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Effects of parathyroidectomy on the biology of bone tissue in patients with chronic kidney disease and secondary hyperparathyroidism

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ABSTRACT

Secondary hyperparathyroidism is a complication of chronic kidney disease that compromises skeletal integrity. In patients with secondary hyperparathyroidism undergoing parathyroidectomy, parathyroid hormone levels dramatically decrease. The effects of parathyroidectomy on bone tissue are poorly understood, especially regarding the proteins expressed by osteocytes, such as fibroblast growth factor 23, dentin matrix protein 1, matrix extracellular phosphoglycoprotein, sclerostin, receptor activator of nuclear factor kappa B ligand (RANKL) and osteoprotegerin, which regulate bone turnover. The objective of this study was to characterize the bone expression of these proteins by immunohistochemistry and correlate these results with those of bone histomorphometry before and after parathyroidectomy. We studied bone biopsies that were obtained from 23 patients before and 12 months after parathyroidectomy. We observed an improvement in bone microarchitecture, but impaired mineralization after parathyroidectomy. We found significant increases in sclerostin and osteoprotegerin expression and a decrease in the RANKL/osteoprotegerin ratio after parathyroidectomy, suggesting that their expression is regulated by parathormone. These proteins correlated with structural and bone formation parameters. We conclude that after parathyroidectomy, significant changes occur in the bone expression of osteocyte proteins and that these proteins potentially regulate bone remodeling.

1. Introduction

Chronic kidney disease (CKD) has a wide range of complications, including disorders of mineral and bone metabolism, which represent an important cause of morbidity and contributes toward worsening the quality of life of the patients affected [1]. These disorders are also associated with the development of vascular calcifications and with increased mortality [2] and are detected from the early stages of CKD [3]. Abnormalities involving calcium (Ca), phosphorus (P), parathyroid hormone (PTH) and vitamin D contribute to the bone alterations observed in patients with CKD [3]. Recent studies have shown that osteocytes and the proteins produced by these cells also participate in these abnormalities [4,5].

Osteocytes regulate mineral deposition in the bone matrix and produce factors that interfere with local chemical processes and distant organs, acting as an endocrine organ. Sclerostin (Scl), receptor activator of nuclear factor kappa B ligand (RANKL) and osteoprotegerin (OPG) are osteocytic proteins that participate in the control of bone formation and resorption [6]. Matrix extracellular phosphoglycoprotein (MEPE), dentin matrix protein 1 (DMP-1) and fibroblast growth factor 23 (FGF23) constitute a group of proteins related to P metabolism and bone mineralization [4].

Among several local and hormonal factors, PTH has been implicated as a regulator of the expression and release of some of these proteins. PTH decreases Scl expression both *in vitro* and *in vivo* [7]. Regulation of FGF23 occurs through mechanisms that are dependent on cyclic AMP and the Wnt signaling pathway [8]. Jain et al. have observed that the serum levels of MEPE correlate negatively with those of PTH in healthy

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subjects [9] and a previous work has demonstrated a negative relationship between DMP-1 and PTH [10]. This hormone also stimulates the expression of RANKL and decreases OPG, regulating osteoclastogenesis [11].

Patients with CKD and secondary hyperparathyroidism (SHPT) undergoing parathyroidectomy (PTX) exhibit changes in PTH from high to controlled levels. Little is known about the effect of this change on the expression of the aforementioned proteins or their influence on bone turnover. The proteins described above, as well as the expression pattern in the bone biopsies of patients with SHPT performed before and 1 year after PTX, have yet to be evaluated.

The aim of our study was to characterize the bone expression of Scl, MEPE, DMP-1, RANKL, OPG and FGF23 by immunohistochemistry and to establish associations between these proteins and bone histomorphometry in SHPT patients before and one year after PTX.

2. Materials and methods

2.1. Study participants

This study was a retrospective cohort study in which we evaluated bone biopsies obtained from biopsy specimens from *Laboratório de Fisiopatologia Renal (LIM 16) - Faculdade de Medicina - Universidade de São Paulo (FMUSP)* and *Hospital do Rim e Hipertensão – Universidade Federal de São Paulo (UNIFESP)*. We included the biopsies of patients with CKD and SHPT, which were performed before and 12 months after PTX (N = 23). All patients were submitted to total parathyroidectomy with autograft. The protocol was approved by the local ethics committee and all patients provided informed consent. Some of these patients had previously been examined in other protocols, which had already been completed and for which free and informed consent had been obtained [12]. The patients were 16 years of age or older. The median age of males (35.5 years; range = 16–57) was not significantly different from females (45 years; range = 34–63; p = 0.12). They had been on hemodialysis for at least 3 months and had PTH levels > 400 pg/mL.

2.2. Biochemical parameters

The laboratory tests described here were the same as those analyzed in the previously completed studies; these tests included total Ca (reference range: 8.8–10.6 mg/dL), P (reference range: 2.7–4.5 mg/dL), alkaline phosphatase (AP; reference range: 35–104 U/L) and intact PTH (reference range: 10–65 pg/mL) (Immulite; DPC-Biermann, Bad Nauheim, Germany).

2.3. Bone biopsy and histomorphometry

All patients were submitted to a transiliac bone biopsy using a 7-mm Bordier trephine after a course of double-labeling tetracycline (20 mg/kg/day) for 3 days, with an interval of 10 days. The biopsy was performed 2–5 days after the last dose of antibiotics.

The specimens were fixed in 70% ethanol, dehydrated, and embedded in methyl methacrylate (MMA). Undecalcified 5-μm-thick sections were cut using a Polycut S equipped with a tungsten carbide knife (Leica, Heidelberg, Germany). Some sections were stained with 0.1% toluidine blue, pH 6.4, and unstained 10-μm slices were obtained for the analysis of dynamic parameters under a microscope with ultraviolet light. All histomorphometric analyses were performed using a semi-automatic image analyzer and Osteomeasure software (Osteometrics, Inc., Atlanta, GA, USA) [13]. The static and dynamic parameters were reported using the nomenclature, symbols, and units recommended by the American Society for Bone and Mineral Research [14].

The following histomorphometric parameters were evaluated: bone volume (BV/TV, %); trabecular separation (Tb.Sp, μm); trabecular number (Tb.N, /mm); trabecular thickness (Tb.Th, μm); osteoid volume (OV/BV, %); osteoid surface (OS/BS, %); osteoid thickness (O·Th, μm); osteoblast surface (Ob.S/BS, %); eroded surface (ES/BS, %); osteoclast surface (Oc.S/BS, %); fibrosis volume (Fb.V/TV, %); mineralizing surface (MS/BS, %); mineral apposition rate (MAR, μm/day); bone formation rate (BFR/BS, $\mu m^3/\mu m^2$/day) and mineralization lag time (Mlt, days).

The reference values used for the static parameters were the values from the normal local controls [13], while the dynamic parameters followed those described in the literature [15,16].

All osteocytes present in the trabecular bone were quantified, and the values obtained were expressed as I) total osteocytes (Ost total, n); II) total osteocytes normalized to the trabecular bone area analyzed (Ost total/B.Ar, n/mm^2); and III) total osteocytes normalized to the tissue area analyzed, which included the trabecular bone and bone marrow (Ost total/T.Ar, n/mm^2).

The bone histomorphometry was categorized according to the newly proposed Turnover/Mineralization/Volume (TMV) classification [17] based on the following parameters: bone turnover (T), defined by BFR/BS, bone mineralization (M), defined by Mlt and bone volume (V), defined by BV/TV.

High turnover is defined as BFR/BS values > 1 SD of the normal range, and low turnover < 1 SD of the normal range (reference range: 0.13 ± 0.07 for men and 0.07 ± 0.03 for women) [15]. Abnormal mineralization is defined as Mlt ≥ 50 days or normal when values are below 50 days [16]. High bone volume is defined as BV/TV values > 1 SD of the normal range, and low bone volume when BV/TV < 1 SD of the normal range, according to each decade for males and females [14].

2.4. Bone protein expression by immunohistochemistry

Bone immunohistochemistry (IHC) was performed as previously described [18]; however, the immunoperoxidase method was adapted to the ABC (Avidin and Biotinylated horseradish peroxidase macromolecular Complex) technique. Briefly, the following steps were performed on bone sections:

1) The removal of MMA with xylene/chloroform (1:1) followed by rehydration with a graded alcohol series; 2) partial decalcification in 1% acetic acid; 3) the blocking of endogenous peroxidase with 3% hydrogen peroxide in methanol; 4) the blocking of endogenous avidin and biotin with the Avidin/Biotin blocking kit (Vector, Burlingame, CA, USA); 5) incubation with primary antibody overnight at 4 °C (anti-human FGF23 produced in mice, kindly provided by Genzyme, 1:200; anti-human DMP1 produced in rabbit, Takara, 1:200; anti-human Scl produced in mice, R&D systems, 1:100; anti-human MEPE produced in goat, R&D systems, 1:200; anti-human RANKL produced in goat, Santa Cruz Biotechnology, 1:80; anti-human OPG produced in goat, Santa Cruz Biotechnology, 1:80); 6) incubation with Vectastain ABC-HRP (Avidin/Biotin Complex-horseradish peroxidase, Vector, Burlingame, CA, USA); 7) another incubation with the AEC (3-amino-9-ethylcarbazole) chromogenic substrate (Sigma-Aldrich, St. Louis, MO, USA); 8) the counterstaining of sections with Mayer's hematoxylin (Merck KGaA, Darmstadt, Germany); and 9) optical microscopic analysis. Negative controls, with the primary antibody omitted, were carried out for each bone section.

To quantify the bone expression of FGF-23, Scl, and RANKL, whose presence in the trabecular bone was limited to osteocyte cells, the staining result for any given osteocyte was classified as positive or negative, and the total number of trabecular osteocytes with positive staining was counted and normalized to the total number of osteocytes analyzed in the trabecular bone section (100 × positive Ost/total Ost, %).

The expression of MEPE and DMP-1 in trabecular bone tissue was quantified using the Image-Pro Plus 7.0 program (Media Cybernetics®, Rockville, USA), where the positive area was normalized to the total analyzed area (100 × positive area/total area, %).

Table 1
Clinical, biochemical, histomorphometric and immunohistochemical observations in patients before and after PTX.

	Pre-PTX (N = 23)	Post-PTX (N = 23)	p	Reference values	
Clinical data					
Age (years)	41.91 ± 12.50	–	–	n/a	
Female (%)	56.60	–	–		
Hemodialysis time (months)	96.96 ± 45.54	–	–		
Biochemistry					
Calcium (mg/dL)	10.18 ± 0.89	9.43 ± 1.06	0.0214	8.80–10.60	
Phosphorus (mg/dL)	6.83 ± 2.01	5.33 ± 1.62	0.0022	2.70–4.50	
Alkaline phosphatase (U/L)	676 (177–2587)	109 (49–233)	< 0.0001	35–104	
PTH-intact (pg/mL)	1960 (597–3587)	58 (1 − 303)	< 0.0001	10–65	
Histomorphometry				Females (n = 41)[a]	Males (n = 47)[a]
BV/TV (%)	14.86 (6.91–53.40)	19.62 (2.90–43.17)	0.38	[d]	[e]
Tb.Th (μm)	111.84 ± 25.27	142.17 ± 34.80	0.0001	126.00 ± 28.80	127.90 ± 29.70
Tb.Sp (μm)	658.19 (86.39–1299)	585.50 (131–3627)	0.0275	498.30 ± 195.90	420.60 ± 124.10
Tb.N (/mm)	1.34 (0.68–5.39)	1.38 (0.30–4.30)	0.0085	1.76 ± 0.52	1.89 ± 0.42
OV/BV (%)	10.57 (1.74–32.40)	4.49 (0.17–17.76)	0.0002	1.55 ± 1.96	2.99 ± 2.75
O.Th (μm)	11.82 ± 3.06	7.84 ± 2.17	< 0.0001	10.80 ± 3.20	11.37 ± 3.50
OS/BS (%)	52.45 ± 16.41	38.78 ± 24.96	0.0119	9.20 ± 8.40	16.10 ± 12.60
Ob.S/BS (%)	13.43 (4.46–44.10)	1.50 (0–8.90)	< 0.0001	1.20 ± 3.20	1.20 ± 1.40
ES/BS (%)	15.77 (3.83–25.50)	1.95 (0–14.60)	< 0.0001	2.30 ± 2.40	1.75 ± 1.21
Oc.S/BS (%)	4.58 (1.20–9.14)	0.34 (0–1.34)	< 0.0001	0.03 ± 0.06	0.03 ± 0.11
Fb.V/TV (%)	6.76 (0.63–19.05)	0.03 (0–0.94)	< 0.0001	0	0
MS/BS (%)	15.52 (3.93–27.70)	2.92 (0.33–21.90)	< 0.0001	11.50 ± 4.50 (n = 29)[b]	18.30 ± 7.50 (n = 12)[b]
MAR (μm/day)	1.20 (0.67–2.25)	0.81 (0.30–1.24)	0.0023	0.65 ± 0.12 (n = 41)[b]	
BFR/BS ($\mu m^3/\mu m^2$/day)	0.17 (0.04–0.43)	0.050 (0.008–0.096)	< 0.0001	0.07 ± 0.03 (n = 29)[b]	0.13 ± 0.07(n = 12)[b]
Mlt (days)	32.3 (10.4–104.6)	101.1 (7.4–809.9)	0.0436	23.5 ± 16.0 (n = 41)[c]	
Ost total (n)	796 (113–2916)	737 (31–2244)	0.229	n/a	
Ost total/B.Ar (n/mm^2)	127.80 ± 45.00	78.70 ± 30.60	< 0.0001		
Ost total/T.Ar (n/mm^2)	27.30 (4.90–75.40)	17.90 (1.60–61.30)	0.0018		
Immunohistochemistry					
DMP-1 (%)	3.47 (0.36–23)	4.02 (0.04–17.31)	0.9636	n/a	
MEPE (%)	1.88 (0.21–7.45)	1.93 (0.70–5.01)	0.3155		
Scl (%)	3.95 (0.25–37.10)	13.5 (3.03–41.42)	0.0009		
FGF23 (%)	6.79 (1.50–28.33)	6 (0.35–74.38)	0.533		
OPG (%)	0.74 (0.17–3.03)	2.54 (0.26–16.12)	0.0001		
RANKL (%)	2.10 (0.61–14.84)	3.62 (0.74–63.63)	0.1752		
RANKL/OPG (UA)	3.39 (0.37–13.95)	1.50 (0.29–14.35)	0.040		

Values are expressed as the mean and standard deviation or median and extremes; n/a: not applicable; PTX: parathyroidectomy; PTH-intact: parathyroid hormone; BV/TV: bone volume; Tb.Th: trabecular thickness; Tb.Sp: trabecular separation; Tb.N: trabecular number; OV/BV: osteoid volume; O.Th: osteoid thickness; OS/BS: osteoid surface; Ob.S/BS: osteoblastic surface; ES/BS: resorption surface; Oc.S/BS: osteoclastic surface; Fb.V/TV: fibrosis volume; MS/BS: mineralizing surface; MAR: mineral apposition rate; BFR/BS: bone formation rate; Mlt: mineralization lag time; Ost total: total number of osteocytes; Ost total/B.Ar: total osteocytes normalized to the trabecular bone area; Ost total/T.Ar: total osteocytes normalized to the tissue area analyzed; DMP-1: dentin matrix protein 1; MEPE: matrix extracellular phosphoglycoprotein; Scl: Sclerostin; FGF23: fibroblast growth factor 23; OPG: osteoprotegerin; RANKL: receptor activator of nuclear factor kappa B ligand; UA: Arbitrary unity; yo: years old.

[a] Reference [3].
[b] Reference [15].
[c] Reference [16].
[d] BV/TV normal values for matched males (11–20 yo, n = 5: 31.32 ± 8.2; 21–30 yo, n = 8: 28.05 ± 5.5; 31–40 yo n = 10: 24.82 ± 5.47; 41–50 yo, n = 11: 24.31 ± 6.26; 51–60 yo, n = 9: 20.78 ± 5.33; 61–80 yo, n = 6: 20.1 ± 5.77) [13].
[e] BV/TV normal values for matched females (11–20, n = 8: 23.06 ± 4.79; 21–30, n = 9: 25.78 ± 4.06; 31–40, n = 10: 26.23 ± 8.32; 41–50 yo, n = 10:18.65 ± 7.17; 51–60 yo, n = 4:17.8 ± 3.33; 61–90 yo, n = 8:17.78 ± 5.52) [13].

2.5. Statistical analysis

Results were expressed as the mean and SD or the median and minimum and maximum values for variables with parametric and non-parametric distributions, respectively. The type of distribution was evaluated with the Kolmogorov-Smirnov test. Biochemical, histomorphometric and immunohistochemical parameters were compared using the paired Student's *t*-test or Wilcoxon's test, where appropriate.

To evaluate the correlations between parameters, we used the significance test for the Spearman correlation coefficient. Fisher's exact test was applied to compare proportions between groups. Statistical analysis was performed with the GraphPad Prism 4 and SPSS for Windows 17.0 programs. Values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. Clinical and laboratory evaluation

The clinical and laboratory characteristics of the patients are presented in Table 1. The patients were relatively young, with a mean age of 42 years; 56% percent of the patients were women, and the mean hemodialysis vintage was 97 months. For the biochemical parameters, the levels of Ca, P, AP and PTH showed a significant drop after PTX. The cumulative doses of elemental calcium and calcitriol during the one-year period after surgery were 135 (73–252) g and 34.3 (5.2–101) μg, respectively.

3.2. Histomorphometric evaluation

The results of the histomorphometric analysis of bone biopsies

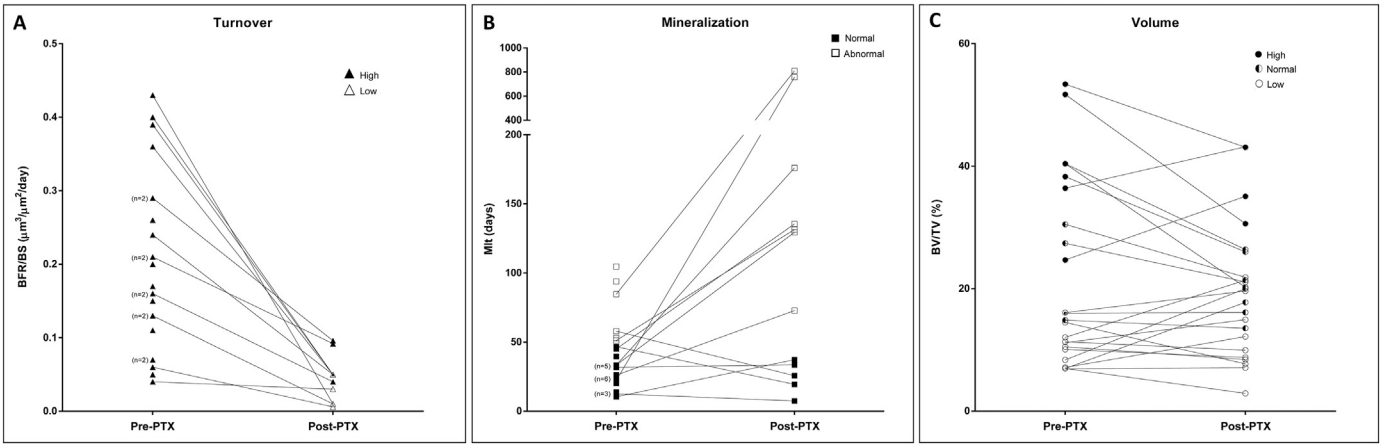

Fig. 1. Turnover, Mineralization and Volume classification according to results of bone histomorphometry obtained in biopsies from patients with secondary hyperparathyroidism pre and 12 months after PTX. One year after PTX we observed a decreased in high Turnover bone disease (A), an increased in abnormal Mineralization (B) and no significant changes in bone Volume (C).

before and after PTX are shown in Table 1. After PTX, structural parameters changed significantly with decreased Tb.Sp and increased Tb.Th and Tb.N, showing improvement in bone microarchitecture. PTX significantly reduced bone formation parameters, OV/BV, O·Th; OS/BS, Ob.S/BS in 52%, 34%, 26%, 88%, respectively, as well as resorption parameters, ES/BS and Oc.S/BS by 78% and 91%, respectively. In addition, marrow fibrosis, that initially was present in all patients, markedly decreased after PTX (Table 1).

We also observed a significant decrease in the number of total osteocytes normalized to the trabecular bone or bone tissue area after PTX (Table 1).

Before PTX, 100% of patients had high Turnover, decreasing to 18% after the surgery (Fig. 1A). Mineralization was normal in 74% of the patients, but only in 22% of them after PTX (Fig. 1B). We also found normal or high bone Volume in 48% of the patients before PTX and in 52% of them after surgery (Fig. 1C). These results are shown in Table 2.

3.3. Immunohistochemistry analysis DMP-1, MEPE, Sclerostin, FGF23, OPG and RANKL bone expression

Osteocyte protein expression was evaluated by immunohistochemistry, and the observed results are described in Table 1.

Analyzing osteocytes in the trabecular bone, we verified that Scl expression increased significantly after PTX (Fig. 2D). Consistent with the fact that this protein is a marker of mature osteocytes, Scl was observed predominantly in the most central osteocytes of the bone trabeculae (Fig. 2D and D1). We also observed a significant increase in OPG expression after PTX (Fig. 2H and H1), which was accompanied by a decrease in the RANKL/OPG ratio.

Table 2
Turnover, Mineralization, and Volume (TMV) classification before and one year after PTX.

	Pre-PTX	Post-PTX	p value[a]
Turnover			
Low	0 (0%)	19 (83%)	< 0.0001
High	23 (100%)	4 (17%)	
Mineralization			
Normal	17 (74%)	5 (22%)	0.0009
Abnormal	6 (26%)	18 (78%)	
Volume			
Low	12 (52%)	11 (48%)	1
Normal or high	11 (48%)	12 (52%)	

PTX: parathyroidectomy; data are expressed as frequency (percentage).
[a] Fisher's exact test.

The distribution of DMP-1, with its most pronounced expression in the cell bodies and dendritic processes of the osteocytes, was diffuse throughout the trabeculae. Like DMP-1, MEPE was also diffusely expressed in the trabeculae and observed in the cell bodies of the osteocytes. The expression of FGF23 was more intense in the osteocytes present in the periphery of the trabeculae. In contrast, the expression of RANKL was observed in osteoblasts close to the osteoid surface, in some medullary cells and in osteocytes. PTX did not alter the bone expression of these proteins.

3.4. Relative changes between pre- and post-PTX: correlations between osteocyte protein expression and bone histomorphometric parameters

As shown in Table 3 and Fig. 3, the changes in Scl bone expression correlated negatively with changes in BV/TV ($r = -0.54$, $p = 0.007$, Fig. 3A), O·Th ($r = -0.50$, $p = 0.0015$, Fig. 3E) and Tb.N ($r = -0.51$, $p = 0.012$, Fig. 3B) and positively with changes in Tb.Sp ($r = 0.51$, $p = 0.012$, Fig. 3C). Correlations between the changes in Scl expression and OV/BV (Fig. 3D), changes in OPG expression with OS/BS variation (Fig. 3F) and the changes in MEPE expression with MS/BS (not shown) where close to the statistical significance. Changes in RANKL expression correlated negatively with BV/TV ($r = -0.45$, $p = 0.046$, Fig. 3G), and Tb.N ($r = -0.55$, $p = 0.012$, Fig. 3H) variations and positively with changes in Tb.Sp ($r = 0.53$, $p = 0.015$, Fig. 3I). A decrease in the RANKL/OPG ratio correlated with an increase in Ob.S/BS ($r = -0.48$, $p = 0.033$, Fig. 3J). Changes in bone expression of DMP-1 and FGF23 did not correlated with any histomorphometric parameter.

We found no correlation between the amount of calcium and calcitriol prescribed during the 1-year period with the biochemical and histomorphometric data and with the expression of bone proteins studied.

4. Discussion

The present study showed correlations between changes in the bone expression of osteocyte proteins, whose alterations are evident in a small group of patients, and laboratory and histomorphometric parameters in SHPT patients subjected to PTX.

The results of the histomorphometric analysis revealed that the majority of the patients had high osteoblastic and osteoclastic activity and highly compromised bone microarchitecture, in addition to extensive areas of fibrosis. These conditions represent one of the histological alterations observed in SHPT. These changes are influenced by the high levels of PTH through multiple mechanisms [19]. The high turnover disease is characterized by an imbalance in remodeling, which

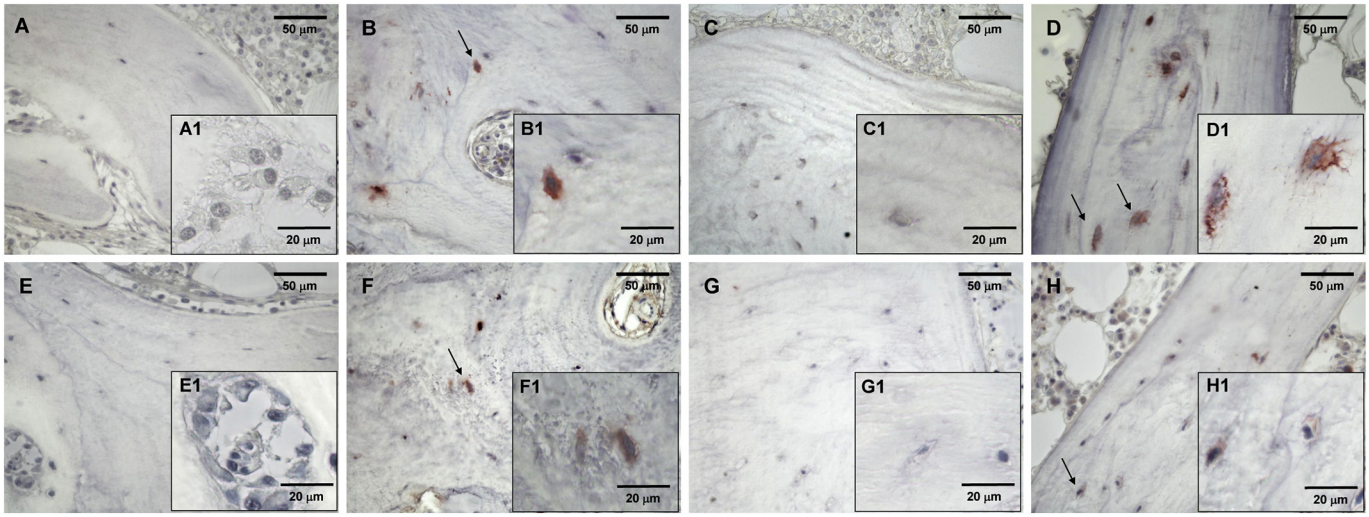

Fig. 2. Representative microscopic features of osteocyte protein expression by immunohistochemistry. Sclerostin negative control before (A, A1) and after (C, C1) PTX. Trabecular bone expression of Sclerostin before PTX was observed in osteocytes deeply embedded in the trabeculae (B, B1). Sclerostin expression increased in trabecular bone after PTX (D, D1). OPG negative control before (E, E1) and after (G, G1) PTX. OPG expression in trabecular bone was high in osteocytes before PTX (F, F1) and decreased after PTX (H, H1). Black arrows identify positively labeled proteins. (A–H: original magnification 400×; A1–H1 original magnification 1000×). Scale bar = 50 μm.

Table 3
Correlations between the relative changes (Δ) in osteocyte proteins and histomorphometric parameters.

	Histomorphometric parameters	r	p
Δ Sclerostin (%)	Δ BV/TV	−0.54	0.007
	Δ OV/BV	−0.41	0.053
	Δ O.Th	−0.50	0.0015
	Δ Tb.N	−0.51	0.012
	Δ Tb.Sp	0.51	0.012
Δ OPG (%)	Δ OS/BS	−0.40	0.059
Δ RANKL (%)	Δ BV/TV	−0.45	0.046
	Δ Tb.N	−0.55	0.012
	Δ Tb.Sp	0.53	0.015
Δ RANKL/OPG (UA)	Δ Ob.S/BS	−0.48	0.033
Δ MEPE (%)	Δ MS/BS	0.37	0.083
Δ DMP-1 (%)	–	–	–
Δ FGF23 (%)	–	–	–

OPG: osteoprotegerin; RANKL: receptor activator of nuclear factor kappa B ligand; UA: arbitrary units; MEPE: matrix extracellular phosphoglycoprotein; DMP-1: dentin matrix protein 1; FGF23: fibroblast growth factor 23; BV/TV: bone volume; OV/BV: osteoid volume; O.Th: osteoid thickness; Tb.N: trabecular number; Tb.Sp: trabecular separation; OS/BS: osteoid surface; Ob.S/BS: osteoblast surface; MS/BS: mineralizing surface; r: Spearman correlation coefficient.

compromises the microarchitecture of bone tissue, with decreased connectivity and increased trabecular separation. Mineralization may also be impaired as the newly formed bone is rapidly removed without sufficient time for mineralization to be adequately complete. Usually, high bone turnover is associated with normal or high bone volume. However, patients submitted to high levels of PTH during a considerable amount of time could present bone loss. Indeed, 8 of our patients has a BV/TV at the time of PTX lower than 11%, which is a threshold value for fracture risk, according to Meunier et al. [20].

Few studies have evaluated the histological characteristics of bone tissue using histomorphometry before and after PTX [21], as in the present study. Yajima et al. investigated patients subjected to bone biopsy one week after PTX. The authors noticed a transient increase in bone formation, whereas osteoclasts disappeared or decreased in number [22]. Several years later, in another study, the same group analyzed bone histology before and two to four weeks after PTX. Simultaneously, the authors observed that in addition to a significant decrease in the osteocyte number that was concomitant with an increase in the number of empty gaps and a reduction in lacunar volume, the osteoclast surface decreased to almost zero, with a substantial increase in osteoid volume and a reduction in fibrosis [23]. We also observed a significant decrease in osteocyte number (per bone area and tissue) after PTX, presumably due to the decrease in cellular signaling by PTH, similar to that observed by Yajima et al. [23]. However, the analysis of the long-term effects of PTX on bone histomorphometry was done only in a small study by Charhon et al. The authors studied bone biopsies of 10 patients with SHPT before PTX and 12 to 16 months after PTX, showing that surgery was associated with a significant reduction in turnover and bone mineralization. These results were similar to those we found. The authors do not refer to changes in trabecular volume nor in trabecular thickness [21]. We found no significant changes in bone volume, but an improvement in bone microarchitecture, characterized by a decrease in trabecular separation and an increase in trabecular number and thickness. We believe that the increase in trabecular thickness could be explained by the minimodeling process that was described some years ago [24]. The authors observed an increase in osteoid volume due to minimodeling 3 to 4 weeks after PTX, and an increase in mineralization 10 to 12 weeks after surgery. However, as our bone biopsies were done 1 year after PTX, we could not observe tetracycline labeling in these areas anymore.

By evaluating osteocytic protein expression, we demonstrated that a significant increase in the bone expression of Scl occurred after PTX. Analyzing pediatric patients with CKD and SHPT who were treated with vitamin D analogue (doxercalciferol), Pereira et al. observed an increase in Scl expression [25]. Thus, SHPT treatment using either PTX or vitamin D analogues likely reduces PTH levels and consequently increases the expression of Scl. It is well known that PTH is an important inhibitor of Scl [26] and that the anabolic effects of PTH on bone tissue are mediated in part by this effect. In this context, the decreases in bone formation rate and osteoblast surface observed after surgery may have been influenced by the increase in Scl expression [27].

We also demonstrated a significant increase in OPG bone expression after PTX. This protein negatively regulates osteoclastogenesis, and increased OPG expression may have contributed to a decrease in the resorption parameters detected after PTX. Previous studies have demonstrated the inhibitory effect of PTH on OPG [28], and the drop in PTH levels after PTX may therefore have influenced this result.

Unexpectedly, we did not observe any difference in RANKL expression after PTX. However, the RANKL/OPG ratio decreased

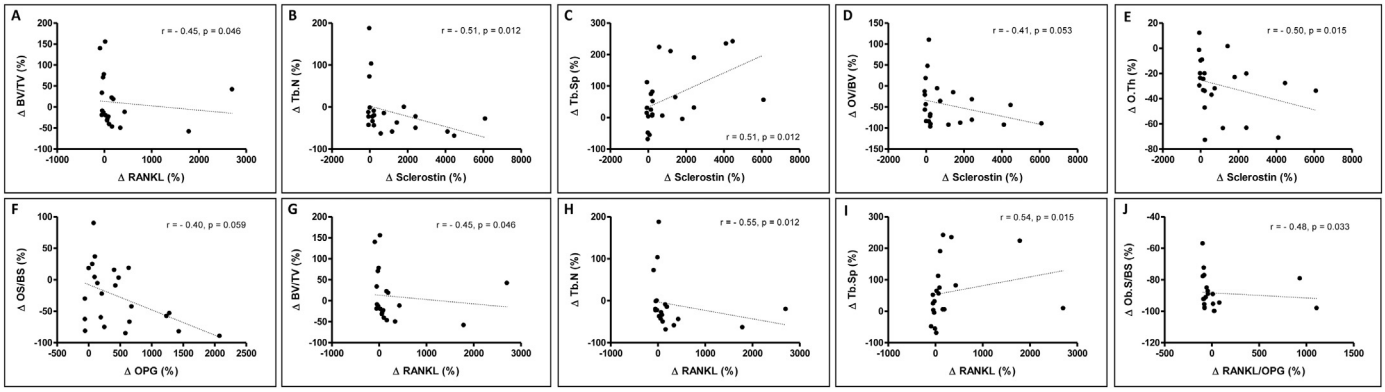

Fig. 3. Association between changes in the osteocytic protein expression and changes in histomorphometric parameters. Changes in Scl bone expression correlated negatively with changes in BV/TV (panel A), Tb.N (panel B), and O.Th (panel E) and positively with changes in Tb.Sp (panel C). Correlations between the changes in Scl expression and OV/BV (panel D), and changes in OPG expression with OS/BS (panel F), where close to the statistical significance. Changes in RANKL expression correlated negatively with BV/TV (panel G), and Tb.N (panel H) and positively with changes in Tb.Sp (panel I). A decrease in the RANKL/OPG ratio correlated with an increase in Ob.S/BS (panel J).

significantly after PTX, an observation in line with the decreases in resorption parameters detected after surgery. RANKL/OPG ratio is a key in the regulation of bone resorption, bone mass and integrity [29]. In the primary hyperparathyroidism scenario, circulating levels of RANKL are also increased and positively correlated with markers of bone resorption. A previous study has shown a decrease in the RANKL/OPG ratio in these patients one year after PTX [30], a finding that it is consistent with our results.

We did not observe a significant change in DMP-1 expression. However, Pereira et al. observed an increase in the expression of the full-length form (98 kDa) and a decrease in the 57-kDa fragment of this protein in pediatric patients with CKD and SHPT treated with doxercalciferol [31]. Notably, we evaluated the N-terminal fragment of this protein, which may explain the differences between our results and those of Pereira et al. [25].

We did not observe significant changes in MEPE expression. Additionally, like MEPE, we did not observe a change in FGF23 expression after PTX. As both systemic and local factors regulate FGF23 activity, the lack of differences in FGF23 expression after PTX might reflect the complex mechanisms that regulate this protein, with several factors both positively and negatively controlling FGF23 expression. Recent studies have shown that FGF23 might have a Klotho-independent effect on bone mineralization through the suppression of tissue non-specific alkaline phosphatase, increasing the concentration of pyrophosphate, a well-known mineralization inhibitor [31]. However we did not detected any differences in FGF23 expression before and after PTX.

We detected association between changes in osteocytic proteins and changes in bone histomorphometric parameters after PTX. Scl plays a central role in the regulation of bone mass by inhibiting the formation of bone tissue. Consistent with this role, our results demonstrated a negative association between Scl and structural (bone volume, trabecular number) and bone formation parameters (osteoid volume) and a positive relationship between Scl and trabecular separation. These results are in agreement with the known effects of Scl in the Wnt pathway [32].

According to our expectations, we demonstrated a negative association between changes in RANKL and changes in structural parameters, such as bone volume and the trabecular number, and a positive association between changes in RANKL and changes in trabecular separation, findings that probably reflect the bone resorption role of this protein. Briefly, after PTX we observed an increase in SOST expression, which would contribute to a decrease in formation and loss of bone mass. On the other hand, decreased RANKL to OPG ratio, which would decrease resorption, and probably the net balance would be a non-significant change in BV/TV.

The main limitations of the present study are the small sample size and a retrospective design. Another limitation is that the patients studied presented very severe SHPT, which is probably no longer observed due to the better control with medications such as calcimimetics and vitamin D receptor activators. We neither had stored samples to measure the serum concentration of these proteins and compare with their bone expression. However, this the first study that evaluated the changes in the bone expression of these proteins after PTX. We could also expand the knowledge of the long-term effects of PTX on bone histomorphometry.

In summary, in this report, we demonstrated the changes in bone proteins expression after PTX, allowing us to discriminate the effects of chronic kidney disease from those of SHPT on Scl, DMP-1, MEPE, FGF23, OPG and RANKL bone expression. Understanding the performance of these proteins throughout an established therapy, such as PTX, allows the identification of potential new targets for therapeutic interventions in the treatment of SHPT.

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