

# RANKL Is a Mediator of Bone Resorption in Idiopathic Hypercalciuria

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**Background and objectives:** This study aimed to determine the expression of osteoprotegerin, receptor activator of nuclear factor  $\kappa$ B ligand, interleukin-1 $\alpha$ , transforming growth factor- $\beta$ , and basic fibroblast growth factor in stone-forming patients with idiopathic hypercalciuria.

**Design, setting, participants, & measurements:** Immunohistochemical analysis was performed in undecalcified bone samples previously obtained from 36 transiliac bone biopsies of patients who had idiopathic hypercalciuria and whose histomorphometry had shown lower bone volume, increased bone resorption, and prolonged mineralization lag time.

**Results:** Bone expression of receptor activator of nuclear factor  $\kappa$ B ligand and osteoprotegerin was significantly higher in patients with idiopathic hypercalciuria *versus* control subjects. Transforming growth factor- $\beta$  immunostaining was lower in patients with idiopathic hypercalciuria than in control subjects and correlated directly with mineralization surface. Interleukin-1 $\alpha$  and basic fibroblast growth factor staining did not differ between groups. Receptor activator of nuclear factor  $\kappa$ B ligand bone expression was significantly higher in patients who had idiopathic hypercalciuria and exhibited higher *versus* normal bone resorption.

**Conclusion:** A higher expression of receptor activator of nuclear factor  $\kappa$ B ligand in bone tissue suggests that increased bone resorption in patients with idiopathic hypercalciuria is mediated by receptor activator of nuclear factor  $\kappa$ B ligand. Osteoprotegerin bone expression might have been secondarily increased in an attempt to counteract the actions of receptor activator of nuclear factor  $\kappa$ B ligand. The low bone expression of transforming growth factor- $\beta$  could contribute to the delayed mineralization found in such patients.

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The most common metabolic abnormality in patients with nephrolithiasis, occurring in up to 50% of patients, is idiopathic hypercalciuria (IH), characterized by an increased intestinal calcium absorption (1,2) and bone resorption (3) and decreased renal tubular calcium reabsorption. Decreased bone mineral density (BMD) in trabecular and cortical bone has been reported in several series of patients with IH (4–10), and some studies also reported an increase of bone resorption markers (7,8). In a population-based retrospective cohort study, the percentage of calcium stone-forming (CSF) patients with a first vertebral fracture was more than four times the expected rates in the general population (11).

Abnormal bone histomorphometry in patients with hypercalciuria was previously described by our group (4–6) as well as by other investigators (12–16). Main findings included increased bone resorption, low bone formation, and a mineralization defect. Literature data suggested that the elevation of

cytokines such as IL-1, IL-6, and TNF- $\alpha$  in peripheral blood monocytes could be responsible for increasing bone resorption, raising the hypothesis that bone resorption could represent the primary mechanism that leads to hypercalciuria (6,7,9,10).

The intrinsic mechanisms that regulate the coordinated sequence of osteoclastogenesis and osteoblastogenesis during bone remodeling are not completely understood. In patients with chronic kidney disease mineral bone disorder (CKD-MBD), we have shown that, at a bone level, cytokines such as IL-1 $\beta$  and TNF- $\alpha$  act in synergism, stimulating bone resorption and inhibiting bone formation, whereas TGF- $\beta$  and basic fibroblast growth factor (bFGF) present anabolic effects (17).

The interaction between bone formation and resorption has been further elucidated by the characterization of osteoprotegerin (OPG) and the receptor activator of NF- $\kappa$ B ligand (RANKL) (18). RANKL plays a central role in osteoclast differentiation and can be inactivated by OPG. Recently, the bone expression of OPG and RANKL system was evaluated by means of an effective immunohistochemistry technique that we developed on the basis of a quick decalcification of bone sections embedded in methylmetacrylate (19); however, there have been no data concerning the role of cytokines in bone tissue of patients who have hypercalciuria and present with normal renal function. This study aimed to evaluate the expres-

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sion of cytokines that are involved in either bone formation or resorption, such as TGF- $\beta$ , bFGF, OPG, RANKL, and IL-1 $\alpha$ , in bone biopsy material of IH stone-forming patients.

## Materials and Methods

Undecalcified bone fragments that were obtained from transiliac bone biopsies that were previously performed (4–6) in a random sample of 36 stone-forming IH patients (21 male; 15 female) and had normal renal function at the Nephrology Division of both Federal and State Universities of São Paulo from 1992 to 2002 were included in this study. Bone material from patients with diseases that affect calcium metabolism, such as hyperparathyroidism, sarcoidosis, diabetes, and CKD, or taking drugs such as corticosteroids or diuretics were not included. Nephrolithiasis was confirmed on the basis of history of calculi voiding, previous surgical or endoscopic removal of stones, or evidence of stones in radiologic images.

IH had been defined by serum calcium within normal limits and 24-h urinary excretion of calcium >250 mg/d (for women) and 300 mg/d (for men) or 4 mg/kg per d for both genders (1) in two nonconsecutive samples, under unrestricted diet, and mean values (2) were used in the data presented. The ethical committee of both institutions approved this study, and all patients signed written consent by the time those biopsies were performed.

Serum measurements of total calcium, inorganic phosphate, creatinine, 1,25(OH) $_2$ D $_3$ , and parathyroid hormone (PTH) were obtained near the date of the bone biopsy. Biochemical tests and PTH were available from all patients, and 1,25(OH) $_2$ D $_3$  was available from 29 patients. Total serum calcium was determined by atomic absorption spectrophotometry (Perkin-Elmer Atomic Spectrophotometer 290B; Perkin-Elmer, Norwalk, CT), and inorganic phosphate was determined by the Fiske and Subbarow method. Serum 1,25(OH) $_2$ D $_3$  and PTH were determined by different assays according to the availability of the methods in our laboratories between 1992 and 2002 (4–6).

### Bone Mineral Density

BMD was assessed by dual-energy x-ray absorptiometry at lumbar spine and femoral sites using a DPX-L apparatus (Lunar Radiation Corp., Madison, WI). The criteria for definition of osteopenia was a hip or spine BMD value >1.0 SD and of osteoporosis by a value >2.5 SD below the mean BMD for young adult population (T-score) according to the World Health Organization criteria (20).

### Bone Histomorphometry

All patients with IH had been submitted to a transiliac bone biopsy and analysis of histomorphometric parameters as described previously (21). Age-matched normal individuals ( $n = 10$ , five men/five women;  $34 \pm 5$  yr of age) were selected as control subjects for static parameters from a large bone histomorphometry database obtained by Dos Reis *et al.* (21) from our laboratory, consisting of iliac biopsies of healthy individuals who were submitted to autopsy immediately after early death. Most had been victims of gunshot or knife wounds, trauma, or traffic accidents and were not known to have any disease; neither were they users of anticonvulsant drugs, corticosteroids, or any medication that interferes with bone metabolism, on the basis of their medical records. Dynamic parameters were compared with the controls described by Melsen *et al.* (22), obtained from 41 volunteers, 12 men (mean age 32 yr) and 29 women (mean age 29 yr). In this study, patients were considered to exhibit high bone resorption when they presented values of eroded surfaces 1 SD above the mean value of this parameter taken from our database (21).

### Immunohistochemistry

Immunostaining for OPG, RANKL, IL-1 $\alpha$ , TGF- $\beta$ , and bFGF was performed using the avidin-biotin complex method on undecalcified bone samples that were embedded in methylmetacrylate and submitted to a brief decalcification and pretreatment with Tween 20 for better epitope exposure, as recently described (19). The primary antibodies used were mouse monoclonal anti-human IL-1 $\alpha$  (R&D Systems, Minneapolis, MN); rabbit polyclonal anti-human TGF- $\beta$  and bFGF (Santa Cruz Biotechnology, Santa Cruz, CA), and goat monoclonal anti-human OPG and RANKL (Santa Cruz Biotechnology). Bone sections labeled for OPG, RANKL, and all other cytokines were counterstained with Mayer's hemalum solution (Merck, Darmstadt, Germany). Toluidine blue had been used in a different section of each fragment to show osteoblast localization. Simultaneous negative controls were carried out by omitting primary antibody in all sections. As positive controls, we used bone sections from patients with CKD-MBD (19). Quantitative expression of OPG, RANKL, and cytokines in bone biopsies were measured by the point-counting technique (23) at a magnification of  $\times 100$  and using a 176-point ocular grid. Counting was performed on 25 microscopic fields for each bone sample. Each point was counted as either "positive" or "negative." The area of immunopositivity in the tissue was determined by the number of positive points in the tissue compared with the total number of points. The results were expressed as a percentage of total tissue. OPG, RANKL, IL-1 $\alpha$ , TGF- $\beta$ , and bFGF immunostaining also was performed for the same age-matched control material selected from our bone biopsy database for histomorphometry (21), as cited previously.

### Statistical Analysis

Results are presented as means  $\pm$  SEM. Parametric unpaired *t* test was used to compare patients who had hypercalciuria with control subjects. Pearson correlations were calculated between serum or urinary values with histomorphometric and immunohistochemical parameters. The level of significance was set at  $P < 0.05$ .

## Results

The group of patients with IH consisted of 21 men and 15 premenopausal women, mean age  $33.8 \pm 11.1$  yr. Mean urinary calcium was  $5.1 \pm 0.1$  mg/kg per d (range 4 to 7 mg/kg per d). One third of these samples presented values >5 mg/kg per d. Mean total serum calcium and phosphorus were  $9.6 \pm 0.0$  and  $3.4 \pm 0.1$  mg/dl, respectively. Hypophosphatemia was observed only in two patients (2.3 mg/dl in both), and none of them presented hypercalcemia. Six of 29 patients presented values of 1,25(OH) $_2$ D $_3$  slightly above normal ranges. All patients presented values of serum PTH within normal ranges. At the time of bone biopsies, 28 (78%) of the 36 patients presented low BMD (19 with osteopenia and nine with osteoporosis).

### Bone Histomorphometry

As shown in Table 1, bone structure was altered in patients with IH, who presented a significantly lower mean bone volume and trabecular number than did control subjects. With respect to indices of bone formation, mineralizing surface was significantly lower, mineralization lag time was significantly increased, and mineral apposition rate was significantly higher. As for bone resorption, both eroded surfaces and osteoclast surfaces were significantly increased. Even when patients were analyzed according to the results of BMD, patients with IH and

Table 1. Histomorphometric parameters<sup>a</sup>

Variable	Patients with Hypercalciuria	Control Subjects	P
<b>Structure</b>			
BV/TV (%)	19.4 ± 7.8 <sup>b</sup>	24.7 ± 6.4	0.020
Tb.N/mm	1.7 ± 0.4 <sup>b</sup>	1.9 ± 0.4	0.008
Tb.Sp (μm)	487.4 ± 166.3	415.3 ± 144.5	NS
Tb.Th (μm)	123.5 ± 29	127.7 ± 28.4	NS
<b>Formation</b>			
OV/BV (%)	2.1 ± 1.6	2.3 ± 2.6	NS
OS/BS (%)	12.5 ± 8.9	12.5 ± 11.6	NS
Ob.S/BS (%)	2.1 ± 2.3	1.4 ± 2.8	NS
Aj.AR (μm/d)	0.40 ± 0.20	0.51 ± 0.20	NS
Mlt/d	45.3 ± 31.4 <sup>b</sup>	23.0 ± 2.4	0.0001
MS/BS (%)	7.6 ± 0.5 <sup>b</sup>	13.8 ± 6.0	0.0001
MAR (μm/d)	0.70 ± 0.30 <sup>b</sup>	0.60 ± 0.12	0.0100
BFR/BS (μm <sup>3</sup> μm <sup>2</sup> /d)	0.08 ± 0.02	0.10 ± 0.03	NS
<b>Resorption (%)</b>			
ES/BS	8.3 ± 5.4 <sup>b</sup>	2.0 ± 1.9	0.001
Oc.S/BS	0.4 ± 0.5 <sup>b</sup>	0.0 ± 0.1	0.010

<sup>a</sup>Data are means ± SEM. Control values from references (21,22). Aj.AR, adjusted apposition rate; BFR/BS, bone formation rate; BV/TV, bone volume; ES/BS, eroded surface; MAR, mineral apposition rate; Mlt, mineralization lag time; MS/BS mineralizing surface; Oc.S/BS, osteoclast surface; Ob.S/BS, osteoblastic surface; OS/BS, osteoid surface; OV/BV, osteoid volume; Tb.N, trabecular number; Tb.Sp, trabecular separation; Tb.Th, trabecular thickness

<sup>b</sup>Versus control, NS (not significant).

with osteopenia ( $n = 28$ ) or without osteopenia ( $n = 8$ ) presented higher eroded surfaces ( $7.3 \pm 4.5$  and  $6.0 \pm 3.8$  versus  $2.0 \pm 1.9\%$ , respectively;  $P < 0.001$ ), osteoclast surfaces ( $0.5 \pm 0.6$  and  $0.3 \pm 0.4$  versus  $0.0 \pm 0.1\%$ ;  $P < 0.002$ ), and mineralization lag time ( $35.6 \pm 25.4$  and  $38.6 \pm 17.6$  versus  $23.0 \pm 2.4$  d;  $P < 0.001$ ) than control subjects (data not shown). The control subjects for dynamic parameters from Melsen *et al.* (22) had similar age as the patients with hypercalciuria from this series and a higher proportion of women. Among these normal dynamic parameters provided by Melsen *et al.*, mineral apposition rate proved to be gender and age independent, whereas bone formation rate and mineralizing surface were found to be higher in men than in women. Although in this study there was a slightly higher proportion of men in the IH group, mineralizing surface was significantly lower rather than higher than that of control subjects, and bone formation rate did not differ between both groups, so we believe that these results were not influenced by gender.

### Immunohistochemistry

Mean values of the quantitative analysis of bone expressions of TGF- $\beta$ , bFGF, IL-1 $\alpha$ , OPG, and RANKL in patients with hypercalciuria and control subjects are shown in Figure 1. TGF- $\beta$  was significantly lower in patients with IH versus control subjects ( $0.52 \pm 0.1$  versus  $1.47 \pm 0.4\%$ ;  $P < 0.003$ ), whereas bFGF was not ( $0.95 \pm 0.2$  versus  $0.84 \pm 0.5\%$ ). IL-1 $\alpha$  immunostaining was slightly higher in patients with IH versus control subjects but without reaching a statistical difference ( $1.18 \pm 0.1$  versus  $0.73 \pm 0.15\%$ ;  $P = 0.08$ ). There was a significantly higher

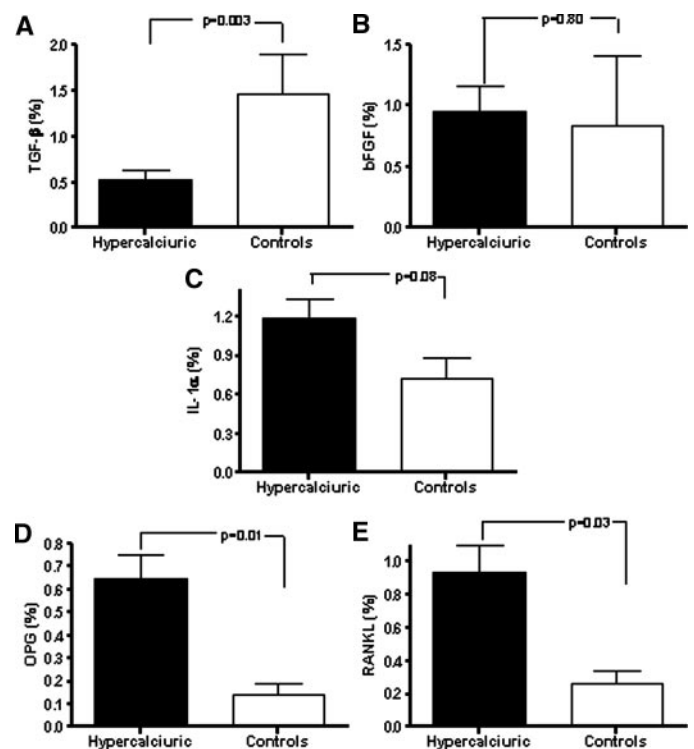


Figure 1. Bone immunostaining quantification. (A) TGF- $\beta$ . (B) Basic fibroblast growth factor (bFGF). (C) IL-1 $\alpha$ . (D) osteoprotegerin (OPG). (E) Receptor activator of NF- $\kappa$ B ligand (RANKL). Mean values are indicated by horizontal bars.

immunostaining for both RANKL and OPG in patients with IH compared with control subjects ( $0.93 \pm 0.16$  versus  $0.27 \pm 0.07\%$ ;  $P < 0.03$ ) and ( $0.65 \pm 0.10$  versus  $0.14 \pm 0.04\%$ ;  $P < 0.01$ ), respectively. As seen in Figure 2, when patients with IH were classified as having high bone resorption ( $n = 27$ ) or normal bone resorption ( $n = 9$ ), RANKL immunostaining was even higher in patients who had IH and exhibited high versus normal bone resorption ( $1.07 \pm 0.1$  versus  $0.33 \pm 0.1\%$ ;  $P = 0.03$ ). Conversely, OPG did not differ between these two subgroups. A significant positive correlation was found between TGF- $\beta$  immunostaining with osteoid surface, osteoid volume, and mineralization surface ( $r = 0.33$ ,  $r = 0.39$ , and  $r = 0.47$ ,  $P < 0.03$ , respectively). There were no significant correlations between OPG and RANKL immunostaining with the degree of hypercalciuria.

Figure 3 illustrates immunoreactivity localization of RANKL, OPG, and TGF- $\beta$  in bone tissue of patients with IH and control subjects. A marked positivity for RANKL (Figure 3A) and OPG (Figure 3B) in osteoblasts next to the osteoid surfaces, some medullary cells, and mature osteocytes is shown in a patient with IH in contrast with the faint staining of these proteins in a control subject (Figure 3, D and E). Weak immunostaining for TGF- $\beta$  was observed in areas adjacent to osteoid surface in a patient with IH (Figure 3C) compared with a control subject (Figure 3F).

### Discussion

IH represents a systemic abnormality in calcium homeostasis in which a dysregulation of calcium transport in the intestine, kidney, and bone takes place (3,24,25). Previous bone histomorphometric studies conducted by our group (4–6) demonstrated increased bone resorption, low bone formation, and prolonged mineralization lag time in patients with IH. Several other investigators also observed abnormal bone histology in IH (12–16), reporting delayed bone mineralization (12–16) and increased bone resorption (12,14–16). Features of altered bone

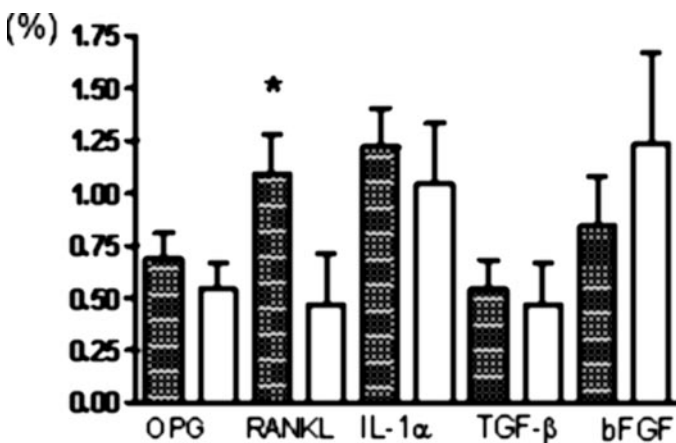


Figure 2. Bone immunostaining quantification of OPG, RANKL, IL-1 $\alpha$ , TGF- $\beta$ , and bFGF (%) in patients with idiopathic hypercalciuria (IH) and high (▨) or normal (□) bone resorption. \* $P < 0.05$  versus normal bone resorption.

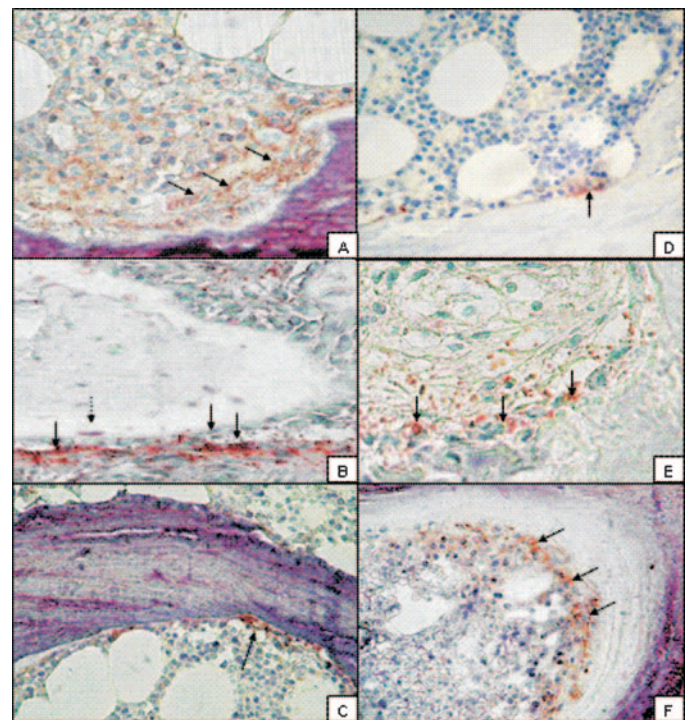


Figure 3. Immunohistochemistry for cytokines in bone biopsies. Positive immunostaining appears as red. Immunostaining of RANKL, OPG, and TGF- $\beta$  in patients with IH is illustrated in A, B, and C, respectively, and in control subjects in D, E, and F. A stronger labeling of RANKL and OPG in osteoblasts next to osteoid surfaces (black arrows), some medullary cells, and mature osteocytes (dashed arrow) is observed in a patient with IH compared with a control subject. Weaker immunostaining of TGF- $\beta$  was observed in areas adjacent to the osteoid surface (black arrows) in a patient with IH compared with a control subject. Magnification,  $\times 100$ .

histomorphometry that we report here were found irrespective of the bone densitometry results indicating osteopenia.

Cytokines can either stimulate or inhibit bone remodeling (26). Although local bone expression of cytokines had already been studied in patients with CKD-MBD by our group (17), this has not been evaluated in patients who have IH and present with normal renal function; therefore, this study was undertaken to evaluate the bone expression of OPG, RANKL, and other cytokines in patients with IH through a pioneering immunohistochemistry technique (19) that we developed, providing a better exposure of epitopes for antibody binding. Such technique presented a unique advantage of enabling bone sections previously obtained to be reanalyzed.

This analysis disclosed a higher bone expression of RANKL and OPG and lower expression of TGF- $\beta$ . TGF- $\beta$  is known as the most important and abundant growth factor in bone matrix, stimulating bone formation, mineralization, and inhibiting bone resorption through a proapoptotic effect on mature osteoclasts and inhibition of osteoclast differentiation (27,28); however, an experimental study in calvarie culture suggested that TGF- $\beta$  may induce bone resorption as well (29). In this series, we observed a significantly lower immunostaining for TGF- $\beta$  in

patients with IH when compared with control subjects. TGF- $\beta$  bone expression was positively correlated with volume and osteoid and osteoblast surface, trabecular number, and mineralization surface and negatively correlated with trabecular separation; therefore, less TGF- $\beta$  might have accounted for the delay in bone mineralization evidenced in the histomorphometric analysis as predicted by its known effects on mineralization (30). Conversely, bFGF, despite presenting some similar effects as TGF- $\beta$  (17), did not present a statistically different immunostaining pattern in patients with IH when compared with control subjects.

Studies by Pacifici *et al.* (7) demonstrated that mononuclear cells from patients with fasting hypercalciuria had an increased spontaneous production of IL-1 $\alpha$ , which correlated negatively with BMD. Subsequently, Weisinger *et al.* (9) demonstrated that not only IL-1 $\alpha$  but also other cytokines, such as IL-6 and TNF- $\alpha$ , had increased expression in patients with IH, with a significant correlation between *in vitro* cytokine production and lumbar spine BMD. On the basis of these findings, which were further corroborated by Ghazali *et al.* (10) and Misael da Silva *et al.* (6), it has been suggested that bone resorption induced by elevated cytokines could represent a primary mechanism that leads to hypercalciuria. In this study, there was a trend for mean value of local bone immunostaining for IL-1 $\alpha$  to be higher in patients with IH (especially in those who presented higher bone resorption) than in control subjects but without statistical significance.

RANKL plays a central role in osteoclastogenesis and can be inactivated by OPG. Thus, the balance between RANKL and OPG controls osteoclastic activity, and factors that stimulate RANKL and/or inhibit OPG will shift the OPG/RANKL ratio toward bone resorption (31).

One of the most marked differences that we found in this study was the much higher bone expression of both RANKL and OPG in patients with IH than in control subjects. The OPG and RANKL bone immunolocalization detected in our study matches with reports of OPG localization by Northern blot analysis in bone cells (32–34) and also with experimental studies showing similar localization of OPG and RANKL performed in paraffin-embedded bone tissue (35,36). Because this study was performed in bone tissue that was retrieved from previous biopsies, Western blot analysis could not be carried out because the specimen had been submitted to fixation.

When we analyzed the results of RANKL according to the level of bone resorption, we observed that patients who had IH with high bone resorption presented even higher RANKL immunostaining than those with normal bone resorption, reinforcing that RANKL may be an important stimulatory local factor for bone resorption in the IH setting. With respect to OPG bone immunostaining, no differences between the subgroups with high or normal bone resorption were depicted. This result was unexpected because OPG inhibits osteoclastogenesis. The association of RANKL with elevated bone resorption has already been suggested in other studies that involved molecular biology techniques and focused on other renal or nonrenal diseases (37–40); however, reports of serum concentration of OPG have shown OPG to be significantly higher rather than lower in women with postmenopausal osteoporosis

than in age-matched control subjects (41,42), what the authors attributed to a compensatory response to the enhanced bone resorption. Because immunohistochemical studies in human bone tissue quantifying RANKL or OPG are scant, the hypothesis of a higher bone expression of OPG counteracting RANKL at a bone level presently observed may be raised on basis of the results of serum levels of cytokines (41).

In this series, serum levels of PTH were within normal range in all patients with IH. Although the negative calcium balance found among patients with hypercalciuria could theoretically lead to secondary hyperparathyroidism, our data, in agreement with other studies (43,44), reinforce the absence of secondary hyperparathyroidism in the majority of patients with IH. It is possible that the putative increase of serum 1,25(OH) $_2$ D $_3$  in patients with hypercalciuria might suppress the secretion of PTH; however, the role of 1,25(OH) $_2$ D $_3$  in IH is still controversial, some authors finding high serum levels of 1,25(OH) $_2$ D $_3$  in patients with IH (45,46), whereas others not (47,48).

In this study, six patients presented serum levels of 1,25(OH) $_2$ D $_3$  slightly above the normal range. Experimental data have shown increased number of vitamin D receptors (VDR) with normal serum levels of vitamin D (25) as well as increased sensitivity to 1,25(OH) $_2$ D $_3$  in duodenum and calvarie of genetic hypercalciuric stone-forming rats (49). Favus *et al.* (47) observed an increased number of VDR in monocytes of patients with IH, although normal serum levels of vitamin D had been found.

We recognize that the high expression of cytokines may be simply associated to the present bone profile and that these findings may not represent a causal relationship. Because it is well established that calcitropic hormones and proresorptive cytokines upregulate messenger RNA expression of RANKL in osteoblasts, helping to promote osteoclast differentiation and activation, present figures of higher expression of RANKL in bone tissue rather than in a circulating form might be the cause of local increased bone resorption or a secondary mechanism to unknown regulating factors, independent of PTH.

On the basis of these findings coupled with the literature data discussed, we speculate on a hypothetical mechanism for the bone involvement in IH. The putative increase of VDR–1,25(OH) $_2$ D $_3$  complexes in the IH setting, in addition to increased intestinal calcium absorption, could possibly stimulate bone expression of RANKL, as suggested by Gonzalez (50), while suppressing PTH. The PTH suppression could then lead to a decreased TGF- $\beta$  bone expression. Less TGF- $\beta$  means less inhibition upon RANKL, magnifying RANKL proresorptive properties, and also contributes to increasing the time lag for mineralization and to reducing bone formation. The increased OPG expression could then represent a compensatory mechanism for higher bone resorption induced by RANKL. The enhanced intestinal calcium absorption and the decreased renal calcium reabsorption induced by a lower PTH may further contribute to urinary calcium losses.

## Conclusions

The higher expression of RANKL in bone tissue suggests that increased bone resorption in patients with IH is mediated by

RANKL. OPG bone expression might have been secondarily increased in an attempt to counteract the actions of RANKL. The low bone expression of TGF- $\beta$  could contribute to the delayed mineralization found in such patients.

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## Disclosures

None.

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