Proinflammatory cytokines and receptor activator of nuclear factor κB-ligand/osteoprotegerin associated with bone deterioration in patients with Crohn's disease

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Objectives **The high incidence of bone disease and the increasing evidence of Crohn's disease (CD) bone decline in corticosteroid users and nonusers suggest that bone metabolism is affected by inflammatory process. The aim of the study was to compare serum levels of proinflammatory cytokines, markers of bone turnover and regulatory molecules of osteoclast biogenesis, receptor activator of nuclear factor κB-ligand (RANKL) and osteoprotegerin (OPG), between naïve and long-standing CD patients.**

Methods **The study included 95 CD patients, 15 of them with newly diagnosed and previously untreated CD. The spine and hip bone mineral density was measured by dual-energy X-ray absorptiometry. Biochemical markers were determined by immunoassay.**

Results **Osteopenia was recorded at diagnosis in 53% of naïve patients and osteoporosis was found in 26% of long-standing CD patients. The newly diagnosed patients showed correlation between TNF-α and soluble RANKL (sRANKL) ($r=0.5$; $P=0.04$), and this positive relationship characterized the study population as a whole ($r=0.3$; $P=0.003$). Analysis of the OPG and sRANKL relationship showed absence of correlation in patients with healthy skeleton, whereas an inverse correlation was found in those with osteopenia ($r=-0.31$; $P=0.033$) and osteoporosis ($r=-0.48$; $P=0.028$). In naïve patients with reduced *T* score, the correlation between sRANKL and OPG was highly inverse ($r=-0.8$; $P=0.02$) and these patients were characterized by lower BMI, significantly higher level of proinflammatory cytokines, elevated C-reactive protein, and increased activity of free sRANKL and OPG.**

Conclusion **Bone disease that accompanies CD at diagnosis suggests that bone metabolism is affected by the underlying inflammatory process *per se*, as probably confirmed by our finding of the central proinflammatory cytokine TNF-α being strongly associated with the osteoclastogenic mediator RANKL, and inversely with bone density.** ***Eur J Gastroenterol Hepatol*** **00:000–000** © **2009 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

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Introduction

Crohn's disease (CD) is a complex multifactorial disease that manifests as a chronic inflammatory disorder of the gastrointestinal tract [1]. Besides environmental factors, genetic predisposition is considered to play a major role in the development of inflammatory bowel disease (IBD), giving rise to an abnormal immune response to the intraluminal antigen. The impaired immune response of the intestinal mucosa seems to be characterized by unregulated activation and proliferation of CD4+ T helper cells with suppressor T lymphocyte deficiency, activation of intestinal macrophages, and release of proinflammatory cytokines [2]. CD is associated with a number of extraintestinal manifestations [3] including low bone mass [4] and an increased risk of bone fractures [5,6]. The overall prevalence of osteoporosis in IBD patients is 15%, based on age and sex *T* scores less than −2.5 scanned by dual-energy X-ray absorptiometry (DXA) [4]. The etiology of metabolic bone disease in CD has not yet been fully clarified; however, chronic remittent inflammation and corticosteroid therapy are known to play a role in its pathogenesis [4,7].

Bone tissue undergoes continuous remodeling throughout one's life. A balanced activity of osteoblasts synthesizing bone matrix, and of osteoclasts, bone resorpting cells, is needed to maintain a normal homeostasis. Disturbances because of osteoclast activity lead to a local or general loss of bone mass. The latest research in the biology of osteoclastogenesis has ascribed a significant role to the signal pathway consisting of receptor activator of nuclear factor κB (RANK), RANK-ligand (RANKL) and osteoprotegerin (OPG) [8,9], members of the tumor necrosis factor (TNF) family. Soluble RANKL (sRANKL) is the main

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stimulatory factor for the formation of mature osteoclasts and is essential for their survival. RANKL is produced by osteoblast cells and activated T lymphocytes. It activates its specific receptor RANK that is located on osteoclasts and dendritic cells. The effects are counteracted by OPG, which is secreted by various tissues and acts as an endogenous soluble receptor antagonist. Proper balance of the RANKL/RANK/OPG signal pathway is necessary to maintain normal bone homeostasis. In addition, functional connection of this signal pathway and immune system has also been described [9–11]. T lymphocytes activated by antigen coupling express RANKL on their membranes, which are then released to the circulation by the action of metalloproteinases. It has been suggested that RANKL derived from T lymphocytes directly stimulates osteoclast maturation and further promotes osteoclastogenic activities in terms of bone matrix resorption [12]. Functional links between bone remodeling and the immune system, in part, mediated by RANKL, however, have only been documented in mouse models of arthritis, periodontitis, and estrogen deficiency but not yet in humans with these conditions or with IBD. As the RANKL/OPG biogenesis may be a consequence of intestinal inflammation and because this signal pathway is shared between the immune and bone systems, we have hypothesized that the action of sRANKL, OPG, and other inflammatory cytokines is not limited to the induction of local inflammation but might be directly or indirectly involved in the activation of bone metabolism. To test the hypothesis, it was necessary to evaluate serum concentrations of RANKL and OPG in naïve and long-standing Crohn's patients, and to correlate these values with proinflammatory cytokines, biochemical markers of bone turnover and bone mineral density (BMD). Indeed, we found that a 50% incidence of low bone mass (BMD reduced ≤ -1 SD) in newly diagnosed Crohn's patients coexisted with a close relationship between TNF-α and RANKL/OPG.

Material and methods

Patients

The study included 95 patients diagnosed with CD free from other diseases that may influence bone mass loss (hyperthyroidism, hyperparathyroidism, and malignant diseases). The diagnosis of CD was based on clinical, radiological, and endoscopic findings, and verified by histological finding of mucosal biopsy. Patients receiving some therapy for osteoporosis (bisphosphonates, calcitonin, estrogen replacement therapy, and selective estrogen receptor modulators) and postmenopausal women were excluded. On study entry, bone status determination and blood sampling were performed on the same day. Serum samples for biochemical measurements were immediately frozen at -80°C. Data collection before bone density measurement included age, sex, weight, height, location and disease duration, current medications, menstrual pattern in women, and history of intestinal resection and previous fractures. Patients were divided into two groups according to corticosteroid therapy: never used and on therapy (≥ 7.5 mg prednisone/day over 3 months or cumulative dose of 675 mg). If corticosteroid therapy was indicated, it was initiated at a high dose (40–60 mg prednisone daily) and then tapered off to minimal dose necessary to maintain remission before ending the application.

Clinical data were collected from hospital records. Laboratory data included: complete blood count, erythrocyte sedimentation rate, C-reactive protein (CRP), and routine biochemical parameters. Body mass index (BMI) was calculated (kg/m^2). The patients filled out a questionnaire on lifestyle habits, that is cigarette smoking, alcohol consumption, physical activity, and family history of osteoporosis.

Ethical considerations

The study was designed and carried out in accordance with the principles of the World Medical Association Declaration of Helsinki Ethical Principles for Medical Research Involving Human Subjects, 2004. The Hospital Ethics Committee approved the study protocol, and an informed written consent was obtained from each patient before entering the study.

Bone status assessment

Lumbar spine (L1–L4) and total hip BMD was measured by absorptiometric technique (DXA) using a Delphi W (S/N 700483) instrument (Hologic Inc., Waltham, Massachusetts, USA). BMD measurements were converted into *T* scores reflecting the number of SDs below the mean for a young healthy population, and *Z* scores reflecting the number of SDs below the mean for age-matched controls (database provided by the manufacturer). According to the WHO guidelines, osteopenia was defined as a *T* score between -1 and -2.5, and osteoporosis as a *T* score less than -2.5. Calcaneal structure was measured in all patients on the left heel using the Sahara bone sonometer (Hologic Inc.).

Receptor activator of nuclear factor κB-ligand and osteoprotegerin measurements

Serum concentrations of sRANKL and OPG were determined by using commercially available specific enzyme-linked immunosorbent assays (ELISAs) according to the manufacturer's instructions (Biomedica GmbH, Vienna, Austria). It should be noted that the ELISA method used on RANKL measurements primarily detects free unbound sRANKL, not complex RANKL–OPG. Free unbound sRANKL is a biologically active form of RANKL molecule, and we only measured the unbound share. 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% confidence interval) 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, California, 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, 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).

Cytokine assay

The sera (aliquots in separate vials and stored at $-80^{\circ}C$) were assayed for the concentrations of TNF-α, IL-1β and IL-6. Serum levels of cytokines were determined by using commercially available high-sensitivity ELISAs according to the manufacturer's instructions. TNF-α, IL-1β and IL-6 were purchased from R & D Systems (Minneapolis, Minnesota, USA). All assays used the quantitative sandwich enzyme immunoassay technique. The minimum detectable limits were as follows: TNF-α 0.12 pg/ml, IL-1β less than 0.1 pg/ml, and IL-6 0.039 pg/ml. Each sample was measured in duplicate.

Statistical analysis

Results for continuous variables are given as mean ± SD or range, and for noncontinuous variables as frequency and percentage. Comparison for differences among variables was tested by one-way analysis of variance (ANOVA) and nonparametric Mann–Whitney *U* test, or Kruskal–Wallis ANOVA. Bivariate correlation was examined by Spearman's rank test or Pearson's correlation matrices. Multiple stepwise regression analysis was used for association among variables. All variables showing independent and significant correlation with bone status on bivariate analyses of correlation were additionally tested by using the multiple regression model. For all these tests, differences were considered significant at *P* value of less than 0.05. Data analysis was performed using the StatSoft statistical package (StatSoft Inc., Tulsa, Oklahoma, USA).

Results

Clinical characteristics

Clinical data and blood samples were obtained from 95 CD patients, of which 81 completed BMD scanning by DXA. Calcaneal structure was measured in all patients using quantitative ultrasound (QUS) technique. Baseline characteristics of patients are shown in Table 1. DXA results indicated reduced spine and/or hip BMD (*T* score ≤ -1 SD) in 72% of all CD patients. Thus, 22% of Crohn's patients had BMD *T* score less than -2.5 and 50% between -1 and -2.5, detected at least at one measured site. Osteopenia (*T* score ≤ -1.0) was found in eight (53%) of 15 newly diagnosed and previously untreated patients. Sixteen (17%) study patients reported fracture after CD diagnosis, involving arm in nine, leg in five and spine in two cases.

Biochemical markers

Receptor activator of nuclear factor κB-ligand/osteoprotegerin concentrations

The mean serum levels of OPG, free sRANKL, proinflammatory cytokines, and markers of bone turnover are presented in Table 2. OPG and sRANKL were significantly higher in CD patients as compared with healthy controls (OPG: 101.6 ± 37 vs. 52 ± 16 pg/ml, $P = 0.001$ and free sRANKL: 10.9 ± 8.2 vs. 5.7 ± 1.9 pg/ml, $P = 0.002$). A weak, inverse, yet significant correlation between free sRANKL and OPG ($r = -0.26$; $P = 0.011$) was found in CD patients but not in controls. On bivariate analysis, free sRANKL correlated solely with TNF-α (Fig. 1), whereas OPG positively correlated with

Table 1 Baseline patient characteristics

Patients	Naïve CD	Long-standing CD	All patients
Crohn's disease (*n*) (men/women)	15 (8/7)	80 (40/40)	95 (48/47)
Age (years) (mean, SD)	24 ± 6	34.7 ± 12.7	33 ± 12
Disease duration (years) (median; range)	At diagnosis	8.2 (0.5–30)	5.2 (0.1–30)
Body mass index (kg/m^2) (mean, SD)	19 ± 3.5	21 ± 4.6	21 ± 4.5
Corticosteroid use (≥ 7.5 mg) for 3 months			
No (*n*)	15	23	38
Yes (*n*)	None	57	57
Fracture postdiagnosis (*n*, %)	None	16 (20%)	16 (17%)

CD, Crohn's disease.

Table 2 Serum concentrations of biochemical markers in 95 patients with Crohn's disease

Parameters	Patients (mean ± SD, range)	Controls (mean ± SD, range)	*P* value
Osteoprotegerin (pg/ml)	101 ± 37 (8–200)	52 ± 16 (30–82)	$P<0.001$
Free soluble RANKL (pg/ml)	10.9 ± 8.2 (0–44)	5.7 ± 1.9 (0–9.6)	$P<0.01$
IL-6 (pg/ml)	7.6 ± 5.3 (0.6–14.7)	1.8 ± 1 (0.4–9.6)	$P<0.001$
TNF-α (pg/ml)	3.6 ± 1.7 (0.7–8.6)	2.1 ± 2 (0–4.7)	$P<0.001$
IL-1β (pg/ml)	2.1 ± 3.7 (0–8.4)	0.54 ± 0.8 (0–2.0)	$P<0.01$
C-reactive protein (mg/ml)	28.8 ± 39 (0–255)	<5.0	$P<0.001$
C-telopeptide (ng/ml)	1.2 ± 1 (0.19–5.19)	0.290 ± 0.195 (0.1–0.750)	$P<0.001$
Osteocalcin (ng/ml)	9.9 ± 9.4 (1.0–47.4)	8.5 ± 5 (3.1–13.7)	NS

C-telopeptide, serum C-telopeptide crosslinked collagen types I; NS, nonsignificant; RANKL, receptor activator of nuclear factor κB-ligand.

IL-6, CRP, and C-telopeptide crosslinked collagen type I. Correlation matrices are presented in Table 3.

Cytokine measurements

All CD patients had higher concentrations of proinflammatory cytokines in the systemic circulation as compared with controls (Table 2). IL-6 and TNF-α were detectable in all patient sera, whereas IL-1β was not measurable in 30% of cases. Stepwise multiple regression analysis of sRANKL and OPG versus proinflammatory cytokines revealed TNF-α to significantly predict sRANKL ($P < 0.001$), whereas IL-6 ($P = 0.001$) and sRANKL ($P = 0.008$) were related to the level of OPG.

Markers of bone turnover

In CD patients, osteocalcin, a marker of bone formation, ranged from 1.0 to 47.4 ng/ml, differing from the reference range for healthy adults of 3.1–13.7 ng/ml (Table 2). A reduced osteocalcin level (< 3 ng/ml) was present in 21 patients, 81% of them on corticosteroid therapy.

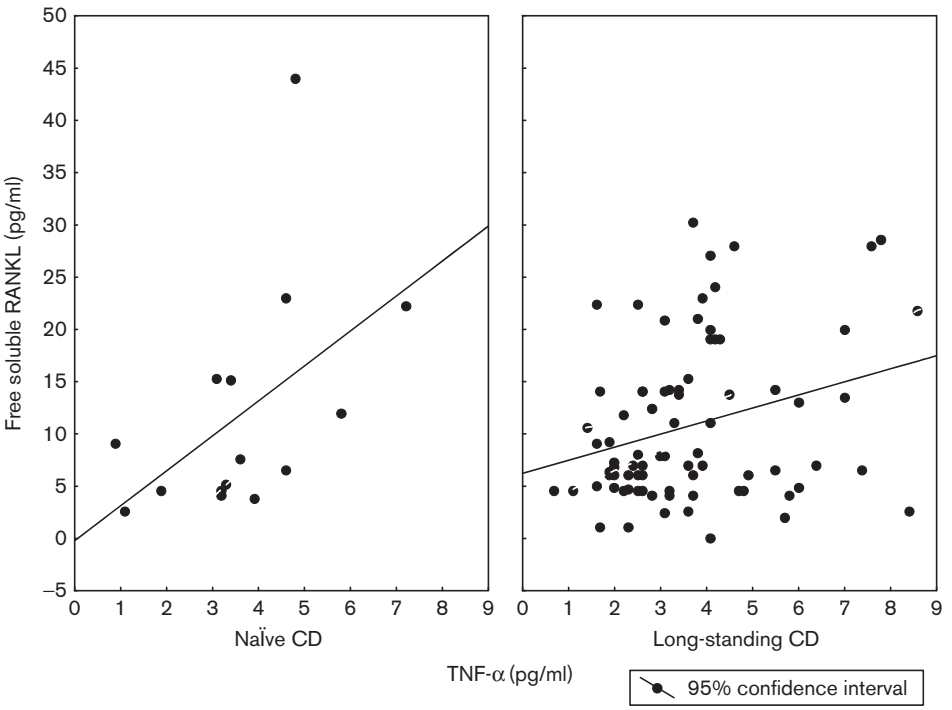
The mean value of serum C-telopeptide crosslinked collagen type I was increased in CD patients versus controls (1.15 ± 1.08 vs. 0.290 ± 0.195 ng/ml; $P = 0.001$). Moreover, in 52% of study patients, the C-telopeptide level exceeded the upper normal limit for healthy males and premenopausal women of 0.750 ng/ml (Table 2). An elevated level of this bone resorption parameter was recorded in both male and female patients. A subgroup of patients with serum C-telopeptide value exceeding 0.750 ng/ml was characterized by higher OPG (110 ± 42 vs. 92 ± 30 ng/ml; $P=0.019$), IL-6 (9.0 ± 5 vs. 6.0 ± 5 pg/ml; $P = 0.009$), TNF-α (4 ± 1.2 vs. 3.3 ± 1.5 pg/ml; $P = 0.04$), and CRP (37.7 ± 44 vs. 19 ± 32 pg/ml; $P = 0.024$) but lower BMI (19.7 ± 3 vs. 22.6 ± 5; $P = 0.005$) as compared with patients with serum C-telopeptide < 0.750 ng/ml.

Table 3 Correlation of clinical and biochemical parameters with serum levels of osteoprotegerin and free soluble RANKL in patients with Crohn's disease

	Osteoprotegerin	Free soluble RANKL
Body mass index	$R_s = -0.34$; $P<0.001$	NS
C-reactive protein	$R=0.54$; $P<0.001$	NS
IL-6	$R_s=0.47$; $P<0.001$	NS
TNF-α	NS	$R=0.5$; $P<0.002$
C-telopeptide	$R=0.49$; $P<0.002$	NS
Osteocalcin	NS	NS
Erythrocyte sedimentation rate	$R_s=0.38$; $P<0.001$	NS
Erythrocytes	$R=-0.40$; $P<0.01$	NS
Hemoglobin	$R=-0.38$; $P<0.01$	NS
Fibrinogen	$R_s=0.22$; $P<0.04$	NS
Calcium	$R=-0.43$; $P<0.006$	NS
LDH	$R_s=-0.24$; $P<0.039$	$R_s=0.33$; $P<0.005$
Albumin	$R=-0.32$; $P<0.047$	NS

C-telopeptide, serum C-telopeptide crosslinked collagen type I; LDH, Lactate dehydrogenase; NS, nonsignificant; *R*, Pearsońs correlation coefficient; RANKL, receptor activator of nuclear factor κB-ligand; R_s, Spearman's rank correlation coefficient.

Fig. 1



A positive correlation between concentrations of central proinflammatory cytokine TNF-α and osteoclastogenic mediator free soluble receptor activator of nuclear factor κB-ligand (RANKL) in the subgroup of naïve patients with Crohn's disease (CD, $n=15$) ($r=0.6$; $P=0.027$), also persisting in the unselected study population of long-standing CD patients ($n=80$) ($r=0.3$; $P=0.009$).

Bone density relationship with biochemical parameters

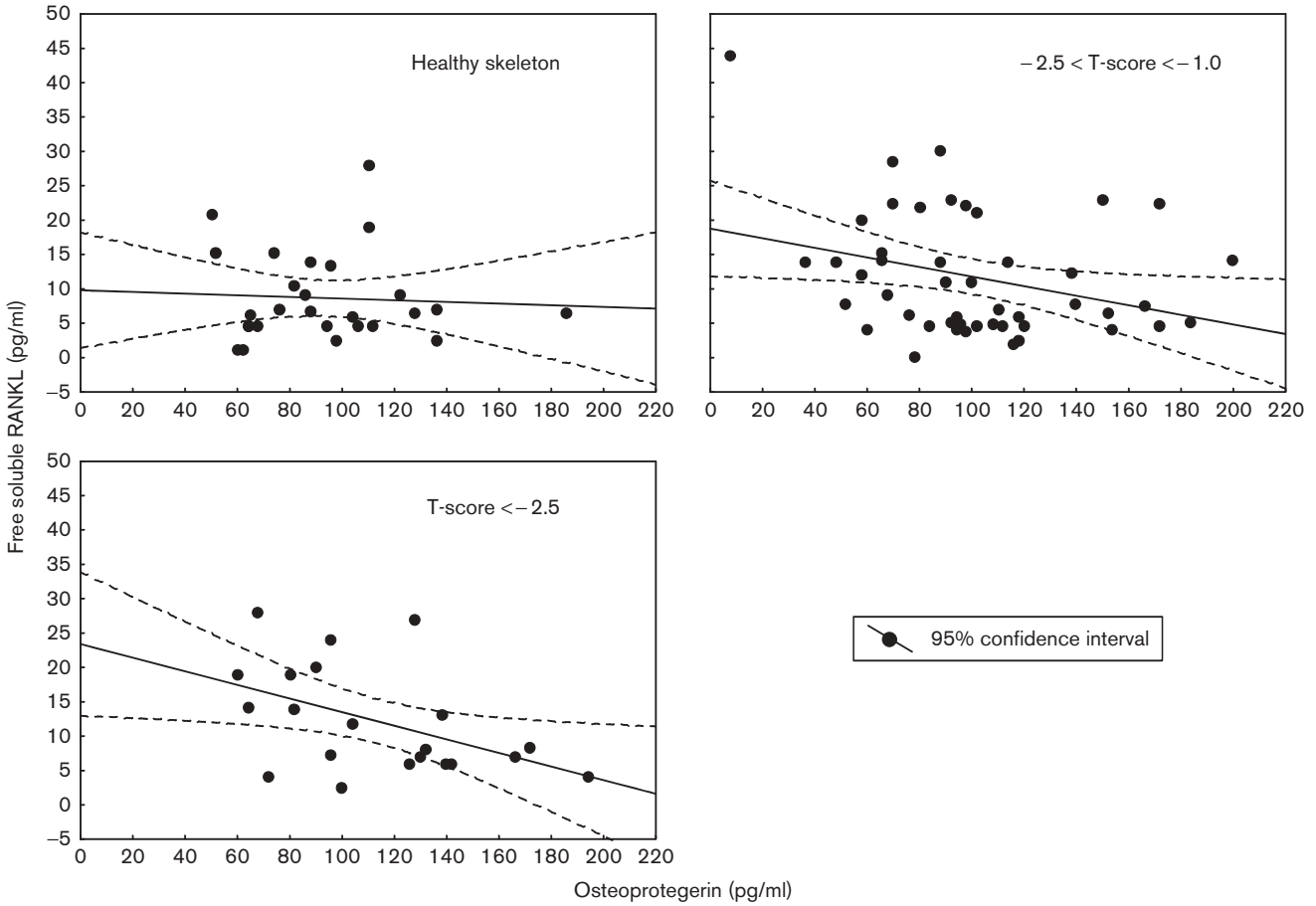
The ANOVA of biochemical markers in patient subgroups according to bone status revealed a statistically significant difference between patients with normal and pathologic BMD *T* scores (Table 4). Elevated concentrations of the osteoclastogenic factor RANKL, OPG, and proinflammatory cytokines were found in patients with low bone mass. In addition, analysis of correlation between OPG and free sRANKL in the three subgroups showed absence of correlation in patients with normal BMD, whereas a significant inverse correlation ($r = -0.31$; $P = 0.033$) was found in patients with BMD *T* scores between -1 and -2.5 and in those with BMD *T* score less than -2.5 ($r = -0.48$; $P = 0.028$) (Fig. 2). The activity of proinflammatory cytokines also differed between the subgroups with normal and pathologic BMD (IL-6, $P = 0.01$; TNF-α, $P = 0.025$), with highest levels recorded in patients with BMD *T* score less than -2.5 (Table 4). Coefficients of regression analysis between clinical or biochemical parameters and BMD at the spine and total hip are presented in Table 5. A significant negative correlation of IL-6 and TNF-α with lumbar spine and total hip BMD were observed, yet it should be noted that the correlation was not strong.

Table 4 Biochemical markers in subgroups of Crohn's disease patients with and without established bone disease

	Bone mineral density scanned by DXA		
Parameters	*T* score > −1.0	−2.5< *T* score ≤ −1.0	*T* score ≤ −2.5
Crohn's disease (n=81)	28%	50%	22%
Disease duration (years)	5.7±6	7±6	8.4±6.6
Osteoprotegerin (pg/ml)	93±30	101±40	113±37*
Free soluble RANKL(pg/ml)	8.7±6	11.7±9	12.2±7*
IL-6 (pg/ml)	5.9±5	7.3±5	10.5±5**
TNF-α (pg/ml)	2.9±1.6	3.8±1.8	3.95±1.4*
C-telopeptide (ng/ml)	1.1±1	1.3±1	0.9±1
Osteocalcin (ng/ml)	7.6±5	11.7±11	8.5±5

ANOVA, analysis of variance; C-telopeptide, serum C-telopeptide crosslinked collagen type I; DXA, dual-energy X-ray absorptiometry; RANKL, receptor activator of nuclear factor κB-ligand.*P<0.05 and **P<0.01.

Fig. 2



Bone status and relationship between free soluble receptor activator of nuclear factor κB-ligand (sRANKL) and its decoy receptor osteoprotegerin (OPG) in systemic circulation of patients with Crohn's disease. Analysis of correlation between free sRANKL and OPG in three subgroups showed absence of correlation in patients with normal bone mineral density, whereas a significant inverse correlation ($r = -0.31$; $P = 0.033$) was found in patients with osteopenia ($-1 \leq T$ score > -2.5) and in those with osteoporosis (T score less than -2.5) ($r = -0.48$; $P = 0.028$).

Table 5 Regression analysis of clinical and biochemical parameters with bone mineral density at the spine and total hip in 81 patients with Crohn's disease

	BMD: L1–L4 spine	BMD: total hip
Duration of Crohn's disease	NS	$R=-0.32$; $P<0.004$
Body mass index	$R=0.28$; $P<0.01$	$R=0.26$; $P<0.022$
Osteoprotegerin	NS	NS
Free soluble RANKL	NS	NS
C-reactive protein	NS	NS
IL-6	$R=-0.22$; $P<0.045$	$R=-0.23$; $P<0.041$
TNF-α	$R=-0.28$; $P<0.01$	$R=-0.28$; $P<0.012$
IL-1β	NS	NS
C-telopeptide	NS	NS
Osteocalcin	NS	NS
Calcium	NS	$R=0.32$; $P<0.008$
Phosphorus	NS	NS
Alkaline phosphatase	$R=-0.25$; $P<0.036$	NS
Albumin	$R=0.32$; $P<0.01$	$R=0.36$; $P<0.003$

AP, alkaline phosphatase; BMD, bone mineral density; C-telopeptide, Serum C-telopeptide crosslinked collagen type I; NS, nonsignificant; *R*, Spearman's rank correlation coefficient; RANKL, receptor activator of nuclear factor κB-ligand.

Multiple stepwise regression model was used to further evaluate the relationship between BMD and metabolic parameters of OPG, sRANKL, IL-6, TNF-α, C-telopeptide crosslinked collagen type I, osteocalcin, and Ca. The analysis pointed to IL-6 as the best predictor of BMD at lumbar spine ($\beta = -0.32$; $P < 0.034$). When the same parameters were taken into account, multivariate regression identified TNF-α ($\beta = -0.31$) and OPG ($\beta = -0.28$) as a significant independent contributor to hip BMD ($P < 0.005$).

Considering biochemical markers of bone turnover, there was no correlation between BMD and serum levels of C-telopeptide or osteocalcin either at lumbar spine or at total hip. The group of patients with normal BMD and balanced bone homeostasis showed positive correlation between C-telopeptide and osteocalcin ($r = 0.38$; $P = 0.04$).This correlation, however, did not exist in patients with low BMD.

Subset analysis

Newly diagnosed Crohn's disease

Fifteen CD patients were newly diagnosed and previously untreated (mean age 24 ± 6 years, range 18–39). Reduced BMD *T* score at diagnosis was found in eight (53%) patients: mean −1.19 ± 0.7 in the spine and −0.82 ± 0.6 in the hip. Five of these patients had lumbar spine BMD *T* score ≤ −1.0; two had hip *T* score ≤ −1.0; and a 25-year-old man had a BMD *T* score ≤ −1.9 at both spine and hip. Patients with reduced *T* scores were characterized by lower BMI (21 ± 3 vs. 17 ± 3; $P = 0.012$), significantly higher serum level of TNF-α (4.3 ± 1.6 vs. 2.6 ± 1; $P = 0.028$) and IL-6 (12.6 ± 2 vs. 5.0 ± 5; $P = 0.003$), increased activity of free sRANKL (15.2 ± 7 vs. 8.2 ± 5; $P = 0.033$) and OPG (107 ± 58 vs. 97 ± 27; $P = 0.047$), and elevated CRP (34.6 ± 28 vs. 21.5 ± 29). In the group of naïva CD patients there was a good correlation between TNF-α and sRANKL ($r = 0.6$; $P = 0.027$) (Fig. 1). Free sRANKL and OPG, both increased in peripheral blood, correlated highly but inversely ($r = -0.85$; $P = 0.02$) in patients with reduced *T* scores, whereas no such correlation was recorded in those with *T* score > −1 SD.

C-telopeptide crosslinked collagen type I, a biochemical marker of bone resorption, was increased in serum of newly diagnosed CD patients in comparison with age-matched and sex-matched healthy controls. Moreover, their mean value of 1.08 ng/ml (confidence interval 0.85–1.31) exceeded the upper normal limit for healthy males and premenopausal women of 0.750 ng/ml, whereas their mean osteocalcin level was within the normal range.

Corticosteroid therapy

Patients treated with corticosteroids showed a significantly lower spine (−1.7 ± 1.6 vs. −0.89 ± 1; $P = 0.011$) and hip (−1.3 ± 1 vs. −0.66 ± 0.9; $P = 0.005$) BMD *T* score as well as calcaneal QUS index (−2 ± 1.2 vs. −1.4 ± 1.4; $P = 0.015$). No difference in the levels of OPG, sRANKL, and proinflammatory cytokines with respect to corticosteroid therapy was observed. No significant difference according to corticosteroid therapy in serum C-telopeptide was observed, but a reduced level of osteocalcin (< 3 ng/ml) was mostly recorded in corticosteroid users (81%).

Fractures

The incidence of fractures from the diagnosis was 17% (16/95). Axial BMD *T* score was not significantly different between the fracture and nonfracture patients, whereas hip *T* score showed better discriminatory ability (−1.7 ± 0.8 vs. −0.9 ± 1; $P = 0.002$). The parameters of calcaneal QUS measurements, broad-beam ultrasound attenuation, and speed of sound identified patients at a high fracture risk ($P < 0.05$), as previously reported [13]. Disease duration was longer in patients with one or more fractures (9.3 ± 6 vs. 6.5 ± 6 years), and 70% of them were on lifelong corticosteroid therapy. No significant difference in biochemical parameters between the patients with and without fractures was observed.

Discussion

The high incidence of bone disease and the increasing evidence for CD bone decline in corticosteroid users and nonusers [14–16] suggest that bone metabolism is affected by the underlying inflammatory process *per se*. This study was conducted in young adults with CD, thus obviating confusion with postmenopausal bone loss. Study results revealed 50% of newly diagnosed and untreated CD patients to have BMD *T* score lower or equal to 1 SD at CD diagnosis. Osteoporosis, with *T* score ≤ −2.5 SD, was found in 26% of long-standing CD patients. Although the results recorded in long-standing CD were consistent with previously reported studies, development of metabolic bone disease detected at the

very diagnosis of CD in young adults was a kind of surprise. The majority of newly diagnosed patients could not state precisely the time elapsed from the first symptoms to the diagnosis of CD. Therefore, we considered that the silent onset of the disease by underlying inflammation might have contributed to the reduction of BMD detected at diagnosis. We reasoned that the action of sRANKL, OPG, and proinflammatory cytokines was not limited to the induction of intestinal inflammation, but might have been, directly or indirectly, involved in the activation of bone metabolism. Study results showed elevated serum levels of free sRANKL, OPG, TNF-α, and IL-6 in both long-standing CD patients with low bone mass and in naïve patients with osteopenia as compared with patients with healthy skeleton. The reduced bone density accompanying CD at the diagnosis in previously untreated patients suggests that bone metabolism is affected by the underlying inflammatory process, as probably confirmed by our finding of the central proinflammatory cytokine TNF-α being strongly associated with the osteoclastogenic mediator RANKL. In addition, TNF-α significantly and inversely correlated with both spine and hip bone density. An inverse correlation between OPG and sRANKL was recorded in patients with reduced bone density but not in patients with healthy skeleton. Recent studies by Moschen *et al.* [17] and Franchimont *et al.* [18] have investigated the production of OPG and RANKL in the colonic mucosa. Moschen *et al.* [17] have not been able to estimate the relative contribution of mucosa derived OPG and sRANKL to their corresponding serum levels. They have observed a 3.4-fold increase in OPG from inflamed colonic mucosa of CD patients as compared with the healthy controls and noninflamed colonic biopsies. (The discrepancy between the reported OPG values is because of the use of different nonstandardized commercially available ELISA kits).

The reason for low serum and mucosal levels of sRANKL can only be speculated. An immediate RANK/RANKL and OPG/RANKL complex formation might be a possible explanation, whereas increased OPG levels may represent homeostatic response aimed at reversing osteopenia and maintaining normal bone mass [17,19,20]. Consequently, the amount of osteoclastically active sRANKL decreases with binding to OPG. Both sRANKL and OPG, produced by intestinal immune cells, share the molecular pathways that underlie the skeletal and immune systems [12]. It is possible that the elevation of these markers in serum may reflect a combination of increased osteoclast activity and intestinal inflammatory activity [18]. This would, in part, explain the strong correlation between serum sRANKL and TNF-α.

TNF-α is a central proinflammatory mediator. Increased TNF-α expression has been shown to directly contribute to the pathogenesis of CD [1]. In newly diagnosed CD patients, we observed a strong relationship between TNF-α and sRANKL, and this positive correlation remained unchanged in the entire unselected study population. Moreover, stepwise multiple regression analysis indicated TNF-α to be the best predictor of sRANKL production. The precise cellular target of TNF-α in bone remodeling and its relationship with RANKL remain to be elucidated. To date, however, it has been shown that the TNF-α to TNF-receptor ligation through p55 adapter protein presumably acts upstream to RANKL [21,22]. Recent data reported by Byrne *et al.* [20] using a mouse model of CD show a direct link between the levels of secreted TNF-α and RANKL induced osteoclast-mediated bone destruction. This could provide a molecular explanation for bone decline associated with diseases having an activated immune system. CD is characterized by an active T cell response to luminal antigens, in which CD4 + cells predominate, resembling Th-1 immune response. Migration of activated T cells from systemic circulation into various tissues has a potential to affect extraintestinal tissues including bone marrow [23]. Consequently, the increased number of activated T cells and enhanced T cell production of TNF-α and RANKL in bone tissue might be the mechanism by which CD induces bone erosion independently of medical treatment [24,25]. Studies investigating the impact of anti-TNF-α treatment on bone turnover in CD patients have shown beneficial effects on bone density [26–29].

Elevated serum level of IL-6 in patients with reduced bone density was found in both newly diagnosed and unselected patient population. Increased IL-6 in systemic circulation of CD patients versus healthy controls has been reported elsewhere [30,31]. A recent study by Sylvester *et al.* [31] suggests IL-6 to mediate the effects of CD serum on in-vitro bone mineralization and may be a contributing factor for osteopenia associated with CD. The concentrations of IL-6 measured in this study group showed close correlation to TNF-α. As TNF-α and IL-1β are known to stimulate the production of IL-6, their enhanced generation during the active stage of the disease may amplify the proinflammatory cytokine network where IL-6 is secreted last. Recent data indicate that IL-6 could influence bone formation in conditions of increased bone turnover [32]. The effects of IL-6 and its soluble receptor on osteoblast proliferation, differentiation, and apoptosis, however, have to be more documented.

In this study, the mean level of osteocalcin did not differ between CD patients and control patients, whereas the bone resorption marker, serum C-telopeptide crosslinked collagen type I, was significantly increased in the former, exceeding the upper normal limit for healthy males and premenopausal women in 52% of study patients. The subgroup of patients with the increased parameter of bone resorption was characterized by elevated levels of TNF-α, IL-6, CRP, and OPG in systemic circulation. It should be noted that 22% of study patients had a reduced

bone formation marker osteocalcin (< 3 ng/ml). Eighty percent of these patients were on corticosteroid therapy, and suppression of bone formation is one of the known pathophysiological effects of corticosteroids. Our data confirmed the bone formation and resorption to be unbalanced in CD patients, and this finding is in agreement with literature data [16,20,33]. Considering the reduced bone density in newly diagnosed patients, the process of bone resorption seems to predominate, whereas corticosteroid therapy could act synergistically by inhibiting de-novo synthesis of bone matrix.

In conclusion, we found reduced bone density in 50% of newly diagnosed and untreated CD patients. A strong relationship was showed between TNF-α and the osteoclastic mediator sRANKL in naïve patients and this positive correlation persisted across the unselected study population. Free sRANKL and OPG showed a highly inverse relationship in patients with reduced bone density but not in those with healthy skeleton. Data on the newly diagnosed patients support the pivotal role of inflammation in bone tissue deterioration. Therefore, bone disease accompanying CD should be considered for therapeutic options already at the diagnosis.

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