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ORIGINAL RESEARCH

The Role of Bone Volume, FGF23 and Sclerostin in Calcifications and Mortality; a Cohort Study in CKD Stage 5 Patients

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Abstract

Chronic kidney disease-mineral and bone disorder has been associated with increasing morbid-mortality. The aim of this study was to determine the prevalence and phenotype of bone disease before transplantation and to correlate FGF23 and sclerostin levels with bone histomorphometry, and study possible associations between FGF23, sclerostin, and bone histomorphometry with cardiovascular disease and mortality. We performed a cross-sectional cohort study of a sample of 84 patients submitted to renal transplant, which were prospectively followed for 12 months. Demographic, clinical, and echocardiographic data were collected, laboratory evaluation, bone biopsy, and X-ray of the pelvis and hands were performed. Patient and graft survival were recorded. We diagnosed low bone turnover in 16 patients (19.5%); high bone turnover in 22 patients (26.8%); osteomalacia in 1 patient (1.2%), and mixed renal osteodystrophy in 3 patients (3.7%). At the end of 12 months, 5 patients had graft failure (5.9%), 4 had a cardiovascular event (4.8%), and 4 died. Age was associated with low remodeling disease, whereas high BALP and phosphorus and low sclerostin with high turnover disease. Sclerostin was a risk factor for isolated low bone volume. High BALP, low phosphorus, and low FGF23 were risk factors for abnormal mineralization. FGF23 appears as an independent factor for severity of vascular calcifications and for cardiovascular events, whereas the presence of valve calcifications was associated with low volume and with turnover deviations. Sclerostin was associated a higher HR for death. Sclerostin and FGF23 seemed to provide higher cardiovascular risk, as well as low bone volume, which associated with extra-osseous calcifications.

Keywords Bone disorders · FGF23 · Mineral metabolism · Sclerostin · Extra-osseous calcifications

Introduction

Chronic kidney disease (CKD) affects millions of people worldwide and has premature death due to cardiovascular (CV) disease as an established complication [1]. One of the major non-traditional risk factors for the extremely high rate mortality observed in CKD patients is the CKD-mineral and bone disorder (CKD-MBD) [2–4] syndrome, which includes a cross-talk between bone and vessels, as changes in biochemical parameters translate into different bone disorders, all of which are associated with extra-osseous calcifications [5].

Non-invasive markers for bone disease have been studied for decades. Studies demonstrated a poor correlation between parathyroid hormone (PTH)/bone alkaline phosphatase (BALP) serum levels and bone turnover, hamper the differential diagnosis of renal osteodystrophy (ROD) in the individual patient [6–9]. Various observations have shown that CKD patients have elevated fibroblast growth factor 23 (FGF23) and sclerostin serum levels [10, 11], making its potential role in ROD a point to be studied. Additionally, FGF23 proved to be a predictor of mortality and CV events in hemodialysis patients [12], although the impact of sclerostin levels are yet to be determined [13].

The aim of this study was (1) to determine the prevalence and phenotype of bone disease before transplantation; (2) to correlate FGF23 and sclerostin with bone turnover,

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mineralization or volume (TMV classification); (3) to correlate FGF23, sclerostin, TMV classification with imaging CV assessment, CV events, and mortality.

Methods

We first conducted a cross-sectional analysis in a cohort of patients that were participating in a prospective observational bone-assessment study of patients admitted for isolated renal transplantation, aged 18 to 66 years old, in our unit (*ClinicalTrials.gov* ID NCT02751099). Next, we followed prospectively those patients for a period of 12 months for mortality and CV events monitoring. The institutions' local ethics committees approved this study. Recruitment of patients (November 1st, 2015 and February 28th, 2018) was done on the day the patient was called for transplant. Written consent was obtained from all participants prior to entering the protocol. The exclusion criteria consisted of admission for double transplantation (liver–kidney or pancreas–kidney), intellectual disability, and age outer the selected range.

Data Collection

At inclusion, data were collected [age, gender, race, etiology of CKD, body mass index (BMI), presence of diabetes, hypertension, hepatitis virus, ovulation status for woman, active medication and doses, and history of parathyroidectomy]; the evaluation of the last echocardiography to access both valve calcifications and left ventricular mass index (LVMI), calculated using the Devereux formula indexed to body surface area, and scoring of vascular calcifications through X-ray of the pelvis and hands by Adragão score [14] was made. Left ventricular hypertrophy (LVH) was defined as a LVMI > 95 g/m^2 in women and > 115 g/m^2 in men. Patients recruited were followed for 12 months, and CV events (myocardial infarction, congestive heart failure, arrhythmia), fractures, rejection episodes, graft loss (and its cause), and death [classified as CV (fatal myocardial infarction, sudden death, and fatal congestive heart failure), infectious, malignancy or others] were recorded.

General laboratory analysis was performed, using standard methods. Intact PTH was measured by immuno-chemiluminescence using a second-generation assay (Immulite 2000; Siemens Medical Solutions Diagnostics, Los Angels, CA). Vitamin D [25(OH)D] was measured with radioimmunoassay provided by IDS (Boldon, UK). Additionally, blood samples were collected, centrifuged, and stored at -80ºC for further analysis of BALP, FGF23, alpha-Klotho, and sclerostin. BALP was measured using an enzyme immunoassay (EIA) utilizing a monoclonal anti-BALP antibody (MIcroVue BAP). FGF23 was measured using a second-generation enzyme-linked immunosorbent assay (ELISA) kit, which detected epitopes within the carboxyl-terminal (C-Term) portion of FGF23 (Immunotopics, San Clement, CA). Alpha-Klotho was determined using a human soluble α-klotho assay kit, consisting of a solid phase sandwich ELISA using 2 kinds of highly specific antibodies (IBL America, MN, USA). Sclerostin was measured using a high sensitivity EIA kit, which is a 96-well immune-capture ELISA (TECOmedical). All measurements were performed according to the manufacturer's instructions.

Before engraftment, a bone biopsy, obtained from the anterior iliac crest in a horizontal approach, was performed in the operating room under general anesthesia, using a 7G trocar (Osteobell T®) by manual puncture. Biopsy specimens of 4.5 mm in diameter by 1.0–1.5 cm in length were fixed in 70% alcohol followed by dehydration in 96% and 99.9% alcohol. The specimens were cleared with xylene and embedded in methyl methacrylate. Serial decalcified 5 µm sections were stained for static histomorphometric parameters evaluation with Masson–Goldner trichrome, Toluidine Blue, von Kossa, acid phosphatase, alkaline phosphatase, Perls, and solochrome. Cortical bone volume and thickness were measured, and cortical porosity higher than 10% was considered abnormal. Trabecular bone volume [normal if bone volume/tissue volume (BV/TV) ≥ 16%], remodeling (normal if osteoblast surface/bone surface (ObS/BS) 0.2%–3.5%, and osteoclast surface/bone surface (OcS/BS) 0.1%–7.5%]; and mineralisation (abnormal if osteoid thickness ≥ 12.5%) [15] were characterized. Bone histomorphometry was analyzed using a semiautomatic technique via Osteomeasure software (Osteometrics, Atlanta, GA). As patients were recruited in the day of the transplant, tetracycline labeling was not performed.

Statistical Analysis

In the cross-sectional evaluation, outcome variables were the bone histomorphometry (turnover, mineralization, volume) and cardiovascular abnormalities (Adragão score, presence of valve calcifications, and LVH). Predictors' variables were FGF23 and sclerostin. In the survival analysis, mortality and CV events were studied.

Continuous variables were expressed as median (interquartile range). Categorical variables were expressed as frequencies. The score of vascular calcification was divided into two groups of severity (1st group–scores 0 and 1; 2nd group–scores ≥ 2). Comparisons between groups were performed using a Fisher exact test or Mann–Whitney test, and associations between variables using linear regression analysis, Fisher exact test or Kruskal–Wallis rank test. Multivariate analysis (logistic regression analysis) was performed to study independent predictors of calcification severity or valve calcifications, and turnover, low volume, and abnormal mineralization. Cut-off values for the outcomes studied

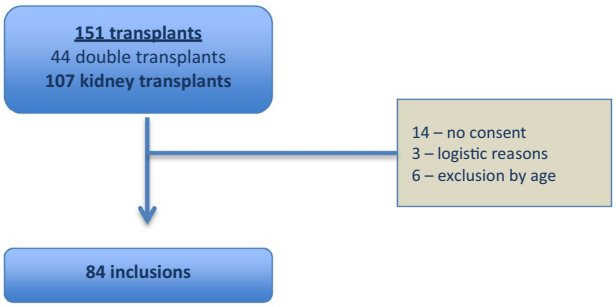

Fig. 1 Flow-chart of the study

were made based on ROC curves, using Youden's index. A survival analysis was performed using Cox proportional hazards model.

All tests were performed using STATA version 13 software package, and a $p < 0.05$ was considered significant.

Results

We included 84 patients in this study (Fig. 1). The demographic characterization of the population is shown in Table 1. This was a middle-aged, mainly male, and Caucasian patients, with median dialysis vintage was nearly 5 years. At the time of the transplant, most patients ($n = 75$, 89.3%) were prescribed bone-related pills. Cardiovascular events occurred in 25% of the population: ischemic event [coronary ($n = 7$), cerebrovascular ($n = 4$), intestinal ($n = 1$), retinal ($n = 1$)], rhythm changes [atrioventricular block ($n = 2$), atrial fibrillation ($n = 1$), other arrhythmias ($n = 2$)], pulmonary thromboembolism ($n = 1$), cardiorespiratory arrest during a surgery ($n = 1$), and bacterial endocarditis with consequent valve changes ($n = 1$).

Metabolic Evaluation

The laboratory results are shown in Table 2. It is worth noting that median values of FGF23 were almost 10 times above the normal range (< 180 RU/mL); sclerostin values were three times above normal, and alpha-klotho had lower than normal values. Although median vitamin D levels were below 30 ng/mL, the patients supplemented with the native hormone had a median value of 27.9 ng/mL (vs. 19.8 ng/mL, $p = 0.008$–for those not supplemented).

Imaging Exams Evaluation

The imaging exams results are present in Table 3. Median Adragão score was 1 (0–2), and the majority of our patients presented a low score (0–36 patients; 1–16 patients; 2–18 patients, and > 2–13 patients). Analyzing each score separately, we found that patients presenting scores of 0/1 had similar demographic characteristics (based on age, gender, dialysis vintage, BMI, and presence of diabetes), and for that reason we divided Adragão score in Group 1 (scores 0 and 1); and Group 2 (scores ≥ 2).

Table 1 Characterization of the population

Demographic characterization	
Age (Years)	53.5 (40.5–61.5)
Gender (M:F)	59 (70.2%):25 (29.8%)
Caucasian race (*n*, %)	65 (77.4%)
PD (previous or current):HD	11 (13.1%):80 (95.2%)
Dialysis vintage (months, IQ range)	55 (42.5–83.5)
Hypertension (*n*, %)	73 (86.9%)
Diabetes (*n*, %)	10 (11.9%)
Hyperparathyroidism (*n*, %)	60 (71.4%)
Parathyroidectomy (*n*, %)	7 (8.3%)
HIV, HBV, HCV (*n*, %)	3 (3.6%): 0: 3 (3.6%)
Etiology of renal disease (*n*, %)	
Unknown	16 (19%)
Hypertensive nephrosclerosis	15 (17.9%)
ADPKD	13 (15.5%)
Diabetic nephropathy (type 1 & 2)	7 (8.3%)
Alport disease	2 (2.4%)
Glomerulonephritis	
Chronic glomerulonephritis	7 (8.3%)
IgA nephropathy|Mensangial proliferation	6 (7.1%)|1 (1.2%)
HIVAN	2 (2.4%)
FSGS	1 (1.2%)
Membranous nephropathy	3 (3.6%)
Lupus nephritis	1 (1.2%)
Vasculitis	
Pauci-immune|Goodpasture	2 (2.4%)|1 (1.2%)
Lithiasis	4 (4.8%)
CAKUT	3 (3.6%)
Bone-related medication (*n*, %)	
Phosphate binders	30 (35.7%)
Cholecalciferol	26 (30.9%)
Vitamin D analogs/calcitriol	55 (65.5%)
Calcimimetics	24 (28.6%)

M:F male:female, *PD* peritoneal dialysis, *HD* hemodialysis, *IQ range* interquartile range, *HIV* human immunodeficiency virus, *HBV* hepatitis B virus, *HCV* hepatitis C virus, *ADPKD* autosomal polycystic kidney disease, *HIVAN* HIV-associated nephropathy, *FSGS* focal segmental glomerulosclerosis, *CAKUT* congenital anomalies of the kidney and urinary tract

Histologic Evaluation

From the 84 bone biopsies, we achieved 81 complete readings (Table 4), as two fragments did not show bone tissue

Table 2 Laboratory evaluation at baseline (median and IQ range)

General laboratory evaluation at baseline	
Hemoglobin (g/dL)	11.6 (10.8–12.6)
Platelets (× 1000/ μL)	210 (181.5–258.5)
Iron (μg/dL)	63.5 (46.0–90.0)
Transferrin saturation (%)	27.1 (18.9–36.4)
Ferritin (ng/mL)	389.5 (259.6–557.4)
Glucose (mg/dL)	88.0 (79.0–101.0)
Urea (mg/dL)	101.0 (65.5–133.5)
Creatinine (mg/dL)	8.1 (5.7–10.7)
Uric acid (mg/dL)	5.1 (3.5–6.8)
Sodium (mEq/L)	139 (137–141)
Potassium (mEq/L)	5.0 (4.5–5.6)
Chloride (mEq/L)	101 (99–103)
Alkaline phosphatase (U/L)	83.0 (62.0–109.5)
Albumin (g/dL)	4.2 (4.0–4.5)
Total cholesterol (mg/dl)	187.5 (154.0–218.5)
Calcium (mg/dL)	9.3 (8.7–9.6)
Phosphorus (mg/dL)	4.1 (3.3–5.1)
Magnesium (mg/dL)	2.3 (2.0–2.5)
Calcitonin (ng/dL)	3.4 (2.0–10.7)
Vitamin D (ng/mL)	21.0 (15.7–34.1)
PTH (pg/mL)	458.1 (237.3–742.4)
Bone alkaline phosphatase (U/L)	33.7 (26.4–46.5)
FGF23 (RU/mL)	1716.4 (599.4–6218.3)
Klotho (pg/mL)	555.3 (367.3–853)
Sclerostin (ng/mL)	1.9 (1.2–2.8)

Normal range for PTH 14.8 to 83.1 pg/mL; vitamin D 4.8 to 52.8 ng/mL; FGF23 ≤ 180 RU/mL; klotho normal values 845 ± 330 pg/mL; BALP is dependent on sex and age: the normal range in premenopausal women is 11.6 to 29.6 U/L, in postmenopausal women 14.2 to 42.7 U/L; in men 15–41.3 U/L; sclerostin is dependent on sex and age, the normal values in premenopausal women is 0.45 ± 0.15 ng/mL, in postmenopausal women 0.51 ± 0.14 ng/mL; in men 0.59 ± 0.13 ng/mL

Table 3 Evaluation of echocardiographic findings & vascular calcifications

Imaging exams	
Echocardiographic findings	
Left ventricular mass index (g/m^2)	108.5 (92.0–129.0)
Interventricular septal thickness (mm)	11.0 (10.0–12.0)
Left ventricular hypertrophy (*n*, %)	32 (39.5%)
Valve calcifications (*n*, %)	19 (23.5%)
LV Fractional shortening (%)	39.0 (34.6–43.0)
Vascular calcification score (Adragão score)	
Hands score	0 (0–2)
Pelvis score	0 (0–1)
Total score	1 (0–2)

Table 4 Histomorphometric results of both cortical and trabecular bone (median and IQ range)

Histomorphometric bone parameters	
Cortical bone	
Porosity (%)	7.8 (5.15–11.9)
> 10% (*n*, %)	31 (36.9%)
Thickness (μm)	737.9 (511.4–949.1)
Trabecular bone	
Bone volume/tissue volume (%)	19.1 (15.5–24.5)
Osteoid surface/bone volume (%)	3.25 (1.8–5.0)
Osteoid thickness	7.9 (6.7–10.3)
Osteoblast surface/bone surface (%)	2.2 (0.8–5.25)
Osteoclast surface/bone surface (%)	1.2 (0.3–2.4)

and one fragment only had cortical bone. ROD was present in 52 (64.2%) patients.

Cortical thickness was not associated with any parameter of trabecular bone. Patients presenting with extreme PTH levels (< 150 pg/mL or > 800 pg/mL) had higher cortical porosity (9% vs. 6.8%, $p = 0.02$) and higher cortical thickness (869.3 μm vs. 698.5 μm, $p = 0.04$).

Most patients had normal remodeling parameters ($n = 43$). Nearly 20% of the population presented low bone turnover ($n = 16$): normal volume in seven patients; low volume (adynamic bone disease) in eight patients; osteomalacia in one. High bone turnover was presented in 22 patients, in the form of hyperparathyroid bone disease in 19 patients (twelve with normal volume and seven with low volume; and as mixed renal osteodystrophy in three patients.

Twenty-five patients had low volume: nine with low turnover; seven with high remodeling disease and an additional nine patients (23.7%) had isolated low bone volume.

Mineralization was abnormal in nine patients: osteomalacia ($n = 1$), mixed renal osteodystrophy ($n = 3$), abnormal mineralization with normal bone turnover, and normal volume ($n = 4$), or low volume ($n = 1$).

Associations Between Bone-Related Markers and Bone Histomorphometry

We aimed to study associations between FGF23 and sclerostin and the bone TMV (turnover–mineralization–volume) classification.

Comparing the three different remodeling categories, high bone turnover was associated with high levels of PTH ($p < 0.001$); high levels of BALP ($p = 0.002$); younger age ($p = 0.002$), and non-Caucasian race ($p = 0.03$). On the other hand, low bone turnover was associated with high levels of sclerostin ($p = 0.002$), low levels of PTH ($p < 0.001$); older age ($p = 0.002$), and diabetes ($p = 0.02$). Shorter dialysis vintage was associated with normal turnover ($p = 0.009$), as

Table 5 Predictors of different bone turnover diseases

	Low turnover ($n=16$)	Normal turnover ($n=43$)	High turnover ($n=22$)	p value
ObS/BS	0.1 (0.0–0.4)	1.9 (0.9–3.5)	7.8 (5.5–12.6)	–
OcS/BS	0.1 (0.0–0.7)	1.1 (0.3–2.0)	3.0 (2.5–4.1)	–
Age (years)	62.5 (50.5–64.0)	54.0 (40.0–60.0)	44.0 (34.0–54.0)	0.003
Male gender	68.7%	67.4%	72.7%	0.948
Caucasian race	87.5%	83.7%	54.5%	0.009
Dialysis vintage (M)	75.0 (43.5–76.5)	49.0 (30.0–64.0)	75.5 (53.0–113.0)	0.009
PD	6.3%	16.3%	13.6%	0.695
Diabetes	31.2%	9.3%	4.5%	0.033
BMI (kg/m^2)	26.1 (23.0–27.7)	24.7 (23.3–28.0)	24.0 (21.5–26.7)	0.225
PTH (pg/mL)	202.8 (78.0–561.0)	411.4 (279.0–671.0)	761.3 (461.5–1026.0)	<0.001
BALP (U/L)	27.7 (22.3–35.8)	31.3 (23.8–43.7)	41.7 (35.4–80.6)	0.002
Sclerostin (ng/mL)	2.2 (1.3–2.9)	1.3 (1.2–1.9)	1.1 (1.1–3.6)	0.002
Klotho (pg/mL)	613.8 (398.0–810.0)	508.0 (334.0–785.0)	784.9 (506.0–1423.0)	0.127
FGF23 (RU/mL)	1075 (528.6–3620)	1887.3 (606–6962)	1610 (613.7–7215)	0.558
Vitamin D (ng/mL)	22.7 (15.6–34.7)	22.2 (18.0–34.1)	17.2 (11.4–29.9)	0.352
Calcium (mg/dL)	9.1 (8.3–10.2)	9.3 (8.7–9.6)	9.3 (9.0–9.6)	0.996
Phosphate (mg/dl)	3.6 (3.0–4.7)	4.1 (3.3–5.0)	4.9 (3.8–5.7)	0.075
Magnesium (mg/dL)	2.1 (2.0–2.3)	2.3 (2.1–2.5)	2.3 (2.1–2.7)	0.202

Statistical analysis performed with Kruskal–Wallis test

ObS/BS osteoblast surface/bone surface, *OcS/BS* osteoclast surface/bone surface, *M* months, *PD* peritoneal dialysis, *BMI* body mass index, *PTH* parathyroid hormone, *BALP* bone alkaline phosphatase, *FGF23* fibroblast growth factor 23

well higher sclerostin levels ($p=0.007$). We did not found differences regarding FGF23 or klotho levels, as presented in Table 5. Concerning medication, the non-use of sevelamer was associated with normal turnover ($p=0.018$).

Comparing low turnover with no-low turnover bone disease in multivariate analysis, just age (OR = 1.1, $p=0.02$) was shown to be an independent risk factor for the disease (model: $p=0.001$, ROC curve 0.82).

Comparing high turnover with no-high turnover bone disease in multivariate analysis, predictors of high bone turnover were longer dialysis vintage ($p=0.026$), black race ($p=0.034$), higher BALP levels ($p=0.038$), higher phosphorus levels ($p=0.002$), and lower sclerostin levels ($p=0.006$), adjusting for age (model: $p<0.001$, ROC curve 0.93), as shown in Supplements Table S1.

Isolated low bone volume was associated with higher levels of sclerostin (2.8 vs. 1.9 ng/mL, $p=0.033$), with no differences in FGF23 (or other demographic data or laboratory data).

Patients with abnormal mineralization had lower phosphorus serum levels [2.8 (1.9–3.2) mg/dL vs. 4.4 (3.5–5.2) mg/dL, $p<0.001$], lower FGF23 levels [436.7 (322.0–555.4) RU/mL vs. 2236.3 (810.5–7088.8) RU/mL $p<0.001$], and higher BALP levels [88.5 (52.5–92.8) U/L vs. 31.7 (25.2–38.9) U/L, $p<0.001$], with no other defining characteristics. Using a multivariate model, adjusted for age and BMI, higher BALP levels, and lower phosphate levels had higher odds for abnormal mineralization comparing to other patients ($p<0.001$; ROC curve 0.91), as we can see in Supplements Table S2.

Associations Between FGF23, Sclerostin, Bone TMV Classification, and Extra-Osseous Calcifications

Vascular Calcifications

Sclerostin and FGF23 related with the severity of Adragão score in univariate analysis ($p<0.05$), in opposition to the other mineral-related parameters (PTH, BALP, Klotho, vitamin D, calcium, phosphorus, magnesium) and bone histomorphometric parameters (Table 6)**.** We noticed that valve calcifications were present mostly in the group of patients with most severe vascular calcifications ($p>0.05$). Medication did not have an impact on vascular calcification score, although there was a trend for calcium-based binders to be associated with the score severity.

In a multivariate model (Supplements Table S3), FGF23 ($p=0.005$) appears as an independent factor for severity of vascular calcifications (namely values above 436.7 RU/mL), as age ($p=0.009$), BMI ($p=0.020$), and presence of diabetes ($p=0.011$) (model: $p<0.001$; ROC curve 0.84).

Table 6 Severity of vascular calcifications and associated factors

	Mild score of vascular calcifications ($n=52$)	Severe score of vascular calcifications ($n=31$)	p value
Adragão score	0.31 ± 0.5	2.9 ± 1.5	–
Valve calcifications	18%	32%	0.143
Male gender	67.3%	74.2%	0.342
Age (years)	47.5 (35.5–58.5)	60 (50–63)	0.001
Caucasian race (%)	69.2	90.3	0.069
BMI (kg/m^2)	24.2 ± 3.1	26.7 ± 3.5	0.003
Diabetes (%)	3.8	25.8	0.005
Hypertension (%)	84.6	90.3	0.350
Dialysis vintage (months)	55 (34.5–81.5)	57 (44–89)	0.127
HD with/out PD (%)	92.3	100	0.147
PD (%)	17.3	6.4	0.140
Parathyroidectomy (%)	5.8	12.9	0.232
Sclerostin (ng/mL)	1.89 ± 0.9	2.45 ± 0.9	0.006
FGF23 (RU/mL)	999.5 (464–5020)	3295.6 (1497–11,737)	0.009
Klotho (pg/mL)	646.8 (407.5–979.0)	494.7 (232.5–834.0)	0.161
PTH (pg/mL)	468.2 (304.2–742.3)	454.8 (209.4–822.6)	0.728
BALP (U/L)	35.8 (26.2–48.4)	33.5 (26.2–44.3)	0.504
Vitamin D (ng/mL)	21.1 (14.1–33.1)	21 (16.3–38.7)	0.575
Magnesium (mg/dL)	2.3 (2.0–2.5)	2.3 (2.1–2.6)	0.713
Calcium (mg/dL)	9.3 (8.8–9.6)	9.4 (8.7–10.2)	0.313
Phosphate (mg/dL)	4.1 ± 1.4	4.7 ± 1.5	0.086
Cortical porosity (%)	8.4 (6.2–12.1)	7.2 (4.3–10.6)	0.285
BV/TV (%)	20.2 (15.7–24.7)	17.6 (14.3–23.2)	0.312
Mineralized volume /TV (%)	19.1 (14.5–24.1)	17.2 (13.6–22.2)	0.363
OcS/BS (%)	1.2 (0.4–2.4)	1.1 (0.1–2.8)	0.732
ObS/BS (%)	1.9 (0.8–5.5)	2.9 (0.8–5.2)	0.793
Calcium-based binders (%)	3.8	16.1	0.064

Statistical analysis performed with Mann–Whitney test

BMI body mass index, *HD* hemodialysis, *PD* peritoneal dialysis, *FGF23* fibroblast growth factor 23, *BV/TV* Bone volume/Tissue volume, *OcS/BS* osteoclast surface/bone surface, *ObS/BS* osteoblast surface/bone surface

Valve Calcifications

The presence of valve calcifications was greater in patients with lower bone volume ($p=0.006$), lower mineralized bone volume ($p=0.013$), and adynamic bone disease ($p=0.001$); on the other hand, normal bone turnover was associated with absence of valve calcifications ($p=0.020$) (Table 7). In multivariate analysis, adjusting for age and diabetes, the only independent variable associated with valve calcifications was adynamic bone disease (OR = 7.3, $p=0.036$).

Follow-Up Assessment in CV Events and Mortality

In the first three months, five patients presented with primary non-function of the kidney graft, and were excluded from the study, and two additional patients died (both of infectious causes). After six and seven months of follow-up, two further patients died (cardiovascular event and infection). During this time-period, three patients presented four cardiovascular events (acute myocardial infarction, congestive heart failure, and arrhythmia).

In a survival analysis, patients that died had significantly higher levels of sclerostin (HR = 3.24, $p=0.041$). We did not find any other predictor. This association maintained (HR 4.5, $p=0.038$) when adjusting for age, gender, and hypertension. We found no predictor of cardiovascular event in a survival analysis.

Discussion

In this article we found that, in a population of patients listed for kidney transplantation, ROD was present in more than half of the patients, if we add remodeling abnormalities (38 patients) to mineralization defects (5 additional patients). Low turnover was associated with low PTH levels only in

Table 7 Risk factors for valve calcifications

	Absence of valve calcification ($n=64$)	Presence of valve calcification ($n=19$)	p value
Adragão score	1 (0–2)	2 (0–3)	0.109
Age (years)	49.5 (39–60)	62 (53–64)	0.012
Male gender (%)	80.0	68.4	0.521
Caucasian race (%)	79.0	73.7	0.644
BMI (kg/m^2)	24.3 (22.7–27.7)	26.7 (21.7–27.8)	0.635
Diabetes (%)	6.4	31.6	0.009
Hypertension (%)	85.5	94.7	0.262
Dialysis vintage (months)	55 (39–82)	53 (43–84)	0.468
HD with/out PD (%)	93.5	100.0	0.335
PD (%)	14.5	10.5	0.497
Parathyroidectomy (%)	9.7	5.3	0.477
Cortical porosity (%)	7.8 (5.6–12.4)	7.7 (3.8–9.8)	0.353
BV/TV (%)	20 (15.8–25.1)	15.7 (11.9–20.2)	0.006
Mineralized volume /TV (%)	19.4 (15–24.7%)	15.3 (11.7–19.5)%	0.013
Normal turnover (%)	87.5%	12.5%	0.020
Adynamic bone disease (%)	25%	75%	0.001
OcS/BS (%)	1.1 (0.3–2.2)	1.8 (0.1–3.7)	0.312
ObS/BS (%)	2.6 (0.8–5.2)	2.2 (0.3–8)	0.972
Sclerostin (ng/mL)	1.9 (1.3–2.9)	2.1 (1.2–2.8)	0.894
FGF23 (RU/mL)	1808.7 (613.7–6962.0)	1574.2 (421.2–3854.8)	0.355
Klotho (pg/mL)	620.1 (363.5–862.0)	479.7 (351.0–846.0)	0.327
PTH (pg/mL)	422.3 (232.3–671.3)	561.8 (279.3–912.7)	0.270
BALP (U/L)	33.5 (26.2–46.2)	35.4 (27.3–61.5)	0.497
Vitamin D (ng/mL)	22.9 (15.5–34.1)	19.8 (15.8–29.9)	0.455
Magnesium (mg/dL)	2.3 (2.1–2.5)	2.3 (2.0–2.5)	0.746
Calcium (mg/dL)	9.3 (8.9–10.0)	9.3 (8.3–9.6)	0.197
Phosphate (mg/dL)	4.1 (3.3–5.0)	4.7 (2.9–5.8)	0.636

Statistical analysis performed with Mann–Whitney test

BV/TV bone volume/tissue volume, *TV* Tissue Volume, *OcS/BS* osteoclast surface/bone surface, *ObS/BS* osteoblast surface/bone surface, *FGF23* fibroblast growth factor 23, *PTH* parathyroid hormone, *BALP* bone alkaline phosphatase

univariate analysis, and in multivariate analysis age was the main factor for the deviation, whereas high turnover was associated with high BALP and phosphorus and low sclerostin levels, and, as demonstrated before, BALP was a better tool than PTH to differentiate high from low or normal bone turnover [9]. Isolated low volume was associated with high sclerostin levels, and abnormal mineralization with low phosphorus and FGF23 levels and high BALP levels. Adragão score was low in this population, and did not correlate with the bone biopsy data, but instead with FGF23 levels, as did age, BMI, and diabetes. The presence of valve calcifications was associated with low bone volume, a lower mineralized bone volume, with adynamic bone disease, and with absence of normal turnover. In multivariate analysis, only adynamic bone disease achieved significance. In our population, higher levels of sclerostin led to a higher HR of death.

We acknowledge that this is a small study, and that patients were prescribed with different bone-related pills that can confound our results. As an observational study, associations do not mean a cause-effect relationship. Because patients were recruited in the day of the transplant, none of these bone biopsies beneficiated from tetracycline labeling, and this could limit remodeling characterization. Also, as this was a population listed for kidney transplantation, meaning that these are the healthiest patients among dialysis patients, the results cannot be generalized for all those patients.

One of the aims of the study was to assess the importance of FGF23 and sclerostin as potential biomarkers for bone turnover, delayed mineralization or low volume. In the comparison of the three types of turnover (low–normal–high), we found that a high level of sclerostin was associated with normal and low turnover, similar to other studies in predialysis patients [16]. Even so, studying low versus no-low

bone turnover, the role of sclerostin was not highlighted, in contrast with the comparisons between high versus no-high bone turnover, in which lower sclerostin levels were associated with high bone turnover. One of our explanations is that the hormone influences the extremes of bone remodeling, but adding the normal bone remodeling will mask the results. Sclerostin, a glycoprotein product of the *SOST* gene in mature osteocytes [17] and a negative regulator of bone metabolism [13], down-regulates the osteoblasts function in a paracrine fashion. It should be remembered that it was demonstrated that sclerostin absolute and fractional excretion is intensified in CKD [18], thus, the production of sclerostin is increased in CKD patients. The sources are not only bone cells (specially osteocytes, but also osteoclast precursors) [10], as we learn that sclerostin mRNA in bone is not increased in CKD patients [19], but additionally vascular cells [10], since increased sclerostin expression has been demonstrated during vascular smooth muscle cell calcification in an animal model [20]. Indeed, sclerostin serves as an inhibitor of bone formation [2, 13, 17] and was the only parameter to predict isolated low bone volume in our population. Some studies hypothesize that sclerostin may negatively influence mineralization via regulation of FGF23 [13] and alteration of vitamin D synthesis in proximal tubular cells [21]. We did not observe that. It is worth emphasizing that our patients with delayed mineralization had mostly mixed uremic osteodystrophy and not osteomalacia, and so higher levels of BALP would be expected.

In order to perform the majority of its functions, FGF23 needs to bind to its receptors, and needs a co-receptor, the klotho protein [22]. None of those factors were associated with LVMI or LVH, contrary to the findings of studies with a larger population [23]. Perhaps the fact that we only had 84 patients enrolled influenced the lack of association. Nevertheless, we identified FGF23 as an independent risk factor for severity of vascular calcification score in this population, which presented, overall, a low Adragão score. A study by Scialla and colleagues [24], which included 3939 patients with mild to severe CKD (GFR of 70–20 ml/min/1.73 m^2) from the Chronic Renal Insufficiency Cohort (CRIC) Study, did not find an association between FGF23 levels and coronary artery calcification. We should acknowledge that our patients are different from those analyzed in the reported study, as all our patients had CKD stage 5, and the median levels of FGF23 were nearly 13 times higher than the levels of the CRIC study patients. Furthermore, our patients with cardiovascular events in a 12-month period had higher levels of FGF23 at baseline, meaning that this hormone is a powerful marker in predicting cardiovascular risk, a finding which is aligned with other reports [12]. As expected, age, diabetes, and dialysis vintage were important factors for calcifications. Curious was the fact that phosphorus was not a risk factor for calcification, probably because the nephrology community is focused on controlling this risk factor.

Although it has been demonstrated that in a population of prevalent hemodialysis patients, Adragão score if ≥ 3 was related to worst outcome [14], we chose the cut-off value for severity when ≥ 2 in this population, as we saw no difference between patients with score 2 or ≥ 3, but we saw a difference between scores 0 /1 and score 2. This is explained by the demographic characteristics of these patients, which are younger and fitter than the usual prevalent hemodialysis older patients.

Valve calcifications were not associated with bone-related minerals or hormones, but with bone histomorphometry, namely volume and turnover. In our multivariate model, low bone turnover, if associated with low volume (adynamic bone disease), was associated with higher odds for the disorder. Other studies also highlighted the importance of mineral and bone metabolism for valve calcifications [25], and the importance of low bone volume to coronary calcifications [26]. In a recent Portuguese study, authors found that higher mineralized bone volume was associated to a lower plain X-ray vascular calcification [27]. We had a similar finding in univariate analysis, concerning valve calcifications.

Although sclerostin was not a strong predictor of severity of vascular calcifications, this hormone was associated with a higher HR for death. This remains to be confirmed, as there were few events to make the association meaningful. Some studies have demonstrated that high levels are associated with better survival in hemodialysis patients [28, 29], suggesting a protective role through inhibition of vascular calcifications [28, 30], while other studies have found an association between high levels and CV mortality in dialysis patients [31], and association with the degree of vascular calcifications [32]. A phase III randomized study to evaluate efficacy and safety of romosozumab in men with osteoporosis, revealed a difference in cardiovascular serious adverse events, with more cases in the treatment group [33], indicating the protective role of the molecule.

Further studies with more patients or data aggregation are needed to confirm these observations. It is the authors' opinion that the creation of a multinational bone biopsy registry could help find more specific biochemical markers of the disease, beyond PTH. It would be interesting to have both BALP and PTH in our clinical daily practice, as PTH seems to offer minimal additional discrimination value to BALP [9].

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Author Contributions ACF and AF concept and design the study; ACF, PC, IA, MM, DN, CS, FC, RS, BC, and GC collected data or perform bone biopsies or performed laboratory evaluations; ACF, MM, RS, BC, GC, FN, AF analyzed the data; ACF drafted the article; PC, IA, MM, DN, CS, FC, RS, BC, GC, FN, AF revised the paper; all authors approved the final version of the paper and agree with all aspects of the work.

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Data Availability The data that support the findings of this study are available upon reasonable request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

Declarations

Conflict of interest Besides receiving a grant from the Portuguese Society of Nephrology and the Portuguese Society of Transplantation/Astellas, we do not have any conflict of interest relating with this manuscript. The results presented in this paper have not been published previously in whole or part, except in abstract format.

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