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Prevalent hypovitaminosis D in Crohn's disease correlates highly with mediators of osteoimmunology

Abstract

Purpose: In the context of osteoimmunology in Crohn's disease, an association was hypothesized among vitamin D and members of the TNF- α family, known as the RANK (receptor-activator of nuclear factor- κ B)-RANK ligand-osteoprotegerin pathway.

Methods: This was a cross-sectional study of 95 patients with Crohn's disease (80 with long-standing disease and 15 newly diagnosed, never treated) and two control groups (healthy volunteers, n=30; and ulcerative colitis patients, n=30). Spine and hip bone mineral density was measured by dual-energy x-ray absorptiometry. Serum 25-hydroxyvitamin-D₃, TNF- α , IL-6, sRANKL, osteoprotegerin levels and biochemical markers of bone turnover were analyzed.

Results: The precursor metabolite, 25(OH)D₃, was measured in 95 young adult CD patients (47 men, 48 women; median age 30 years). A suboptimal 25(OH)D₃ level was observed in 90% of CD patients, of whom 40% had a serious deficiency. There was no significant difference in 25(OH)D₃ levels between CD patients and those with ulcerative colitis. Analysis revealed an association between 25(OH)D₃ deficiency and the increased biogenesis of osteoclastically-active sRANKL (p=0.014) and the proinflammatory cytokines TNF- α (p=0.015) and IL-6 (p=0.029). CD patients with low bone mineral density had a mean 25(OH)D₃ (35 $\pm$ 18 nmol/l) in the range of serious deficiency to insufficiency, whereas mean 25(OH)D₃ was higher (49 $\pm$ 28 nmol/l) in patients with healthy bone status, although levels were still inadequate (p=0.004). The logistic model reported low levels of 25(OH)D₃ to be a significant predictor of bone disease [odds ratio=2.66(6.8), p<0.009]. In the multivariable analysis, adjusted for several confounding factors, 25(OH)D₃, sRANKL, IL-6 and TNF- α were independently associated with a likelihood of bone disease [odds ratio (range): 1.02(2.75); 1.09(3.71); 1.27(6.95) respectively, p=0.001].

Conclusion: The presented findings suggest that a 25(OH)D₃ deficiency accompanying an inflammatory state in CD is a high risk condition for metabolic bone disease.

Manuscript submitted 16th, January 2014
Manuscript accepted 21st April, 2014

Clin Invest Med 2014; 37 (3): E154-E162.

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Crohn's disease-related bone loss is a serious comorbid condition characterized by varied and complex aetiology [1]. The risk factors can be related to patient genetics, the impact of the inflammatory processes *per se*, environmental factors and/or long-term corticosteroid therapy [2, 3]. The high incidence of bone disease and increasing evidence that Crohn's disease (CD) affects bone status in corticosteroid users and non-users suggest that bone metabolism is strongly affected by the underlying inflammatory state [4].

Vitamin D plays a well-known role in bone formation and mineralization *via* its signalling pathway. Recent findings have revealed that vitamin D also acts as an important regulator of immune system function. The immunomodulatory effects of vitamin D, by down-regulating the T helper-1-driven immune response, suggest its plausible role in autoimmune disorders [5, 6]. Several lines of evidence have implicated vitamin D in deregulated immune response, known as the main characteristic of inflammatory bowel disease (IBD). Firstly, there are epidemiological reports of subclinical vitamin D deficiencies in IBD patients, measured by the precursor metabolite 25-hydroxyvitamin D₃ which is the major circulating form of vitamin D [7–12]. Secondly, numerous studies using various animal models have suggested that vitamin D signalling *via* the vitamin D receptor (VDR) plays an important role in the pathophysiology of the immune system in IBD [13–17]. Finally, experiments on immune-cell populations and inflammatory mediators have made substantial progress in understanding the mechanisms of vitamin D immunomodulatory effects [18–21].

It is becoming evident that the vitamin D signalling pathway is shared between the immune and bone systems [22, 23]. The interplay between these two systems is termed osteoimmunology. In the context of osteoimmunology in CD, we hypothesized that there is an association between vitamin D status and members of the TNF- α family: RANK (receptor activator of nuclear factor κ B), RANK-ligand (RANKL) and osteoprotegerin (OPG). These three TNF- α family members are involved in both the osteoclastogenesis and innate immune response [24–26]; therefore, the main objectives of the present study were *i*) to evaluate serum concentrations of 25(OH)D₃ and free soluble RANKL and OPG levels in newly diagnosed and long-standing Crohn's patients and *ii*) to correlate these values with proinflammatory cytokines - biochemical markers of bone turnover and bone mineral density (BMD) for predicting metabolic bone disease in Crohn's patients.

Methods

Patients

The precursor metabolite, 25-hydroxyvitamin D₃, was measured in 95 subjects either with long-standing (n=80) or newly diagnosed and previously untreated (n=15) CD. The diagnosis of CD was based on clinical, radiological and endoscope findings, and was verified by histological findings of the mucosal biopsy. Patients with other diseases or conditions that may influence bone mass loss (hyperthyroidism, hyperparathyroidism, malignant diseases and postmenopausal women) were excluded. Upon study entry, bone status was measured by dual-energy x-ray absorptiometry and blood was sampled on the same day. Prior to bone density measurements, data were collected on patient age, sex, weight, height, location and disease duration, current medications, menstrual pattern in women, and history of intestinal resection and previous fractures. Of the 80 patients with long-standing CD, 22 had disease duration over 10 years and another five patients had duration of over 20 years. According to the Montreal classification of disease location, upon diagnosis there were 31 patients in the L1 group, 16 in L2 and 42 in L3 (ileocolitis). Data are lacking for six patients. Among the previously diagnosed patients (n=80), 22 had undergone surgery, 10 for small bowel resection and another seven for large bowel resection. Another five patients had combined small and large bowel resection at least once. The group of newly diagnosed and previously untreated CD (n=15) had mild and moderate disease activity according to the CDAI and CRP levels.

Patients were divided into two groups according to corticosteroid therapy: never used and on therapy. If corticosteroid therapy was indicated, it was initiated at dose of 0.75mg/kg daily and then tapered off. Of the 80 patients with long-standing CD, 39 had been treated with steroids over a two month period at least once in their course of the disease. For the remainder of the 41 patients in the previously diagnosed group (n=80), there were no reliable data on steroid consumption and they could not therefore be considered as a steroid naïve group. With regard to steroid treatment, 31 previously diagnosed patients had received treatment with azathioprine, two with methotrexate and 16 had an overlap in both the steroid and azathioprine groups. Three years prior to this study, five patients with perianal disease were treated in a clinical trial with infliximab for remission induction (application of three doses) and, without maintaining remission (if achieved), with anti-TNF therapy.

Laboratory data included complete blood count, basic biochemistry and specific parameters such as free soluble

TABLE 1. Characteristics and biochemical markers in subpopulations of Crohn's disease patients according to 25-hydroxyvitamin D₃ levels

	Adequately supplied 25(OH) _{D3} >75 nmol/l	Deficient 25(OH) _{D3} 30–75 nmol/l	Seriously deficient 25(OH) _{D3} <30 nmol/l	p-value
N, (Female/Male)	9, (5/4)	48, (28/20)	38 (15/23)	
Age (y) mean (95% CI)	29 (19.8–38.4)	32.6 (29–36)	34.5 (30–38.9)	NS
Disease duration (y)	3.5±4.2	8.0±6.7	6.3±6.1	p=0.003
Treatment naïve/Long-standing CD	5/4	4/44	6/32	
Glucocorticoid therapy (yes/no)	4/5	30/18	22/15	
Body mass index (kg/m ²)	20.1±2.6	21.7±5.0	20.4±4.3	NS
25-hydroxyvitamin D ₃ (nmol/l)	89.1±9.6	45.2±12.4	19.7±7.1	p<0.001
Interleukin-6 (pg/ml)	6.15±6.1	6.9±5.3	8.82±5.1	p=0.029
Tumour necrosis factor-α (pg/ml)	3.1±1.3	3.8±1.8	3.53±1.8	p=0.015
C-reactive protein (mg/ml)	35.6±33.4	26.3±42	30.3±38.7	NS
Osteoprotegerin (pg/ml)	109.4±50.6	93.9±33.4	109.5±38.3	NS
Free soluble RANKL (pg/ml)	4.98±2.2	11.8±9.3	11.3±6.9	p=0.014
C-telopeptide (ng/ml)	1.53±1.3	0.95±0.79	1.31±1.3	NS
Osteocalcin (ng/ml)	9.1±6.4	9.2±7.0	10.8±12.2	NS
BMD: L1-L4 Spine z-score	-0.733±1.3	-1.192±1.5	-1.384±1.5	NS
BMD: Total hip z-score	-0.633±1.1	-0.758±1.0	-1.09±1.1	0.043
Bone disease (yes/no)	3/6	34/14	31/7	

RANKL, receptor activator of nuclear factor κB-ligand; C-telopeptide, serum C-telopeptide crosslinked collagen types I; BMD, bone mineral density; Values are expressed as mean±SD; Log-transformed values were used for ANOVA, analysis of variance

RANKL, OPG, TNF-α, IL-6, osteocalcin and C-telopeptide levels. Body mass index (BMI) was calculated (kg/m²). For newly diagnosed and untreated patients, the blood test and data on 25(OH)_{D3}, OPG, sRANKL, IL-6 and TNFα were obtained during their first hospitalization and before any treatment. For other patients, blood samples were taken upon their first admission to this hospital.

To obtain a control group of healthy individuals that was age-sex matched to the CD-patients study group, 30 healthy volunteers (median age 33.5, range 21–45 years) who were not on medication or suffering from conditions affecting bone density and did not have any known disease were selected. The local reference population was recruited from hospital personnel and their friends. As Croatia is a Mediterranean country with high levels of sunlight, and there was no reason to question vitamin D levels in the standard population.

Patients with ulcerative colitis (n=30) had a different disease duration and were considered as second control group. A total of 21 patients with proctitis / left sided colitis and nine with extensive colitis according to Montreal classification were

included. None of the patients were on anti-TNFs or taking vitamin D supplements. In the groups, 12 and seven patients, respectively, were treated with steroids over a two-month period, at least once in their course of the disease. No significant differences in 25(OH)_{D3} levels were found between the steroid users and non-users.

Ethics

The Hospital Ethics Committee approved the study protocol, and informed written consent was obtained from each subject before entering the study.

Bone Status Assessment

The lumbar spine (L1-L4) and total hip BMD was measured by absorptiometry (DXA) using a Delphi W (S/N 700483) instrument (Hologic, Inc., Waltham, , USA). BMD measurements were converted into T-scores reflecting the number of standard deviations below the mean for a young healthy population, and Z-scores reflecting the number of standard deviations

TABLE 2. Determinants of 25-hydroxyvitamin D₃ obtained by forward stepwise multiple regression analysis

Parameters	Bivariate analysis β	<i>p</i> value	Multivariate regression $\beta^{\#}$	<i>p</i> value
Age (y)	-0.396	0.025		
*CD duration (y)	0.05	NS		
*BMI (kg/m ²)	0.164	NS		
Glucocorticoid therapy	-0.05	NS		
*Osteoprotegerin (pg/ml)	-0.07	NS		
* Free soluble RANKL (pg/ml)	-0.313	0.013	-0.242	0.038
*CRP (mg/L)	0.018	NS		
*Interleukin-6 (pg/ml)	-0.385	0.007	-0.412	0.003
* Tumour necrosis factor- α (pg/ml)	0.263	0.043	0.249	0.036
C-telopeptide (ng/ml)	0.093	NS		
* Osteocalcin (ng/ml)	-0.051	NS		
BMD-lumbar	-0.896	NS		
BMD-hip	-0.113	NS		

RANKL, receptor activator of nuclear factor κ B-ligand;

C-telopeptide, serum C-telopeptide crosslinked collagen types I;

BMD, bone mineral density

β bivariate coefficient

$\beta^{\#}$ multivariate regression coefficient

*log-transformed values were used

NS not significant

tions below the mean for age-matched controls (database provided by the manufacturer). According to the International Society for Clinical Densitometry - 2007 ISCD Official Positions, a Z-score ≤ -2 SD is defined as "below the expected range for age". A reduced BMD was considered for Z-scores ≤ -1 SD.

Vitamin D measurement

For the diagnosis of vitamin D deficiency, the precursor metabolite 25-hydroxyvitamin D₃ was measured by the ELISA test purchased from Immunodiagnostik AG (Bensheim, Germany). The assay utilizes a competitive ELISA technique with a selected monoclonal antibody recognizing 25(OH)D₃. Expected values given by the manufacturer are as follows: seriously deficient <30 nmol/l, deficient 30–75 nmol/l and adequately supplied 75 nmol/l.

Cytokine Assay

The sera (aliquots in separate vials stored at -80°C) were assayed for the concentrations of tumour necrosis factor- α

(TNF- α), interleukin-1 β (IL-1 β) and interleukin-6 (IL-6). Serum levels of cytokines were determined using commercially-available high sensitivity ELISAs according to manufacturer's instructions. TNF- α , IL-1 β and IL-6 were purchased from R&D Systems (Minneapolis, MN, USA). All assays employed the quantitative sandwich-enzyme immunoassay technique. The minimum detectable limits were as follows: TNF- α 0.12 pg/ml, IL-1 β <0.1 pg/ml, and IL-6 0.039 pg/ml. Each sample was measured in duplicate.

RANK-ligand and Osteoprotegerin Measurements

Commercially-available specific ELISAs were used according to the manufacturer's instructions (Biomedica GmbH, Vienna, Austria) to determine serum concentrations of soluble RANKL (sRANKL) and OPG. It should be noted that the ELISA method used for RANKL measurements primarily detects free, unbound sRANKL, not the RANKL-OPG complex. Free, unbound sRANKL is the biologically active form of RANKL, and only the unbound state was measured in this study. The low detection limit of the OPG assay was 2.8 pg/ml

and that of free sRANKL 1.6 pg/ml. Each sample was measured in duplicate.

Normative values for free sRANKL and OPG were determined in a control group of 30 healthy volunteers matched by age and sex to the study group of CD patients. The established own normal value (mean, 95% CI) was 5.7 (4.7–6.6) pg/ml for free sRANKL and 52 (44–60) pg/ml for OPG.

Biochemical Parameters of Bone Turnover

Serum tests for bone formation and resorption included osteocalcin and collagen type I C-terminal crosslink. Serum osteocalcin was measured by the Immulite® Osteocalcin (Diagnostic Products Corporation, Los Angeles, CA, USA) immunoassay using a chemiluminescent substrate and reference range for healthy adults of 3.1–13.7 ng/ml.

Collagen type I C-terminal crosslink, a breakdown product of type I collagen secreted to the bloodstream, was measured by a competitive CrossLabs ELISA (Nordic Bioscience Diagnostics A/S, Herlev, Denmark). The expected values given by the manufacturer for various populations are (mean, range; ng/ml): males 0.294 (0.115–0.748), premenopausal women 0.287 (0.112–0.738), and postmenopausal women 0.439 (0.142–1.351).

Statistical analysis

Variables with a skewed distribution (i.e., 25(OH)_D₃, RANKL, OPG, IL-6, TNF- α , CRP, osteocalcin and BMI) were logarithmically transformed prior to further analyses. Differences between continuous variables were evaluated by ANOVA or Mann-Whitney U test, as appropriate. Categorical variables were expressed as percentages and compared using the chi-square test. Linear regression analysis was used to examine the extent to which 25(OH)_D₃ levels were associated with proinflammatory cytokines, RANKL, OPG, biochemical markers of bone turnover, as well as with bone mineral density (BMD) and z-scores. Each variable that showed an independent and significant correlation in the univariate correlation analyses were additionally tested by multiple regression analysis conducted by a stepwise model. Logistic regression analysis was used to identify the predictors of bone disease. In this study, bone disease was set as a dependent variable, and the independent variables used were those with previous statistical significance and those with proven clinical relevance.

Results

Vitamin D and Crohn's disease

Adequate 25(OH)_D₃ levels (>75 nmol/l) were observed in only 9% of the CD patients in the study group (n=95). Suboptimal 25(OH)_D₃ levels were observed in 91% of participants and 40% had a serious deficiency (<30 nmol/l). Patients with inadequate 25(OH)_D₃ levels tended to be older (although this was not significant) and had a longer disease duration (p=0.003) (Table 1). There was no significant difference in 25(OH)_D₃ levels between CD patients (n=95) and those with ulcerative colitis (n=30). Interestingly, 75% of newly diagnosed CD patients (n=15) showed a serious deficiency of 25(OH)_D₃ (<30 nmol/l) at diagnosis. The prevalence of 25(OH)_D₃ deficiency in corticosteroid users and non-users was not statistically significant (p=0.384). Table 1 shows the basic characteristics and biochemical markers in the three subgroups of CD patients according to 25(OH)_D₃ level. Analysis revealed that the significantly increased production of the osteoclastical factors, free sRANKL (p=0.014), and the proinflammatory cytokines TNF- α (p=0.015) and IL-6 (p=0.029) were associated with 25(OH)_D₃ deficiency.

Vitamin D in inflammatory conditions

Table 2 shows the results of univariate and multivariate regression of circulatory 25(OH)_D₃ determinants. Proinflammatory free sRANKL and IL-6 were associated with 25(OH)_D₃ levels inversely and this association was highly significant (Fig 1). In the multivariate model using stepwise regression, a significant association was confirmed between the proinflammatory cytokines and regulatory molecules of osteoclastogenesis, (as independent predictors), and 25(OH)_D₃; free sRANKL (regression coefficient β =-0.24, p=0.038), IL-6 (β =-0.412, p=0.003) and TNF- α (β =0.249, p=0.036) (Table 2).

25(OH)_D₃ insufficiency was additionally analysed as the logistic function of the explanatory variables. In this model, the set of proinflammatory parameters (OPG, free sRANKL, IL-6, CRP and TNF- α) were identified as significant (p=0.015) independent contributors of 25(OH)_D₃ insufficiency, whereas the variables of bone turnover, bone status assessment and patients basic characteristics (age, disease duration, BMI and steroid treatment) were not relevant.

Vitamin D relationship to bone status

CD patients (n=68) with low BMD (mean lumbar z-score, -1.88, total hip z-score, -1.33) had a mean level of 25(OH)_D₃ of 35±18 nmol/l, in the range of serious deficiency to insuffi-

FIGURE 1. Association among levels of 25-hydroxyvitamin D₃, osteoclastically active free soluble RANKL (Fig 1A) and inflammatory marker interleukin-6 (Fig 1B) in cohorts of Crohn's patients (n=95). This relationship was inversely significant; 25(OH)D₃ and free sRANKL ($r = -0.3$, $p = 0.013$); 25(OH)D₃ and IL-6 ($r = -0.4$, $p = 0.007$). Log-transformed values were used.

ciency, whereas patients with normal BMD ($n=27$) had higher levels of 49 ± 28 nmol/l, though this is still within the range of inadequate levels (ANOVA $p=0.004$). The analysis showed a significantly higher production of IL-6 ($p=0.008$), TNF- α ($p=0.007$) and free sRANKL ($p=0.046$) in patients with established bone disease (Figure 2). The logistic regression model was used to analyse the predicting outcome of bone disease (dependent variable). The level of 25(OH)D₃, as an independent variable, was classified as adequately supplied, deficient or seriously deficient. The chi-square result (6.78) for the difference between the intercept and current model was highly significant ($p < 0.009$), with an odds ratio = 2.66 (range 6.8) and constant $B_0 = -1.28$. Thus, the low 25(OH)D₃ is a significant predictor of the likelihood of bone disease. In the multivariable analysis adjusted for several confounding factors, 25(OH)D₃ levels, together with free sRANKL, IL-6, TNF- α , OPG, and steroid therapy, were independently associated with a likelihood of bone disease [odds ratio (range): 1.04(6.24); 1.07(2.63); 1.22(4.95); 1.0(6.02); 3.17(3.17), respectively, $p=0.0001$].

Sixteen (17%) of the study patients reported fractures after CD diagnosis. 25(OH)D₃ insufficiency *per se*, was not found to be associated with the incidence of bone fractures. None of the patients or control group received bisphosphonates, vitamin D or calcium supplements, with the exception of three patients with long standing CD and established osteoporosis, who were treated with bisphosphonates, vitamin D and calcium supple-

ments. Two of these patients reported bone fractures prior to initiating bisphosphonate treatment. These subjects were included in the study as there was no impact on results, even when they were excluded from the study.

Discussion

The present study is focused on the association between 25-hydroxyvitamin D₃ levels and objective osteoimmunology markers, which may aid in better predicting bone disease in IBD. Serum 25-hydroxyvitamin D₃ levels were used to evaluate vitamin D status in study patients. 25(OH)D₃ is the major circulating form of vitamin D and numerous studies have proven it to be the best indicator for monitoring vitamin D status [7, 35, 36, 37]. Although 1,25(OH)₂D₃ represents the biologically-active form of vitamin D, its level is regulated by the parathyroid hormone, calcium and phosphorus levels, and its measurement does not reflect vitamin D status [7]. The Clinical Guidelines of the Endocrine Society suggest the measurement of serum 25-hydroxyvitamin D levels by a reliable assay as the initial diagnostic test in patients at risk for vitamin D deficiency [35]. The prevalence of 25(OH)D₃ deficiencies in the study group was relatively high. A total of 90% of Crohn's disease patients had suboptimal concentrations of 25(OH)D₃; including 40% with a serious deficit. Previous studies of vitamin D status in the Crohn's population reported a deficiency

FIGURE 2. Proinflammatory cytokines, free soluble RANKL and 25-hydroxyvitamin D₃ status in Crohn's patients with and without concomitant metabolic bone disease. ANOVA analysis showed a significantly higher production of IL-6 (8.3 ± 5.2 vs 5.8 ± 5.3 pg/ml, $p=0.008$), TNF- α (3.9 ± 1.7 vs 2.99 ± 1.6 pg/ml, $p=0.007$), free sRANKL (11.9 ± 6.6 vs 8.6 ± 6.4 pg/ml, $p=0.046$), and decreased circulatory level of 25(OH)D₃ (35 ± 18 vs 49 ± 28 nmol/l, $p=0.004$) in patients with established bone disease. Log-transformed values were used for the ANOVA analysis.

prevalence of 22–70% [9, 27], although a deficiency prevalence of up to 91% was also observed [28]. Given the present and previous findings regarding the association between vitamin D deficiency and disease activity, this may reflect the fact that all patients in this study were under hospital treatment and experiencing a graver form of the disease than the general CD population.

The findings of this study show a particularly high association among low 25(OH)D₃ levels and high levels of inflammatory markers and the regulatory factor of osteoclastogenesis. The results revealed the significantly increased production of osteoclastical free soluble RANKL and proinflammatory cytokines TNF- α and IL-6 to be associated with a serious deficiency or suboptimal levels of 25(OH)D₃. In fact, this relationship was characterized by an inverse correlation. Osteoclastically-active sRANKL, its receptor RANK and osteoprotegerin share the molecular pathway that underlie the skeletal and immune systems [22]. In inflammatory states, both B- and

T-cells produce RANKL. We reasoned that the local production of a cascade of proinflammatory cytokines and RANKL biogenesis was not limited to the induction of intestinal inflammation, but might be involved in the activation of bone metabolism [26, 29]. Our clinical data are in good agreement with the experimental observations, in which vitamin D deficiency induces RANKL-mediated osteoclastogenesis [30]. A serious deficiency of 25(OH)D₃ was found in 75% of treatment naïve Crohn's disease patients at diagnosis, and this observation was accompanied by reduced bone mineral density. Decreased 25(OH)D₃ in the systemic circulation of naïve Crohn's disease patients has been reported elsewhere [11]; however, it is unclear whether 25(OH)D₃ deficiency plays a causative role in pathogenesis or is a consequence of intestinal malabsorption.

The knowledge of the role of vitamin D in immunomodulation in IBD has been expanded and a body of evidence has documented its implication in disease pathogenesis [31, 32]. Immunologically, CD appears to be a result of deregulation of

innate intestinal immunity. In this deregulation process, NOD2 encodes a protein that recognizes muramyl dipeptide, the lysosomal breakdown product of bacterial peptidoglycan [33]. The protein, NOD2, has been associated with numerous regulatory roles in the mucosal immune system. The NOD2/CARD15/IBD gene is the locus associated with the strongest risk of development of CD and major NOD2 mutations that encode proteins incapable of recognizing muramyl dipeptide [34]. It is known that all immune system cells can express the vitamin D receptor, a member of the nuclear receptor family of ligand-regulated transcription factors. Recent data indicate that the active form of vitamin D (1,25-dihydroxyvitamin D₃) acts as a VDR-ligand in promoting transcription of the NOD2 gene, thus highlighting an important link between the vitamin D axis and pathogenesis of IBD [18, 31].

The present study has several limitations. This was a cross-sectional study with a heterogeneous population of 95 Crohn's patients, with a limited number of 15 newly-diagnosed patients who had not previously received treatment. Half of the 80 patients were diagnosed with CD in other hospitals and referred to our institution later in the course of the disease; therefore, medical records and the initial disease activity grading were absent for some patients. As Croatia is a Mediterranean country with plenty of sun, the focus was not placed on dietary intake of vitamin D. Periodic, though not necessarily daily, exposure to sunlight should be sufficient to prevent vitamin D deficiency. Changes in the patient's dietary intake of food rich in calcium or vitamin D would be difficult or impossible to track. Regardless of the limitations, we can conclude that vitamin D deficiency reflects CD itself rather than the background population in Croatia.

Metabolic bone disease is a well-recognised complication of CD. The pivotal role of vitamin D in skeletal homeostasis and its interplay with the osteoimmunology pathway is believed to affect bone status. The clinical data presented here show very good agreement between low levels of 25(OH)D₃ and low BMD z-scores and these 25(OH)D₃ values correlated inversely with elevated osteoimmunology markers. The multivariate analysis showed that 25(OH)D₃ deficiency, together with elevated levels of free soluble RANKL, IL-6, TNF- α , OPG, and steroid therapy, was an independent predictor of bone disease outcomes. Our present results strongly suggest that 25(OH)D₃ deficiency, accompanying an inflammatory state in CD, is a high risk condition for metabolic bone disease.

Financial Support

This work was supported by the Ministry of Science RH, grant no. 045-0165.

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