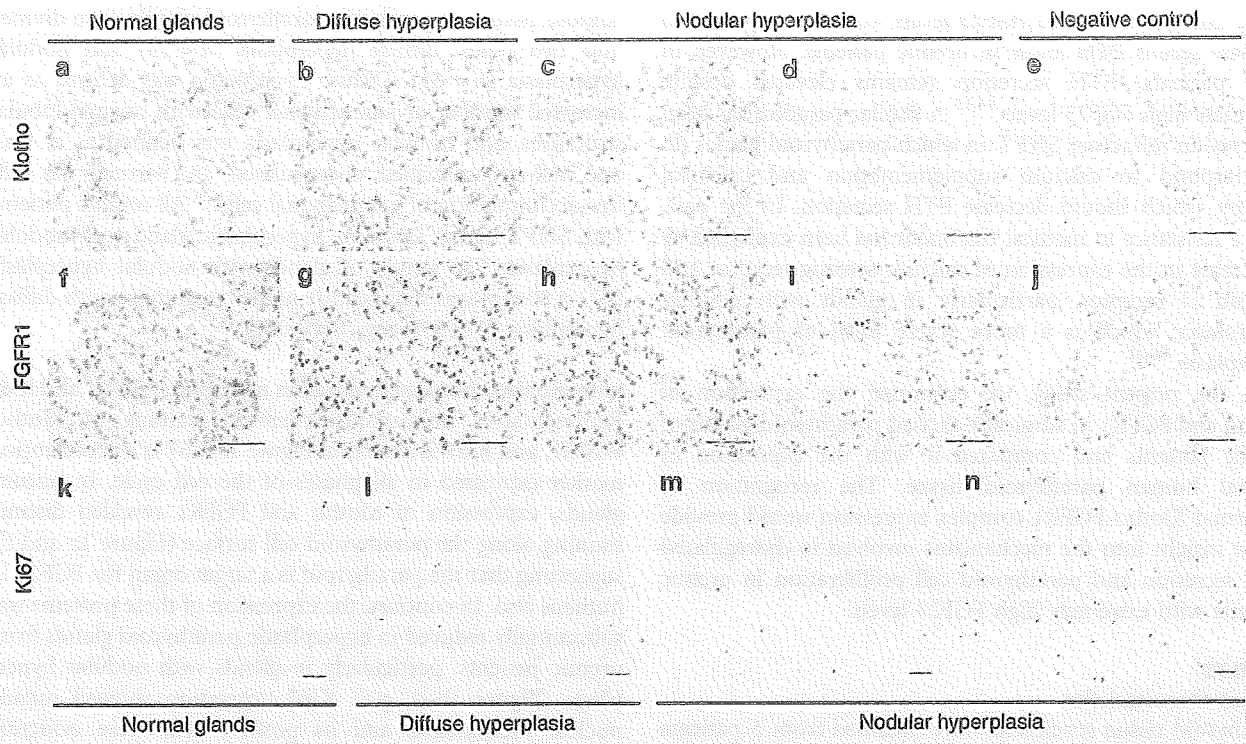

Figure 1 | Representative immunohistochemical staining of Klotho, fibroblast growth factor receptor 1 (FGFR1), and Ki67. (a–e) Klotho staining (e; negative control); (f–j) FGFR1 staining (j; negative control); (k–n) Ki67 staining. (a, e, f, j, k) Normal parathyroid glands; (b, g, l) diffuse parathyroid hyperplasia; (c, d, h, i, m, n) nodular parathyroid hyperplasia. Note the distinct staining of Klotho and FGFR1 along the cell surface in normal parathyroid glands. These expressions were substantially reduced in hyperplastic glands from uremic patients, particularly in glands with nodular hyperplasia. Ki67-positive cells were evidently increased in uremic parathyroid hyperplasia. Such an increase in Ki67-positive cells was particularly remarkable in nodular hyperplasia. Each area was scored as follows: grade 3, (a and f); grade 2 (b and g); grade 1 (c and h); and grade 0 (d and i). Original magnifications: (a–j), × 400; (k–n), × 200. Bars = 100 μm.

(Figure 3c). Ki67 score positively correlated with parathyroid gland weight ($r = 0.33$, $P = 0.003$). Notably, there was a significant correlation between Klotho and FGFR1 scores ($r = 0.71$, $P < 0.001$). This agrees with the fact that Klotho is co-expressed with FGFR1.[16] Klotho score was negatively correlated with parathyroid gland weight ($r = -0.24$, $P = 0.033$), and there was a nearly significant negative correlation between FGFR1 score and parathyroid gland weight ($r = -0.21$, $P = 0.057$). However, no significant correlation was found between either Klotho or FGFR1 score with Ki67 score.

DISCUSSION

In the present study, we evaluated Klotho and FGFR1 expression in parathyroid glands from uremic patients with severe SHPT and from control patients with normal kidney function, and showed that these expressions were significantly downregulated in hyperplastic parathyroid glands compared with normal parathyroid tissue. Furthermore, the reduction in Klotho and FGFR1 expression was more remarkable in glands with nodular hyperplasia than those with diffuse hyperplasia.

FGF23 is a hormone that has a broader role in the pathogenesis of alterations in mineral and bone metabolism[25] by interacting with FGFR in the presence of Klotho.[8,9] Klotho directly binds with FGFR1c, 3c, and 4, and increases their affinity to FGF23,[8] whereas Klotho-dependent FGF23 signaling defined by upregulation of the gene, early growth responsive 1 (*Egr-1*), is restricted to interaction with FGFR1c.[9] Indeed, a recent study showed that neither FGFR3 nor FGFR4 is the principal mediator of FGF23 effects in the kidney, suggesting that the Klotho–FGFR1 complex is the main target for FGF23.[10]

Importantly, Klotho and FGFR1 are also co-expressed in parathyroid glands.[16] A previous study showed that FGF23 administration increases *Egr-1* expression in the parathyroid glands, implying that the parathyroid is a physiological target for FGF23.[8] A subsequent study using rats and *in vitro* parathyroid cultures showed that FGF23 suppresses PTH secretion.[16] Other researchers have also reported similar findings using primary bovine parathyroid cell cultures.[17] Thus, it is clear that FGF23 is a negative regulator of PTH secretion at least in normal physiology. Nevertheless, in uremic patients undergoing dialysis therapy, PTH hypersecretion and parathyroid cell proliferation is observed despite elevated FGF23 levels.[14,15] In this context, our finding that the Klotho–FGFR1 complex is severely depressed in hyperplastic glands may shed light on the inability of extremely

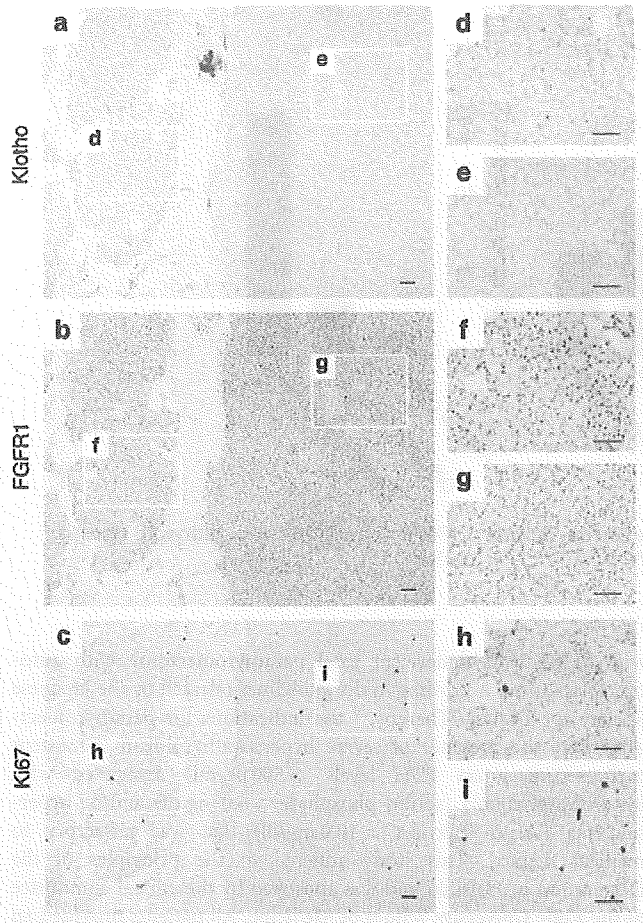

Figure 2 | Immunohistochemical staining of nodular lesions surrounded by diffuse hyperplastic parathyroid tissue. (**a**, **d**, **e**) Klotho staining; (**b**, **f**, **g**) fibroblast growth factor receptor 1 (FGFR1) staining; (**c**, **h**, **i**) Ki67 staining. Boxed areas are shown at higher magnification to right of each frame, as indicated. Both Klotho and FGFR1 expressions were markedly decreased in nodular areas, whereas diffuse hyperplastic cells outside the nodular lesion showed weak but definitive positive staining. Ki67-positive cells were observed predominantly in nodular areas. Original magnification: (**a**–**c**), × 100; (**d**–**i**), × 400. Bars = 100 μm.

high FGF23 levels in sufficiently suppressing PTH secretion in uremic patients. Recent studies suggest that FGF23 levels increase as kidney function declines and are associated with early and progressive calcitriol deficiency, and thus, may contribute to the development of SHPT.[4–6] In addition, progressive depression of the Klotho–FGFR1 complex in hyperplastic parathyroid glands may also have a role in SHPT progression by inducing resistance to the inhibitory effect of FGF23 on PTH secretion. In this study, however, we were unable to directly confirm that the depressed expression of Klotho and FGFR1 cause functional impairment of FGF23 on the parathyroid glands. Future experimental studies are needed to investigate fully the mechanisms underlying the resistance of PTH hypersecretion to extremely high FGF23 levels in uremia. Specifically, whether or not systemic or local upregulation of Klotho could mediate abnormal PTH secretion and parathyroid cell proliferation is of interest and worthy of further research.

The mechanism by which Klotho and FGFR1 expression is reduced in uremic parathyroid hyperplasia is unclear. A similar reduction in Klotho expression also occurs in parathyroid adenoma from patients with primary hyperparathyroidism.[26] In that study, parathyroid Klotho mRNA levels were inversely correlated to serum calcium level, which is in agreement with a previous study showing that low extracellular calcium is a stimulus for Klotho-mediated PTH secretion.[27] Relevantly, Klotho expression is also decreased in the kidney obtained from chronic kidney disease patients.[28] Studies in animal models have shown that systemic administration of calcitriol or phosphorus-restricted diet enhanced the renal expression of Klotho.[29,30] Whether these factors modulate the expression of Klotho and/or FGFR1 in the parathyroid glands needs to be determined in future research.

Our study also showed that even in the same patient, both Klotho and FGFR1 expressions were more severely reduced in nodular hyperplasia compared with diffuse hyperplasia. Thus, it is clear that the severity of parathyroid hyperplasia itself is associated with a reduction in the Klotho–FGFR1 complex in uremic patients. In the past, progressive reduction in calcium-sensing receptor and vitamin D receptor expression has been observed during the course of parathyroid hyperplasia.[18–22] A reduction in vitamin D receptor expression may reportedly precede the onset of parathyroid cell proliferation,[31] and more recent studies suggest that activation of the epidermal growth factor receptor by tumor growth factor-α is the cause for both hyperplastic growth and vitamin D receptor reduction.[32] On the other hand, reduced calcium-sensing receptor in the parathyroid glands may follow the development of hyperplasia and then contribute to further parathyroid growth.[33] Future studies should elucidate whether depressed expression of the Klotho–FGFR1 complex has a role in the pathogenesis of SHPT or it is only a secondary change caused by the progression of parathyroid hyperplasia.

We previously reported that serum FGF23 levels predict future refractory SHPT in dialysis patients.[14,15] Although the mechanism of this finding is unclear, it is possible that chronic phosphate retention, as reflected by elevated FGF23 levels, may contribute to further progression of parathyroid hyperplasia, because high phosphate level directly stimulates PTH secretion and parathyroid cell proliferation.[34,35] Another possibility is that high levels of FGF23 at baseline may be a consequence of prolonged active vitamin D administration for severe hyperparathyroidism,[13] which may be related to future resistance to vitamin D therapy. Furthermore, the results of this study propose that increased levels of FGF23 may reflect depression of the Klotho–FGFR1 complex, which is associated with a more severe form of parathyroid hyperplasia. These possibilities should be examined in future studies.

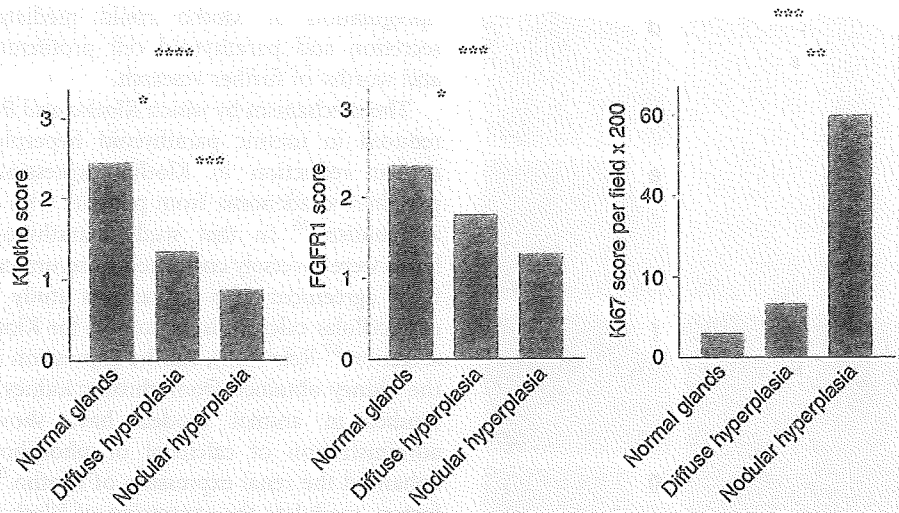

Figure 3 | Semi-quantification expression of Klotho, fibroblast growth factor receptor 1 (FGFR1), and Ki67 in parathyroid tissue of each group. The values shown are means ± s.e.m. *$P<0.1$; **$P<0.05$; ***$P<0.01$; ****$P<0.001$.

Finally, we did not find a significant association of either Klotho or FGFR1 expression with parathyroid cell proliferation as evaluated by Ki67 score, although the expression of the Klotho–FGFR1 complex was markedly downregulated in parallel with the severity of parathyroid hyperplasia and an increase in parathyroid gland weight. The precise reason for these findings is unclear; however, it is possible that the inclusion of patients who have a history of cinacalcet treatment may have influenced the results, as calcimimetics prevent excessive parathyroid cell proliferation and gland hyperplasia in uremic rats.[36,37] Further studies are needed to examine whether FGF23 mediates parathyroid cell proliferation in the presence of the Klotho–FGFR1 complex and whether such an effect is altered in uremia.

In conclusion, Klotho and FGFR1 expression decreased significantly in uremic parathyroid hyperplasia, particularly in glands with nodular hyperplasia. The results of this study suggest that the depressed expression of the Klotho–FGFR1 complex in hyperplastic glands may explain, at least in part, the resistance to extremely high FGF23 levels that would be expected to decrease the serum PTH levels. Additional studies are needed to determine whether downregulation of the Klotho–FGFR1 complex has a role in abnormal PTH secretion and parathyroid growth in uremia. Further insights into the FGF23–Klotho–FGFR1 system is important in understanding the role of FGF23 in the pathogenesis of SHPT, and in developing therapeutic approaches to treat SHPT in uremic patients who have extremely elevated FGF23 levels.

MATERIALS AND METHODS

Parathyroid gland tissues

Normal parathyroid glands were obtained in conjunction with thyroid surgery from patients with normal kidney function, and hyperplastic parathyroid glands were obtained from dialysis patients with SHPT who underwent total parathyroidectomy with autotransplantation. According to the guidelines released by the Japanese Society for Dialysis Therapy,[38] the indications for parathyroidectomy were the presence of severe hyperparathyroidism (persistent high serum intact PTH levels >500 pg/ml) associated with hyperphosphatemia (serum phosphate >6.0 mg/dl) and/or hypercalcemia (serum calcium >10.0 mg/dl) that was refractory to medical therapy. This study adhered to the principles of the Declaration of Helsinki and was approved by the ethical committee of the Kobe University School of Medicine. All patients provided an informed consent.

Antibodies

A polyclonal anti-human Klotho antibody was kindly provided by Kyowa Hakko Kirin (Tokyo, Japan). This antibody was generated by immunizing rabbits with a synthesized peptide (PLQPATGDVASDSYNNVFRDT) corresponding to a sequence in the human Klotho protein (amino acids 116–138). Affinity-purified antibody was obtained by extraction from antiserum using the peptide immobilized on agarose gel (SulfoLink kit; Pierce, Rockford, IL, USA). A rabbit anti-FGFR1 polyclonal antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and a mouse monoclonal anti-human Ki67 antibody was purchased from DAKO (Glostrup, Denmark).

Immunohistochemistry

Parathyroid glands were fixed in 10% formalin and embedded in paraffin. The sections (3 μm) were deparaffinized in xylene and rehydrated through an ethanol series. Sections were heated in a microwave for 20 min in Target Retrieval Solution at pH 6.0 (DAKO) for Klotho staining and in 0.01 mmol/l citrate buffer at pH 6.0 for Ki67 staining. Endogenous peroxidase was inactivated with hydrogen peroxide for 5 min. After blocking with 10% goat serum for 15 min, the sections were incubated with anti-Klotho antibody (1:2500 dilution, 60 min), anti-FGFR1 antibody (1:150 dilution, 30 min), and anti-Ki67 antibody (1:75 dilution, 30 min). Universal Negative Control Rabbit (DAKO) was used as a negative control. Sections for Klotho staining were incubated for 30 min with

Table 2 | Primers used for RT-PCR

Gene	Forward primer	Reverse primer
FGFR1	CCATCGACCATGGATGGTTTC	TGGGATTACAGGCGTGAGCA
Klotho	TGAGGTCCTGTCTAAACCCTGTGTC	ATGTGCAAGGCCCTCAACAAG
GAPDH	GCACCGTCAAGGCTGAGAAC	TGGTGAAGACGCCAGTGGA

FGFR1, fibroblast growth factor receptor 1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; RT, reverse transcription.

peroxidase-labeled polymer conjugated to goat anti-rabbit immunoglobulins (EnVision System/HRP; DAKO), whereas sections for FGFR1 and Ki67 staining were incubated with anti-rabbit/mouse secondary antibody for 15 min and with streptavidin–horseradish peroxidase for 15 min (LSAB2 System-HRP; DAKO). Finally, all the sections were stained with 3,3′-diaminobenzidine tetrahydrochloride for 5 min and counterstained with hematoxylin for 1 min. Every step was followed by three washes with phosphate-buffered saline for 5 min, and all the procedures were performed at room temperature.

Semi-quantification

Semi-quantitative immunohisochemical analysis was performed as previously described.[21] In brief, the immunoreactivities for Klotho, FGFR1, and Ki67 antigen were evaluated in six areas that were randomly selected by one observer. The Klotho and FGFR1 signals were scored according to the percentage of positive staining along the cell surface using the following grading criteria: grade 3, more than 75%; grade 2, 50–75%; grade 1, 25–50%; and grade 0, below 25% (Figure 1). The number of Ki67-positive cells in each area, counted at a magnification of $\times 200$, was designated as the Ki67 score. This analysis was performed by three independent observers (the intra- and inter-observer coefficients of variation were all $<10\%$). After taking an average of the scores, Klotho, FGFR1, and Ki67 scores were assigned to each gland.

RNA isolation and quantitative real-time reverse transcription PCR

Parathyroid tissues were immediately freeze-dried in liquid nitrogen and stored at −80 °C until RNA isolation. Total RNA was isolated using Isogen (Nippon Gene, Tokyo, Japan). First-strand cDNA was synthesized using a TaKaRa RNA PCR kit (AMV) (Takara Biochemicals, Osaka, Japan). Quantitative real-time PCR analysis was performed by using the Bio-Rad CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) and the FastStart Universal SYBR Green Master mix (Roche, Indianapolis, IN, USA). The relative gene expression was normalized to the gene expression of glyceraldehyde 3-phosphate dehydrogenase in the same sample. Sequences of primers used for quantitative real-time reverse transcription PCR are listed in Table 2. Specificity of the PCR products was verified by melting curve analysis and agarose gel electrophoresis.

Laboratory methods

Blood samples were obtained 1 or 2 days before parathyroidectomy. Samples were stored for <2 h at 5 °C until centrifugation. On arrival at the laboratory, the blood samples were centrifuged at 3000 r.p.m. for 10 min, aliquoted, and stored at −80 °C until analysis. Serum PTH(1–84) levels were measured using a third-generation PTH assay (Whole PTH; Scantibodies Laboratories, Santee, CA, USA). Serum FGF23 levels were determined using a sandwich ELISA kit (Kainos Laboratories, Tokyo, Japan) that exclusively detects the full-length FGF23 peptide. Serum calcium, phosphorus, albumin, alkaline phosphatase, blood urea nitrogen, and creatinine were measured using standard methods. The measured serum calcium levels were adjusted to albumin levels using the following equation: corrected calcium = serum measured calcium + (4 − albumin).

Statistical analysis

All values are expressed as means ± s.e.m. Statistical significance was determined by Student's *t*-test and Fisher's exact test for two-group comparisons and by one-way ANOVA (analysis of variance) for multiple-group comparison followed by Bonferroni's *post-hoc* test. Pearson's correlation coefficient analyses were used to examine the relationships between each parameter. $P<0.05$ was considered statistically significant. All computations were performed using Dr SPSS II for Windows version 11.01 J (SPSS Japan, Tokyo, Japan).

DISCLOSURE

All the authors declared no competing interests.

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REFERENCES

1. Drueke TB. Cell biology of parathyroid gland hyperplasia in chronic renal failure. *J Am Soc Nephrol* 2000; **11**: 1141–1152.
2. Silver J, Kilav R, Naveh-Many T. Mechanisms of secondary hyperparathyroidism. *Am J Physiol Renal Physiol* 2002; **283**: F367–F376.
3. Fukagawa M, Nakanishi S, Kazama JJ. Basic and clinical aspects of parathyroid hyperplasia in chronic kidney disease. *Kidney Int* 2006; **70**(Suppl 102): S3–S7.
4. Larsson T, Nisbeth U, Ljunggren O *et al.* Circulating concentration of FGF-23 increases as renal function declines in patients with chronic kidney disease, but does not change in response to variation in phosphate intake in healthy volunteers. *Kidney Int* 2003; **64**: 2272–2279.
5. Shigematsu T, Yamashita T, Fukumoto S *et al.* Possible involvement of circulating fibroblast growth factor 23 in the development of secondary hyperparathyroidism associated with renal insufficiency. *Am J Kidney Dis* 2004; **44**: 250–256.
6. Gutierrez O, Isakova T, Rhee E *et al.* Fibroblast growth factor-23 mitigates hyperphosphatemia but accentuates calcitriol deficiency in chronic kidney disease. *J Am Soc Nephrol* 2005; **16**: 2205–2215.
7. Liu S, Zhou J, Tang W *et al.* Pathogenic role of FGF23 in Hyp mice. *Am J Physiol Endocrinol Metab* 2006; **291**: E38–E49.
8. Urakawa I, Yamazaki Y, Shimada T *et al.* Klotho converts canonical FGF receptor into a specific receptor for FGF23. *Nature* 2006; **444**: 770–774.
9. Kurosu H, Ogawa Y, Miyoshi M *et al.* Regulation of fibroblast growth factor-23 signaling by Klotho. *J Biol Chem* 2006; **281**: 6120–6123.
10. Liu S, Vierthaler L, Tang W *et al.* FGFR3 and FGFR4 do not mediate renal effects of FGF23. *J Am Soc Nephrol* 2008; **19**: 2342–2350.
11. Shimada T, Hasegawa H, Yamazaki Y *et al.* FGF-23 is a potent regulator of vitamin D metabolism and phosphate homeostasis. *J Bone Miner Res* 2004; **19**: 429–435.
12. Perwad F, Zhang MY, Tenenhouse HS *et al.* Fibroblast growth factor 23 impairs phosphorus and vitamin D metabolism *in vivo* and suppresses 25-hydroxyvitamin D-1alpha-hydroxylase expression *in vitro*. *Am J Physiol Renal Physiol* 2007; **293**: F1577–F1583.
13. Nishi H, Nii-Kono T, Nakanishi S *et al.* Intravenous calcitriol therapy increases serum concentration of fibroblast growth factor 23 in dialysis patients with secondary hyperparathyroidism. *Nephron Clin Pract* 2005; **101**: c94–c99.
14. Nakanishi S, Kazama JJ, Nii-Kono T *et al.* Serum fibroblast growth factor-23 levels predict the future refractory hyperparathyroidism in dialysis patients. *Kidney Int* 2005; **67**: 1171–1178.

15. Kazama JJ, Sato F, Omori K *et al.* Pretreatment serum FGF-23 levels predict the efficacy of calcitriol therapy in dialysis patients. *Kidney Int* 2005; **67**: 1120–1125.
16. Ben Dov IZ, Galitzer H, Lavi-Moshayoff V *et al.* The parathyroid is a target organ for FGF23 in rats. *J Clin Invest* 2007; **117**: 4003–4008.
17. Krajisnik T, Bjorklund P, Marsell R *et al.* Fibroblast growth factor-23 regulates parathyroid hormone and 1alpha-hydroxylase expression in cultured bovine parathyroid cells. *J Endocrinol* 2007; **195**: 125–131.
18. Fukuda N, Tanaka H, Tominaga Y *et al.* Decreased 1,25-dihydroxyvitamin D3 receptor density is associated with a more severe form of parathyroid hyperplasia in chronic uremic patients. *J Clin Invest* 1993; **92**: 1436–1443.
19. Kifor O, Moore Jr FD, Wang P *et al.* Reduced immunostaining for the extracellular Ca2g-sensing receptor in primary and uremic secondary hyperparathyroidism. *J Clin Endocrinol Metab* 1996; **81**: 1598–1606.
20. Gogusev J, Duchambon P, Hory B *et al.* Depressed expression of calcium receptor in parathyroid gland tissue of patients with hyperparathyroidism. *Kidney Int* 1997; **51**: 328–336.
21. Yano S, Sugimoto T, Tsukamoto T *et al.* Association of decreased calcium-sensing receptor expression with proliferation of parathyroid cells in secondary hyperparathyroidism. *Kidney Int* 2000; **58**: 1980–1986.
22. Tokumoto M, Tsuruya K, Fukuda K *et al.* Reduced p21, p27 and vitamin D receptor in the nodular hyperplasia in patients with advanced secondary hyperparathyroidism. *Kidney Int* 2002; **62**: 1196–1207.
23. Sato T, Tominaga Y, Ueki T *et al.* Total parathyroidectomy reduces elevated circulating fibroblast growth factor 23 in advanced secondary hyperparathyroidism. *Am J Kidney Dis* 2004; **44**: 481–487.
24. Tominaga Y, Tanaka Y, Sato K *et al.* Histopathology, pathophysiology, and indications for surgical treatment of renal hyperparathyroidism. *Semin Surg Oncol* 1997; **13**: 78–86.
25. Quarles LD. Endocrine functions of bone in mineral metabolism regulation. *J Clin Invest* 2008; **118**: 3820–3828.
26. Björklund P, Krajisnik T, Akerström G *et al.* Type I membrane klotho expression is decreased and inversely correlated to serum calcium in primary hyperparathyroidism. *J Clin Endocrinol Metab* 2008; **93**: 4152–4157.
27. Imura A, Tsuji Y, Murata M *et al.* α-Klotho as a regulator of calcium homeostasis. *Science* 2007; **316**: 1615–1618.
28. Koh N, Fujimori T, Nishiguchi S *et al.* Severely reduced production of klotho in human chronic renal failure kidney. *Biochem Biophys Res Commun* 2001; **280**: 1015–1020.
29. Imai M, Ishikawa K, Matsukawa N *et al.* Klotho protein activates the PKC pathway in the kidney and testis and suppresses 25-hydroxyvitamin D3 1alpha-hydroxylase gene expression. *Endocrine* 2004; **25**: 229–234.
30. Morishita K, Shirai A, Kubota M *et al.* The progression of aging in klotho mutant mice can be modified by dietary phosphorus and zinc. *J Nutr* 2001; **131**: 3182–3188.
31. Taniguchi M, Tokumoto M, Matsuo D *et al.* Parathyroid growth and regression in experimental uremia. *Kidney Int* 2006; **69**: 464–470.
32. Arcidiacono MV, Sato T, Alvarez-Hernandez D *et al.* EGFR activation increases parathyroid hyperplasia and calcitriol resistance in kidney disease. *J Am Soc Nephrol* 2008; **19**: 310–320.
33. Ritter CS, Finch JL, Slatopolsky EA *et al.* Parathyroid hyperplasia in uremic rats precedes down-regulation of the calcium receptor. *Kidney Int* 2001; **60**: 1737–1744.
34. Slatopolsky E, Finch J, Denda M *et al.* Phosphorus restriction prevents parathyroid gland growth. High phosphorus directly stimulates PTH secretion *in vitro*. *J Clin Invest* 1996; **97**: 2534–2540.
35. Almaden Y, Hernandez A, Torregrosa V *et al.* High phosphate level directly stimulates parathyroid hormone secretion and synthesis by human parathyroid tissue *in vitro*. *J Am Soc Nephrol* 1998; **9**: 1845–1852.
36. Wada M, Furuya Y, Sakiyama J *et al.* The calcimimetic compound NPS R-568 suppresses parathyroid cell proliferation in rats with renal insufficiency. Control of parathyroid cell growth via a calcium receptor. *J Clin Invest* 1997; **100**: 2977–2983.
37. Chin J, Miller SC, Wada M *et al.* Activation of the calcium receptor by a calcimimetic compound halts the progression of secondary hyperparathyroidism in uremic rats. *J Am Soc Nephrol* 2000; **11**: 903–911.
38. Guideline Working Group, Japanese Society for Dialysis Therapy. Clinical practice guideline for the management of secondary hyperparathyroidism in chronic dialysis patients. *Ther Apher Dial* 2008; **12**: 514–525.

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ORIGINAL ARTICLE

The impact of lifestyle factors on serum 25-hydroxyvitamin D levels: a cross-sectional study in Japanese women aged 19–25 years

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Abstract Insufficient levels of serum 25-hydroxyvitamin D [25(OH)D] lead to low bone mineral density (BMD) by increasing serum levels of intact parathyroid hormone (PTH), and are associated with a high mortality rate. Therefore, the 25(OH)D level is used as an indicator of frailty in older persons. To obtain higher serum 25(OH)D levels, management of lifestyle habits and nutrient intake is important beginning in a person's younger years. This study evaluated the degree of association between serum 25(OH)D concentrations and lifestyle factors in young Japanese women. A cohort study was conducted from December 2003, and the survey was finished by February 2004. The subjects were 274 Japanese women aged 19–25 years old. The parameters evaluated in these subjects included: (1) serum concentrations of 25(OH)D, intact PTH, calcium, and phosphorus; (2) BMD in the lumbar spine and hip; and (3) lifestyle factors (nutrient intake, physical activity, and duration of sunlight exposure). The serum 25(OH)D level was negatively associated with the intact PTH level (Spearman; $r = -0.17$, $P = 0.006$). The BMD was significantly higher in the high 25(OH)D and low intact PTH group than the other group ($P < 0.05$). The serum 25(OH)D level was significantly correlated with daily intake of dietary vitamin D ($r = 0.20$, $P = 0.001$), the mean number of steps taken per day ($r = 0.16$, $P = 0.010$) and the mean time spent in sedentary activity ($r = -0.14$, $P = 0.018$) among the lifestyle factors evaluated. Multiple regression analysis showed the degree of association between lifestyle factors and serum 25(OH)D to be small ($R^2 = 0.084$). Daily intake of dietary vitamin D and daily walking may be useful for increasing the serum 25(OH)D level in young Japanese women.

Keywords 25-Hydroxyvitamin D · Nutrient intake · Physical activity · Japanese women

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Introduction

Vitamin D in the body is primarily produced in basal epidermis by ultraviolet radiation, and it is also supplied from intake of foods [,]. Vitamin D is then transported to the liver where it is metabolized to 25(OH)D. 25(OH)D is converted in the kidneys to active 1,25 dihydroxyvitamin D [1,25$(OH)_2$D], and exerts its effects as a bone metabolic hormone with intact parathyroid hormone (PTH) [ ].

It is well established that serum 25(OH)D concentration is the best clinical indicator of vitamin D status. A lower level of serum 25(OH)D leads to bone fractures [,] and serum 25(OH)D concentration is reported to fall [ ] in the elderly, with the vitamin D deficiency being common in the

elderly in the range of 30–90% in the Western population [,]. The serum 25(OH)D concentration is related to lifestyle factors such as vitamin D intake and sunlight exposure [,], and the circulating 25(OH)D level serves as an indicator of vitamin D sufficiency [ ].

In a previous study in a Japanese population, 4.6% of the subjects had low serum 25(OH)D levels in the peri-/postmenopausal period [ ], but women in their twenties had significantly lower serum 25(OH)D concentrations than those in their thirties and older [ ], and 40.3% of the subjects had vitamin D insufficiency as college students [ ]. Additionally, low serum concentrations of 25(OH)D and high serum concentrations of intact PTH were found to predispose young individuals to low bone mineral density (BMD) [ ].

It is reported that the primary dietary sources of vitamin D in food are fish and eggs in Japanese [ ], but there is no report on the relationship between 25(OH)D and nutrient intake or other lifestyle factors in Japanese women.

The aim of this study was to clarify the relationship between 25(OH)D concentrations and lifestyle factors, such as nutrient intake, physical activity and duration of exposure to sunshine, in young Japanese women.

Subjects and methods

Study participants

The present Kawada-cho Peak Bone Mass Study is a cohort study in young Japanese women in Tokyo, Japan [ ]. The participants consisted of healthy female volunteers who were students at the School of Nursing (college-degree four-year course) and the Nursing Vocational School (non-college-degree three-year course) of Tokyo Women's Medical University, Tokyo, Japan. We obtained written consent from 348 candidate study subjects who agreed to participate voluntarily. Participants were excluded if they had systemic or metabolic disorders or medications with known effects on bone metabolism and had abnormalities in hormonal regulation or nutritional habits, including menstrual disturbance and eating disorders. Of these, 274 women finally participated in the study. The study protocol was approved by the Ethics Committee of Tokyo Women's Medical University School of Medicine.

Study design

The baseline survey was carried out from December 2003 to February 2004. Each participant completed a questionnaire about background information including age, weight, birth weight, age at menarche, and current menstrual status, along with the questionnaires described below.

Laboratory assessments

All blood samples were taken when the participants gathered to receive the questionnaires, and to undergo blood chemistry tests for serum calcium, phosphorus, and albumin. Serum 25-hydroxyvitamin D concentrations were determined by the Nichols Advantage Chemiluminescence protein-binding assay (CLPBA) method [ ]. Intact PTH was measured as a marker for vitamin D insufficiency by using a two-site immunoradiometric assay (Nichols Institute Diagnostics).

Bone mineral density measurements

BMD at the lumbar spine (L2–L4) was measured by dual-X ray absorptiometry (DXA) using the QDR-4500 absorptiometer (Hologic Inc, Bedford, MA). The manufacturer's lumbar spine phantom was scanned daily for quality control and to correct for instrument drift. As previously reported, coefficient of variation in our measurements was <0.7% for the day-to-day quality control scans. BMD was reported as grams per square centimeter.

Lifestyle factors

Assessment of food and nutrient intakes

Dietary habits during the past month were assessed with a validated, self-administered Diet History Questionnaire (DHQ) [,], which was completed by each participant at home and was checked by ≥2 dietitians. The DHQ is a 16-page structured questionnaire that consists of the following 7 sections: general dietary behavior, major cooking methods, frequency of consumption of 6 alcoholic beverages as well as their portion sizes, semi-quantitative frequency of intake of 121 selected foods and nonalcoholic beverage items, dietary supplements, frequency of consumption of 19 staple foods (rice, bread, noodles, and other wheat foods) and *miso* (fermented soybean paste) soup as well as their amounts, and open-ended food items consumed regularly (≥1 time/week) not listed in the DHQ. The food and beverage items and portion sizes in the DHQ were derived primarily from data in the National Nutrition Survey of Japan and several recipe books on Japanese dishes [ ]. Dietary intake of 147 food and beverage items, energy, fat, total carbohydrate, alcohol, and dietary fiber were calculated by using an ad hoc computer algorithm developed for the DHQ, which was based on the Standard Tables of Food Composition in Japan [ ].

Information on dietary supplements and data from the open-ended questionnaire items were not used for calculation of dietary intake. Detailed descriptions of the methods used for calculating dietary intake and the validity

of the DHQ were published elsewhere [,]. Spearman's correlation coefficients between the DHQ and the 3rd estimated dietary records were 0.48, 0.48, 0.55, and 0.48, respectively, for energy, protein, fat, and carbohydrate in 47 women [ ]. In addition, Pearson's correlation coefficients between the DHQ and the 16th semi-weighed dietary records were 0.32, 0.30, 0.52, 0.46, 0.43, 0.30, and 0.40, respectively, for energy, protein, fat, carbohydrate, calcium, phosphorus, and vitamin D in 92 women, with the Spearman's correlation coefficients being 0.39, 0.65, and 0.32, respectively, for fish, meats, and eggs (unpublished observation, S. Sasaki, 2006). For analysis of intake levels, we used energy-adjusted values, i.e., percentage of energy accounted for by protein, fat, and carbohydrate, and amount per 1000 kcal of energy for other nutrients and foods.

Assessment of physical activity

JALSPAQ Information about the subjects' participation in exercises, household activities, walking and cycling for transportation, as well as their occupational type were assessed with a self-administered Japan Arteriosclerosis Longitudinal Study Physical Activity Questionnaire (JALSPAQ). JALSPAQ is a 2-page structured questionnaire that consists of the following five activities and four additional questions: sleep, work related activities, traveling to and from places (walking and cycling), housework (cooking, laundry, cleaning, caring for one's children and elderly), exercise and non-exercise leisure time activities.

Data on leisure time activities were collected from free-response items. Questions included: (1) exercise duration per session; (2) frequency of sessions per month; and (3) intensity of sessions. Activities were coded with the Compendium of Physical Activity [,], which reflects the type and MET intensity of each activity.

Summary estimates of physical activity energy expenditure were calculated in terms of standard metabolic equivalents (METs) as MET-hours/day. MET values were obtained by multiplying the hours spent on each of the categories evaluated and the products summed to give kilocalories per kilogram per day. Total energy expenditure was estimated as the sum of energy expended in the 5 activity categories. The validity of the JALSPAQ was assessed using a sample of 271 volunteers. The correlation between the 24-h physical activity reported and that reported on the JALSPAQ was 0.36 in men and 0.38 in women. The correlation between the values registered by the uni-axial accelerometer and those reported on the JALSPAQ was 0.38 in men and 0.38 in women.

Accelerometer Lifecorder EX, a uniaxial accelerometer (Suzuken Co. Ltd, Nagoya, Japan), measures acceleration in the vertical direction. The accelerometer was designed to detect movements of the body trunk by being attached to the waist, and to record the number of steps taken and the intensity of physical activity registered on a unique scale of 1–9 at 4-s intervals. Detailed descriptions of the algorithm used for calculating TEE and the validity of the Lifecorder have been published elsewhere [ ].

Assessment of exposure to sunlight

The estimated duration of exposure to sunlight was calculated based on the following information obtained from the JALSPAQ: time spent on traveling to and from places (i.e., to work, for shopping) and outdoor leisure time activities considered to involve exposure to sunlight.

Statistical analysis

Continuous variables were expressed as a mean and SD to describe the status of the participants. To evaluate the relationship between serum 25(OH)D, intact PTH concentrations and the lumbar spine (L2–L4) BMD, Wilcoxon's rank sum test was used. The participants were then divided into four groups by median values for serum 25(OH)D and intact PTH concentrations. All continuous variables of interest (background information, physical activity, and nutrient intake) were analyzed for correlation with serum 25(OH)D concentrations, using Spearman's rank correlation coefficient and stepwise multiple regression analysis. All statistical analyses were performed by using the JMP (Japanese version 5.1.2, SAS Institute, Cary, NC).

Results

The participant characteristics are presented in Table . The serum calcium concentration was significantly positively correlated with the serum 25(OH)D concentration (Spearman; $r = 0.23$, $P < 0.001$), and was also inversely correlated with the serum intact PTH concentration (Spearman; $r = -0.21$, $P = 0.001$,). The serum 25(OH)D and intact PTH concentrations were significantly inversely correlated (Spearman; $r = -0.17$, $P = 0.006$, Fig.). Other background characteristics (age, birth weight, age at menarche, and BMI) were not correlated with 25(OH)D or intact PTH. Seventy-six participants were found to comprise the group showing the high 25(OH)D (≥median of 18.0 ng/mL) and low intact PTH (<median of 40.3 pg/mL) concentrations, and were considered to combine the most appropriate conditions. The lumbar spine BMD was significantly higher in the high 25(OH)D and low intact PTH group ($n = 76$, mean ± SD = 1.02 ± 0.10 g/cm^2) than the other group ($n = 198$, 0.99 ± 0.11 g/cm^2: Wilcoxon,

Table 1 Basic characteristics of the participants[a]

Item	Value	Range
Age (y)	20.6 ± 1.4	19–25
Birth weight (g)	3143.2 ± 446.5	1800–4800
Age at menarche (y)	12.0 ± 1.3	9–17
BMI (kg/m^2)	21.2 ± 2.7	15.2–31.2
BMD (g/cm^2)		
Lumbar spine (L2–L4)	1.00 ± 0.11	0.74–1.30
Total proximal femur	0.90 ± 0.10	0.63–1.24
Intact parathyroid hormone (pg/mL)	40.5 ± 11.6	15.0–71.1
25(OH)D (ng/mL)	18.7 ± 4.8	5.8–32.3

[a] All values are mean ± SD; range in parentheses. $n = 274$ except for birth weight ($n = 261$) and age at menarche ($n = 272$)

BMD bone mineral density, *25(OH)D* 25-hydroxyvitamin D

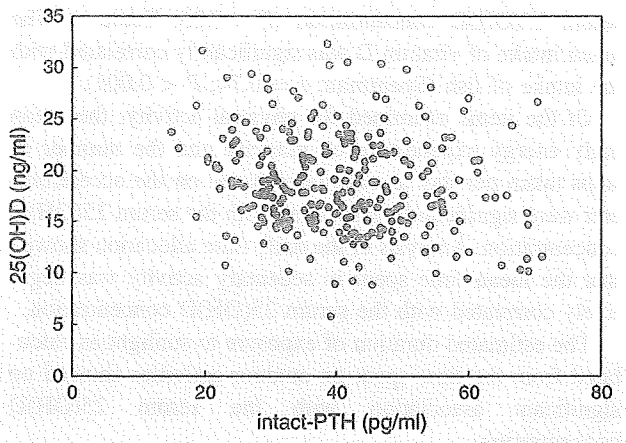

Fig. 1 The relationship between the serum intact PTH and 25(OH)D concentrations. The serum intact PTH and 25(OH)D concentrations were significantly inversely correlated (Spearman; $r = -0.17$, $P = 0.006$)

$P = 0.038$). The same results observed in the hip BMD (0.92 ± 0.10 vs. 0.89 ± 0.10 g/cm^2; $P = 0.049$). Age, BMI, serum phosphorus, serum bone metabolic markers, birth information and age at menarche were not significantly different between the two groups ($P > 0.05$).

Analysis of the values for lifestyle factors is shown in Table . To exclude the influence of intake volume, nutritional intake values were stratified by total consumption calories. The mean daily energy expenditure as calculated from the JALSPAQ questionnaire was 1786.8 ± 300.7 kcal, and was found to be consistent with the accelerometer values.

Of the nutrients examined, vitamin D (including supplements) and fish showed a significant correlation with the

Table 2 Daily nutrient intake and physical activity

Item	Value	Range
Energy, and selected nutrient and food intakes assessed by the DHQ		
Energy (kcal/day)	1863.4 ± 629.3	685.9–6134.3
Proteins (% of energy)	13.4 ± 2.4	4.3–21.7
Fat (% of energy)	28.9 ± 6.0	12.8–46.5
Carbohydrates (% of energy)	54.5 ± 6.9	15.0–80.2
Calcium (mg/1000 kcal)	424.1 ± 207.9	76.9–1508.1
Vitamin D (μg/1000 kcal)	9.9 ± 6.7	0.4–46.4
Fish (g/1000 kcal)	46.0 ± 36.7	0–279.3
Egg (g/1000 kcal)	20.0 ± 15.6	0–111.2
Physical activity		
As assessed by the JALSPAQ		
Total energy expenditure (METs-h/day)	33.3 ± 2.6	29.1–48.3
Sleep (h)	6.4 ± 1.2	4.0–12.0
School curriculum (h)	5.1 ± 1.3	0–10.7
Traveling to and from places (h)	1.2 ± 0.8	0.2–4.5
Housework (h)	0.8 ± 0.7	0–3.6
Exercise (h)	0.1 ± 0.2	0–1.5
Leisure (h)	0.4 ± 0.8	0–4.9
Sedentary activity (h)	10.0 ± 2.1	3.1–17.3
As assessed by the accelerometer[a]		
Total energy expenditure (kcal/day)	1820.4 ± 171.0	1364.0–2300.0
Energy expenditure for exercise (kcal/day)	222.3 ± 75.8	56.0–497.0
Steps (steps/day)	8839.5 ± 2638.8	2273–18022
Exposure to sunlight[b] (h/day)	1.2 ± 0.8	0.17–4.5

All values are mean ± SD; $n = 274$ except for accelerometer ($n = 267$), daily time allocation ($n = 273$)

DHQ the Diet History Questionnaire, *JALSPAQ* the Physical Activity Questionnaire by the Japan Arteriosclerosis Longitudinal Study

[a] Lifecorder EX, a uniaxial accelerometry sensor by Suzuken Co., Ltd

[b] Duration of exposure to sunlight was calculated from the questionnaire responses: amount of time spent on traveling to and from places (i.e., to work, for shopping), outdoor leisure time activities considered as involving exposure to sunlight

serum 25(OH)D concentration ($P < 0.05$; Table). The mean intake of vitamin D was significantly correlated with the intake of fish (Spearman; $r = 0.74$, $P < 0.001$).

Of the items examined for physical activity, the mean daily energy expenditure on exercise and the number of steps taken per day as calculated based on the accelerometer were significantly associated with the serum 25(OH)D concentration. Analysis of the daily time allocation showed that the mean time spent in sedentary activity was negatively correlated with the serum 25(OH)D concentration.

The estimated duration of exposure to sunlight as calculated from the time spent on outdoor activities showed no significant association with the serum 25(OH)D concentration.

The vitamin D intake, the steps taken per day and the time spent in sedentary activity were chosen for stepwise multiple regression analysis, with the 25(OH)D concentration as the outcome variable ($P < 0.05$). As a result, each of these factors was found to significantly impact the 25(OH)D concentration (Table), while the r values were small.

Table 3 Correlation coefficients (r) for serum 25(OH)D levels versus lifestyle factors

Variable	r	P
Selected nutrient and food intakes assessed by the DHQ		
Calcium (mg/1000 kcal)	0.11	0.077
Vitamin D (μg/1000 kcal)	0.20	0.001
Fish (mg/kcal)	0.18	0.002
Egg (g/1000 kcal)	0.07	0.249
Physical activity		
As assessed by the JALSPAQ		
Total energy expenditure (METs-h/day)	0.08	0.164
Sedentary activity (h)	–0.14	0.018
As assessed by the accelerometer[a]		
Total energy expenditure (kcal/day)	0.07	0.265
Energy expenditure for exercise (kcal/day)	0.15	0.016
Steps (steps/day)	0.16	0.009
Exposure to sunlight[a] (h/day)	0.04	0.487

Spearman's rank correlation coefficient

DHQ Diet History Questionnaire, *JALSPAQ* the Physical Activity Questionnaire by the Japan Arteriosclerosis Longitudinal Study

[a] Duration of exposure to sunlight was calculated from the questionnaire responses: amount of time spent on traveling to and from places (i.e., to work, for shopping), outdoor leisure time activities considered to involve exposure to sunlight

Table 4 Lifestyle factors showing significant correlation to serum 25(OH)D

Variable	Parameter estimate	Standard estimate	P	R^2	Model R^2
Vitamin D (μg/1000 kcal)	0.258	3.724	0.001	0.037	0.084
Steps (number/day)	0.000	2.147	0.010	0.024	
Sedentary activity (h)	–0.287	–2.039	0.038	0.015	

Stepwise multiple regression analysis

Discussion

Vitamin D and PTH have an important role in controlling the plasma calcium concentration. Any fall in the ionized calcium concentration is detected by the calcium receptor of the parathyroid gland, followed by the secretion of PTH by the parathyroid gland. PTH then activates vitamin D production, which in turn promotes calcium absorption from the intestines, increases bone resorption by the osteoclasts and compensates for the plasma calcium concentration which is accompanied by the reduction of calcium accumulated in the bone [ ].

Insufficient intake of vitamin D is known to cause untoward conditions, such as secondary hyperparathyroidism and decreased BMD [ ], and vitamin D deficiency is known to be a significant risk factor for osteoporosis and secondary hyperparathyroidism. Vitamin D, as it results from both cutaneous production and from dietary intake, reflects the conditions of daily living. Around 80–90% of (the precursor of) vitamin D is absorbed through the intestines or produced at the skin through exposure to sunlight, becoming a biologically active hormone after hydration [ ]. It is thus recommended that hands, face and arms, or arms and legs, be exposed to sunlight for a period equal to 25% of the time required to cause a light pinkness to the skin [ ]. Vitamin D intake varies from country to country [ ]. The standard value recommended for intake of dietary vitamin D is 5 μg for 15–18-year-olds in Japan.

Serum 25(OH)D concentration is the best clinical indicator of the vitamin D concentration in blood. The serum 25(OH)D concentration is lower in the elderly [,], lower in women than in men [ ] and lower in winter than in the other seasons [,]. Low concentrations of 25(OH)D, defined as below 25 nmol/L, lead to an increase in the serum PTH concentration and to increased bone resorption [ ]. Insufficiency of 25(OH)D in youth is associated with low BMD of the forearm [ ] and hampers acquisition of maximum peak bone mass at the lumbar spine [ ]. In addition, it is reported in a study evaluating BMD of the calcaneus that low levels of 25(OH)D may adversely affect bone strength [ ].

In this study, we measured serum 25(OH)D levels using Nichols Advantage CLPBA. It detects serum 25(OH)D2 with much less sensitivity than serum 25(OH)D3. In Japan, vitamin D2 preparations are not prescribed for patients and vitamin D2 supplements are less used. Furthermore, we had

reported that the ratio of 25(OH)D2 to total serum 25(OH)D in Japanese was extremely small [ ]. Therefore, there is no doubt that the 25(OH)D2 levels as measured on the Nichols Advantage did not affect our study results.

We investigated the association between serum 25(OH)D, intact PTH levels and BMD. The serum 25(OH)D concentration is negatively correlated with intact PTH. The low intact PTH and high 25(OH)D group showed higher serum calcium concentrations and BMD than the other group. Background data including age, BMI, serum parameters and birth information were not significantly different between the two groups. High 25(OH)D levels were assumed to control the intact PTH level, and to contribute toward an increase in calcium absorption and, consequently, in BMD.

Analysis of the lifestyle factors showed that exposure to sunlight had no impact on serum 25(OH)D. Previous study reports indicated positive correlation between sunlight exposure and serum 25(OH)D [,]. But this study indicated no correlation between them. We estimated the reasons for this discrepancy as follows. First, the amount of vitamin D synthesis by sunlight reaches the upper limit of normal in Tokyo, at 35° north latitude [ ]. Furthermore, Hollis et al. reported that an adequate UVB exposure level (18–20 mJ/cm^2) in sunlight to induce pre-vitamin D on the epithelium is not generally reached during winter in the northern United States above latitude 40° [ ]. Second, the measurement of sunlight exposure time may have some methodological problems. However, our results showing no association between the estimated time of exposure to sunlight and the serum 25(OH)D level did not contradict the positive correlation between sunlight exposure and serum 25(OH)D. Landin-Wilhelmsen et al. have reported that physical activities are often associated with being outdoors, and active individuals should therefore have a better chance of having sun exposure [ ]. On the contrary, our study showed that there was no significant correlation between sunlight exposure and serum 25(OH)D levels. We might speculate that our participants may have applied some ultraviolet protection cosmetics when they exercised, though we did not check on it. That's likely the reason why only physical activities correlated with 25(OH)D.

Dietary intake of vitamin D (including supplements) and fish had an impact on serum 25(OH)D (Table). The participants consumed 56.9 ± 45.4 g of fish per day, which was found to be significantly correlated with vitamin D. The steps taken per day or energy expenditure on exercise had a positive impact, while the time spent in sedentary activity (watching TV, playing computer games) had a negative impact on serum 25(OH)D, suggesting that physical activity acted in an additive manner with vitamin D intake in Japanese young women. Although there have been reports showing correlation between physical activity and serum 25(OH)D [,], the present study was too small to draw any conclusion in this regard. Calcium is the most abundant of minerals available in the human body, of which 99% is found in bone with the rest in blood and muscle. Vitamin D participates in the contraction of muscle and is known to maintain myodynamia by transporting calcium from bone to muscle when it is calcium-deficient. Moreover, Kwon et al. reported that concomitant low serum albumin and vitamin D levels are associated with decreased muscle strength and balancing capability in elderly people [ ].

The present study had several limitations. First, this cohort study was confined in geographical coverage to Tokyo only. Therefore, the distribution of the research parameter sunlight exposure could have been narrow. Second, participants were only students or nurses by occupation, possibly suggesting a similar lifestyle pattern among the participants. And third, since sunlight exposure was estimated from the JALSPAQ, the use of ultraviolet protection cosmetics was not able to be ruled out.

However, this is the first report investigating the association between the impact of lifestyle factors and serum 25(OH)D levels in Japanese young women which appears to partially explain the correlation between the steps taken per day and the serum 25(OH)D level. Further research is needed to verify the reported correlation between physical activity and serum 25(OH)D.

In conclusion, the serum 25(OH)D concentration was positively affected by dietary vitamin D or fish intake and the mean steps taken per day or energy expenditure on exercise, and was negatively affected by the time spent in sedentary activity. These findings may suggest that lifestyle modification at an early age may contribute to preventing osteoporosis or frailty in later years.

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References

1. Holick MF (1995) Environmental factors that influence the cutaneous production of vitamin D. Am J Clin Nutr 61:638S–645S
2. Working Group on the Nutritional Status of the Population of the Committee on Medical Aspects of the Food Nutrition Policy: subgroup on the Nutritional Aspects of Bone Health. 49 Nutrition and bone health: with particular reference to calcium and vitamin D. Report of the Subgroup on Bone Health (1998) Rep Health Soc Subj. The Stationary Office, London, iii–xvii, p 1–24
3. Sahota O, Mundey MK, San P, Godber IM, Lawson N, Hosking DJ (2004) The relationship between vitamin D and parathyroid hormone: calcium homeostasis, bone turnover, and bone mineral density in postmenopausal women with established osteoporosis. Bone 35:312–319