

Results

The baseline characteristics and data from blood examination are shown in Table 1. CD patients were younger, but had longer disease duration than those with UC. Although body mass index (BMI) was not significantly different between these groups, nutritional indices such as serum albumin and total cholesterol levels were lower and serum inflammatory marker, C-reactive protein (CRP), was higher in patients with CD than those with UC. Serum calcium level was not different between the two groups. Plasma concentrations of PK and MK-7 were significantly lower, and serum PIVKA-II and ucOC levels were reciprocally higher in CD patients than those with UC. We have then performed the multiple regression analysis to identify factors affecting serum ucOC level, since it may be subject to altered bone turnover. Serum BAP and plasma PK were both significant predictors for serum ucOC level ($R^2=0.453$; $\beta=0.442$, $p=0.036$ and $\beta=0.415$, $p=0.044$). More detailed consideration on plasma vitamin K levels will be done in the "Discussion" section, since no definite reference values are available at present.

Current concept holds that plasma 25OH-D levels of less than 20 ng/mL and between 21 and 29 ng/mL indicate vitamin D deficiency and insufficiency, respectively [28,

29]. Average plasma 25OH-D concentration was 15.7 ng/mL in IBD patients as a whole, 11.2 and 20.2 ng/mL in CD and UC patients, respectively. Plasma 25OH-D level was below 20 ng/mL in all patients with CD and approximately 60% of patients with UC. Serum PTH concentration was significantly higher in CD than in UC patients, and above the cut-off value of 55 pg/mL in approximately 40% and 20% of patients with CD and UC, respectively. Serum BAP and NTX-I were higher in CD than UC patients, although statistically not significant.

BMD measurement

Considering that CD patients were significantly younger than UC subjects, comparison of BMD in these groups was made principally based on Z scores, which was significantly lower than zero in all measurement sites in CD and distal one-third of radius in UC. Thus, the Z score in the CD group was significantly lower than that in the UC group, except at the distal one-third of radius where Z score was decreased in both groups. Results expressed as T score are basically the same, although the difference between CD and UC was not so marked than expressed as Z score, probably reflecting the younger mean age in the CD group. T scores at the distal one-third of radius were below -2.5 SD in 39%

Table 1 Background profiles and results from blood tests in patients with CD and UC

	IBD (n=70)	CD (n=29)	UC (n=41)	p value
Age (years)	36.4±12.4 (34.0)	32.2±6.7 (31.0)	39.3±14.6 (37.0)	0.008 ^a
Sex (male/female)	44/26	20/9	24/17	—
Body mass index (kg/m ²)	20.4±3.0 (20.3)	20.1±2.8 (19.5)	20.7±3.2 (20.8)	0.401 ^a
Disease duration (years)	9.8±8.3 (9.0)	12.7±6.6 (12.0)	7.8±8.8 (5.0)	0.001 ^b
Glucocorticoid therapy (n)	25	8	17	—
Immunosuppressive therapy (n)	27	19	8	—
Infliximab therapy (n)	3	3	0	—
C-reactive protein (mg/dL)	1.4±2.8 (0.3)	2.4±3.2 (0.8)	0.7±2.2 (0.2)	<0.001 ^b
Albumin (g/dL)	4.1±0.6 (4.1)	3.9±0.5 (3.9)	4.3±0.6 (4.4)	0.001 ^b
Total cholesterol (mg/dL)	153.0±42.3 (145.5)	126.1±26.3 (120.0)	175.8±40.1 (177.0)	<0.001 ^b
Calcium (mg/dL)	8.9±0.4 (9.0)	8.8±0.4 (8.8)	9.0±0.3 (9.1)	0.095 ^a
PK (ng/mL)	0.735±0.533 (0.570)	0.462±0.281 (0.470)	0.985±0.591 (0.890)	0.002 ^b
MK-7 (ng/mL)	3.282±4.414 (1.369)	1.989±3.824 (0.470)	4.472±4.657 (2.190)	0.001 ^b
PIVKA-II (mAU/mL)	22.77±8.54 (22.0)	25.75±9.34 (24.50)	19.79±6.57 (18.50)	0.020 ^b
ucOC (ng/mL)	8.52±7.96 (5.84)	12.26±9.65 (9.08)	4.94±3.21 (3.93)	<0.001 ^b
25OH-D (ng/mL)	15.69±6.71 (15.5)	11.20±4.20 (11.00)	20.18±5.68 (19.50)	<0.001 ^a
PTH (pg/mL)	50.76±21.58 (45.8)	57.00±22.74 (42.90)	44.53±18.80 (41.20)	0.031 ^b
Serum BAP (μg/L)	15.0±7.2 (12.5)	16.3±7.7 (12.9)	12.6±5.5 (10.3)	0.190 ^b
Serum NTX-I (nmol BCE/L)	15.0±6.8 (14.3)	16.8±7.9 (15.2)	12.8±4.4 (11.9)	0.077 ^b

Data are expressed as the mean±SD with the values in parentheses showing the median.

PK phyloquinone, MK-7 menaquinone-7, PIVKA-II protein induced by vitamin K antagonist, ucOC under carboxylated osteocalcin, BAP bone specific alkaline phosphatase

^a Comparison of indices between patients with CD and those with UC were done by unpaired *t* test depending on normality

^b Comparison of indices between patients with CD and those with UC were done by Mann-Whitney test depending on normality

of CD patients and 18% of UC patients and between -2.5 and -1.0 SD in 50% and 55% of subjects with CD and UC, respectively (Table 2).

Multiple regression analyses for variables associated with BMD Z scores at various sites

Multiple regression analyses were done for BMD including BMI, plasma concentrations of PK, MK-7, and 25OH-D as independent variables. Serum PTH level was excluded since coinclusion of 25OH-D and PTH caused multicollinearity to skew the results. As shown in Table 3, BMI was a significant predictor of BMD at weight-bearing sites such as the lumbar spine, femoral neck, and total hip. Plasma MK-7 and 25OH-D concentrations were significant predictors of femoral neck BMD. Plasma PK concentration was a significant predictor of BMD at the distal one-third of radius and lumbar spine.

Analysis of food intake in CD and UC patients

Food intake could be evaluated in 25 patients (15 with CD and 10 with UC). Fat intake was significantly lower and protein intake was significantly higher in patients with CD than those with UC. The results were similar when expressed as the percentage of total energy intake. The adequate intakes (AI) for calcium in Japan are 600–650 mg for men and 550–600 mg for women. AI for vitamin K is 75 µg for men and 65 µg for women, respectively, and that for vitamin D is 5 µg [30]. As a whole, although the

Table 3 Multiple regression analyses for the determination of independent factors for BMD

Sites	R ²	Variable	β coefficient	p value
Lumbar spine	0.529	BMI	0.663	0.005
		Plasma PK	0.612	0.035
Femoral neck	0.748	BMI	0.363	0.028
		Plasma MK-7	0.295	0.036
		Plasma 25OH-D	0.484	0.037
Total hip	0.731	BMI	0.438	0.012
Distal one-third of radius	0.388	Plasma PK	0.813	0.016

Only significant predictors are shown. Determinants of independent predictors for BMD at each site were analyzed by multivariate analysis with forced entry. Variables included were BMI, plasma 25OH-D, PK, and MK-7

average calcium intake was below AI, vitamin K and D intakes apparently exceeded AI (Table 4).

Ten patients with CD were on enteral nutrition (EN) with almost fat-free formula; Elental® (Ajinomoto Pharma, Tokyo, Japan) with 18.8%, 1.4%, and 79.8% of total energy contributed by protein, fat, and carbohydrate, respectively. One patient with UC was on total parenteral nutrition. When nutrient intake was compared between CD patients with EN and those without EN, the former had higher protein and carbohydrates intakes and lower fat intake than the latter. Regarding other nutrients intake, there was no significant difference between the two groups except calcium. There were no significant differences in plasma vitamin K and 25OH-D concentrations between these groups (data not shown).

Multiple regression analyses for plasma vitamin K and 25OH-D concentrations

Multiple regression analyses revealed that fat intake was a significant determinant of plasma PK and 25OH-D levels. Vitamin K intake was a significant predictor for plasma MK-7 level (Table 5).

Discussion

In this study, we have studied the IBD-induced osteoporosis in relation to vitamin K and D status of the patients. Decreased BMD and high-turnover bone was far more pronounced in patients with CD than those with UC.

Although glucocorticoid treatment is one of the postulated pathogenic factors for osteoporosis in IBD [1, 3, 31, 32], current use of glucocorticoid was not associated with decreased BMD in the present study. Unfortunately, the possible involvement of glucocorticoid could not be

Table 2 BMD in patients with CD and UC

	CD (n=18)	UC (n=22)	p value
BMD (g/cm ³)			
Lumbar spine (L1-4)	0.880±0.072	0.931±0.138	0.152
Femoral neck	0.697±0.105	0.768±0.126	0.064
Total hip	0.801±0.120	0.910±0.136	0.012
Distal one-third of radius	0.634±0.066	0.664±0.084	0.222
Z scores			
Lumbar spine (L1-4)	-0.96±0.57**	-0.14±1.13	0.005
Femoral neck	-1.00±0.78**	-0.09±1.16	0.005
Total hip	-0.85±0.91**	0.27±1.11	0.001
Distal one-third of radius	-2.19±0.94**	-1.29±1.79**	0.064
T scores			
Lumbar spine (L1-4)	-1.18±0.59	-0.79±1.06	0.155
Femoral neck	-1.14±0.85	-0.56±1.05	0.067
Total hip	-0.95±0.97	-0.11±1.08	0.014
Distal one-third of radius	-2.31±1.00	-1.83±1.81	0.055

Values represent the mean±SD, and comparison between CD and UC groups was made with unpaired t test

**p<0.01, statistically significant difference from zero with one-sample t test in the Z score

Table 4 Food intake in CD and UC patients

	IBD (n=25)	CD (n=15)		UC (n=10)	p value	EN p value
		EN therapy (n=10)	Non-EN therapy (n=5)			
Energy (kcal)	1,707±479 (1,580)	1,961±465 (1,796)	1,412±320 (1,501)	1,602±466 (1,524)	0.338 ^a	0.055 ^a
Energy intake from EN (kcal)	–	810±318 (750) (min 300–max 1200)	–	–	–	–
Proportion of total energy intake from EN (%)	–	42.0±16.8 (39.1) (min 20–max 77)	–	–	–	–
Protein (g)	68.2±19.3 (62.8)	81.9±21.1 (79.8)	60.3±12.3 (61.9)	58.5±11.8 (57.0)	0.022 ^b	0.028 ^b
Fat (g)	29.9±13.9 (28.3)	22.1±10.0 (24.0)	29.1±7.5 (30.7)	38.1±15.8 (38.1)	0.030 ^b	0.164 ^b
Carbohydrates (g)	287.8±98.4 (274.3)	359.0±85.3 (339.3)	223.5±60.3 (242.1)	248.9±85.5 (258.9)	0.098 ^b	0.005 ^b
Calcium (mg)	483±250 (431.0)	662±230 (675)	380±144 (351)	356±214 (354.5)	0.032 ^b	0.014 ^b
Vitamin K (µg)	131.1±124.6 (73.0)	96.8±68.8 (66.0)	207.0±220.9 (73.0)	127.5±102.2 (97.0)	0.846 ^a	0.337 ^b
Vitamin D (µg)	9.6±10.4 (6.9)	9.3±7.4 (7.4)	10.2±13.3 (1.5)	9.6±12.5 (6.6)	0.782 ^a	0.893 ^b
Macronutrient (% energy)						
Protein	16.2±2.9 (15.6)	16.7±2.0 (16.0)	17.4±2.4 (17.4)	15.2±3.7 (14.4)	0.008 ^a	0.617 ^b
Fat	16.4±7.7 (14.9)	10.1±4.0 (10.6)	18.8±3.9 (19.0)	21.5±7.6 (21.1)	0.009 ^b	0.004 ^b
Carbohydrates	66.5±8.7 (66.1)	73.4±6.4 (72.7)	62.7±5.1 (61.3)	61.5±7.8 (61.9)	0.017 ^b	0.005 ^b

Values represent the mean±SD with values in parentheses being the median. "p value" and "EN p value" represent the comparison between CD and UC patients and the comparison between CD subjects with EN and those without EN, respectively

^aComparisons between CD and UC patients and that between CD with EN and without EN were done with unpaired *t* test depending on normality

^bComparisons between CD and UC patients and that between CD with EN and without EN were done with Mann–Whitney test depending on normality

evaluated in more detail, since most of them were referred to the university hospital from another hospital and cumulative dose of glucocorticoid could not be precisely calculated. We believe, however, that glucocorticoid use is unlikely to be mainly responsible for the decreased BMD in the current subjects based on the following consideration. Trabecular bone is mainly affected in glucocorticoid-induced osteoporosis (GIO) [33]. In GIO, decreased BMD is most prominent at the lumbar spine with trabecular predominance [33]. In contrast is the present finding that decreased BMD was most marked at the distal one-third of radius, a site of cortical predominance.

Table 5 Multiple regression analyses for the predictor(s) of plasma 25OH-D, PK, and MK-7 levels

	R ²	Variable	β coefficient	p value
Plasma PK	0.586	Fat intake	0.620	0.030
Plasma MK-7	0.464	Vitamin K intake	0.708	0.036
Plasma 25OH-D	0.452	Fat intake	0.584	0.046

Only significant predictors are shown. Independent predictor for plasma PK, MK-7, or 25OH-D concentrations was analyzed by multivariate analysis with forced entry. Serum CRP level and intakes of protein, fat, and carbohydrates 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

Another possible factor includes disease severity. IBD is associated with increased production of inflammatory cytokines, e.g., IL-1, IL-6, and TNF-α which are potent stimulators of osteoclastic bone resorption [34–36]. Although circulating concentration of these cytokines could not be measured, serum level of CRP was evaluated as an inflammation marker. Although serum CRP level was higher in CD patients, it was not associated with BMD (data not shown).

Low BMI is another factor to be associated with IBD-related osteoporosis [5, 37], but the current results that the average BMI was in the normal range and BMD at nonweight-bearing site was also decreased, which make it unlikely that the reduced BMD in these subjects is related to their BMI.

Then, we focused our attention to the possible involvement of vitamin K and D deficiency. Unfortunately, no single measure can represent the vitamin K status with PK and MK-7 being the two major circulating forms. PK is rich in green vegetables, whereas MK-7 content is extraordinarily high in fermented soy "natto," which is a common food in Japan, but not elsewhere [38, 39]. Large standard deviation in plasma MK-7 concentration probably reflects that some Japanese favors, but some dislike "natto." Indeed, a large geographic difference in plasma MK-7 concentration in Japan was reported to be due to the frequency of natto intake [40]. Since most vitamin K intake

comes from green vegetables in America and Europe [9, 10], previous reports on the plasma concentration of vitamin K from outside Japan focused on PK [11, 40]. Although circulating vitamin K levels have been measured with various methods, 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]. In our recent report from the Nagano study using the same assay procedure, mean plasma PK level was 1.52 ng/mL, 1.74 ng/mL, and 1.29 ng/mL in healthy women aged 30–49, 50–69, and over 70 years, respectively [41]. Thus, blood level of vitamin K was much lower in IBD patients than that in the healthy Japanese measured by the same assay. The data in the Nagano study may be higher than those in the average Japanese, since many participants in the Nagano study were farmers with much vegetable consumption, for which further discussion will be made in the next paragraph.

Then, we considered the physiological relevance of the above data. We measured serum levels of PIVKA-II and ucOC as the sensitive markers of vitamin K deficiency in the liver and bone, respectively, with the cut-off values being 28 mAU/mL for PIVKA-II and 4.5 ng/mL for ucOC. Both levels were significantly higher in CD patients than those with UC. These results, together with the decreased plasma levels of PK and MK-7 in CD patients, strongly suggest that circulating vitamin K levels are decreased at least in patients with CD. Decreased plasma levels of 25OH-D, PK, and MK-7 are likely to have physiological significance considering that they were determinants of BMD at some measurement sites as shown in Table 3, as well as the above-mentioned elevated concentrations of PIVKA-II and ucOC.

The average and median concentration for ucOC, but not for PIVKA-II, was above the cut-off value in these subjects, especially CD patients. Serum PIVKA-II level exceeded the cut-off level in only 25% and 4% of patients with CD and UC, respectively. In contrast, serum ucOC concentration was above the cut-off value in 92% and 36% of patients with CD and UC, respectively. These differences could be explained by a pharmacokinetic feature called "first-pass effect." Vitamin K absorbed from the gastrointestinal tract is transported to the liver via the portal vein where it is used for the γ -carboxylation of clotting factors [42, 43]. Only the vitamin K unutilized in the liver will be available to the bone. Therefore, the bone is likely to be much more susceptible to vitamin K deficiency than the liver. Thus, serum ucOC level well reflects the skeletal vitamin K deficiency, but needs to be interpreted with caution that it is also affected by bone turnover as exemplified with its association with BAP.

The average serum concentration of 25OH-D was 11.5 and 20.2 ng/mL in CD and UC patients, respectively.

Serum PTH concentration was reciprocally higher in CD than in UC. Thus, most IBD patients, especially those with CD, were considered to be vitamin D deficient.

The next consideration relates to the factor(s) responsible for the deficiency of these vitamins. As shown in Table 4, there was no significant difference in vitamin K and D intakes between CD and UC, which suggests that the difference in blood levels of these vitamins could not be ascribed to the difference in their intake. Malabsorption of these vitamins would be the most likely explanation for the apparent discrepancy, which is compatible with the previous report that the absorption of exogenously administered vitamin D₂ was severely disturbed in CD, but not in UC [23].

As the basis for the malabsorption of vitamin K and D, compromised ability of the intestine to absorb these vitamins would be the most fundamental because of intestinal inflammation or intestinal resection in some cases. In the current study, multiple regression analyses revealed that fat intake was a significant determinant of plasma concentrations of both PK and 25OH-D. Many patients in the current study were under nutritional therapy with restricted fat intake, since excessive fat intake is considered to worsen the intestinal inflammation in IBD patients. These results suggest that restricted fat intake could be another factor responsible for the impaired absorption of vitamin K and D, which, however, is not supported by some previous studies. For example, Tangpricha et al. [44] reported that vitamin D dissolved in fat-free orange juice was effectively absorbed from the intestine and indicated that fat content of the diet little influenced vitamin D absorption. Thus, further studies, favorably the intervention ones, are required on the role of fat restriction on the absorption of fat-soluble vitamins.

Unlike PK, vitamin K intake was the significant predictor for plasma MK-7 level. The difference between two vitamin K analogs may reflect their pharmacokinetic difference such as the far longer half-life of MK-7 than PK [38], although further detailed studies are needed. Actually, this study is a baseline valuation. Follow-up study is now under way to evaluate the patients' vitamin status and BMD with milder food restriction with more use of immunosuppressants and biomodulators.

In the present study, vitamin K and D status of IBD patients was both studied, which was not adopted before. The intake of vitamins and their plasma concentration were simultaneously evaluated, which was not usually the case in the previous studies. These would be the strength of the current study. We have to mention two limitations of this study. First, the number of subjects studied was not so large. Thus, it could not be determined whether vitamin K and D deficiency observed in the current study was associated with increased fracture risk as reported in the

previous report [45]. Next, the patients were under nutritional therapy with restricted fat intake. Thus, further studies with larger number of subjects with wider variety of background profiles are necessary to generalize the present findings.

In summary, BMD was decreased and plasma concentrations of PK, MK-7, and 25OH-D were quite low in patients with IBD, especially CD, despite apparently sufficient intake of these vitamins. Impaired intestinal absorption of these fat-soluble vitamins is likely to be associated with vitamin K and D deficiency and bone loss in IBD.

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Conflicts of interest None.

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