were digested with acids either for a short or long time, or incinerated at 550 °C, their analytical values for Mo were coincident with the certified values. In addition, recoveries of the amount of Mo added to milk and serum samples were almost 100% with dry incineration. The limit of detection (LOD) was 0.1 μg/L when nebulizing to ICP-MS.

## 14.3. MOLYBDENUM IN FOOD

### 14.3.1. Molybdenum Concentration in Food

Molybdenum concentrations in various food groups are summarized in Table 14.2 [10, 13, 14]. The Mo concentration in food is highly different for each food group. Food groups with high Mo concentration are plant foods, such as cereals, pulses, nuts, and their products, whereas the Mo concentration in animal foods, such as meats, fish, and dairy products, is extremely low. The highest Mo concentration is observed in pulses, nuts, and their products.

Molybdenum concentrations in plants change not only as a function of the Mo concentration in soil, but also with soil pH [15]. Therefore, even in the same food commodity, a remarkable difference in Mo concentration can occur due to differences in the area where the plants grew, for example, a value of almost 10 μg/g was observed for the Mo concentration in US soybeans while a value of less than 1 μg/g was observed in Chinese soybeans [16].

### 14.3.2. Speciation of Molybdenum in Food

There is a little information of the chemical species of Mo in food. Since Mo enzymes exist in animal tissues, it is thought that animal foods include Mo bound to protein; however, no research has been devoted so far to the amount of Mo existing as a

**TABLE 14.2 Molybdenum Concentration (μg/g Fresh Weight) in Foods Consumed in USA, France, and Japan**

| Food Group | USA [13] | France [14] | Japan [10] |
|---|---|---|---|
| Wheat and wheat products | 0.32 | 0.29 | 0.23 |
| Rice and rice products | 0.29 | 0.11 | 0.63 |
| Pulse, nuts, and pulse products | 1.55 | 1.06 | 1.32 |
| Potatoes (starchy vegetables) | 0.07 | 0.42 | 0.08 |
| Vegetables | 0.05 | 0.17 | 0.12 |
| Fruits | 0.03 | 0.01 | 0.04 |
| Meat and meat products | 0.03 | 0.02 | 0.05 |
| Poultry | 0.05 | 0.14 | 0.03 |
| Fish and fish products | 0.01 | 0.08 | 0.02 |
| Eggs | 0.09 | 0.03 | 0.40 |
| Milk | 0.05 | 0.04 | 0.04 |
| Cheese | 0.10 | 0.07 | 0.08 |

**TABLE 14.3 Estimated Mo Intake (μg/day per person) in Populations of Several Countries**

| Countries | Method of Estimation | |
|---|---|---|
| | Calculation | Analysis of Diet |
| USA | 120–240 [13] | |
| Mexico | | 185 [17] |
| France | 275 [14] | |
| | 114 [18] | |
| Germany | | 95 [17] |
| | | 175[a] [17] |
| Japan | 225 [10] | 217 [9] |
| | | 147[b] [9] |
| | | 318[c] [9] |
| Korea | 11 [19] | |

[a] Vegetarian diets.
[b] Diets without soybean products.
[c] Diets rich in soybean products.

protein-bound form in animal food. Such enzymes also exist in plants; however, it is assumed that most Mo in soybeans is the inorganic low molecular weight compound molybdophosphate [16].

### 14.3.3. Molybdenum Intake in Human Population

Table 14.3 shows the daily Mo intake from food in several human populations. The Mo intake in most countries ranges from 100 to 300 μg/day. In the Dietary Reference Intake (DRI) of the US/Canada, the Recommended Dietary Allowance (RDA) and the tolerable upper limit (UL) of Mo intake have been set at 45 μg/day and 2 mg/day, respectively [20]. Accordingly, Mo intake in these countries is thought to be adequate. The Mo intake of Korean people was estimated to be only 11 μg/day [19]; however, this figure is doubtful because the analytical values adopted in this report were one order of magnitude lower than those of other reports. Since cereals and pulses contain Mo at a very high level, the Mo intake of vegetarians [17] and people who eat a large amount of soybeans [9] is higher than that of the general population.

## 14.4. MOLYBDENUM IN HUMAN SAMPLES

### 14.4.1. Molybdenum in Urine

Table 14.4 shows the urinary Mo concentration for two healthy populations in Europe [21, 22]; these two studies give similar analytical values. The urinary concentration in the two European populations showed a logarithmical normal distribution rather than a normal distribution. In a Mo balance study of healthy Japanese young women, urinary Mo excretion was highly correlated to Mo intake, as

**TABLE 14.4 Molybdenum Concentration in Spot Urine Samples from Healthy Italian and Danish Adults**

| | Italy [21] | Denmark [22] |
|---|---|---|
| Number of subjects | 51M, 49F | 71M, 57F |
| Age | M 44.1 ± 12.7<br>F 41.6 ± 13.3 | 40–70 years |
| Analytical method | ICP-MS | ICP-MS |
| Analytical values (μg/L) | | |
| Range | 11.1–155.8 | 3.7–180.7 |
| Mean | 54.1 | 42.0 |
| SD | 33.9 | 26.8 |
| Geometrical mean | 44.0 | 34.8 |
| Median | 46.2 | 36.2 |
| 10th percentile to 90th percentile | 16.5–101.8 | – |
| 95% reference interval[a] | – | 10.2–106.1 |
| Analytical values (μg/g creatinine) | | |
| Range | 7.4–137.0 | 3.9–127.8 |
| Mean | 44.8 | 34.1 |
| SD | 23.8 | 22.6 |
| Geometrical mean | 39.0 | 27.8 |
| Median | 39.3 | 27.3 |
| 10th percentile to 90th percentile | 23.0–72.3 | – |
| 95% reference interval[a] | – | 7.2–102.8 |

[a] Nonparametric 95% reference value.

shown in Figure 14.1 [9]; therefore, the urinary Mo concentration of these populations in the two European regions where the dietary pattern is similar may show a common distribution. Urinary Mo concentration in vegetarians or Asian people who eat more cereals and pulses than general Europeans may be somewhat higher than in these two populations.

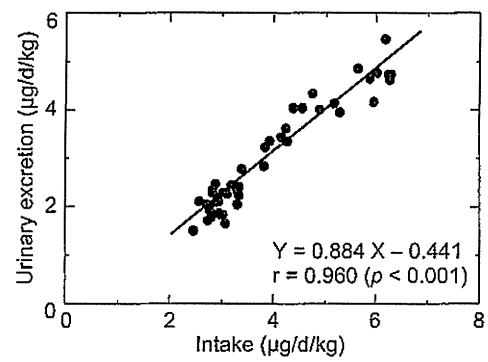


**FIGURE 14.1** Relationship between Mo intake and urinary Mo excretion in healthy young Japanese women in balance study [9].

TABLE 14.5 Molybdenum Concentration in Healthy Human Blood

| | Number of Subjects | Age | Concentration (μg/L)[a] | Analytical Method | Reference |
|---|---|---|---|---|---|
| Serum/plasma | | | | | |
| Japan | 22M, 33F | 20–59 | 0.70 (0.27–1.81)[b] | ICP-MS | [11] |
| Belgium | 27M, 23F | 18–75 | 0.55 ± 0.21 | NAA | [23] |
| USA | 2M | Unknown | 0.5 ± 0.1 | ICP-MS | [24] |
| Whole blood | | | | | |
| UK | 44 | Unknown | 0.62 ± 0.29 | ICP-MS | [5] |
| Germany | 50M, 80F | 18–70 | 0.43 (0.14–1.1)[c] | ICP-MS | [25] |
| Venezuela | 244M, 174F | 18–27 | 2.66 ± 0.66 | AAS | [26] |

[a] Values are arithmetic means ± SD unless otherwise noted.
[b] Geometric mean with SD range in parentheses.
[c] Arithmetic mean with 5th percentile to 95th percentile in parentheses.

### 14.4.2. Molybdenum in Blood

Table 14.5 shows the Mo concentration in healthy human blood measured in various countries. Serum Mo concentration in Japanese subjects showed a logarithmic normal distribution [11], while the variation coefficient of serum Mo in Belgian subjects was comparatively low [23]. The average values of plasma or serum Mo concentration were similar among Japanese, Belgian, and American [24] subjects although several Japanese subjects showed slightly higher values. Because the plasma Mo concentration was correlated to the Mo intake [8], this higher serum Mo concentration in Japanese is probably derived from their high consumption of soybean products rich in Mo.

The values of whole blood Mo in German [25] and British [5] subjects were similar and also similar to the values of serum Mo; however, the Mo concentration of whole blood from Venezuelan subjects [26] was obviously higher than those of European subjects. The Venezuelan study used the AAS method while the two European studies used the ICP-MS technique for Mo determination; however, since the dietary pattern and Mo intake of Venezuelan people are unclear, it cannot be concluded that differences in the analytical method influence the analytical values of whole blood Mo concentration.

### 14.4.3. Molybdenum in Milk

The Mo concentration of human milk decreased rapidly from 15 μg/L on day 1 to 4.8 μg/L at 7 to 10 days postpartum and 2.6 μg/L by 1 month [27]. Table 14.6 shows the Mo concentration in matured human milk samples from various countries. The Mo concentration in human milk from mothers in European countries was lower than in other areas. The US/Canadian DRI has adopted a value of 2.00 μg/L as the average human milk Mo concentration [20]. The human milk Mo of mothers in Japan, Guatemala, and Zaire are similar to this value. On the other hand, the human milk Mo level in the Philippines is markedly high compared to that in other areas.

**TABLE 14.6 Molybdenum Concentration in Matured Human Milk**

| Country | Number of Samples | Median (μg/L) | Range (μg/L) | Analytical Method | Reference |
|---|---|---|---|---|---|
| Germany[a] | 19 | 0.53 | 0.18–0.81 | ICP-MS | [28] |
| Guatemala | 14 | 2.12 | <0.3–9.00 | NAA | [29] |
| Hungary | 13 | <0.3 | <0.3–3.88 | NAA | [29] |
| Japan | 79 | 3.18 | <0.1–25.91 | ICP-MS | [12] |
| Nigeria | 9 | 2.65 | 0.34–9.71 | NAA | [29] |
| Philippines | 15 | 10.37 | 6.75–35.41 | NAA | [29] |
| Sweden | 10 | 0.40 | <0.3–5.87 | NAA | [29] |
| Zaire | 15 | 1.39 | <0.3–5.81 | NAA | [29] |
| USA/Canada | – | 2.00[b] | – | – | [20] |

[a] Include samples from mothers who live in the Czech Republic and Poland.
[b] Values adopted in DRI to calculate the adequate intake (AI) of Mo for US/Canadian infants.

## 14.5. CLINICAL SIGNIFICANCE OF SERUM AND PLASMA MO

### 14.5.1. Index of Dietary Molybdenum Intake

Turnlund and Keyes examined variations of the plasma Mo concentration in four young American subjects who consumed five levels of dietary Mo for 24 days each [8]. As shown in Figure 14.2, which is based on this study, the plasma Mo strongly correlated to the dietary Mo intake. This indicates that the serum/plasma Mo concentration is an index of dietary Mo intake. As shown in Table 14.3, a higher dietary Mo intake (more than 300 μg/day) occurs in vegetarians or Japanese people with a high consumption of soybean products, but dietary Mo intake more than

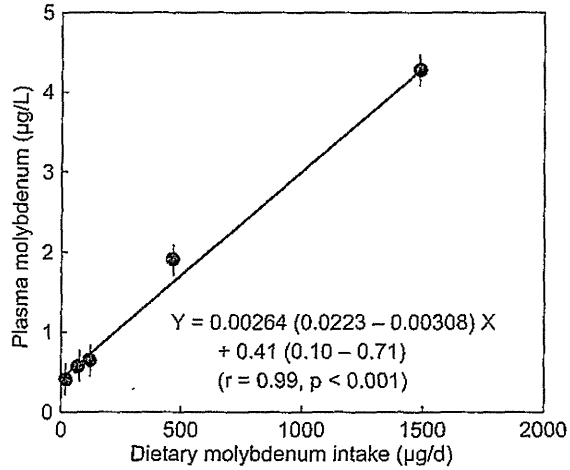


**FIGURE 14.2** Relationship between Mo intake and serum Mo concentration of four American male adults in a balance study performed by Turnlund and Keyes [8]. Values within parentheses indicate 95% confidence intervals.

500 μg/day cannot occur. When the highest dietary Mo intake is estimated to be 500 μg/day, the highest serum/plasma Mo concentration (95% confidence interval) is in the range 1.22–2.25 μg/L. Accordingly, when the serum/plasma Mo concentration is more than 2.25 μg/L, a metabolic abnormality or abnormal exposure must be considered. This criterion is reasonable when considering the serum Mo concentration of Japanese healthy adults [11].

### 14.5.2. Index of Molybdenum Exposure

***14.5.2.1. Occupational Exposure*** There are several reports on occupational exposure to Mo. Twenty-five workers employed at a molybdenite roasting plant in Denver in the US were exposed to Mo dust at an 8 h time-weighted average value of 9.47 $mg/m^3$ [2]. This Mo exposure caused a large elevation of serum ceruloplasmin and smaller increases in serum uric acid levels. Joint pains, backaches, headaches, and nonspecific hair and skin changes were the most frequent complaints of the workers. Plasma Mo concentration of the workers ranged from 9 to 330 μg/L. This plasma Mo concentration is markedly higher than the upper limit of serum/plasma Mo in healthy adults established above. Accordingly, measurement of serum/plasma Mo can demonstrate occupational exposure to Mo.

***14.5.2.2. Artificial Joint*** Joint-replacement surgery has revolutionized the treatment of osteoarthritis as the most effective therapy. Due to the possibility that several trace elements are released from the prosthesis into blood, it is necessary to monitor the trace element levels in blood and to examine the long-term effects of trace element exposure. Since a metal-on-metal prosthesis is frequently used in current joint-replacement surgery, the chance of exposure to several trace elements is deemed to increase. This metal is used as a material for several prostheses, as well as Al, Co, Cr, Ni, Ti, and V. In fact, there are several reports on the monitoring of blood Mo in patients with artificial joints [30, 31]. Luetzner et al. determined changes in serum levels of Co, Cr, and Mo in 41 patients after cemented unconstrained total knee arthroplasty without patellar resurfacing, 18 with unilateral total knee arthroplasty, and 23 patients with bilateral total knee arthroplasty surgeries. While serum Co and Cr increased in patients compared to the controls without implants, the serum Mo in patients (median, 2.55 μg/L) was not significantly higher than in controls (median, 2.11 μg/L). Since the control values in this study are close to the upper limit of serum/plasma Mo in healthy adults (2.25 μg/L), a problem in the analysis of serum Mo may exists in this study. Thus, no evidence of Mo exposure by artificial joints using blood Mo has been found; however, it is thought that measuring blood (serum/plasma or whole blood) Mo can monitor exposure to this metal from artificial joints.

### 14.5.3. Index of Various Diseases

***14.5.3.1. Liver Dysfunction*** In a Belgian study, serum Mo concentration was significantly increased in subjects with various types of liver disease and significant correlations between serum Mo and activities of serum aspartate aminotransferase

(AST) and alanine aminotransferase (ALT) were observed [23]. Significant correlations between serum Mo and the activities of AST and ALT were also found in the Japanese study [11]. Since Mo accumulates in the liver at a level of more than 300 μg/kg [32], high serum Mo in subjects with liver dysfunction may be derived from a liver leakage. Similarly to serum Mo, serum levels of xanthine oxidase, a molybdoenzyme, were significantly higher in patients with liver disease than in healthy controls [33]. Thus, serum Mo is increased by liver disease and is a suitable index for liver function.

***14.5.3.2. Renal Failure*** Serum Mo levels of 60 patients with chronic renal failure were examined before and after hemodialysis [34]. The serum Mo significantly decreased from 27 ± 19 μg/L before hemodialysis to 14 ± 7 μg/L after hemodialysis. The correlations between serum Mo and serum β2-microglobulin, serum parathyroid hormone, serum calcium, and duration of hemodialysis were significant. Serum Mo in patients markedly exceeded the upper limit of healthy adults even after hemodialysis. Since the main excretion route of Mo is urine, subjects with renal failure show remarkably high serum Mo values [9, 35]. However, subjects with high serum urea in a medical examination did not show high serum Mo (Yoshida, M. et al., unpublished data). Therefore, serum Mo is not an index of early-stage renal dysfunction, but an index of renal failure.

## 14.6. CONCLUSIONS

The determination of Mo in biological materials can be performed using ICP-MS. Food groups with high Mo concentration are plant foods, such as cereals, pulses, nuts, and their products. The Mo intake in most countries ranges from 100 to 300 μg/day and is thought to be adequate. A large part of Mo in diet is readily absorbed in intestine and then excreted to urine. Serum Mo and urinary Mo are strongly associated with Mo intake. When the Mo concentration in serum is more than 2.25 μg/L, a metabolic abnormality or abnormal exposure should be assumed.

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*Chapter 8*

# PATIENTS WITH CROHN'S DISEASE HAVE HYPOVITAMINOSIS D AND K, WHICH IS INDEPENDENT OF GENERAL MALNUTRITION

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## INTRODUCTION

Inflammatory bowel disease (IBD) consisting of Crohn's disease (CD) and ulcerative colitis (UC) is an inflammatory disease affecting the gastrointestinal tract. As its name implies, UC mainly affects large intestine. In contrast, CD can affect all part of the gastrointestinal tract from mouth to anus, but in most cases with CD, small intestine is involved. Nutritional complications are common in patients with IBD, especially those with CD[1,2]. Deficiencies of proteins, calories, and vitamins are quite prevalent in subjects with CD, which may be caused by such factors as inadequate dietary intake, intestinal loss of protein, or malabsorption[3-6]. Previous studies have reported the high prevalence of hypovitaminosis D or K in patients with IBD [7-11].

Vitamin D, amongst its diversity of actions, enhances intestinal absorption of calcium and phosphorus as its fundamental action [12]. Its deficiency causes mineralization defect; rickets and osteomalacia[13]. Its inadequacy, even in its milder form (insufficiency) increases the risk of fracture through negative calcium balance and secondary hyperparathyroidism[14]. Recently, the skeletal action of vitamin K has come to our attention, although its sole action of clinical significance has long been considered to be the one as the co-factor for γ-

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carboxylation of four of the clotting factors in the liver [15]. Skeletal vitamin K insufficiency has been demonstrated to be a risk factor for fracture [16,17].

Osteoporosis is a common complication of IBD [18-22]. It has also been reported that the incidence of fracture among patients with IBD was greater than that in general population [23]. Thus, hypovitaminosis D and K is likely to be of great importance in the evaluation and possibly the treatment of osteoporosis associated with IBD.

In our recent report [24], CD patients had significantly lower plasma vitamin K and D concentrations, and significantly higher serum levels of markers for the inadequacy of these vitamins; parathyroid hormone (PTH), protein induced by vitamin K absence (PIVKA)-II and undercarboxylated osteocalcin (ucOC), which are indicators for vitamin D insufficiency, hepatic vitamin K insufficiency, and skeletal vitamin K insufficiency, respectively. Subjects with CD had significantly lower BMD scores at almost all measurement sites than those with UC. Plasma levels of vitamin D and K correlated with BMD and the patients' fat intake, but not with their intake of these vitamins. Multiple regression analysis revealed that low plasma concentrations of vitamin D and K were independent risk factors for low BMD at radius. These results suggested that maintaining vitamin D- and K-adequacy is necessary for the bone health. The insufficiency of these vitamins is likely to arise from their malabsorption probably due to restricted fat intake as well as the compromised intestinal ability to absorb the nutrients.

However, nutritional indices such as serum albumin and total cholesterol levels were also lower in patients with CD than those with UC. Thus one could argue against the role of these vitamins by stating that decreased blood concentrations of these vitamins merely reflect the overall malnutrition.

In this paper, we have attempted to demonstrate that decreased blood concentrations of vitamin D and K in IBD patients are independent of general malnutrition by use of one of the multivariate analyses; principal component analysis (PCA).

## SUBJECTS AND METHODS

### Study Subjects

The study subjects were 128 patients with IBD (male:78, female:50) consisting of 60 patients with CD (male:36, female:24) and 68 patients with UC (male:42, female:26) visiting the Gastroenterology Clinic of Kyoto University Hospital. Consent to participate in this study was obtained after explanation of the objective and protocol of this study.

### Bone Mineral Density (BMD) Measurement

BMD was measured at various skeletal sites; lumbar spine, femoral neck, total hip, distal one-third of radius, ultradistal radius with dual energy X-ray absorptiometry (DXA) using Hologic QDR-2000 (Bedford, MA). Data were expressed as z-value, which shows a standard deviation from age- and sex-adjusted average based on the Japanese reference database.

## Blood Examination

Blood was obtained after overnight fasting. After centrifugation, plasma or serum samples were stored at –30°C with protection from light until analysis. Serum concentration of 25-hydroxyvitamin D (25OH-D) was measured by radioimmunoassay (RIA) (DiaSorin, Stillwater, MN, USA). 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), HPLC analyses were conducted with a HPLC system (Shimadzu, Kyoto, Japan), Mass spectrometry was performed with API3000 LC-MS/MS System (Applied Biosystems, Foster City, CA). $^{18}O$-labeled vitamin K was the internal standard in the analysis of plasma vitamin K [25]. Serum intact-PTH was measured by an electro immunoradiometric assay (IRMA) (Scantibodies Laboratory, Santee, CA, USA), with 14-66 pg/ml as the reference range.

Other biochemical markers were measured by auto-analyzer. Approximately half of serum calcium is bound to albumin, and its serum concentration is affected by alteration in serum albumin level. Therefore, serum calcium concentration was corrected as follows; corrected calcium=calcium – (albumin – 4).

## Statistical Analysis

Statistical analyses were done with SPSS 17.0J (SPSS Japan, Tokyo). Comparison of independent two groups was done with Student's t-test or Mann-Whitney test depending on normality. Multiple regression analyses were performed to investigate the determinants for BMD at each site. Data on various nutritional indices and circulating vitamin D- and K-levels were analyzed with principal component analysis (PCA), which is a statistical method to summarize the various parameters into small number of summary factors (components). These components are obtained in such a way that 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. Thus, they are independent of each other. Components with the eigenvalue greater than 1 were adopted as is used in the usual practice.

# RESULTS

## Biochemical Markers

The baseline characteristics of the study subjects are shown in Table 1. Patients with CD were significantly younger than those with UC. CD patients had higher CRP level, and lower nutritional indices such as BMI, hemoglobin, serum albumin and cholesterol concentrations than those with UC. Other indices were not significantly different between two groups, and largely fell into the reference ranges.

**Table 1. Baseline characteristics of the study subjects**

| | IBD (n=128) | CD (n=60) | UC (n=68) | p value |
|---|---|---|---|---|
| Age (years) | | 34.4+/-7.5 | 41.7+/-16.4 | <0.01 |
| Sex (Male/Female) | | 36/24 | 42/26 | - |
| Body mass index (kg/m$^2$) | | 19.2+/-2.7 | 21.1+/-3.0 | <0.01 |
| C-reactive protein (mg/dl) | | 0.7+/-1.0 | 0.3+/-0.6 | 0.01[b] |
| White blood cell (x10$^3$) | | 6.1+/-1.9 | 5.9+/-1.9 | NS |
| Red blood cell ( | | 4.3+/-0.5 | 4.5+/-0.6 | NS |
| Hemoglobin (g/dL) | | 11.8+/-1.9 | 13.1+/-2.0 | <0.01 |
| Total protein (g/dL) | | 6.8+/-0.7 | 7.1+/-0.4 | 0.01 |
| Albumin (g/dl) | | 3.9+/-0.4 | 4.3+/-0.4 | <0.01 |
| Triglyceride (mg/dL) | | 95.4+/-40.7 | 101.3+/-77.6 | NS |
| Total cholesterol (mg/dl) | | 131.7+/-25.2 | 176.5+/-36.6 | <0.01 |
| Alkaline phosphatase | | 228.9+/-72.3 | 227.5+/-95.8 | NS |
| Corrected calcium (mg/dl) | | 8.8+/-0.3 | 8.8+/-0.3 | NS |
| Phosphorus | | 3.3+/-0.7 | 3.4+/-0.4 | NS |

Data are expressed as mean+/-SD.

The p value indicates the statistical difference between CD and UC patients based on Student's t-test or Mann-whitney test depending on normality.

**Table 2. Serum concentrations of 25(OH)D, PTH, and vitamin K in IBD patients**

| | IBD (n=128) | CD (n=60) | UC (n=68) | p value |
|---|---|---|---|---|
| 25OH-D (ng/ml) | | 14.1+/-8.5 (12.6) | 18.5+/-7.2 (18.2) | <0.01 |
| PTH (pg/ml) | | 30.6+/-12.4 (28.0) | 26.0+/-9.7 (25.5) | 0.04 |
| PK (ng/ml) | | 0.60+/-0.72 (0.45) | 0.86+/-0.54 (0.78) | <0.01 |
| MK-7 (ng/ml) | | 2.70+/-7.49 (0.45) | 6.43+/-10.1 (1.67) | <0.01 |

Data are expressed as mean+/-SD with the values in parentheses showing the median.

The p value indicates the statistical difference between CD and UC patients based on Student's t-test or Mann-whitney test depending on normality.

## Circulating Levels of Vitamins D and K

Serum 25OH-D concentration was significantly lower and PTH level was significantly higher in CD patients than UC subjects. There is a general consensus that serum 25OH-D concentration less than 20 ng/mL indicates hypovitaminosis D. Serum 25OH-D level was below 20 ng/ml in 82% of patients with CD and 57% of patients with UC. Most of the subjects had serum PTH level within the reference range (≤66 pg/mL). Serum concentrations of PK and MK-7 were significantly lower in patients with CD than those with UC (Table 2). In our recent report using the same assay procedure, mean plasma PK level was 1.74 ng/ml, and plasma MK-7 level was 4.96 ng/ml in healthy Japanese women aged 30-49 [26]. Therefore, circulating levels of vitamin K were considered to be extremely lower in IBD patients than that in the healthy subjects.

**Table 3. BMD in patients with CD and UC**

| | CD (n=27) | UC (n=31) | p value |
|---|---|---|---|
| Lumbar spine (L1-4) | -0.52+/-0.64** | -0.45+/-1.15* | NS |
| Femoral neck | -1.02+/-0.85** | -0.45+/-1.12 | NS |
| Total hip | -0.87+/-0.81** | -0.27+/-1.08 | 0.03 |
| Distal one-third of radius | -1.85+/-1.15** | -1.79+/-1.51** | NS |
| Ultra distal radius | -1.87+/-0.86** | -1.20+/-1.36** | NS |

Data are shown as the z-score normalized by gender and age and expressed as mean +/- SD. The p value denotes the statistical significance in BMD in CD and UC subjects. The asterisks show the statistically significant difference from zero (*: $p<0.05$, **<0.01).

## BMD Measurement

In Table 3 are shown the data on BMD. Although patients with CD had z-score significantly lower than zero at all measurement sites, UC subjects had z-score below zero at lumbar spine, distal one-third and ultradistal radius. BMD in CD patients was significantly lower at total hip

## Principal Component Analysis (PCA)

PCA was performed with the parameters included for analysis being hemoglobin serum albumin, total cholesterol, 25OH-D level and plasma PK, MK-7 concentrations (Table 4). Two components were obtained and explained 56.7% of the variance. The first component was composite of high albumin, total cholesterol and hemoglobin, and second component consisted of high 25OH-D, PK and MK-7. The interpretation of each component was made as follows; the first component representing overall nutritional status, the second component, vitamin D and K status. When CD and UC subjects were analyzed separately, vitamin D and K status was also independent of overall nutritional status (data not shown).

**Table 4. Principal component analysis of nutrition indices**

| | Component 1 | Component 2 |
|---|---|---|
| Serum Albumin | **0.828** | 0.040 |
| Serum total cholesterol | **0.675** | 0.056 |
| Hemoglobin | **0.786** | 0.241 |
| Serum 25OH-D | 0.309 | **0.730** |
| Plasma PK | -0.125 | **0.820** |
| Plasma MK-7 | 0.130 | **0.498** |

Factor loadings to four components after varimax rotation are shown. Loadings greater than 0.35 are shown in bold

Two components thus obtained were considered to represent the following nutritional status; component 1 overall nutritional status, component 2 vitamin status

**Table 5. Comparison of two parameters obtained from PCA.**

| | CD (n=60) | UC (n=68) | P value |
|---|---|---|---|
| First Component (Overall Nutritional Status) | -0.54+/-0.87 | 0.47+/-0.86 | <0.001 |
| Second Component (Vitamin D and K Status) | -0.25+/-1.08 | 0.22+/-0.87 | 0.006 |

The p value indicates the statistical difference between CD and UC patients based on Student's t-test.

Next, these two summary scores were compared between CD and UC subjects. Both of them were significantly lower in CD patients than UC subjects, which suggests that patients with CD are both under-nourished and in vitamin D- and K-insufficiency (Table 5).

## Multiple Regression Analyses for Factors Associated with BMD Z Scores At Various Site

Multiple regression analyses were done to study to what extent the BMD (z-score) could be explained by these summary scores (Table 6). BMD at all measurement sites except lumbar spine and distal one-third radius were significantly contributed by these two summary scores, with first component (overall nutritional status) contributing to BMD at ultradistal radius, and second component (vitamin status) significantly contributed to BMD at femoral neck, total hip, and ultradistal radius.

**Table 6. Multiple Regression Analyses for BMD.**

| Sites | $R^2$ | p value | Variable | β | p value |
|---|---|---|---|---|---|
| Lumbar spine | 0.06 | 0.07 | Overall Nutritional Status | 0.10 | 0.44 |
| | | | Vitamin D and K Status | 0.27 | 0.04 |
| Femoral neck | 0.21 | <0.01 | Overall Nutritional Status | 0.11 | 0.45 |
| | | | Vitamin D and K Status | 0.47 | <0.01 |
| Total hip | 0.18 | <0.01 | Overall Nutritional Status | 0.22 | 0.09 |
| | | | Vitamin D and K Status | 0.36 | <0.01 |
| Distal one-third of radius | 0.02 | 0.65 | Overall Nutritional Status | 0.10 | 0.51 |
| | | | Vitamin D and K Status | 0.07 | 0.63 |
| Ultradistal radius | 0.14 | <0.01 | Overall Nutritional Status | 0.28 | 0.04 |
| | | | Vitamin D and K Status | 0.26 | 0.05 |

Abbreviations are as follow; β for β coefficient and p for p value. Determinants of independent predictors for BMD at each site were analyzed by multivariate analysis with stepwise method. Variables included were two parameters obtained from PCA.

## DISCUSSION

The insufficiency of vitamin D and K are associated with increased risk of fracture[14,27-29]. However, the importance of vitamin insufficiency is often overlooked. Classical vitamin deficiency diseases are easily diagnosed, since they are accompanied by phenotypic abnormalities such as rickets or osteomalacia in vitamin D deficiency, and bleeding tendency in vitamin K deficiency. In contrast, vitamin insufficiency could not be diagnosed for the individual subjects phenotypically, and the increased risk of chronic diseases due to vitamin insufficiency becomes apparent only by the epidemiological studies.

Thus each subject could only be evaluated by the surrogate markers. Serum concentration of 25OH-D best reflects the vitamin D status. It is a general consensus that its concentration below 20 ng/mL is associated with increased fracture risk, although recent evidences indicate that higher concentration around 30 ng/mL is necessary for fracture prevention[30,31]. In the case of vitamin K, there are some methodological problems. First, vitamin K consists of phylloquinone (vitamin $K_1$) and menaquinones (vitamin $K_2$), the latter further composed of several analogs with different length of side chain. Therefore, vitamin K status cannot be evaluated by a single measurement of blood level of one of the vitamin K analogs. In addition, although circulating vitamin K levels have been measured with various methods, results are different between assay procedures. The present data were obtained with our newly developed LC-APCI-MS/MS procedure with stable isotope-labeled internal standard yielding high sensitivity and specificity [25]. Thus, measurement of circulating levels of these vitamins aids us to evaluate the subjects' vitamin status, especially vitamin insufficiency.

In the present study, patients with IBD had hypovitaminosis D and K, especially those with CD. Patients with CD had also lower overall nutritional status than those with UC. We have recently reported that hypovitaminosis D and K were associated with decreased BMD in IBD subjects[24]. However, osteoporosis is a common complication of IBD, for which many factors have been reported to be responsible; low body weight, inflammatory process, and therapeutic glucocorticoid use[18-22].

Thus, it must be decided whether the decreased concentrations of vitamin D and K are merely the reflection of overall malnutrition or not. Confounders are serious challenges in the clinical and epidemiological studies. In the intervention studies, randomized controlled trial (RCT) would largely eliminate the interference by confounders. Of the observational studies, cohort study would be less sensitive to the confounding variables. In the cross-sectional studies, eliminating the interference by confounders is a tough methodological challenge.

The main strategies to control for confounding in observational epidemiological investigations and, in particular, in case-control studies, are restriction, matching, stratification and fitting of regression models. In the present study, we have attempted to discriminate hypovitaminosis D and K from general malnutrition by use of another statistical method; principal component analysis (PCA). It is a useful statistical procedure to summarize the complex and diverse data into the small number of independent components (summary score). In the epidemiological or clinical studies, PCA has been employed in the dietary pattern analysis [32,33].

Two components or the summary scores obtained in the current study were considered to represent the overall nutritional status, and vitamin D- and K-status, respectively. Both of them were lower in patients with CD than those with UC. Since they are independent of each

other, these results suggested that subjects with CD had both hypovitaminosis D and K, and general malnutrition, but the former is not merely a reflection of the latter. The results by multiple regression analyses indicated that insufficiency of these vitamins would negatively affect BMD at various skeletal sites.

We believe that our current report carries significance both clinically and methodologically, it is not free from limitation. Although the multiple regression analysis has identified vitamin D and K status as the significant contributor to BMD at femur and radius, the $R^2$ value was low, which indicates that the current model could explain only a small portion of variation. Further studies are required for the additional determinants for BMD in IBD.

In conclusion, patients with CD have hypovitaminosis D and K independent of general malnutrition, and analyzing data from cross-sectional studies with PCA would be of help in eliminating the interference by confounding variables.

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