



Comparison of clinical, biochemical and histomorphometric analysis of bone biopsies in dialysis patients with and without fractures

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Abstract

Chronic kidney disease-mineral bone disorders (CKD-MBD) are associated with increased risk of fracture. Studies report about 3% of fractures in CKD patients, and these occur earlier than in the general population, namely 16 and 13 years earlier for men and women, respectively. Better understanding of the pathophysiology of fractures would probably contribute to new therapeutic approaches. This study aimed to evaluate report of long bone fractures from a bone biopsies bank from patients on hemodialysis and compare clinical and biochemical characteristics, as well as the results of the histomorphometric analysis of trabecular and cortical bone of these patients with a control group (without fractures), paired for age, gender, and time on hemodialysis. Bone proteins (SOST, DMP1 and MEPE) were evaluated by immunohistochemistry. Seventeen patients with fracture and controls were studied. Fracture prevalence was 0.82/1000 patients/year. Serum phosphorus levels were significantly lower in the fracture group. Histomorphometric analysis revealed that all the patients had high turnover disease, and the fracture group had smaller volume and trabecular thickness, greater osteoid surface, smaller eroded surface, smaller mineralizing surface, formation rate and longer mineralization lag time when compared to controls; the DMP1 expression in the cortical bone was smaller and the SOST in the trabecular bone was higher in fractured patients. As conclusion, we found low prevalence of fractures. Both groups had high turnover disease, but the fractured ones presented more impaired bone microarchitecture, as well as lower formation and greater mineralization defect. Bone proteins expression correlated with parameters involved in bone remodeling.

Keywords Fracture · CKD-MBD · Bone turnover · Histomorphometry · Immunohistochemistry

Introduction

The bone structure, which distinguishes vertebrates from other animals, has developed over thousands of years, becoming what we know as the skeleton, a specialized form of connective tissue. The skeleton performs several

functions, such as protection of organs, locomotion, mineral reserve of calcium and phosphorus, defense against acidosis, among others [1]. Some diseases are capable of damaging the well-orchestrated functioning of bone tissue, making it less resilient and responsive to external aggressions; one of which is kidney disease [2]. This pathology is becoming increasingly common in the population due to aging and the high prevalence of hypertension and/or diabetes mellitus.

The incidence of non-traumatic fracture, whether in patients with chronic kidney disease (CKD) or not, has been increasing significantly in the world [3, 4].

Alterations in mineral metabolism, microarchitecture and reduction of bone mass appear early and gradually during loss of renal function, so that when patients initiate dialysis, about 50% of them refer to a history of fracture [2].

Data from the *Third National Health Examination Survey* (NHANES) indicate that the risk of hip fracture is twice as high in patients with glomerular filtration rate

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(GFR) of less than 60 mL/min when compared to those with GFR > 60 mL/min [2].

In patients on dialysis, the incidence of hip fracture is 17.4 times higher than in the general population [5]. It is noteworthy that in these patients the mortality rate is 64% in 1 year, compared to the rate of 15–20% in the general population. In addition, the fracture occurs at an earlier age than that of the general population, 16 and 13 years for men and women, respectively [2].

Understanding the mechanisms that increase bone fragility in patients with CKD would help improve prevention and consequently the treatment of these patients.

Bone histomorphometry has proven to be an excellent tool for understanding turnover, bone mineralization and volume changes [6]. The same can be said about immunohistochemistry in undecalcified bone tissue by evaluating the expression of in situ proteins [7].

Until now, no study has associated these two techniques to evaluate the bone biopsy characteristics of patients with CKD and fracture. The objective of this study was to compare the clinical, biochemical and histomorphometric characteristics of the trabecular and cortical bone of patients with chronic kidney disease and fracture with that of patients without fractures, paired for age, sex, and time on hemodialysis, as well as compare the expression of proteins involved in the formation (sclerostin—SOST) and bone mineralization (Dentin matrix acid phospho protein 1—DMP1, and matrix extracellular phosphoglycoprotein—MEPE) of these patients.

Materials and methods

Patients

Retrospective study using a Brazilian biopsy bank from *Laboratório de Fisiopatologia Renal (LIM 16)—Faculdade de Medicina—Universidade de São Paulo (FMUSP)* from 2004 to 2015. The inclusion criteria was hemodialysis patients with history of femur or humerus fracture, as well as controls paired for sex, age and time on hemodialysis. The exclusion criteria included aluminum intoxication (more than 20% of trabecular recovered by aluminum), bad biopsy quality, absence of clinical information, previous kidney transplantation, and parathyroidectomy.

We also analysed the medications used in the treatment of CKD-MBD at the time of bone biopsy in both groups of patients.

The local ethics committee had previously approved the study under number n° 13445.

Biochemical parameters

Total calcium, phosphorus, alkaline phosphatase (AP) and parathormone (PTH) data was collected from request biopsy form.

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, separated by an interval of 10 days. The biopsy was performed 2–5 days after the last dose of antibiotics. The vast majority of biopsies were performed in the first month after fractures.

The specimens were fixed in 70% ethanol, dehydrated, and embedded in methylmethacrylate. Undecalcified 5- μ m-thick sections were cut using a Polycut S equipped with a tungsten carbide knife (Leica, Heidelberg, Germany), and some sections were stained with 0.1% toluidine blue, pH 6.4, and unstained 10- μ m slices were obtained for analysis of dynamic parameters under a microscope with ultraviolet light. All histomorphometric analyses were performed using a semiautomatic image analyzer and Osteomeasure software (Osteometrics, Inc., Atlanta, GA, USA) [8]. The static and dynamic parameters were reported using the nomenclature recommended by the American Society for Bone and Mineral Research [9].

The following histomorphometric parameters were measured: bone volume (BV/TV, %); trabecular separation (Tb.Sp, μ m); trabecular thickness (Tb.Th, μ m); osteoid volume (OV/BV, %); osteoid surface (OS/BS, %); osteoid thickness (O.Th, μ m); osteoblast surface (Ob.S/BS, %); eroded surface (ES/BS, %); osteoclast surface (Oc.S/BS, %); fibrosis volume (Fb.V, %); bone formation rate (BFR/BS, μ m³/ μ m²/day); mineralization lag time (Mlt, days); mineral apposition rate (MAR, μ m/day); and mineralizing surface (MS/BS, %). We analyzed cortical bone parameters: thickness and porosity. Cortical porosity (Ct.Po, %) was defined as the percentage of the cortical porosity area by the cortical area, and cortical thickness (Ct.Th, μ m).

The reference ranges used for static parameters were obtained from our normal laboratory controls [10], whereas the ranges for the dynamic parameters were the same as those described according to Rehman et al. study [11].

The bone histology was categorized according to the newly proposed turnover/mineralization/volume (TMV) system [12]. TMV classification was based on the following parameters: bone turnover (T), defined by BFR/BS (normal values for males and females from decades:

31–40, 41–50, 51–60 and 61–70) [11]; bone mineralization (M), defined by MIt (normal values for males and females from the decades: 31–40, 41–50, 51–60 and 61–70) [11]; and bone volume (V), defined by BV/TV (normal values for males: $24.0 \pm 6.1\%$; for females: 21.8 ± 7.2) [10]. High turnover defined as BFR/BS values > 1 SD above the normal range, and low turnover as BFR/BS values > 1 SD below the normal range. Abnormal mineralization defined as MIt ≥ 50 days. High bone volume defined as BV/TV values > 1 SD above the normal range, and low bone volume when BV/TV > 1 SD below the normal range.

Bone protein expression by immunohistochemistry

Bone immunohistochemistry (IHC) was performed as previously described [7]. In brief the following steps were performed:

- (1) Removal of methacrylate (MMA) with xylene/chloroform followed by rehydration with graded alcohol solutions; (2) partially decalcified in 1% acetic acid; (3) blocking of endogenous peroxidase with 3% hydrogen peroxide in methanol; (4) blocking of nonspecific proteins with Dako-Cytomation Protein Block X0909 (Dako, CA, USA); (5) incubation with primary antibody overnight at 4 °C (mouse monoclonal anti-human sclerostin; R&D System; dilution 1:100; goat IgG anti-human MEPE; R&D System; dilution 1:200 and rabbit polyclonal anti-human DMP1; TAKARA; dilution 1:200; (6) incubation with Biotinylated Link followed by streptavidin-HRP conjugate (Universal Dako LSAB Dako K 0690); (7) followed by another incubation with AEC (3-Amino-9-ethylcarbazole) substrate chromogenic (Sigma-Aldrich, Saint Louis, MO, USA); (8) and then counterstained with Mayer's hematoxylin (Merck KGaA, Darmstadt, Germany); and (9) optical microscopy analysis. Negative controls were performed for each bone section by omitting the primary antibody. Bone sclerostin expression was quantified by counting the positively stained osteocytes in relation to the analyzed bone area. MEPE and DMP1 expression were quantified using the Image-Pro Plus 7.0 program. In the trabecular bone, 25 fields were evaluated. In cortical area, the analysis was performed in the complete area. MEPE and DMP1 values were expressed as percentage (positive area/total area $\times 100$).

Statistical analysis

Continuous parametric variables are expressed as the mean \pm standard deviation, and nonparametric variables are expressed as median and 25th–75th percentiles. For the univariate analysis, we used the Mann–Whitney and *t* Student tests. For correlation tests, the Spearman test was used.

Statistical analyses were conducted using Statistica software Version 8.0 (Tibco Software Inc., Palo Alto, CA,

USA), and all tests were performed with a two-sided 0.05 level of significance.

Results

We evaluated 1888 bone biopsies from the database of the *Laboratório de Fisiopatologia Renal (LIM 16)—Faculdade de Medicina—Universidade de São Paulo (FMUSP)*. In these, 115 fractures were reported, we excluded 59 because they presented aluminum intoxication. Of these patients, 76.3% had high bone turnover and 23.7% low bone turnover. We also excluded 31 patients with fractures found in bones other than those of the study. Of these, 14 patients had vertebral fractures (71.4% with high and 28.6% with low bone turnover), nine had rib fractures (77.8% with high and 22.2% with low bone turnover), four had clavicle fractures (75% with high and 25% with low bone turnover), one had calcaneal fracture (high bone turnover), one had scapular fracture (high bone turnover), one had metatarsal fracture (high bone turnover) and one had fracture of the wrist (high bone turnover). Three biopsies were from transplant patients. In nine cases, clinical and/or laboratory data were incomplete, and 15 cases were excluded because of poor quality of fragment. Some patients had more than one criteria of exclusion. Thus, we selected 17 patients with low impact fractures: 14 with femoral and three with humerus fractures.

Regarding the demographic characteristics of the patients (Table 1), we found a predominance of women (9/52.94%), younger than 60 years (12/70.59%), with less than 10 years on hemodialysis (10/58.83%).

Among those with fracture (group 1), hypertension (8/47.05%) was the main cause of renal disease, followed by glomerulonephritis (4/23.52%). In the group without fracture (group 2), glomerulonephritis (5/29.41%) and unknown (5/29.41%) were predominated.

In group 1, we found 7 (41.17%) patients with low bone volume (BV/TV $< 17.9\%$ for men and BV/TV $< 14.6\%$ for women) and in group 2 only one patient (5.88%).

Regarding the biochemical laboratory data (Table 1), we found statistical difference regarding the values of phosphorus; being higher in group 2 (6.8 ± 1.34 vs. 5.4 ± 1.85 ; $p = 0.02$). Concerning to total calcium, alkaline phosphatase and parathormone serum evaluation, they were similar between the groups.

Histomorphometric analysis results performed on the bone biopsies (Table 1), group 1 had a lower bone volume (20.8 ± 9.5 vs. 27.3 ± 8.3 ; $p = 0.04$), and trabecular thickness (105.9 ± 29.31 vs. 125.3 ± 25.4 ; $p = 0.04$) greater osteoid surface (60.3 ± 17.6 vs. 44.6 ± 19.9 ; $p = 0.02$), and mineralization lag time [229 (102.2–1381) vs. 57.3 (33.2–97); $p = 0.0002$], smaller mineralizing surface (4.8 ± 2.7 vs. 9.4 ± 5.3 ; $p = 0.01$), bone formation rate

Table 1 Comparison between fracture and non-fracture groups

	Fracture (<i>n</i> = 17)	Non-fracture (<i>n</i> = 17)	<i>p</i>
General characteristics			
Age < 60 years, <i>n</i> (%)	12 (70%)	12 (70%)	*
Female, <i>n</i> (%)	9 (52.94%)	9 (52.94%)	*
Dialysis length < 10 years, <i>n</i> (%)	10 (60%)	10 (60%)	*
Laboratory characteristics			
iCa (mg/dL)	9.2 ± 1.0	9.4 ± 1.5	0.72
P (mg/dL)	5.5 ± 1.8	6.8 ± 1.3	0.02
tAP (UI/L)	326 ± 297.7	366.5 ± 346.6	0.72
iPTH (pg/mL)	1441.8 ± 1182.8	1284.3 ± 737.8	0.64
Medications in use ^a			
Sevelamer	7 (41.2%)	10 (58.9%)	0.49
CaCO ₃	5 (29.4%)	2 (11.8%)	0.40
Active vitamin D analogues	7 (41.2%)	4 (23.5%)	0.46
Cholecalciferol	2 (11.8%)	0	0.49
Histomorphometry			
BV/TV (%)	20.8 ± 9.5	27.3 ± 8.3	0.04
Tb.Sp (μm)	378.2 (255.9–680.6)	386.1 (260.7–520.3)	0.33
Tb.N (/mm)	2.0 ± 0.9	2.3 ± 0.98	0.42
Tb.Th (μm)	105.9 ± 29.3	125.3 ± 25.4	0.04
OV/BV (%)	13.4 (3.7–28.0)	8.6 (4.0–16.3)	0.31
OS/BS (%)	60.3 ± 17.6	44.6 ± 19.9	0.02
O.th (μm)	12.8 ± 6.2	12.7 ± 5.3	0.95
BFR/BS (μm ³ /μm ² /day)	0.04 ± 0.04	0.1 ± 0.09	0.005
Mlt (days)	229.0 (102.2–1381.0)	57.3 (33.2–97.0)	0.0002
MAR (μm/day)	1.0 ± 0.3	1.1 ± 0.4	0.32
MS/BS (%)	4.8 ± 2.7	9.4 ± 5.3	0.01
Oc.S/BS (%)	2.5 ± 2.1	3.8 ± 2.7	0.13
ES/BS (%)	8.6 ± 4.5	15.0 ± 5.3	0.0005
Ct.Th (μm)	381.4 ± 183	486.8 ± 211.7	0.16
Ct.Po%	10.5 ± 4.8	9.5 ± 3.9	0.53

Values are mean ± SD or median (25th, 75th percentiles)

BV/TV bone volume, *Tb.Sp* trabecular separation, *Tb.N* trabecular number, *Tb.Th* trabecular thickness, *OV/BV* osteoid volume, *OS/BS* osteoblast surface, *O.Th* osteoid thickness, *BFR/BS* bone formation rate, *Mlt* mineralization lag time, *MAR* mineralization apposition rate, *MS/BS* mineralizing surface, *Oc.S/BS* osteoclast surface, *ES/BS* eroded surface, *Ct.Th* cortical thickness, *Ct.Po* cortical porosity

*NS-paired for sex, age and time on hemodialysis

^aFor treatment of CKD-MBD

(0.04 ± 0.04 vs. 0.1 ± 0.09; *p* = 0.005), and eroded surface (8.6 ± 4.5 vs. 15 ± 5.3; *p* = 0.0005).

We did not find differences between the two groups regarding the histomorphometric analysis of the cortical bone (Table 1).

Regarding bone protein expression (Table 2), we found lower expression of DMP1 in the cortical bone in group 1 [1.3 (0.7–2.0) vs. 2.9 (1.5–7.8); *p* = 0.02] (Fig. 1c), as well as higher SOST expression in trabecular bone [5.2 (1.6–8.9) vs. 1.0 (0.5–3.9)] (Fig. 1d). The expression of the other proteins analyzed did not present statistical difference between the groups.

Most patients with fracture (60%) were on HD treatment for less than 10 years.

We evaluated the correlations between the results of the biochemical analysis (Ca, P, AP and PTH) and histomorphometric parameters. We observed that the phosphorus presented a direct correlation with eroded surface (*r* = 0.52; *p* = 0.001) and indirect correlation with mineralization lag time (*r* = − 0.35; *p* = 0.04). AP correlated positively with bone volume (*r* = 0.38; *p* = 0.02), bone formation rate (*r* = 0.36; *p* = 0.03), eroded surface (*r* = 0.56; *p* = 0.0007), osteoclast surface (*r* = 0.59; *p* = 0.0003), osteoblast surface (*r* = 0.47; *p* = 0.005), fibrosis volume (*r* = 0.45; *p* = 0.012)

Table 2 Comparison among DMP1, MEPE e SOST protein expression on cortical and trabecular bone of patients with and without fracture

	Fracture (<i>n</i> = 17)	Non-fracture (<i>n</i> = 17)	<i>p</i>
DMP1 cortical (%)	1.3 (0.7–2.0)	2.9 (1.5–7.8)	0.02
DMP1 trabecular (%)	1.8 (1.3–5.9)	3.5 (2.3–7.2)	0.09
MEPE cortical (%)	3.4 (2.4–3.6)	2.5 (1.8–5.2)	0.66
MEPE trabecular (%)	3.4 ± 2.2	4.6 ± 3.7	0.28
SOST cortical (n/mm ²)	8.9 (3.0–45.3)	12.6 (5.0–19.0)	1.0
SOST trabecular (n/mm ²)	5.2 (1.6–8.9)	1.0 (0.5–3.9)	0.035

Values are mean ± SD or median (25th, 75th percentiles)

DMP1 dentin matrix acidic phosphoprotein 1, *MEPE* matrix extracellular phosphoglycoprotein, *SOST* sclerostin

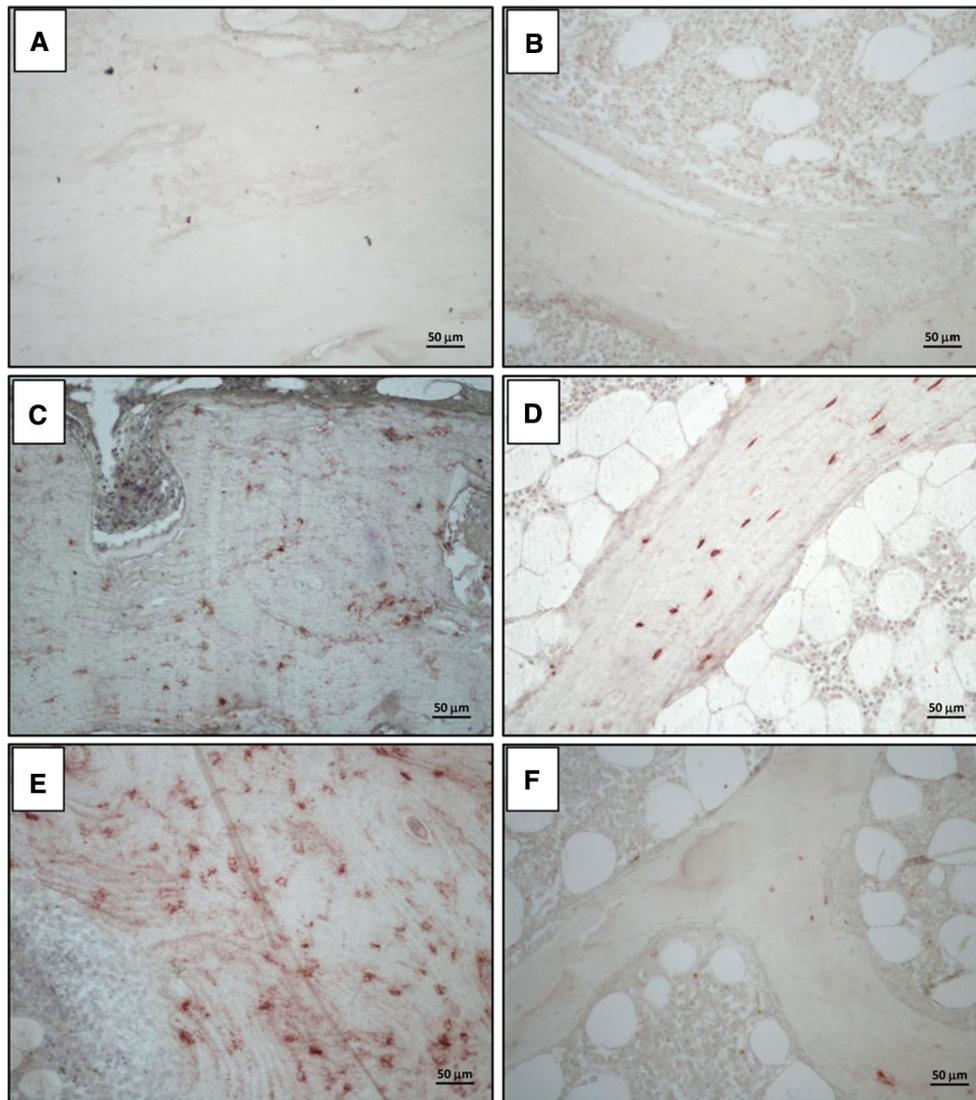


Fig. 1 Representative microscopic features of osteocytes protein expression (DMP1 and SOST) by immunohistochemistry. **a** DMP1 negative control in cortical bone; **b** SOST negative control in trabecular bone; **c** DMP1 expression in fractured patient's cortical bone; **d**

SOST expression in fractured patient's trabecular bone; **e** DMP1 expression in no fractured patient's cortical bone; **f** SOST expression in no fractured patient's trabecular bone (magnification ×200; scale bar = 50 μm)

and PTH level ($r = 0.62$; $p = 0.0001$). PTH presented a direct correlation with bone volume ($r = 0.61$; $p = 0.0001$), osteoid thickness ($r = 0.47$; $p = 0.005$), mineral apposition rate ($r = 0.42$; $p = 0.02$), bone formation rate ($r = 0.42$; $p = 0.013$), osteoclast surface ($r = 0.64$; $p = 0.0001$), osteoblast surface ($r = 0.66$; $p = 0.0002$) and fibrosis volume ($r = 0.58$; $p = 0.0001$). This was correlated inversely with the cortical thickness ($r = 0.62$; $p = 0.0001$).

We also evaluated the correlations between the expression of bone proteins (DMP1, MEPE and SOST) and histomorphometric analysis results. We observed that SOST expressed in trabecular bone was inversely correlated with trabecular thickness ($r = -0.38$; $p = 0.008$) and eroded surface ($r = -0.48$; $p = 0.006$). SOST expression in cortical bone was inversely correlated with trabecular thickness ($r = -0.46$; $p = 0.015$), mineralizing surface ($r = -0.41$; $p = 0.04$), and bone formation rate ($r = -0.48$; $p = 0.01$). The expression of MEPE in the trabecular bone was positively correlated with osteoid volume ($r = 0.42$; $p = 0.016$), osteoid thickness ($r = 0.35$; $p = 0.04$), osteoclast surface ($r = 0.44$; $p = 0.01$) and with alkaline phosphatase levels ($r = 0.40$; $p = 0.02$); while its expression in cortical bone correlated positively only with osteoid thickness ($r = 0.46$; $p = 0.018$).

Discussion

In this study, we reviewed 1888 reports of bone biopsies of CKD patients on hemodialysis for less than 10 years, we found 6% of patients with fractures, higher than that described in the DOPPS study [13]. We analyzed only patients with low impact bone fractures (femur and humerus) and excluded those with fractures in other places, with aluminum intoxication, renal transplantation, previous parathyroidectomy, and those with bone biopsy fragment with technical problems or missing data. After the exclusion, the percentage was reduced to 0.9%. The patients included were predominantly young (less than 60 years old), with time on dialysis of less than 10 years, and histomorphometric analysis of the biopsies revealed that they had high turnover disease with significant impairment of microarchitecture and mineralization.

The DOPPS study [13], which in its three stages studied 36,337 patients on hemodialysis in 12 countries, showed that 3% of patients had fractures during the follow-up of 1.6 years, 491 of femurs and 643 of other sites. In this study the incidence of any type of fracture ranged from 12/1000 patient years in Japan to 45/1000 patient years in Belgium. Specifically the femur fracture ranged from 3/1000 patients year in Japan to 20/1000 in Sweden. Our results, 0.82/1000 patient years, for this type of fracture, differ from those described in DOPPS, but we evaluated a smaller number of patients in a longer follow-up time.

Our results do not reflect the prevalence of fractures in patients on hemodialysis in Brazil. We performed a retrospective study with information obtained from biopsy referral reports; we did not analyze vertebral fractures, which although they are the most frequent, are often not diagnosed because they present few symptoms [14]. Among the excluded cases, we also found a predominance of high bone turnover (76.1%), which may reflect a characteristic of the dialysis population in our environment where control of hyperparathyroidism leaves much to be desired [15].

The prevalence of CKD is increasing worldwide, as is osteoporosis. These increases are due, in part, to the greater aging of the population. Fractures occurring in the different stages of renal disease are due both to the different forms of renal osteodystrophy and the presence of osteoporosis, a difficult-to-diagnose coexistence.

A recent review [14] report that, in the general population, osteoporosis is increasingly underdiagnosed and undertreated, which is due to the reduction, for economic reasons, of routine bone densitometry, non-appreciation and non-treatment of asymptomatic vertebral fractures what would prevent new fractures, and the fear of complications related to the osteoporosis treatment, especially with bisphosphonates, that can lead to jaw necrosis and atypical fractures. If these facts occur in the general population what about patients with CKD and osteoporosis? Only recently, KDIGO [16] has suggested bone densitometry to be used to assess fracture risk. According to Miller [17], patients with CKD stage 1–3 the diagnosis and treatment of osteoporosis may follow the same guidelines given to patients with osteoporosis without CKD. In patients with CKD stage 4 and 5, however, bone biopsy would be necessary. It is noteworthy that there are practically no studies on the effect of drugs in the osteoporosis treatment of CKD patients on stage 4, 5 and 5D.

Among the patients with fracture we found 51% occurred with aluminum intoxication (59/115). Classically, aluminum intoxication was accompanied by osteomalacia. However, our results showed that aluminum was detected in all histological types and predominantly in high turnover disease (osteitis fibrosa and mixed bone disease) suggesting that the metal did not necessarily interfere with bone mineralization. It is noteworthy that, in most cases, the presence of the metal in mineralization front was not as massive as observed in the biopsies of patients with osteomalacia. The main sources of aluminum intoxication continue to be the excessive concentration of the metal in dialysate and the use of aluminum-based phosphate binders. In the biopsy reports, all dialysis centers had reverse osmosis for water treatment and the most commonly used phosphate binder was sevelamer, followed by calcium salts, at least at the time of bone biopsy. Since a large number of patients had been on dialysis for a long time, it was not possible to correctly identify the source of

contamination. In a recent review Chappard et al. [18] suggests other sources of aluminum, such as biomaterials used in heart valves, bone implants and orthopedic prothesis, aluminum fluorosilicate bone cements, parenteral nutrition, and food additives. We do not have data to evaluate these other sources of contamination in the studied patients.

The results of the histomorphometric analysis revealed that both groups (fractured patients and controls) had high turnover bone disease. What distinguished the two groups was that those with fracture presented greater impairment of microarchitecture and bone mineralization. But in both groups the bone lesions are very serious.

Mineralization is also compromised as the newly formed bone is quickly removed without allowing time for the mineralization to complete properly. Increased osteoid matrix and less rigid bone trabeculae diminish the hardness of the bone tissue facilitating the fracture [19, 20].

Chronic PTH elevation decreases cortical thickness and increases bone trabeculae, but, as we have already mentioned, this increase occurs because of the increased osteoid matrix. We did not find differences in cortical thickness and cortical porosity when comparing patients with and without fractures, but in four patients with fractures, we did not analyze the cortical bone because of the difficulty in adequately separating them from the trabecular bone. However, we found an inverse correlation between PTH levels and cortical thickness when the patients were analyzed together, which suggests that excessive PTH contributed to decrease its thickness. Since the cortical bone is responsible for about 80% of the bone mass [21], reduction of its thickness may cause reduction of resistance in this region, increasing the risk of fracture.

When analyzing the correlations between biochemical analysis results and histomorphometric parameters including all the patients, we observed that they reflect the high bone turnover that would be expected, since we did not have patients with low bone turnover. The inverse association between serum phosphorus levels and the mineralization lag time, although weak, is interesting, as it suggests that in the face of an increased bone turnover, a lower concentration of phosphorus probably contributes to mineralization not being done correctly.

Serum levels of calcium and phosphorus, ion constituents of hydroxyapatite, as well as the various hormones involved in the regulation of these ions (FGF-23, calcitriol, PTH) are essential for bone mineralization to occur properly. All are profoundly affected by CKD making it difficult to evaluate, in isolation, the role of each one in bone mineralization. We did not find a significant difference in the use of phosphorus binders between the groups. Unfortunately, we do not have information about the nutritional status of the patients that could contribute to the differences between the levels of phosphorus between the two groups. It has recently been

demonstrated that daily dialysis patients develop hypophosphatemia and osteomalacia [22], confirming the importance of adequate levels of phosphorus so that mineralization is done correctly.

Studies have evaluated the results of histomorphometric analysis with the expression of osteocytes proteins in order to better elucidate the biology of bone tissue, its participation in bone turnover, and types of renal osteodystrophy [8, 23].

Pereira et al. [23], studying bone biopsies of children and adolescents with CKD, showed increased expression of FGF-23 and DMP1 in the early stages of the disease, in addition to an inverse association between them and bone mineralization, while MEPE expression was inversely associated with bone volume. Graciolli et al. [8], however, analyzing a larger number of patients, also at different stages of CKD, compared the results of the histomorphometric analysis with biochemical markers of bone mineral metabolism disorders, markers of bone specific proteins, as well as their expression in bone tissue. The results showed that with the progression of the disease there is increased bone resorption, decreased bone formation rate and worsening of bone mineralization, besides the already known sequence of events on bone mineral metabolism disorders. Serum levels of FGF-23 correlated with its expression in bone tissue. Increased expression of SOST and parathormone receptor 1 occur in the early stages, whereas FGF-23 and phosphorylated B catenin occur in the later stages. This important study showed that osteocyte dysfunction is early in CKD and the understanding of it will contribute to clarify the pathophysiology, improve the diagnosis and probably the treatment of the bone complications of these patients.

Our results also showed interesting associations between the expression of proteins produced by osteocytes and bone turnover. When we compared the expression of DMP1 in patients with and without fractures, we detected lower expression in the cortical bone and also in the trabecular bone, being close to the significance in this compartment. This protein, among other functions, acts on mineralization by regulating the formation of type I collagen and hydroxyapatite crystals [24] suggesting that its decrease may have contributed to weakening the two compartments, facilitating fracture. We also observed increased expression of SOST in trabecular bone of fractured patients. This protein acts by inhibiting bone formation by inhibiting the Wnt pathway [25]; which is corroborated by the lower bone formation rate, lower bone volume and trabecular thickness observed in these patients.

Regarding the MEPE expression, our results showed a positive association with the osteoid matrix (volume and thickness), which would favor a delay in mineralization, which has already been demonstrated in a study performed in vivo and in vitro by Addison et al. [26].

These results contrast with the findings of Pereira et al. [23], where MEPE was inversely associated with structural parameters (bone volume and trabecular thickness). It is noteworthy that these authors studied children and did not show the isolated results of patients with high and low bone turnover. Our patients, besides being adults, all had high bone turnover, which can modify the expression of the proteins studied.

Our study presents several limitations among them being a retrospective study, therefore with limited information and cannot be extrapolated to the general dialysis population. Exclusion criteria allowed only a small number of biopsies to be analyzed and all of them with high turnover bone disease, which prevents these findings from being generalized to patients with fracture and low bone turnover. The lack of information about bone mineral density is also one of the limitations of the study. The patients data was obtained from a biopsy database between the years 2004 and 2015 in which there are no reports of bone densitometry. Recently, KDIGO [16] started to recommend densitometry in patients with stage V CKD. Future studies with densitometry and bone biopsies will be important to evaluate the role of these tests as both predictors of fracture and guidance in the treatment of patients with CKD.

However, our study is probably the first to describe the histomorphometric alterations of bone biopsies in patients with CKD and fracture, as well as to “enter” the bone tissue to analyze the proteins expressed by osteocytes and their associations with bone turnover.

The osteocytes functions begin to be better understood, and the proteins expressed by these cells constitute a large puzzle that begins to be deciphered.

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Compliance with ethical standards

Conflict of interest Rosa M. A. Moysés, Vanda Jorgetti and Aluizio B. Carvalho have received lecture fees and are consultant to Sanofi, Abbott do Brasil, and Amgen. All other authors have no conflict of interest.

References

- Clarke B (2008) Normal bone anatomy and physiology. *Clin J Am Soc Nephrol* 3:S131–S139
- Jamal SA (2010) Bone mass measurements in men and women with chronic kidney disease. *Curr Opin Nephrol Hypertens* 19:343–348
- Cooper C, Campion G, Melton LJ 3rd (1992) Hip fractures in the elderly: a world-wide projection. *Osteoporos Int* 2:285–289
- Burge R, Dawson-Hughes B, Solomon DH, Wong JB, King A, Tosteson A (2007) Incidence and economic burden of osteoporosis-related fractures in United States, 2005–2025. *J Bone Miner Res* 22:465–475
- Coco M, Rush H (2000) Increased incidence of hip fracture in dialysis patients with low serum parathyroid hormone. *Am J Kidney Dis* 36:1115–1121
- De Oliveira RA, Barreto FC, Mendes M, dos Reis LM, Castro JH, Britto ZM, Marques ID, Carvalho AB, Moysés RM, Jorgetti V (2015) Peritoneal dialysis per se is a risk factor for sclerostin-associated adynamic bone disease. *Kidney Int* 87:1039–1045
- Gomes SA, dos Reis LM, de Oliveira IB, Noronha IL, Jorgetti V, Heilberg IP (2008) Usefulness of a quick decalcification of bone sections embedded in methyl methacrylate: an improved method for immunohistochemistry. *J Bone Miner Metab* 26:110–113
- Gracioli FG, Neves KR, Barreto F, Barreto DV, dos Reis LM, Canziani ME, Sabbagh Y, Carvalho AB, Jorgetti V, Elias RM, Schiavi S, Moyses RMA (2017) The complexity of chronic disease-mineral and bone across stages of chronic kidney disease. *Kidney Int* 91:1436–1446
- Dempster DW, Compston JE, Drezner MK, Glorieux FH, Kanis JA, Malluche H, Meunier PJ, Ott SM, Recker RR, Parfitt AM (2013) Standardized nomenclature, symbols, and units for Bone Histomorphometry: a 2012 update of the report of the ASBMR Histomorphometry Nomenclature Committee. *J Bone Miner Res* 28:2–17
- Dos Reis LM, Batalha JR, Muñoz DR, Borelli A, Correa PH, Carvalho AB, Jorgetti V (2007) Brazilian normal static bone histomorphometry: effects of age, sex and race. *J Bone Miner Metab* 25:400–406
- Rehman MTA, Hoyland JA, Denton J, Freemont AJ (1994) Age related histomorphometric changes in bone in normal British men and women. *J Clin Pathol* 47:529–534
- Moe S, Drüeke T, Cunningham J, Goodman W, Martin K, Olgaard K, Ott S, Sprague S, Lameire N, Eknoyan G (2006) Definition, evaluation, and classification of renal osteodystrophy: a position statement from Kidney Disease: Improving Global Outcomes (KDIGO). *Kidney Int* 69:1945–1953
- Tentori F, McCullough K, Kilpatrick RD, Bradbury BD, Robinson BM, Kerr PG, Pisoni RL (2014) High rates of death and hospitalization follow bone fracture among hemodialysis patients. *Kidney Int* 85:166–173
- Miller PD (2016) Underdiagnoses and undertreatment of osteoporosis: the battle to be won. *J Clin Endocrinol Metab* 101:852–859
- Goldenstein PT, Elias RM, Pires de Freitas do Carmo L, Coelho FO, Magalhães LP, Antunes GL, Custódio MR, Montenegro FL, Titan SM, Jorgetti V, Moysés RM (2013) Parathyroidectomy improves survival in patients with severe hyperparathyroidism: a comparative study. *PLoS One* 8:e68870
- Kidney Disease: Improving Global Outcomes (KDIGO) CKD-MBD Update Work Group (2017) KDIGO 2017 clinical practice guideline update for the diagnosis, evaluation, prevention, and treatment of chronic kidney disease–mineral and bone disorder (CKD-MBD). *Kidney Int Suppl* 7:1–59
- Miller PD (2014) Chronic kidney disease and osteoporosis: evaluation and management. *Bonekey Rep* 3:542
- Chappard D, Bizot D, Mabileau G, Hubert L (2016) Aluminum and bone: review of new circumstances associated with Al³⁺ deposition in the calcified matrix of bone. *Morphologie* 100:95–105
- Zheng CM, Zheng JQ, Wu CC, Lu CL, Shyu JF, Yung-Ho H, Wu MY, Chiu JJ, Wang YH, Lin YF, Lu KC (2016) Bone loss in chronic kidney disease: quantity or Quality? *Bone* 6:57–70
- McNerny EMB, Nickolas TL (2017) Bone quality in chronic kidney disease: definitions and diagnosis. *Curr Osteoporos Rep* 15:207–213

21. Barger-Lux MJ, Recker RR (2002) Bone microstructure in osteoporosis: transilial biopsy and histomorphometry. *Top Magn Reson Imaging* 13:297–306
22. Hanudel MR, Froch L, Gales B, Jüppner H, Salusky IB (2017) Fractures and osteomalacia in a patient treated with frequent home hemodialysis. *Am J Kidney Dis* 70:445–448
23. Pereira RC, Juppner H, Azucena-Serrano CE, Yadin O, Salusky IB, Wesseling-Perry K (2009) Patterns of FGF-23, DMP1, and MEPE expression in patients with chronic kidney disease. *Bone* 45:1161–1168
24. Suzuki S, Haruyama N, Nishimura F, Kulkarni AB (2012) Dentin sialophosphoprotein and dentin matrix protein-1: two highly phosphorylated proteins in mineralized tissues. *Arch Oral Biol* 57:1165–1175
25. Winkler DG, Sutherland MK, Georghegan JC, Yu C, Hayes T, Skonier JE, Shpektor D, Jonas M, Kovacevich BR, Staehling-Hampton K, Appleby M, Brunkow ME, Latham JA (2003) Osteocyte control of bone formation via sclerostin, a novel BMP antagonist. *EMBO J*. 22:6267–6276
26. Addison WN, Masica DL, Gray JJ, McKee MD (2010) Phosphorylation-dependent inhibition of mineralization by osteopontin ASARM peptides is regulated by PHEX cleavage. *J Bone Miner Res* 25:695–705