HLA-B27 rats develop osteopaenia through increased bone resorption without any change in bone formation

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Abstract

Osteopaenia is a common complication of inflammatory bowel diseases (IBD). However, the mechanisms of bone loss are still the subject of debate. The aims of this study were to investigate bone loss in HLA-B27 transgenic rats, a spontaneous model of colitis and to compare the results provided by the usual markers of bone remodelling and by direct measurement of bone protein synthesis. Systemic inflammation was evaluated in HLA-B27 rats and control rats from 18 to 27 months of age. Then bone mineral density, femoral failure load, biochemical markers of bone remodelling and protein synthesis in tibial epiphysis were measured. Bone mineral density was lower in HLA-B27 rats than in controls. Plasma osteocalcin, a marker of bone formation, and fractional protein synthesis rate in tibial epiphysis did not differ between the two groups of rats. In contrast, urinary excretion of deoxypyridinoline, a marker of bone resorption, was significantly increased in HLA-B27 rats. The present results indicate that bone fragility occurs in HLA-B27 rats and mainly results from an increase in bone resorption. Systemic inflammation may be the major cause of the disruption in bone remodelling homeostasis observed in this experimental model of human IBD.

Keywords: Bone Resorption, Inflammatory Bowel Disease, Osteopaenia, Transgenic Model

Introduction

It is well known that inflammatory bowel disease (IBD) is associated with an increased risk of osteopaenia and osteoporosis1. Bone loss has been reported in Crohn’s disease and ulcerative colitis, leading to an increased incidence of fracture among IBD patients2,3. Many factors have been suggested as potential contributors to low bone mineral density such as corticosteroid therapy, nutritional deficiency, especially calcium and vitamin D, or decreased physical activity1. In addition, inflammatory factors such as cytokines, released in large amounts in any inflammatory disease, can modulate bone turnover through alteration of both bone formation and resorption1.

The mechanisms of bone loss associated with IBD have not been clearly defined. Contradictory results have been reported in this type of patient. Some studies suggest that bone loss occurs mainly through suppression of bone formation, without any change of bone resorption5. Other data reported increased bone resorption, without any decrease of bone formation6,7. A greater increase in bone resorption than in bone formation can also lead to bone loss8. These discrepancies may result from the large variability in the clinical characteristics and treatments of the patients studied. Moreover, it is difficult to know the respective contribution of the inflammatory disease and of the treatment, such as steroids, to bone loss and the modifications of bone remodelling observed in patients. A few studies have been conducted using animal models of IBD, indicating either a predominant role of decreased bone formation9,10 or of increased bone catabolism11.

Bone formation and resorption are generally assessed by indirect methods or markers that reflect the overall rate of bone turnover. These methods need to be validated against direct measurements. Recently, bone collagen synthesis measured directly using the incorporation of labelled proline was shown to increase in response to feeding, whereas plasma osteocalcin suggested no effect of food intake on bone.

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formation\textsuperscript{12}. The aims of this study were to determine the mechanisms of bone loss in transgenic HLA-B27 rats that spontaneously develop colitis and osteopaenia\textsuperscript{13}. The inflammatory status of the rats was evaluated on the basis of acute phase proteins and \textit{ex vivo} whole blood cytokine production. The originality of this study was that it compared the results provided by usual markers of bone remodelling to those obtained by direct measurement of bone protein synthesis.

**Material and methods**

**Animals and experimental design**

All procedures were performed according to current legislation on animal experimentation in France. Seven male HLA-B27/\textit{\beta}-2-microglobulin transgenic rats of the 33-3 line aged 12 weeks (M&B A/S Bomholtgård, Ry, Denmark) and 8 male Fischer 344 control rats of the same age (Iffa Credo, Saint Germain sur l’Arbresle, France) were housed in a temperature-controlled room (22-23°C) in individual wire-bottom cages with a 12h-12h light-dark cycle. From week 12 until week 18, the rats had free access to water and to a standard pellet diet (A04 C, UAR, France). Then they were fed a semi-synthetic diet supplying the following (g/kg): carbohydrate acid requirements), lipids 60, crude fibres 50 and minerals 670, casein 140 and methionine 1 (balanced to meet all amino acid requirements), lipids 60, crude fibres 50 and minerals plus vitamins sufficient for adult rat maintenance, as previously detailed\textsuperscript{14}. Food intake and body weight were measured twice a week until the end of the experiment (27-weeks-old). A blood sample was harvested from a tail vein on EDTA to measure blood cells and to collect plasma at various times during the experimental period. Plasma levels of acute phase proteins, i.e., fibrinogen, orosomucoid were assayed to monitor systemic inflammation. At the end of the experimental period, the animals received a bolus injection of L-[1-\textsuperscript{13}C] valine (150 \textmu mol/100 g body wt, 80\% MPE, 0.5 ml/100 g body wt) in a lateral tail vein to measure protein synthesis rate. Animals were anaesthetized with sodium pentobarbital (6 mg/100 g body weight) and killed by blood puncture in the abdominal aorta at 10, 12, 14, 16, 18, 20, 22 or 24 minutes after the injection. Blood was collected on heparin; 6 ml were immediately incubated to measure LPS-stimulated \textit{ex vivo} cytokine production. The remaining blood was centrifuged and the plasma was collected for analyses of L-[1-\textsuperscript{13}C] valine enrichment by gas chromatography-mass spectrometry on a HP-5890 quadrupole spectrometer (Hewlett-Packard, Paris, France). Valine was measured as N(O,S) ethoxycarbonyl ethyl ester derivative under electron-impact ionisation\textsuperscript{17}. The remaining pellet containing proteins was washed twice with 10\% TCA, twice with a 0.2 M perchloric acid solution and dissolved in 0.3 N NaOH for one hour at 37°C. An aliquot was then collected for protein assay\textsuperscript{15} and the pellet was hydrolysed in 6 N HCl at 110°C for 48 hours. HCl was evaporated; the pellet was diluted in 0.1 N HCl and desalted by cation-exchange chromatography. Amino acids were eluted with 4 M NH\textsubscript{4}OH, dried by evaporation and resuspended in 0.1 N HCl. N(O,S) ethoxycarbonyl ethyl ester derivatives of valine were prepared for measurement of [\textsuperscript{13}C]-valine incorporated in proteins by gas chromatography/combustion/isotope ratio mass spectrometry (MAT252, Finnigan MAT, Bremen, Germany)\textsuperscript{17,18}.

**Analytical methods**

Blood cell counts were performed using an automatic analyser (Cobas Minos Vet, ABX, Montpellier, France). Fibrinogen concentration was measured by a turbidimetric method with a commercial kit (Biodirect, Les Ulis, France). The assessment of orosomucoid was performed by the single radial immunodiffusion method using anti-rat AGP produced in the laboratory as already described\textsuperscript{15}.

Plasma osteocalcin concentrations were measured by homologous radioimmunoassay (RIA) using rat osteocalcin standard (Biochemical Technologies kit; Biochemical Technologies, Stoughton, MA, USA). Sensitivity was 0.01 ng/ml and intra and interassay accuracies were 7 and 9\%, respectively. Urine deoxypyridinoline was measured by a radioimmunoenzymatic assay using a Pyrilinks-D kit (Metra Biosystems, Mountain View, CA, USA). Sensitivity was 2 nM and intra and interassay accuracies were 4 and 6\%, respectively. Results were expressed as nmol of deoxypyridinoline per mmol of creatinine to adjust values for variation in urine volume. Creatinine was assayed according to the Jaffé method (kit bioMérieux SA, Marcy-l’Étoile, France) in which picric acid forms a coloured solution in the presence of creatinine.

**Protein synthesis rate was measured as previously described\textsuperscript{15,16}. Briefly, frozen epiphyses were powdered in liquid nitrogen with a ball mill (Dangoumeau, Prolabo, Paris, France) and homogenised in eight volumes of a 10\% trichloroacetic acid solution (TCA). Tissue homogenates were centrifuged to separate the acid-soluble fraction. Supernatants containing free amino acids were purified by cation-exchange chromatography in minidisposable columns (Amberlite AG50X8, 100-200mesh, H\textsuperscript{+} form; Bio-Rad, Richmond, CA, USA). Amino acids were eluted with 4 M NH\textsubscript{4}OH. After evaporation, the eluate was resuspended in 0.1 N HCl and used for measurement of free [\textsuperscript{13}C]-valine enrichment by gas chromatography-mass spectrometry on a HP-5890 quadrupole spectrometer (Hewlett-Packard, Paris, France). Valine was measured as N(O,S) ethoxycarbonyl ethyl ester derivative under electron-impact ionisation\textsuperscript{17}. The remaining pellet containing proteins was washed twice with 10\% TCA, twice with a 0.2 M perchloric acid solution and dissolved in 0.3 N NaOH for one hour at 37°C. An aliquot was then collected for protein assay\textsuperscript{15} and the pellet was hydrolysed in 6 N HCl at 110°C for 48 hours. HCl was evaporated; the pellet was diluted in 0.1 N HCl and desalted by cation-exchange chromatography. Amino acids were eluted with 4 M NH\textsubscript{4}OH, dried by evaporation and resuspended in 0.1 N HCl. N(O,S) ethoxycarbonyl ethyl ester derivatives of valine were prepared for measurement of [\textsuperscript{13}C]-valine incorporated in proteins by gas chromatography/combustion/isotope ratio mass spectrometry (MAT252, Finnigan MAT, Bremen, Germany)\textsuperscript{17,18}.

**Whole blood culture and cytokine assays**

Blood was diluted 1/10 in RPMI 1640 (Gibco, Paisley, Scotland, UK) with 2 mM glutamine, 100 U/ml penicillin and 100 \mu g/ml streptomycin and then distributed in 12-well plates (Falcon\textsuperscript{\textregistered}, 1 ml/well). Whole blood cultures were stimulated with lipopolysaccharide (LPS 055:B5, Sigma Aldrich, Saint-Louis, USA) at a final concentration of 10 \mu g/ml. Plates were incubated at 37°C with 5\% CO\textsubscript{2} for 8 hours. Preliminary experiments have shown that these conditions resulted in

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optimal cytokine production. After incubation, plates were centrifuged at 900 g for 10 minutes. Supernatants were recovered and kept at -80°C until cytokine assay. TNF-α and IL-6 concentrations were determined by specific enzyme-linked immuno-sorbant assay (DuoSet, R&D systems, Minneapolis, USA) according to the manufacturer’s instructions. The lowest level of detection was 12.5 pg/ml for TNF-α and 30 pg/ml for IL-6. Each sample was analysed in duplicate at the appropriate dilution.

Bone characteristics

Femoral mechanical testing

Immediately after collection, femurs were stored in 0.9% NaCl at 4°C before testing mechanical resistance. The femur length and mean diaphyseal diameter were measured with a precision calliper. The femoral failure load was determined with a Universal Testing Machine (Instron 4501, Instron, Canton, MA) 24 hours later, according to a three-point bending test. The 2 lower supports were separated by a distance of 20 mm to guarantee that 85-90% of the bone flexure was due to bending. The cross head speed was 0.5 mm/min.

Bone mineral density

Dual-energy X-ray absorptiometry measurements were performed using a Hologic QDR-4500 A X-ray bone densitometer (Hologic France, Massy, France). Bone mineral content (BMC) was determined for the whole femur. Bone mineral density (BMD) was determined for the total femur (T-BMD) and for two subregions; the distal metaphyseal zone (M-BMD), rich in trabecular bone; and the diaphyseal zone (D-BMD), rich in cortical bone.

Calculations

Fractional protein synthesis rate in tissue (FSR) was calculated as previously described from the following formula: 

\[ \text{FSR} = \frac{(E_b - E_n) \times 100}{(E' \times t)} \]

where \( E_b \) is the enrichment of protein-bound valine at the end of the incorporation period, \( E_n \) is the natural enrichment of protein-bound valine measured in rats fed the same diet but not injected with labelled valine, \( E' \) is the 13C enrichment of free valine calculated halfway between injection and killing, and \( t \) the time between injection and killing. FSR was expressed in %/d.

Data analysis and statistics

Data are given as means±SEM in 8 control and 7 HLA-B27 rats. The differences between the two groups were tested using an unpaired bilateral Student’s t-test. D-BMD and M-BMD data were analysed using ANOVA for repeated measurement, with group as the inter-individual factor and bone zone as the intra-individual factor. Statistical significance was set as \( P<0.05 \). \( P \) values between 0.05 and 0.1 were considered representative of a trend towards significance.

Results

Throughout the study, HLA-B27 rats were lighter than the control ones, with the difference reaching 16% at the end of the experiment (323±11 and 384±7 g, respectively, \( P<0.05 \)). Between 18 and 27 weeks of age, their growth rate and food efficiency were not significantly different (0.61±0.06 and 0.75±0.06 g/d for HLA-B27 and control rats, 0.039±0.005 and 0.043±0.003 g of growth/g of food intake for HLA-B27 and control rats, respectively). HLA-B27 rats had higher white blood cell counts and plasma levels of orosomucoid and fibrinogen throughout the experiment, as shown by the values observed at the age of 27 weeks in Table 1. Moreover, these parameters fluctuated over time in HLA-B27 rats, as illustrated for orosomucoid in Figure 1. As far as cytokines are concerned, TNF-α and IL-6 productions in LPS-stimulated whole blood cultures were

<table>
<thead>
<tr>
<th>Variables (means±SEM)</th>
<th>Control</th>
<th>HLA-B27</th>
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<tr>
<td>White blood cells (x10³/mm³)</td>
<td>8.7±0.3</td>
<td>28.4±1.3**</td>
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<tr>
<td>Orosomucoid (g/l)</td>
<td>36±2</td>
<td>219±21**</td>
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<tr>
<td>Fibrinogen (g/l)</td>
<td>3.15±0.08</td>
<td>4.57±0.22**</td>
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<tr>
<td>TNF-α production (ng/ml blood)</td>
<td>0.69±0.26</td>
<td>1.84±0.43*</td>
</tr>
<tr>
<td>IL-6 production (ng/ml blood)</td>
<td>1.62±0.21</td>
<td>6.32±1.71*</td>
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*\( P<0.05 \), **\( P<0.0001 \) vs. control rats, \( n=8 \) for control and 7 for HLA-B27 rats.

Table 1. Plasma concentration of acute phase proteins and ex vivo cytokine production by LPS-stimulated whole blood at 27 weeks.

Figure 1. Time course of plasma concentration of orosomucoid. Values are means±SEM. *\( P<0.03 \) vs. control rats (\( n=8 \) for control and 7 for HLA-B27 rats).
greater for HLA-B27 rats than for controls (Table 1).

Concerning bone parameters, femoral BMC was 22% lower in HLA-B27 than in control rats (Table 2). T-BMD, D-DMB and M-BMD were 15, 12 and 15% lower in HLA-B27 rats. The difference observed in D-DBM tended to be lower than that for M-BMD (P=0.057, for interaction). Femoral failure load was 12% lower in HLA-B27 than in control rats. Plasma osteocalcin concentration did not differ between the two groups of animals, while urinary excretion of deoxypyridinoline was 34% greater in the HLA-B27 than in the control rats (Table 3). The bone remodelling index, calculated as serum osteocalcin divided by creatinine-corrected deoxypyridinoline, was 41% lower in HLA than in control rats. Protein concentration and fractional synthesis rate measured in the tibial epiphysis were not significantly different between the two groups of rats (Table 4).

### Discussion

HLA-B27 transgenic rats of the 33-3 line spontaneously develop inflammatory lesions, mainly in the distal part of the gut. Because of this, they are commonly used as an experimental model of human IBD. An increased risk of osteopaenia and osteoporosis is currently reported in patients suffering from IBD. This fragility can increase the risk of fractures that is observed in IBD patients. Osteopaenia was also described in various experimental models of colitis, such as trinitrobenzenesulphonic acid (TNBS)-treated rats and interleukin 10-deficient mice. Alveolar bone loss and decreased bone strength were already reported in HLA-B27 rats. Accordingly, reductions in BMD and in bone strength in HLA-B27 rats were observed in this study. Therefore these transgenic rats, which spontaneously develop IBD, can be confidently used as an experimental model of IBD-associated osteopaenia. We additionally showed that their bone fragility is linked to increased bone resorption without any modifications in either osteoblast activity, as shown by plasma osteocalcin levels, or bone protein synthesis rate.

Since commensal gut flora, which is dependent on environmental conditions, plays an important role in the pathogenesis of B27-associated gut inflammation, it is important to summarize the mean features of HLA-B27 rats bred in our conditions. As far as the intestinal parameters were concerned, caecum and colon weights were greater in HLA-B27 than in controls and lesions were apparent in these parts of the gut. Indeed, intestinal disease was supported by macroscopic and histological modifications in the caecum and colon from HLA-B27 rats. The thickness of the wall was increased, mainly at the level of the mucosa, due to an increased depth of the crypts and infiltration of mono- and poly-nuclear monocytes. Myeloperoxidase activity, an indicator of leucocyte infiltration, was increased in the colon of HLA-B27 rats, which all developed diarrhoea. The inflammatory state in the colon of HLA-B27 rats was also characterized by T cell activation and increased cytokines production. A higher expression of INF-γ, TNF-α and IL-6 has been reported by us and others. Gut inflammation resulted in an increase of systemic markers of inflammation such as leucocyte number and acute phase proteins. Fluctuations of such markers suggest a development of the disease by episodes of attack and remission. Accordingly, TNF-α and IL-6 cytokine production by circulating cells was shown to be higher in HLA-B27 than control rats in this study. We clearly observed that the disease is also associated with metabolic modifications in non-digestive tissues. In particular, we previously described muscle atrophy, mainly due to increased proteolysis.

In our experimental conditions, lower femoral mass (BMC), T-BMD, M-BMD, D-BMD were observed in HLA-B27 rats as compared to controls (Table 2). Osteopaenia can result from

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<tr>
<td>BMC (g)</td>
<td>0.496±0.013</td>
<td>0.388±0.012*</td>
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<tr>
<td>T-BMD (g/cm³)</td>
<td>0.2406±0.0027</td>
<td>0.2056±0.0057**</td>
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<tr>
<td>D-BMD (g/cm³)</td>
<td>0.2204±0.0040</td>
<td>0.1936±0.0024**</td>
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<tr>
<td>M-BMD (g/cm³)</td>
<td>0.2342±0.0026</td>
<td>0.2001±0.0024**</td>
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<td>Femoral failure load (N)</td>
<td>121.4±4.3</td>
<td>106.4±2.6*</td>
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*P<0.02, **P<0.0001 vs. control rats, n=8 for control and 7 for HLA-B27 rats.

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<tr>
<td>Plasma osteocalcin (ng/ml)</td>
<td>42.4±3.2</td>
<td>35.2±2.2</td>
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<tr>
<td>Urine deoxypyridinoline (nmol/mmol creatinine)</td>
<td>183±11</td>
<td>246±15*</td>
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<tr>
<td>Bone remodelling index</td>
<td>4.13±0.40</td>
<td>2.43±0.19*</td>
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*P<0.01 vs. control rats, n=8 for control and 7 for HLA-B27 rats.

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<tr>
<td>Proteins (mg/g tibial epiphysis)</td>
<td>79.8±2.4</td>
<td>86.2±3.6</td>
</tr>
<tr>
<td>Fractional synthesis rate (%/d)</td>
<td>19.4±1.8</td>
<td>24.5±2.2</td>
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n=8 for control and 7 for HLA-B27 rats.
altered bone formation and/or bone resorption. The rate of formation or resorption of the bone matrix is often estimated through components released in the circulation or in the urine during either osteoblast or osteoclast activities. In our study, plasma osteocalcin and urinary deoxypyridinoline excretion were used as markers of bone formation and resorption, respectively. Osteocalcin is a protein synthesized by osteoblasts and incorporated into the extracellular bone matrix but a fraction of newly synthesized osteocalcin is released into the circulation. Deoxypyridinoline is a non-reducible pyridinium cross-link resulting from post-traductional modification of collagen, which is not metabolized in vivo but excreted in urine. So in our study, low D-BMD and decreased femoral failure load revealed bone fragility in HLA-B27 rats. Changes in 3-D microarchitecture may explain our D-BMD results. Using a micro-computed tomography device and mechanical testing, it has recently been shown that the majority of bone structural and strength parameters were significantly lower in 8-month-old HLA-B27 rats as compared with control littermates. Indeed, significant lower bone volume to total volume fraction (39%) and trabecular thickness (13%) and greater trabecular spacing (67%) were observed in the distal femur from HLA-B27 rats as compared to controls. HLA-B27 rats have less bone volume in the trabecular bone and greater spacing between the trabeculae. These data supported our findings of lower M-BMD BMC (bone mass) in HLA-B27 rats as compared to controls. This result is consistent with previous observations on a model of colitis induced by TNBS in rats. However, decreased bone formation as measured by histomorphometric analysis was reported in TNBS-treated rats and in interleukin 10-deficient mice, another experimental model of colitis. These discrepancies could be partly due to the different models used. Indeed, we previously found different mechanisms of muscle atrophy in HLA-B27 rats and those treated with dextran sulphate sodium to induce colitis. In humans, differences have been observed for the mechanisms involved in the osteopaenia present in Crohn’s disease and ulcerative colitis, as well. Another point is related to the biochemical markers used as indicators of bone formation or resorption, whose major limits are related to their metabolic clearances and specificity as already discussed. Moreover, these parameters provide static measurements and are devoid of any kinetic aspect. Our direct measurements of bone protein synthesis in this study confirm that bone loss was not due to changes in osteoblast activity in HLA-B27 rats.

The biochemical marker of bone resorption used here is actually an index of bone protein degradation, for which no direct measurement is available. This catabolic process appears to be higher in HLA-B27 than in control rats, as already observed for skeletal muscle. Consequently, mechanisms of regulation of protein degradation in bone and in skeletal muscle could share some features.

The aetiological factors involved in bone loss in colitis are multiple, but cytokines probably play a major role. The mucosal inflammation present in IBD is associated with local immune activation and increased in situ production of inflammatory mediators such as TNF-α, interleukins or interferon γ. However, IBD can be considered as a systemic disease. Indeed, increased TNF-α and IL-6 production by circulating cells and plasma concentrations of acute phase proteins were evident in this study, meaning that the HLA-B27 rats clearly exhibited systemic inflammation. Activation of the immune system leads to many extra intestinal manifestations and osteopaenia is one of them. The role of cytokines in bone remodelling and the process of osteopaenia have been well documented, in particular in osteoporosis associated with the menopause. Indeed, treatments with specific cytokine antagonists have been shown to prevent bone loss and bone resorption in ovariectomized animals. Infliximab (an antagonist of TNF-α) treatment of patients with Crohn’s disease was reported to allow an increase in bone formation and actually a decrease in glucocorticoid dose. Both TNF-α and IL-6 are likely implicated in the increase in bone resorption observed in HLA-B27 rats, since there is evidence that these cytokines play a pathogenic role in accelerated bone remodelling and excessive bone resorption. In fact, TNF-α and IL-6 are known to up-regulate the TNF-family molecule receptor activator of NFκB ligand (RANKL), which plays a major role in the development and activation of osteoclasts through the RANKL/RANK/osteoprotegerin signalling pathway.

In conclusion, this study, using a spontaneous model of IBD without any therapeutic treatment and no major malnutrition disorders, confirms that this pathology by itself induces bone loss. The direct measurements of tibial epiphysis protein synthesis rate confirm the results obtained for plasma osteocalcin, a habitual marker of osteoblast activity. Taken together, these results suggest a predominant role of bone resorption, probably mediated by the systemic inflammation, in the bone loss occurring in this experimental model of human IBD.

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