BONE-DERIVED HORMONES, BONE DISORDERS, AND CARDIOVASCULAR DISEASE IN KIDNEY TRANSPLANTED PATIENTS

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A thesis submitted in partial fulfillment of the requirements for the Doctorate Degree in Medicine, in the specialty of Clinical Investigation

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THE IMPORTANT THING IS NOT TO STOP QUESTIONIG.
CURIOSITY HAS ITS OWN REASON FOR EXISTING.
Albert Einstein

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GLOSSARY

ARIC: Atherosclerosis Risk in Communities
BALP: Bone alkaline phosphatase
BFR: Bone formation rate
BMD: Bone mineral density
BMI: Body mass index
BV/TV: Bone volume / tissue volume
CaSR: Calcium-sensing receptor
CHULC: Centro Hospitalar e Universitário de Lisboa Central
CKD: Chronic kidney disease
CKD-MBD: Chronic kidney disease–Mineral and bone disorder
CRIC: Chronic Renal Insufficiency Cohort
CT: computed tomography
CV: Cardiovascular
DOPPS: Dialysis Outcomes and Practice Patterns Study
DXA: Dual-energy X-ray absorptiometry
eGFR: estimated glomerular filtration rate
EDTA: ethylenediaminetetraacetic acid
EIA: Enzyme immunoassay
ELISA: Enzyme-linked immunosorbent assay
ESRD: End-stage renal disease
FGF23: Fibroblast growth factor 23
FRAX: Fracture Risk Assessment Tool
GFR: Glomerular filtration rate
HR: Hazard ratio
HR-pQCT: High-resolution peripheral computed tomography
KDIGO: Kidney Disease: Improving Global Outcomes
LRP: Low-density lipoprotein receptor-related protein
LVH: Left ventricular hypertrophy
LVMI: Left ventricular mass index
MAR: Mineral apposition rate
MBV/TV: Mineralized bone volume / tissue volume
MESA: Multi-Ethnic Study of Atherosclerosis
MLT: Mineralization lag time

MS/BS: Mineralizing surface / bone surface

NHANHES III: Third National Health and Nutrition Examination Survey

NPV: Negative predictive value

ObS/BS: Osteoblast surface / bone surface

OcS/BS: Osteoclast surface / bone surface

OR: Odds ratio

OtV/BV: Osteoid volume / bone volume

OtS/BS: Osteoid surface / bone surface

PINP: Procollagen type 1 N- terminal propeptide

PT-MBD: Post-transplant mineral and bone disorder

PTH: Parathyroid hormone

RANK-L: Receptor activator of NF-kB ligand

ROD: Renal Osteodystrophy

T0: Time 0 – baseline

T1: Time 1 – 1-year after baseline

TBS: Trabecular bone score

TNF: Tumor necrosis factor

TMV: Turnover-mineralization-volume

TRAPS5b: Tartrate-resistant acid phosphatase 5b

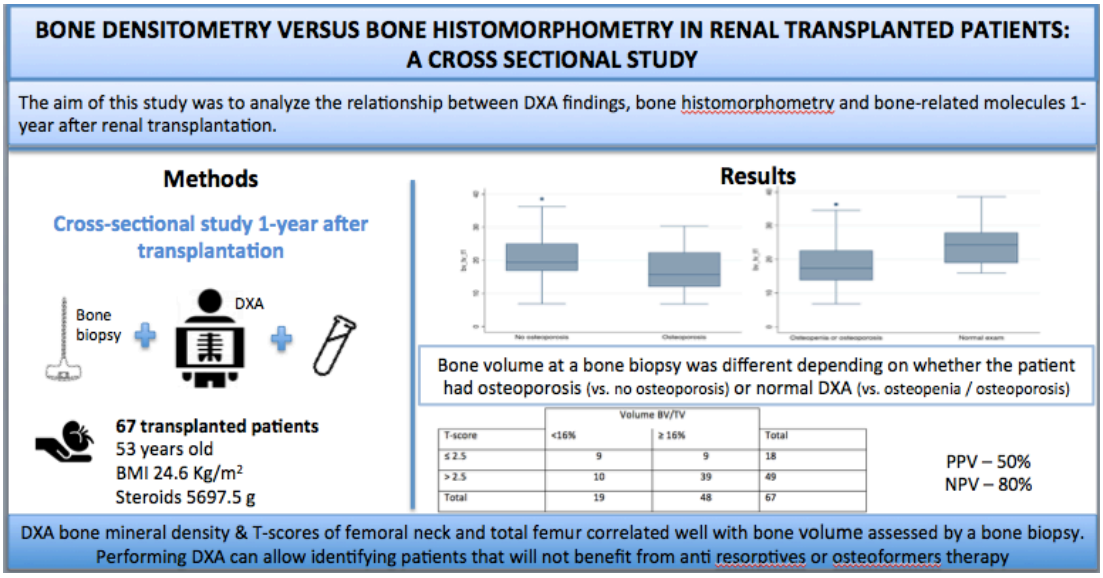
VDR: Vitamin D receptors

WHO: World Health Organization

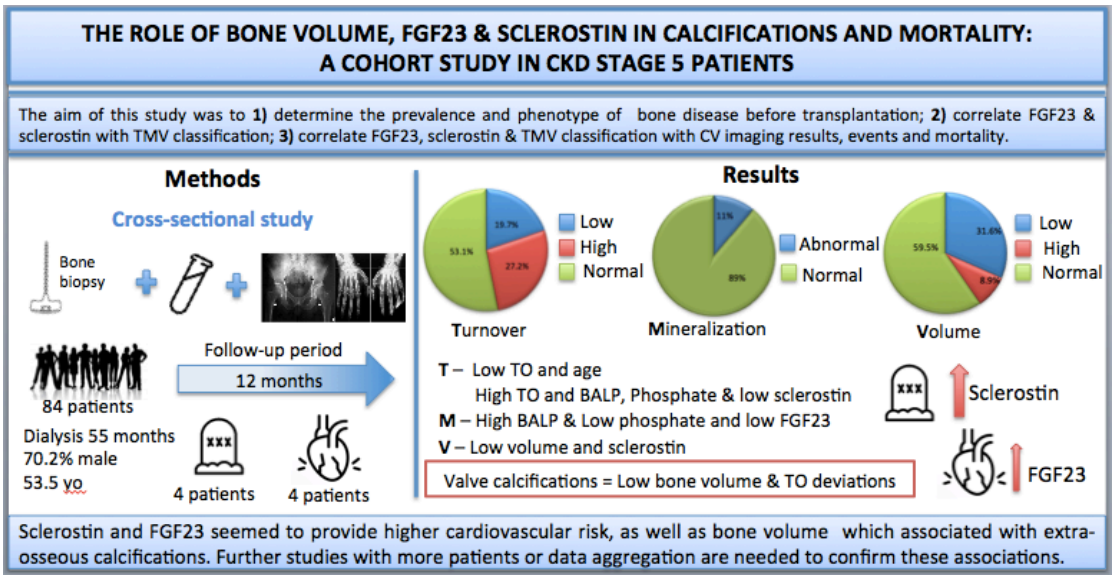
RELATED PUBLISHED ARTICLES

Published original articles based on the results obtained by our investigation

1. **Ferreira AC**, Mendes M, Silva C, Cotovio P, Aires I, Navarro D, Caeiro F, Salvador R, Correia B, Cabral G, Nolasco F, Ferreira A. Bone densitometry versus bone histomorphometry in renal transplanted patients: a cross-sectional study. *Transpl Int* 2021;34:1065-1073 doi: 10.1111/ tri.13888

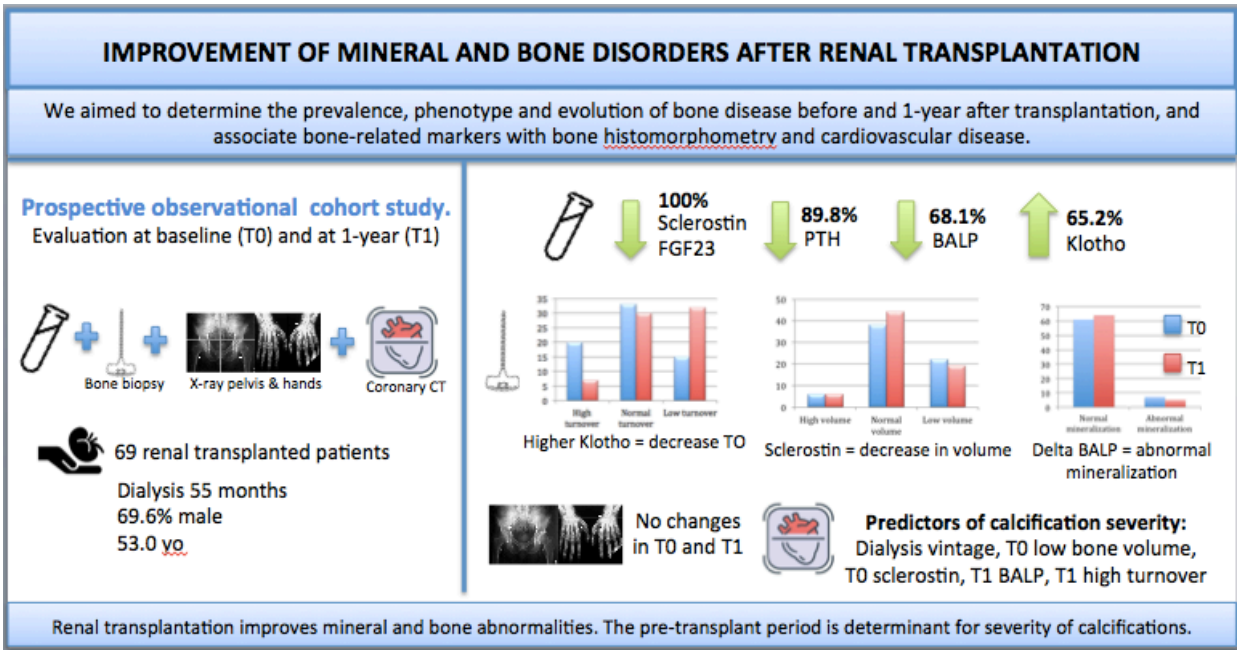


2. **Ferreira AC**, Cotovio P, Aires I, Mendes M, Navarro D, Silva C, Caeiro F, Salvador R, Correia B, Cabral G, Nolasco F, Ferreira A. The role of bone volume, FGF23 and sclerostin in calcifications and mortality: a cohort study in CKD stage 5 patients. *Calcif Tissue Int* 2022;110:215-224
doi: 10.1007/s00223-021-00910-8



3. **Ferreira AC**, Ferreira A. The importance of the first year of kidney transplantation in the presence of left ventricular hypertrophy. *Port J Nephrol Hypert* 2021;35:270-271 doi.org/10.32932/pjnh.2021.12.165

4. **Ferreira AC**, Mendes M, Silva C, Cotovio P, Aires I, Navarro D, Caeiro F, Ramos R, Salvador R, Correia B, Cabral G, Nolasco F, Ferreira A. Improvement of mineral and bone disorders after renal transplantation Transplantation. 2022; online ahead of print doi: 10.1097/TP.4099



Other published articles whose contents have been used in the elaboration of the thesis

5. **Ferreira AC,** Cohen-Solal M, D'Haese P, Ferreira A. The role of bone biopsy in the management of CKD-MBD. *Calcif Tissue Int* 2021;108:528-538 doi: 10.1007/s00223-021-00838-z
6. Torregrosa JV, **Ferreira AC**, Cucchiari D, Ferreira A. Bone Mineral Disease after Kidney Transplantation. *Calcif Tissue Int* 2021;108:551-560 doi: 10.1007/s00223-021-00837-0
7. **Ferreira AC,** Cohen-Solal M, D'Haese P, Ferreira A. The role of bone biopsy in the management of CKD-MBD: CKD-related osteoporosis or CKD-MBD / Osteoporosis? *Calcif Tissue Int* 2021;109:112 doi: 10.1007/s00223-021-00854-z
8. **Ferreira AC**, Ferreira A. Mineral and bone disease in renal transplantation. *Port J Nephrol Hypert* 2021;35:93-95 doi.org/10.32932/ pjnh.2021.07.131

9. Matias PJ; Laranjinha I; Azevedo A; Raimundo A; Navarro D; Jorge C; Aires I; Mendes M; **Ferreira AC;** Amaral T; Gil C; Ferreira A. Bone fracture risk factors in prevalent hemodialysis patients. *J Bone Miner Metab* 2020;38:205-212 doi: 10.1007/s00774-019-01041-9
10. Matias P, Jorge C, **Ferreira AC**, Borges M, Aires I, Amaral T, Gil C, Cortez J, Ferreira A. Cholecalciferol supplementation in hemodialysis patients: effects on mineral metabolism, inflammation, and cardiac dimension parameters. *Clin J Am Soc Nephrol* 2010;5:905-911 doi: 10.2215/CJN.06510909
11. Matias P, **Ferreira AC**, Jorge C, Borges M, Aires I, Amaral T, Gil C, Cortez J, Ferreira A. 25 hydroxyvitamin D3, arterial calcifications and cardiovascular risk markers in haemodialysis patients. *Nephrol Dial Transplant* 2009;24:611-61 doi: 10.1093/ndt/gfn502
12. **Ferreira AC**, Matias P, Jorge C, Borges M, Aires I, Amaral T, Gil C, Cortez J, Ferreira A. Vitamin D, inflammation and malnutrition in prevalent haemodialysis patients – is there a link? *Port J Nephrol Hypert*, 2008;22: 305-312

ETHICS AND INFORMED CONSENT

The institutions' local ethics committees (from Nova Medical School and from Centro Hospitalar Universitário de Lisboa Central) approved this study and the National Committee for Data Protection approved data collection and treatment.

The physician who called the patient for transplantation was responsible for explaining the research project and asking for informed consent. The study characteristics were explained to the potential participants and the patient information sheet was given to those who expressed an interest in the study. All patients could clarify doubts and all patients had at least 2 hours to decide whether to participate. Written consent was obtained from all participants prior to entering the protocol. Consent to take part in the study was recorded in each patient's notes and in the study records.

INTRODUCTION

Chronic kidney disease – mineral and bone disorder syndrome in CKD stage 5 patients

Chronic kidney disease (CKD) is a growing problem that affects millions of people worldwide and has premature death due to cardiovascular (CV) disease as an established complication[1]. Indeed, CV disease in this population is 10 to 20-fold greater than in the general population, representing, at least, a 15 – 25% per year mortality rate[2]. CV events constitute more than 50% of the mortality risk in CKD patients, and a relative excess mortality is observed in young patients, below 45 years of age[3].

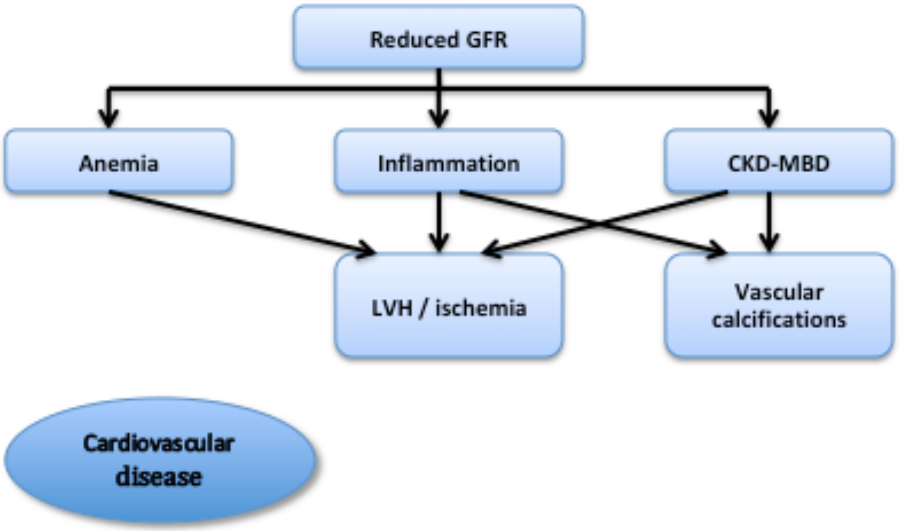


Figure 1 – Risk factors for cardiovascular events in CKD patients

GFR – glomerular filtration rate; CKD-MBD – chronic kidney disease – mineral and bone disorder; LVH – left ventricular hypertrophy

A decrease in glomerular filtration rate (GFR) leads to accumulation of nitrogen products, uremic toxins, and changes in handling mineral products [calcium, phosphorus, parathyroid hormone (PTH), vitamin D levels, among others], which translates into different bone disorders (related to bone turnover, mineralization, and volume) and all these are associated with extra-skeletal calcifications[4]. This cross-talk between bone and vessels constitutes a systemic syndrome known as **CKD-mineral and bone disorder (CKD-MBD)** [5, 6] which is present in almost all end-stage renal disease (ESRD) patients. CKD-MBD is thought to be one of the major non-traditional risk factors for the extremely high rate of mortality observed in CKD patients[7]. Bone disease at any stage of CKD is associated with CV morbidity[7].

Additionally, and as a consequence of the disturbed mineral and bone metabolism, CKD-MBD patients are at increasing risk of low trauma fractures, which are also associated with increased morbid-mortality. Indeed, the risk of fracture-related mortality increases with the severity of CKD. Several studies along the decades have shown us that CKD patients fracture more than the general population[8-11], and the estimated risk is up to 25.6 / 1000 patients year[9]. The exact mechanism for bone fragility is not clearly established, but MBD is definitively a contribution to this phenomenon.

MINERAL ABNORMALITIES IN CKD

During the course of CKD, serum levels of phosphate will increase and serum levels of calcium will decrease, leading to maladaptive responses in the controlling hormones of calcium and phosphorus. Currently, three hormones are of utmost importance in controlling those minerals: fibroblast growth factor 23 (FGF23); PTH, and vitamin D. In addition, other molecules modify their own levels and contribute to the mineral and bone pathology: more well-known ones, such as alkaline phosphatase, or more recently discovered ones, such as sclerostin. Despite the actions of the three main hormones, the accumulation of minerals, mainly phosphorus, continues to occur in CKD, as kidneys fail to respond to hormonal stimulation.

A recent study from the Dialysis Outcomes and Practice Patterns Study (DOPPS) highlighted that combined abnormalities in phosphorus, calcium, or PTH were responsible for higher mortality and all-cause hospitalizations in comparison with patients without abnormal biochemical analysis in those three parameters. These results were particularly important for those aged ≥65 years old[12].

The controlling hormones

- ✓ FGF23 and klotho

FGF23 acts as a phosphaturic hormone, a suppressor of vitamin D, and has a non-consensual effect on PTH[13-15]. Although the molecule has been considered as the prototype of a phosphatonin, a recent study in a cohort of individuals with normal renal function demonstrated that FGF23 supresses vitamin D and reduces renal calcium excretion, but, surprisingly, did not associate FGF23 to renal phosphate excretion[16].

This molecule is a member of the FGF family, and is secreted by osteocytes (and osteoblasts in a small fraction), in response to several physiological stimuli (including $1,25(OH)_2D$, PTH, and phosphate load)[17], circulating as a hormone. To perform the majority of its functions, FGF23 binds to its specific receptors (FGF receptor 1), and needs a co-factor, klotho protein, which acts as a co-receptor[15]. Klotho is expressed in kidneys and in the parathyroid gland[13]. Inflammation, oxidative stress, and FGF23 levels decrease expression of klotho[18].

FGF23 is elevated in the early stages of CKD, and is remarkably high in end-stage renal disease (by a factor of 1000[19]), and its role in phosphorus excretion is given as the main reason for this event. It should be stressed that in CKD klotho levels are low, influencing resistance to FGF23 activity. Other reasons for the high levels of FGF23 in CKD could be the retention of FGF23 (caused by the decrease in GFR) and vitamin D treatment[20].

Phosphaturia is a consequence of FGF23 actions, and this is achieved by two major mechanisms: 1) through the inhibition of renal tubular phosphate reabsorption, mediated by the inhibition of luminal sodium-phosphate co-transporters – NaPi - 2a and 2c in the proximal tubule, specifically in the apical membrane of those tubular cells; 2) through the reduction of phosphate and calcium absorption in the intestine, mediated by the suppression of vitamin D production, via the inhibition of the activating enzyme D-1alfa-hydroxylase and stimulation of the catabolic enzyme D-24-hydroxylase[17,21]. In reducing vitamin D action, PTH levels would rise, as calcium absorption would decline. Nevertheless, some studies have shown that the complex FGF23-klotho inhibits secretion of PTH, another phosphaturic hormone[13,14].

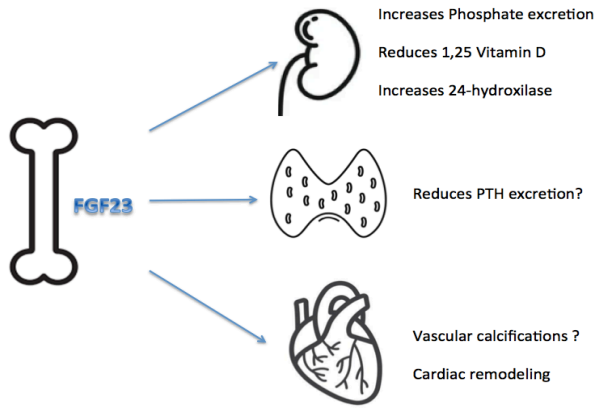


Figure 2 – Functions of FGF23 (adapted) [22]

It is interesting to note that FGF23 seems to inhibit bone mineralization through vitamin D inhibition[15].

While it seems that the main function of FGF23 is to decrease calcitriol, calcemia, phosphatemia, and PTH[23], high serum levels of FGF23 are associated with vascular calcifications and left ventricular hypertrophy (LVH)[24]. Pathologically elevated levels of FGF23 may exert α-klotho-independent effects on non-traditional organs, such as the heart, liver, and cells of the immune system[17].

These maladaptive effects may thus contribute to cardiovascular disease, kidney disease progression, and mortality[17]. Indeed, FGF23 is a predictor of mortality and CV events in hemodialysis patients[25]. It should be stressed, however, that a large sample from the general population found associations between FGF23 serum levels and all-cause mortality only, rather than associations with vascular function or incident CV disease[26].

It should be noted that some difficulties in the interpretation of FGF23 serum levels have been associated with different methods and proteins quantified: intact FGF23 versus truncated FGF23 fragments. Nevertheless, it seems that both are well correlated[27].

- ✓ Calcidiol and Calcitriol

The substrates for vitamin D, ergocalciferol (or vitamin D_2, obtained by nutrition) and cholecalciferol (or vitamin D_3, synthetized by our skin in response to UV light, but also obtained from some foods), are transformed into calcidiol [25(OH)-vitamin D] in the liver, by the action of 25-α-hydroxylase. In its turn, calcidiol is transformed into the active form of vitamin D, calcitriol [1.25$(OH)_2$D], by 1-α-hydroxylase present in proximal tubule cells and in extra-renal sites[28]. PTH, calcitonin, growth hormone, estrogens, prolactin, hypocalcemia, and hypophosphatemia stimulate 1-α-hydroxylase. In its turn, calcitriol, metabolic acidosis and FGF23 inhibit the enzyme. In order for vitamin D to perform its action, vitamin D receptors (VDR) must be activated.

Although the active form is calcitriol, in clinical practice it is calcidiol that is normally evaluated, due to its longer half-life (3 weeks)[29], as opposed to calcitriol's half-life, estimated at 4 to 6 hours, and whose serum levels are 1000 inferior to calcidiol's. Also,

as 1-α-hydroxylase is also present in extra-renal tissues and peripheral production of 1.25$(OH)_2$D takes place, it seems that local concentration of 25(OH)D influences its production[30].

This hormone is vital for mineral homeostasis, as it regulates renal and intestinal calcium and phosphorus handling; regulates bone turnover, inhibiting PTH levels, and has a direct effect on osteoblasts and osteoclasts activity.

In addition to mineral metabolism, vitamin D also has non-classical functions, as it regulates cellular proliferation, plays a part in immune function (in innate and adaptive immunity), and has endocrine effects (in inflammation, insulin-resistance, muscular function, and in the renin-angiotensin-aldosterone axis, affecting blood pressure)[31].

Vitamin D insufficiency (25-hydroxyvitamin D_3 < 30 ng/mL) and deficiency (25-hydroxyvitamin D_3 < 15 ng/mL) are common features in CKD patients[19]. The determinants of vitamin D status are intake, sun exposure, age, nephrotic syndrome (and proteinuria), diabetes and liver disease[32]. In CKD patients, other factors are vital for its low levels: 1) the reduced kidney function and low activity of renal 1-α-hydroxylase; 2) the high levels of FGF23 and consequent activation of 24 α-hydroxylase, which converts the active form into inactive metabolites. Conversely, PTH stimulates vitamin D activity, and simultaneously increases bone turnover, releasing calcium and phosphorus from the bone. For this reason, low levels of vitamin D are one of the main factors for secondary hyperparathyroidism[33]. The guidelines from Kidney Disease: Improving Global Outcomes (KDIGO) recommend evaluating calcidiol serum levels and supplementing, if necessary[34].

We published two manuscripts on the role of calcidiol levels in inflammation, and cardiovascular disease in hemodialysis patients[35,36] and one on the effects of supplementation of cholecalciferol in hemodialysis patients, and realized that supplementation was a simple, safe, and cost-effective action[37].

- ✓ PTH

PTH is increased in the majority of ESRD patients, and parathyroid hyperplasia is an early finding in CKD.

The main function of PTH is to maintain calcium levels within a narrow range, and this is accomplished by: 1) its action in the bone, increasing calcium and phosphorus efflux from this organ; 2) its action in the renal distal tubule, increasing the reabsorption of calcium and excretion of phosphorus; 3) its action in the renal proximal tubule and vitamin D metabolism, stimulating the enzyme D-1alfa-hydroxylase, increasing calcitriol production. Calcium-sensing receptor (CaSR) is a major regulator of PTH transcription and secretion, inhibiting the hormone. VDR also suppresses PTH transcription, but not PTH secretion. As a consequence of its actions, and in order to prevent positive phosphorus balance (efflux of phosphorus from the bone and intestinal reabsorption of phosphorus through vitamin D effects), PTH acts secondarily in the proximal tubules, decreasing luminal sodium-phosphate co-transporters activity, and thus increasing phosphorus excretion.

Secondary hyperparathyroidism is a consequence of the low serum calcium levels and high serum phosphorus levels observed in CKD patients. Other causes for the increased PTH levels in CKD include vitamin D deficiency, reduced expression of CaSR and VDR, maybe the elevated levels of FGF23, and the peripheral resistance to PTH actions. This disease is associated with renal osteodystrophy, fractures, vascular calcifications, cardiovascular mortality, and all-cause mortality[38].

Although PTH seems to correlate with bone remodeling, it must be remembered that this hormone is not a bone-derived hormone, and that its variations are mainly related to serum calcium levels.

It is important to remember that the way PTH is measured is of extreme importance. Initially, PTH was measured through antibodies against C-terminal or against the midregion of the molecule, which were inconsistent laboratory methods. The methods of measuring of the intact hormone evolved, using a second-generation assay with a double antibody. Nevertheless, this method still interacts with C-terminal fragments, namely the 7-84 PTH fragments[39]. Nowadays, third-generation assays are available, but are more expensive. Third-generation assays uses specific antibodies for the N-terminal portion of the hormone, measuring the whole PTH[40]. This method still lacks proper validation.

- ✓ Bone-specific alkaline phosphatase

Alkaline phosphatases are enzymes and membrane-bound glycoproteins responsible for the hydrolysis of inorganic pyrophosphate. Humans have four types of alkaline phosphatase (placental, intestinal, germ cell, and tissue non-specific, and bone-specific alkaline phosphatase is a subtype of this last one, along with liver and kidney isoforms)[41,42].

Bone alkaline phosphatase (BALP) is a subtype of the circulating alkaline phosphatases, encoded by the ALPL gene, and usually represents less than 50% of the total circulating alkaline phosphatase. It is produced by osteoblasts during bone formation; its levels are not affected by renal function[43], and it possibly reflects bone turnover. BALP has four different isoforms identified by liquid chromatography (B/I, B1, B1x, B2), with B1 predominant in cortical bone and B2 predominant in trabecular bone[44].

One of its actions is the inactivation of pyrophosphate, which is an inhibitor of mineralization[45], and the inactivation of osteopontin, a calcification inhibitor[42]. It is curious that FGF23 supresses the activity of tissue non-specific alkaline phosphatase, decreasing the degradation of pyrophosphate, and thus decreasing phosphorus levels[42].

Similarly to PTH, BALP levels are also associated with fractures and mortality in dialysis patients[46,47], as levels are associated with pathological mineralization (vascular calcifications)[42], but those observation were not seen in the general population or in renal transplanted patients.

<u>New players in mineral and bone metabolism</u>

- ✓ Sclerostin

Sclerostin is a glycoprotein product of the *SOST* gene in mature osteocytes[23] and is a negative regulator of bone metabolism[48]. This protein is a soluble Wnt pathway antagonist (Wingless-type mouse mammary tumor virus integration site), via binding to co-receptors low-density lipoprotein receptor-related protein (LRP) 5/6, inhibiting their association with Wnt receptors[49,50]. The Wnt pathway is very important in such biological processes as proliferation, migration, and differentiation of cells. The Wnt

signalling activates three different pathways; one of those is the Wnt/beta-catenin pathway, which inhibits the activity of the beta-catenin degradation complex in cytoplasm. Genetic mutation on the Wnt/beta-catenin pathway leads to premature coronary disease and severe osteoporosis, providing evidence of the importance of the Wnt signalling in the bone-vessels axis[51].

By the inhibition of the Wnt pathway, sclerostin down-regulates the osteoblasts function in a paracrine fashion (reduction of their activation, proliferation, and increasing their apoptosis), and serves as an inhibitor of bone formation[7,23,48].

Various observations have shown that CKD patients have moderately elevated sclerostin serum levels[51,52], and so its potential role in renal osteodystrophy is a point to be studied. Indeed, various laboratory studies point out that sclerostin contributes to low-turnover osteodystrophy, and that this hormone may negatively influence mineralization via regulation of FGF23[48] and alteration of vitamin D synthesis in proximal tubular cells[53]. This is yet to be proved.

The synthesis of sclerostin and the factors that promote its elevation are currently being studied. Although its secretion is in the main exclusively from osteocytes, osteoclasts precursors, renal and vascular cells can also secrete sclerostin[51]. Ageing, male gender, diabetes, high fat mass, and mechanical unloading of the skeleton are possible factors for increased sclerostin serum levels[23]. Other known stimulators include calcitriol, phosphate, glucocorticoids, low PTH (as PTH down-regulates sclerostin), and calcitonin[23,50,54,55]. Mineral abnormalities in CKD may be a stimulus for sclerostin production, and we know that sclerostin serum levels increase in CKD along with increasing rates in renal elimination of sclerostin[56]. Serum levels decrease rapidly after renal transplantation[57].

In terms of CKD patients, Oliveira and co-workers described that pre-dialysis patients treated with sevelamer presented with decreased serum levels of sclerostin, and also FGF23[58]. On the other hand, Cejka and co-workers negatively correlated serum levels of sclerostin with histomorphometric parameters of bone turnover, osteoblast number and function in hemodialysis patients[59]**.** A very recent study demonstrated that, in CKD stage 3 and 4, sclerostin serum levels correlated with low-turnover bone disease[60]. Other authors have pointed out the potential role of sclerostin in inducing

bone resistance to PTH[54]. The relationship between serum sclerostin, serum FGF23, klotho, and PTH was studied in a recent work, and a positive correlation with FGF23 was found, but not with the other variables. Nevertheless, this was not the primary aim of the work in question[54]. Further, sclerostin has been shown to be a suppressor of bone-alkaline phosphatase activity[50,61]. Relationships between sclerostin and calcitonin in CKD patients have not yet been studied so far[54].

The impact of high sclerostin levels on CV disease and mortality are yet to be determined[48]. Wnt signalling has been involved in vascular calcifications, and increased sclerostin expression has been demonstrated during vascular smooth muscle cell calcification in an animal model[23,62]. In humans, particularly in CKD patients, different studies have reached different conclusions. Some studies have demonstrated that sclerostin high levels are associated with better survival in hemodialysis patients[49,50], suggesting a protective role through inhibition of vascular calcifications[49,63], while other studies have found an association between high levels and CV mortality in dialysis patients[54], justified by the propensity for vascular calcifications via low-bone-turnover disease[48], leading investigators to speculate that a U-shaped dose effect could be the cause of these findings[23]. A recent study performed in end-stage renal disease patients showed that sclerostin was associated with the degree of vascular calcifications[61]. Nevertheless, the role of sclerostin in CV health is very important to clarify as a sclerostin antibody (romosozumab) is being evaluated for osteoporosis treatment in post-menopausal women[64], and initial studies have not shown evidence of enhanced vascular or valve calcifications after three years of treatment with romosozumab[65]. Nevertheless, a recent study comparing the use of romosozumab vs. alendronate revealed a higher number of serious adverse events in the romosozumab therapy group (2.5% vs. 1.9%)[66] in a 12-month study. Another study, in this case in osteoporotic men, revealed more cardiovascular serious adverse events compared to placebo (4.9% vs. 2.5%) over a 12-month period[67].

- ✓ Calcitonin

Calcitonin is a hormone released by C-cells of thyroid gland in acute hypercalcemia. Its main function is to decrease calcium serum levels, by decreasing osteoclast activity, and facilitation of the deposition of calcium and phosphorus in bone (especially in post-prandial state). This hormone also stimulates vitamin D production in pregnancy and lactation. Laboratory studies have suggested that the absence of

calcitonin could increase bone mass and bone formation[68]. Nevertheless, an older study in adults with CKD showed no correlation between calcitonin and bone disease in renal patients.[69]

The kidney is the primary site for calcitonin metabolism, and serum levels of the hormone can be elevated in CKD patients. Nevertheless, studies are lacking both in CKD and renal transplanted populations.

The minerals in CKD

- ✓ Phosphorus

Renal dysfunction leads to incapacity to excrete phosphorus in a proper way, resulting in its accumulation. This event stimulates FGF23 and PTH, in an attempt to achieve a new balance in phosphorus levels.

As we saw, FGF23 promotes excretion of phosphorus, but also promotes an inhibition of calcitriol synthesis and probably PTH, with consequent hypocalcemia. On the other hand, hypocalcemia will stimulate PTH excretion. Hyperphosphatemia promotes secondary hyperparathyroidism in four major ways: 1) high levels of phosphorus combine with calcium, leading to calcium-phosphate deposition in the form of bioapatite in vessels, cardiac valves, and myocardium[70], and promoting hypocalcemia; 2) hyperphosphatemia leads to inhibition of calcitriol production, either *per se* or via the action of FGF23; 3) hyperphosphatemia associates with resistance of calcitriol action in the parathyroid glands, inducing bone resistance to PTH action; 4) hyperphosphatemia *per se* also seems to increase PTH secretion.

Although hyperphosphatemia is classically associated with mortality in CKD patients[71-73], a study released in 2014 showed that in a population of 142069 receiving in-center hemodialysis, magnesium modified the mortality risk associated with hyperphosphatemia, and higher magnesium levels attenuated mortality, in the same way that lower magnesium levels boosted hyperphosphatemia-related mortality[74].

- ✓ Calcium

Calcium is the most abundant positively charged ion in the human body and has many functions. The most part of this ion is within the bones and only 1% is in intra

and extracellular sites. PTH and vitamin D mainly perform regulation of calcium, through CaSR. PTH mobilizes calcium (and phosphorus) from bones, raises calcium reabsorption from the renal Henle loop and distal tubules (and excretes phosphorus), and stimulates renal synthesis of calcitriol, whereas calcitriol increases intestinal calcium (and phosphorus) absorption. Other hormones, such as calcitonin, estrogen, and prolactin also regulate calcium levels, but these hormones are less studied.

During the course of CKD, total calcium levels tend to decrease as a consequence of hyperphosphatemia, and of reduction of calcitriol synthesis (subsequent to decreased renal function, FGF23 action, and some skeletal resistance to PTH). This stimulates PTH secretion in the parathyroid glands and induces the development of secondary hyperparathyroidism[38].

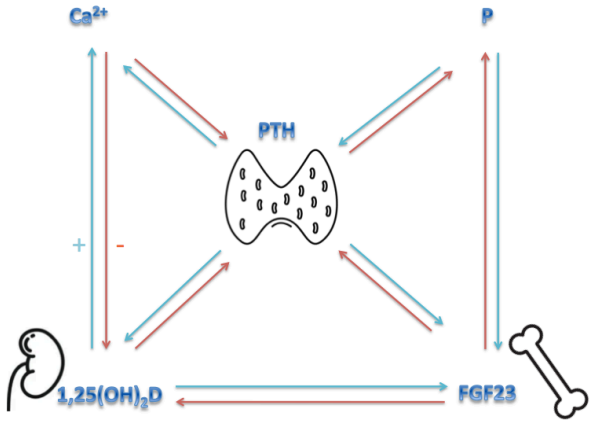


Figure 3 – Feedback loops and mineral metabolism (adapted)[75]

BONE DISORDERS IN CKD

Bone is composed of two components: inorganic bone and organic matrix, or osteoid. Both components are remodeled in a three-month cycle by osteoblasts and osteoclasts.

Osteoblasts are cells derived from the mesenchymal bone marrow linage[76] and are responsible for osteoid formation, mineralization, and activation of osteoclasts. When surrounded by mineralized matrix, osteoblasts turn to osteocytes, cells similar to neurons, responsible for the secretion of factors that regulate bone formation, namely FGF23 and sclerostin[77].

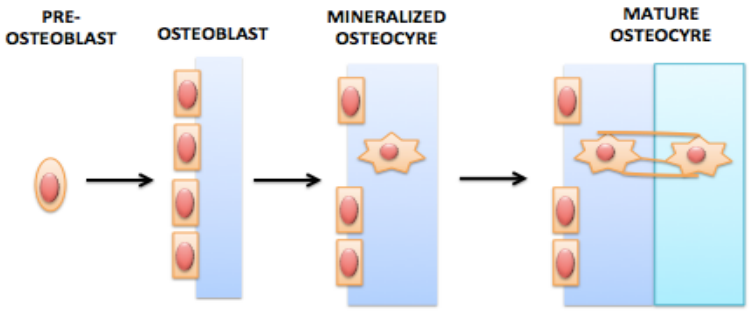


Figure 4 – Osteoblast-to-osteocyte ontogeny (adapted)[77]

Osteoclasts, derived from hematopoietic cells[76], express several receptors, including the receptor activator of NF-kB ligand (RANK-L), and are responsible for reabsorption of mineral and matrix bone components.

Briefly, resorption of bone is a function of osteoclasts; formation of new bone is a task of osteoblasts (triggered by PTH and vitamin D), and osteocytes control both processes. The remodeling sequence is established: 1) osteoclast activation; 2) bone resorption; 3) osteoblast recruitment and activation; 4) osteoid syntesis; 5) osteoid mineralization; 6) osteocyte appearance[78].

While mechanical function of the bone is operated by cortical bone (and decreased bone strength leads to fracture), which accounts for almost 80% of human bone mass[79], metabolic function is served by trabecular bone. Abnormal metabolic activity is hypothesized to be related to extra-skeletal calcifications[80]. The bone disorders observed in CKD patients are part of the CKD-MBD syndrome and are known by the name of Renal Osteodystrophy.

Renal Osteodystrophy (ROD)

ROD is common in ESRD patients and is associated with bone pain, fractures, proximal myopathy, vascular calcifications, calciphylaxis, anemia, pruritus, neurotoxicity, and cardiomyopathy. A bone biopsy is necessary in order to classify ROD[81]. Classically, ROD was organized into four major groups[82,83], according to histological bone abnormalities:

1. Hyperparathyroid bone disease: a high-turnover disease, with normal mineralization and variable volume

2. Adynamic bone disease: a low-turnover disease, characterized by normal mineralization and decreased bone volume
3. Osteomalacia: a low-turnover disease with reduced mineralization and variable bone volume
4. Mixed renal osteodystrophy: high-turnover bone disease along with decreased mineralization.

Since 2006, authors have tried to classify bone disease according to turnover, mineralization, and volume[6], the TMV classification. Turnover can be high, normal or low; mineralization abnormal or normal; and volume high, normal or low. To summarize, bone turnover is altered in hyperparathyroid bone disease, mixed renal osteodystrophy, adynamic bone disease, and osteomalacia. Mineralization is halted in osteomalacia and in mixed disorder. Loss of bone volume occurs when reabsorption exceeds bone formation and gain of bone volume occurs when formation exceeds reabsorption. Changes in the volume are mainly present in hyperparathyroid bone disease and adynamic bone disease[83].

ROD is almost universal in ESRD patients, is heterogeneous, and has changed over the last few decades. Indeed, low-turnover bone disease has increased over the past two decades[84], since hyperparathyroidism is aggressively treated in uremic patients, physicians use iron to treat anemia; use high-flux dialysis membranes, and a good proportion of dialysis patients are diabetics and old.

Correct diagnosis of the CKD bone-related disorder is necessary for its correct treatment, because treatment depends on the type of bone disease. For instance, high-turnover disease is treated with calcimimetics, calcitriol, or vitamin D analogs; low-turnover disease is difficult to treat, and physicians can lower calcium load and suspend all types of vitamin D, and demineralization is treated with phosphate supplementation and vitamin D replacement therapy[83]. Some authors have suggested treating adynamic bone disease with a human recombinant PTH (teriparatide)[85], and this is explained by the fact that osteoblasts have PTH receptors, and this hormone increases the number, lifespan, and function of the osteoblasts[19].

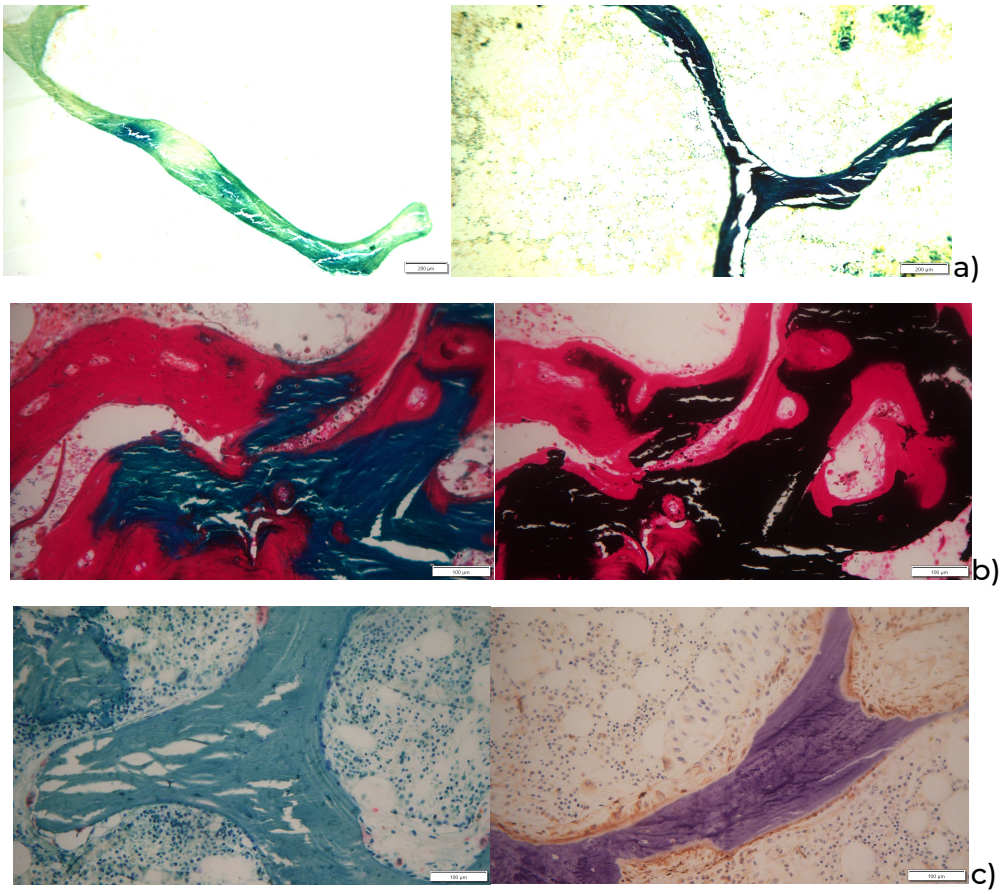


Figure 5 – Histologic bone abnormalities (a – low-turnover bone disease; b – mineralization defect; c – high-turnover bone disease)

Although almost all patients with CKD present changes in turnover and/or mineralization; isolated changes in bone volume (osteopenia or osteoporosis) can also be present, though probably in a minority of patients. In this sense, an older patient with low bone mineral density and/or a history of fragility fracture may have osteoporosis or another MBD related to CKD. As the most important factors for osteoporosis are advanced age and glucocorticoid therapy, it is not difficult to imagine that our CKD patients are at risk of the disease. The World Health Organization (WHO) defines osteoporosis as "a systemic skeletal disease characterized by low bone mass and microarchitecture deterioration of bone tissue, with a consequent increase in bone fragility and susceptibility to fracture"[86,87], and in clinical practice, the diagnosis is based upon dual-energy X-ray absorptiometry (DXA) measurements, and osteoporosis is diagnosed when a T-score -2.5 standard deviation or more below the young-adult mean bone mineral density (BMD) is found[88]. There are risk factors for osteoporosis, with the most important ones advanced age, previous fragility fracture history, and steroid therapy[88]. Age is the most relevant

factor, and for any kind of T-score on DXA, the risk of fracture will rise in aged persons[88]. This is especially true in post-menopause women. Menopause is related with bone remodeling changes; the resorption increases 50 to 75%[89], leading to an imbalance between resorption of old bone and formation of new bone. The decline in estrogen is one of the main factors for this evolution, leading to increase production of tumor necrosis factor (TNF) α, which leads osteoblasts to release several cytokines, ending in an osteoclastogenesis cascade. The production of the RANK-L is increased and binds to the receptor RANK in osteoclasts, increasing osteoclasts activity[90]. Furthermore, osteoprotegerin secretion, a natural antagonist of RANK-L, is also decreased in menopause[89].

In CKD, various studies emphasize the relationship between a low GFR and the risk of osteoporosis and fractures. In a study from 2003, a data analysis from the Third National Health and Nutrition Examination Survey (NHANHES III) showed that both women and men with osteopenia or osteoporosis were within the lowest GFR groups[91]. Using the same database, it was shown that the prevalence of CKD was superior in the group of patients that reported having a fracture event[92]. In addition, a DOPPS study reported that the incidence of fractures was higher for dialysis patients than the general population, and that the event was related to a 3.7-fold increase of the relative risk of death[93]. Equally so, a study using the Atherosclerosis Risk in Communities (ARIC) participants associated low GFR and high albuminuria as risk factors for fractures[9]. A recent review states that hip fractures are four-fold higher for dialysis patients than for the general population, adjusting for age, gender, and ethnicity. In addition, it highlighted that fractures are more frequent in Caucasian patients, and in women[94].

We also published an article on this topic, and found an incidence rate of 31 episodes of fractures per 1000 person-years in a median dialysis vintage of 47 months, with a correlation with BALP levels and very high (> 800 pg/mL) or very low (< 300 pg/mL) PTH levels[95]. This article is available upon request.

Bone volume is challenging in terms of treatment, and lifestyle measures (exercise, smoke and alcohol eviction) are essential. Hormone replacement therapy in premenopausal women with CKD and amenorrhea is a good option, but this therapy in postmenopausal women is associated with important and negative side effects,

such as stroke or breast cancer[96]. Antiresorptive agents, including raloxifene, bisphosphonates, or denosumab, and bone stimulatory agents, such as teriparatide or romosozumab, are not approved for use in dialysis patients, not even in advanced cases of CKD. Data on safety and efficacy of antiresorptive treatments are inconclusive, and these drugs should be used with caution in those patients, ideally after appropriate diagnosis of the bone disease.

Bisphosphonates induce a switch-off of bone resorption (inactivating osteoclasts by inhibition of farnesyl pyrophosphatase synthase), diminish bone formation (almost inducing a low-bone-turnover disease) and increase bone mineralization[97]. As bisphosphonates have a high affinity to hydroxyapatite, it can accumulate in bone for decades, especially in CKD patients, as the kidney excretes the fraction not retained in bone. It is worth emphasizing that longer duration of bisphosphonates use increases the risk of atypical femur fractures, and the risk decreases rapidly after bisphosphonates discontinuation[98]. It should be noted that bisphosphonates are also nephrotoxic, can potentially accelerate the progression of kidney disease, can accumulate in patients with low estimated glomerular filtration rate (eGFR), and the severity of side-effects can be dependent on the renal function. Denosumab, a humanized monoclonal antibody against the receptor activator of NF-kB ligand, has similar effects, as it supresses osteoclast activity. Both decrease the risk of osteoporotic fractures (hip, vertebral, and non-vertebral). These agents cause suppression of bone turnover, and thus have to be used with caution in advanced stages of CKD and in ESRD, because the use of these agents can theoretically increase the risk of fractures in patients with ROD, as they can aggravate low-bone-turnover disease. The kidney does not clear denosumab, making the use of this drug in CKD and transplanted patients more attractive[99]. Nevertheless, the use of denosumab in ESRD patients is associated with hypocalcemia, which can be a serious adverse event[100]. Another note of concern is the fact that denosumab withdrawal may be associated with an increased risk of vertebral fractures[101].

Nevertheless, it is very difficult to diagnose osteoporosis in patients with CKD. The WHO diagnosis criterion used in the general population can be applied in patients with an eGFR ≥ 60ml/min, or even in patients with eGFR between 60 and 30 ml/min, provided that other CKD-MBD are excluded, since experts agree that DXA measurements for bone mineral density are appropriate in CKD stage 3 and 4

patients[102]. Furthermore, those patients with a history of fragility fracture are also suitable for the diagnosis. Below an eGFR of 30 ml/min, osteoporosis diagnosis is challenging. First of all, DXA cannot differentiate cortical and trabecular bone; cannot assess microarchitecture or bone turnover, and finally DXA measures areal BMD, and not volumetric BMD. Also, applicability is limited in the presence of vascular calcifications[94]. Still, recently, in 2017, the updated CKD-MBD KDIGO guidelines reviewed the utility of performing DXA image tests in CKD patients, based on the new data[103-106], and recommended bone mineral density testing in CKD patients for fracture risk assessment, if the results would impact treatment decisions[107].

The screening for fracture risk is possible through the Fracture Risk Assessment Tool (FRAX), a tool developed by WHO in 2008. This tool estimates the individual's 10-year fracture risk (both major fracture and hip fracture) based on eleven variables (age, gender, weight, height, smoking, alcohol abuse, previous fracture, parent fractured hip, glucocorticoids, rheumatoid arthritis, and secondary osteoporosis) plus an optional bone mineral density measurement obtained at the femoral neck. Nevertheless, there are concerns over the use of this tool in CKD patients, as it doesn't adjust the risk for the eGFR. Some experts defend that clinicians should consider doubling the absolute fracture risk in patients with eGFR less than 45 mL/min[108,109]. However, a recent study of more than 10,000 individuals showed that bone mineral density testing and FRAX were good tools to discriminate fracture risk in patients with non-dialysis CKD, as they are in the general population[110], in line with a previous study into 320 CKD stage 3-5d patients[103]. It is important to note that in CKD patients hip fracture incidence increases with progressive CKD[111,112], and this is associated with an increase in mortality in the dialysis population[113].

There are other new techniques that can provide a non-invasive three-dimensional evaluation of bone microarchitecture. High-resolution peripheral computed tomography (HR-pQCT) is one of those, and has some advantages as it differentiates trabecular from cortical bone and provides information on volumetric density, cortical porosity, and bone resistance (not given even by a bone biopsy), Nevertheless, this expensive technique does not differ from DXA in predicting the risk of fractures[106] in CKD patients. Another innovative tool to predict the risk of fractures is the Trabecular Bone Score (TBS), a software-based tool that draws trabecular bone based on the DXA images. According to this software, the higher the TBS, the more compact the bone[114].

There is still debate about the role of this score in CKD patients, with some studies showing advantages in its use for the prediction of non-vertebral fractures in dialysis patients[115], while others do not show any benefit[116]. Despite these new techniques, DXA remains the standard method for predicting fracture risk.

The gold standard for the diagnosis of CKD-associated bone disease is a bone biopsy, which is an invasive method[81]. With a bone fragment, we can evaluate turnover, mineralization, and volume. Indeed, this is the only method that quantifies and evaluates bone mineralization, making it possible to distinguish osteoporosis from hyperabsorption and osteoporosis from deficient bone formation. This technique assesses both cortical and trabecular bone, and it gives both static and dynamic information, quantifying bone formation (using osteoid surface, osteoid volume, osteoid thickness, and osteoblast surface), bone resorption (using osteoclast surface and resorption surface), bone formation rate (based on mineralizing surface and mineral apposition rate obtained with tetracycline labeling) and bone volume, trabecular thickness, trabecular separation, and trabecular number. There is currently no substitute for bone biopsy. However, this is an expensive, invasive, and one-shot procedure, and is performed in only a few centers worldwide[4,82,117], due to the expertise needed in: 1) obtaining the bone fragment; 2) performing the sampling procedure (which is prolonged); 3) interpreting the histopathological analysis (which is a time-consuming process).

Our center wrote a review article on the topic of bone biopsy[81], available upon request.

As bone disease is a major determinant of death in ESRD patients, non-invasive markers of bone disease have been studied for decades. The first biomarkers studied were PTH and BALP[118]. Studies from recent years have demonstrated a poor correlation between PTH/BALP serum levels and bone turnover, and this lack of specificity and sensitivity does not allow the differential diagnosis of renal osteodystrophy in a individual patient[118-121] based only on these markers. A recent study showed that the use of BALP, intact procollagen type 1 N- terminal propeptide (PINP) and tartrate-resistant acid phosphatase 5b (TRAP5b) could differentiate low from non-low turnover bone disease. Nevertheless, new biomarkers are needed and osteocyte-derived factors, such as FGF23 and sclerostin, are seen as promising answers.

The future could combine newer imaging techniques with the newer laboratory biomarkers toward a virtual bone biopsy[122].

VASCULAR CALCIFICATIONS IN CKD

Extra-skeletal calcifications are the third feature of CKD-MBD, and identification of vascular calcification in CKD patients has tremendous impact on CV risk stratification and therapeutic guidance[123]. Different exams, including, among others, X-ray, CT, and MR can analyze the vascular health of CKD patients. The plain X-ray of the pelvis and hands for evaluation of vascular calcifications is a simple and inexpensive tool. Using this method, we can apply a very simple (and Portuguese) CKD-validated calcification score (the Adragão score)[123,124], which has proved to be an independent predictor of CV death, CV hospitalizations, and vascular disease in dialysis patients[123].

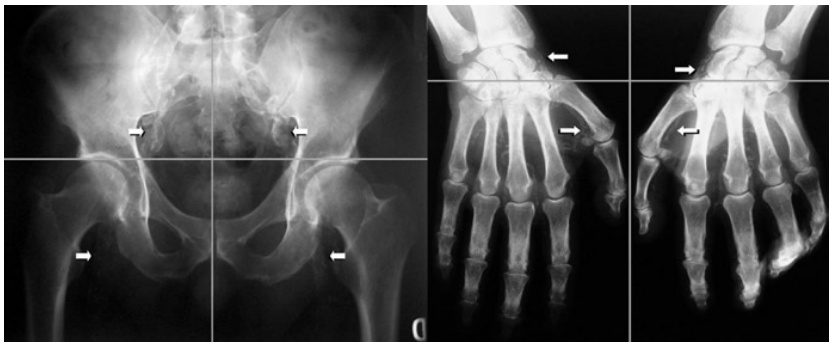


Figure 6 – Simple vascular calcification score – Adragão score

However, coronary artery calcifications or coronary artery calcium score assessed by non-contrast computed tomography are proven to be the best predictors of mortality, and associated with measures of left ventricular systolic and diastolic function[125] in hemodialysis patients. This method is the gold standard for risk stratification and cardiovascular events prediction in CKD patients[126], and also for evaluation of vascular calcifications progression. The percentiles can be calculated using the Multi-Ethnic Study of Atherosclerosis (MESA)[127] website, which can be found in the following webpage: http://www.mesa.nhlbi.org/Calcium/input.aspx

Vascular calcifications are common in aging patients, in diabetes, dyslipidemia, genetic diseases, and in CKD patients[128]. In those (and in diabetic patients), both intimal and media muscular layer can be calcified, and are predictors of cardiovascular events and all-cause mortality over conventional risk factors[128]. We

know that vascular calcifications progress faster in ESRD patients than in the general population, increasing the risks of its consequences.

Coronary Artery Calcium Score			
Absolute Values		Adjusted Percentile*	
0	Absence of calcification	0	Absence of calcification
1 – 10	Minimal	1 – 25	Minimal
11 – 100	Mild	26 – 50	Mild
101 – 400	Moderate	51 – 75	Moderate
401 – 1000	Severe	76 – 89	Severe
> 1000	Extremely severe	> 90	Extremely severe

Table 1 – Coronary artery calcium score

* Adjusted for age and gender

Valve calcifications, especially in aortic and mitral valves, are another point to be explored in ESRD patients, as they are eight times more prevalent in dialysis patients than in the general population[129], and occur 10 to 20 years earlier in these patients[130]. In addition to the patients' genetics, mechanical stress, inflammation, or even endocarditis, abnormalities of mineral and bone metabolism are important risk factors for valve calcifications[130].

The mechanisms of developmental and progression of vascular calcifications in uremic patients have been studied by several experimental and clinical studies. Hyperphosphatemia and changes in plasma PTH (low and high abnormal values) are two recognized risk factors. Potential new players are:

- FGF23 and klotho: numerous studies have demonstrated correlations between serum levels of FGF23 and coronary artery calcifications [131-134], although others found no association [135]; klotho is considered an anti-aging protein and to have cardiovascular protection properties[136], and its deficiency observed in CKD patients seems to stimulate the osteoblastic transformation of vascular smooth muscular cells[136].
- Sclerostin: this is a negative regulator of bone metabolism[48], and seems to be involved in vascular calcifications. Some studies have demonstrated that high levels are associated with better survival in hemodialysis patients[49,50], suggesting a protective role through inhibition of vascular calcifications[49,63],

while other studies have found an association between high levels and CV mortality in dialysis patients[54], and association with the degree of vascular calcifications[61].

- Bone turnover: it is postulated that excess bone reabsorption is associated with vascular calcifications, as some of the mineral content from the skeletal system is deposited in the vessel wall[136]. In addition, osteoporosis diagnosed by DXA correlated with coronary artery calcification and its progression in a recent study[134]. Nevertheless, bone biopsies studies correlating bone turnover and mineralization with vascular calcifications scores are needed.

Mineral and bone disorder after renal transplantation

Kidney transplantation is the treatment of choice for established CKD stage 5. In the last two decades, five-year allograft survival improved from 75% to 85% and from 55% to 80% (living-related and deceased donor) and more than 50% at 10 years[83,117].

With improved long-term outcomes, CV disease and fractures have emerged as events that reduce both quality of life and survival of renal transplanted patients[13], increasing the requirement for medical assistance, and post-transplant mineral and bone disease (PT-MBD) is considered one of the major causes for these two outcomes. Indeed, cardiovascular disease is the leading cause of death after renal transplantation, with these patients at twice the risk of it compared to the general population, with a 3.5 to 5% annual risk of fatal and non-fatal cardiovascular events[128]. Even with correction of uremia, abnormal bone mineralization and accelerate bone loss are known features of PT-MBD[4], perhaps causing cardiovascular events[82], mainly due to disturbed mineral metabolism.

Apart from these, recent studies associate PT-MBD to allograft dysfunction and subsequent return to dialysis, and to new-onset diabetes after transplant[7], both conditions coupled with the loss of quality of life, and billions spent on healthcare costs, and higher mortality.

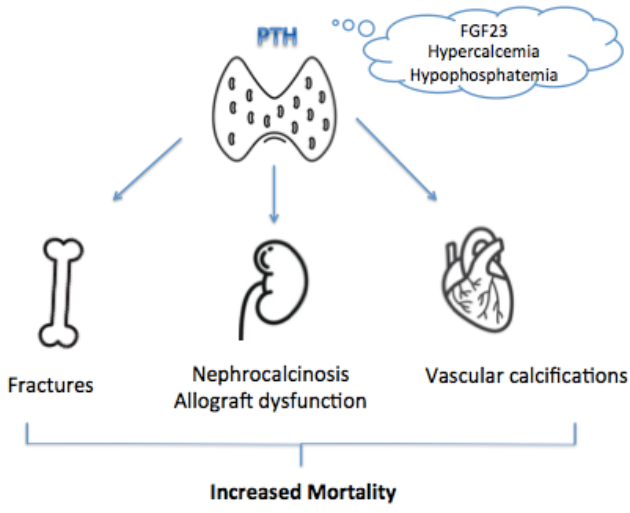


Figure 7 – Potential renal transplant outcomes in patients with MBD (adapted)[19]

We reviewed bone and mineral disease after renal transplantation in two recent papers[137,138], which are available upon request.

DISTURBED MINERAL METABOLISM

Disturbed mineral metabolism has been investigated as a probable cause of CV events and fractures in renal transplanted patients, as happens in CKD patients. Contrary to CKD, hypophosphatemia, hypercalcemia, and hypomagnesemia are frequent metabolic complications in the early period post-kidney transplant, and controlling hormones of these minerals tend to decrease, although in many patients normal values will not be achieved.

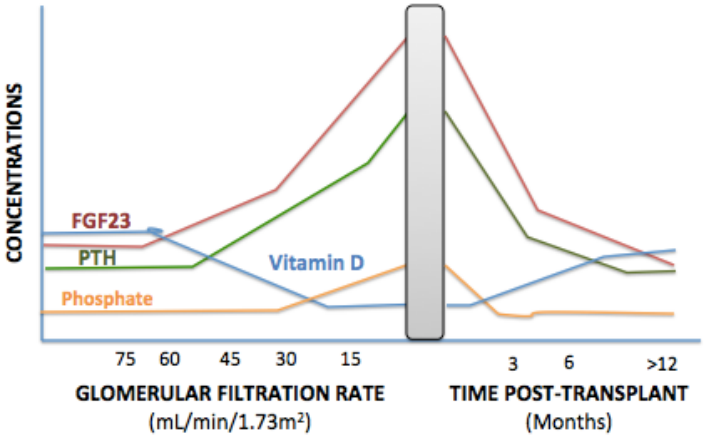


Figure 8 – Temporal aspects of disordered mineral metabolism in CKD and after renal transplantation (adapted)[19]

- ✓ FGF23

Although serum levels of FGF23 decrease after renal transplantation, these patients still present higher levels of the hormone than the general population, even with hypophosphatemia[13]. In that sense, FGF23 may lead to gradual bone demineralization in transplanted patients, as can steroids and vitamin D deficiency[13], as suggested by a recent study that revealed the association of high FGF23 and low PTH serum levels at time of transplantation with bone mineral density loss in the first year after transplantation[139]. Conversely, one study based on 19 pediatric renal transplanted patients, showed no difference between the serum levels of FGF23 in those patients and in a control CKD-population based on eGFR[140]. Nevertheless, this was performed in a very small sample size; the match case – control was performed on a 1:1 basis; only a univariate analysis was executed, and all these limitations decrease the power of the study, and limit its conclusions.

Similarly to that seen in CKD patients, some authors have pointed out endothelial dysfunction and cardiovascular disease mediated by FGF23 in the transplant setting[141]. Further, transplant loss (through nephrocalcinosis) and mortality were related to FGF23 high serum levels in other studies[14,142].

Studies relating FGF23 and bone histology in transplantation are lacking, and we still do not know the role of FGF23 in MBD in transplanted recipients.

- ✓ PTH

PTH usually declines after a successful renal transplant, rapidly in the first 3 to 6 months, and more gradually afterwards. The main factors for this improvement are correction of phosphorus, calcium, and vitamin D levels[83]. Nevertheless, maintenance of high serum PTH levels, although renal function is normal, is observed in nearly 25% of patients[83], and this is known as tertiary hyperparathyroidism. One of the reasons for disturbed PTH levels in the transplant setting is that parathyroid cells have a long lifespan of approximately 20 years[19], and monoclonal glandular hyperplasia can be occurring[82]. Other factors include longer dialysis vintage, along with high serum levels of phosphorus and PTH pre-transplant, as well as low levels of 25 hydroxyvitamin D_3 and 1,25 di-hydroxyvitamin D_3. Tertiary hyperparathyroidism leads to hypophosphatemia, hypercalcemia, and even nephrocalcinosis[19]. The optimal range in the PTH level in renal transplanted patients is unknown[19].

- ✓ Calcidiol and Calcitriol

In the first months post-transplantation, hypophosphatemia, inappropriate PTH high levels, and recovery of kidney function increases calcitriol serum levels, through activation of 1-α-hydroxylase. On the other hand, maintenance of high FGF23 levels activates 24-α-hydroxylase, reducing its levels. The equilibrium between these two feedback loops, as well as kidney function and immunosuppression, determines its serum levels. As a result of all these factors, vitamin D deficiency (25-hydroxyvitamin D_3 < 30 ng/mL) is still common in renal transplanted patients[19,99].

- ✓ Sclerostin

Observations in renal transplanted recipients are somewhat surprising. Some authors have shown high levels of sclerostin along with high levels of PTH[7]. Information on

relationships between sclerostin serum levels and bone biopsy data, as well as CV disease, are lacking in this group of patients.

- ✓ Phosphorus

Hypophosphatemia affects nearly 90% of the renal transplanted patients[143,144]. However, this event is self-limited, and nearly 6 – 27% will maintain low serum levels of phosphorus[13]. The trigger for this episode was thought to be tertiary hyperparathyroidism, but inappropriate phosphate wasting can occur despite low levels of PTH[13]. The discovery of FGF23 suggested novel mechanisms to interpret these findings, and tertiary hyperphosphatonism[144,145], due to synergic actions of PTH and FGF23, is currently believed to happen[19]. In addition, renal denervation is a factor for hyperphosphaturia observed in these patients[83]. It is important to note that imbalances in phosphorus levels after transplantation are associated with mortality risk[82] and defective mineralization[99], which can be complicated by osteomalacia. Progressive bone demineralization can predispose patients to fractures[137].

- ✓ Calcium

Initially, serum calcium levels decrease and this feature usually normalizes in the first weeks after transplantation. In 5 to 55% of patients, a biphasic pattern is observed, and hypocalcemia immediately post-transplantation is replaced by hypercalcemia in the 1st week post-transplantation[19,83]. It should be noted that during the first year of transplant, the mineral will continue to decrease, and only 5 to 10% of patients will have persistently high calcium levels[137]. Despite this observation, a study performed in 2007 showed no relation between high serum levels of calcium and abnormalities in bone turnover, suggesting defective tubular calcium handling as an additional cause for this[143], besides high PTH levels. It seems that persistent high calcium levels are associated with interstitial microcalcification and worse graft survival[99] and, importantly, premature death[146].

DISTURBED BONE METABOLISM

It is postulated that post-transplant MBD reflects the pre-existing CKD-MBD at the time of renal transplantation, the effects and consequences of immunosuppression, and the effects of renal dysfunction after transplantation[83]. Kidney dysfunction after renal transplantation is common, and, as in non-transplanted patients, is associated with CKD-MBD syndrome, which encloses bone disorders. Bone disorders are

accompanied by bone pain and deformity, osteoporosis, osteonecrosis, and fractures, leading to poor outcomes related to both graft and patient[82].

Post-transplantation bone disease

It is assumed that accelerated bone mineral density loss occurs within the first 6 to 12 months of transplantation, especially in trabecular bone, and that bone loss occurs in 11 – 56% of the renal transplanted recipients, due to altered mineral metabolism. Additionally, younger age, longer dialysis vintage, and low BMI (<23) seem to be risk factors for this event[4]. Other risk factors for bone loss in transplantation are immunosuppressive agents, such as high steroid doses.

- ✓ Steroids

Briefly, and as demonstrated in Figure 9, glucocorticoids reduce bone formation mediated by direct inhibition of osteoblasts function, impair osteoblastogenesis, increasing osteoblasts and osteocytes apoptosis (the latter inducing osteolysis), and increase osteoclastogenesis[147], by raising the expression of the receptor activator of nuclear factor –kB ligand[19,148]. In addition, glucocorticoids reduce muscle mass leading to a greater risk of falls[148]; decrease secretion of estrogens and androgens, and activate vitamin D catabolism[149], reducing the absorption of calcium from the gastrointestinal tract and renal tubular cells, resulting in a negative calcium balance[83], leading to maintenance or development of secondary hyperparathyroidism[147]. Finally, glucocorticoids up-regulate Wnt antagonists[19], aggravating bone loss.

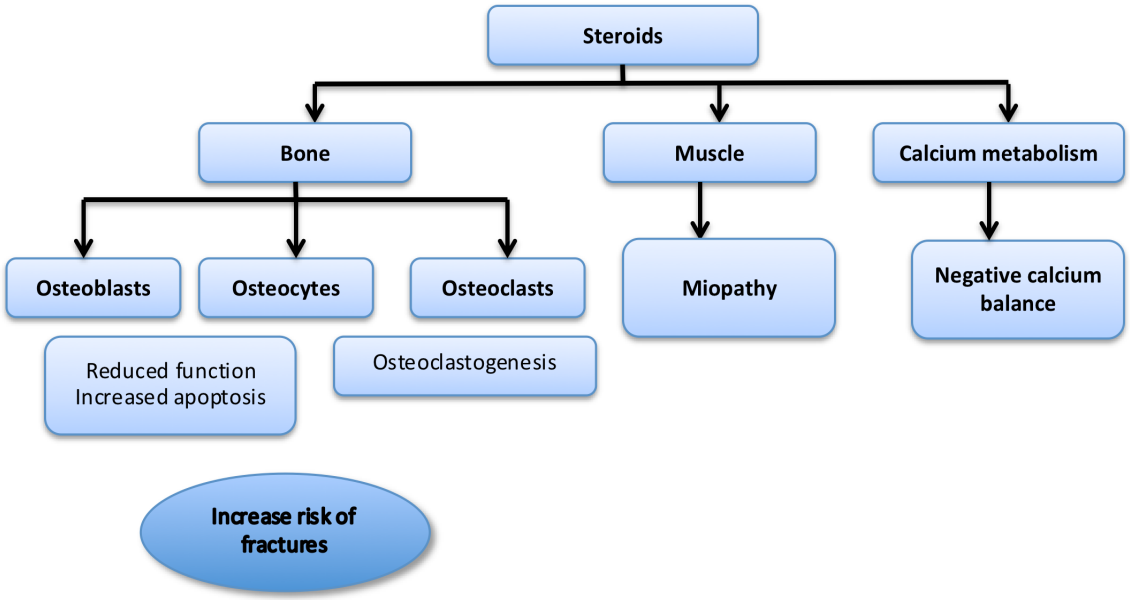


Figure 9 – Steroid therapy and Osteoporosis

It is important to note that the risk of fractures rapidly decreases after discontinuation of glucocorticoids, and that bone strength promptly improves[148].

- ✓ Calcineurin inhibitors, mTOR inhibitors and anti-metabolites

The actions of calcineurin inhibitors on bone are less well defined[19], but it seems that both cyclosporine and tacrolimus activate osteoclasts cells in murine models[83] and induce vitamin D resistance through VDR down-regulation[149]. These effects contribute to an increase in PTH levels[99]. Sirolimus inhibits osteoclasts, and seem to be a sparing bone immunosuppressive agent[19,149], like azathioprine, mycophenolate mofetil, and induction therapies[83].

Although we assume disturbed mineral metabolism and bone loss early post-transplantation, the evolution of bone disease after transplantation is not well-defined, and studies on bone histomorphometry are contradictory[19,143,150-156], and this may mirror not only the different time points of the bone biopsy, but also that many studies rely on only one bone biopsy. In that context, early biopsies in the post-transplant period can only reflect abnormalities in the pre-transplant period[19].

Cueto-Manzano and co-workers[157] performed a bone-biopsy study in 1999 in 43 renal transplanted patients with at least 2 years of transplantation and stable renal function. They found a moderate increase in osteoclast function, as well as a retardation of mineral apposition and bone formation rates. They conclude that renal transplanted patients have a net bone loss, best predicted by age and male gender[157].

One year later, two contradictory studies looked for the abnormalities in bone in the post-transplantation period through biopsies. The first, by Munier-Faugere[158], performed bone biopsies from 6 months to 27 years post-transplant and described that bone abnormalities were in line with the time post-transplantation, mainly due to the negative impact of corticoid therapy in those patients. Those authors also described abnormal mineralization in 90% of cases and decreased turnover and volume in more than 45% and 50%, respectively. The second study, by Carlini and colleagues[151], was performed in 25 renal transplanted patients with a mean time of 7.5 years post-transplant. All parameters studied improved with time, and the more serious abnormalities presented sooner after transplantation, contrasting with the

previous report. In addition, most patients presented with high-turnover bone disease.

Understanding that turnover status before the transplant could dictate the evolution of TMV in the post-transplant period, Abdallah and colleagues[155] examined the natural course of 12 patients with adynamic bone disease proven by a bone biopsy and observed that, after 12 months, 9 out of 12 showed improvements in turnover and bone formation rate. The expected degree of bone mass loss was not achieved.

On the other hand, Borchhardt and co-workers[143] performed a bone biopsy study in 17 renal transplanted patients with hypercalcemic hyperparathyroidism (defined by calcium > 10.4 mg/dl, PTH > 85 pg/ml, eGFR > 30 mL/min). These authors showed either low or high-turnover bone disease among these 17 patients, despite hypercalcemia, proving that without a bone biopsy, it is impossible to guide the correct treatment. It must be noted that a PTH > 85 pg/mL is not that high.

These studies, although important, showed the limitations of not performing double bone-biopsy studies after renal transplantation. The first double bone-biopsy study dates back to 1991[159], and encompassed 20 patients biopsied at baseline and after a six-month period. The results showed normalization from high bone turnover, with a reduction in the bone formation rate, even so without low volume. In 2003, Rojas *et al* [153] biopsied 11 patients at baseline and two to three months after, demonstrating early apoptosis of osteoblasts and decreased osteoblastogenesis, with PTH being a protective factor for osteoblast survival.

A study from Portugal, published in 2015, was based on the findings of double bone biopsies in seven patients submitted to renal transplantation. The first biopsies were performed two months after engraftment while the second 24 months to five years after the first. Low-turnover bone disease emerged as a potential concern in this population[79].

In 2016, Evenepoel and co-workers[160] performed a double bone biopsy at baseline and after 12 months in 36 renal-transplanted patients. Half the patients presented with normal turnover; almost 45% showed low bone turnover disease and near 3% high turnover disease; 8% had low volume; abnormal mineralization was present in more

than 16%. Twelve months after, no volume or mineralization abnormalities were found, hypercalcemic hyperparathyroidism patients showed normal or low turnover disease and loss of trabecular bone was related to corticoid therapy cumulative dosage.

More recently, a study from Brazil with 32 bone biopsies at baseline and 31 after 12 months showed that kidney transplantation was associated with a decrease in bone formation, an improvement in cortical porosity and thickness, but no improvement in trabecular bone (even with worsened trabecular microarchitecture) with no significant alterations in mineralization, but mineralization defects increased[161].

A third prospective study from Finland enrolled 27 renal-transplanted patients submitted to a first bone biopsy 15 months before the transplant, while on dialysis, and a second bone biopsy after 2 years of transplantation[162]. In this study, a low-turnover disease replaced high-turnover disease in most patients (63% to 19% for the first and 26% to 52% for the second), with no major changes in bone volume and no demineralization. Further, DXA and bone turnover markers did not correlate with the histomorphometric findings.

These studies as a whole lead us to assume that high-turnover bone disease decreases and that adynamic bone disease is more frequent and accounts for a nearly of 50% of the pathology observed after renal transplantation[4]. Also, current treatment practices, such as steroid-sparing immunosuppression protocols along with supplementation of vitamin D, can enhance bone mass in transplant[161] in the central skeleton, with bone losses limited to peripheral sites[163], as a result of decreased bone formation[99]. It seems that the catabolic action of PTH is limited to cortical rather than trabecular bone[99]. Osteomalacia is uncommon, less than 5%, after renal transplantation[4].

Fractures

Bone loss, with osteopenia or osteoporosis, reflects the imbalance between bone formation and bone resorption post-transplantation, which increases the risk of fractures[164]. Diagnosis of osteoporosis in the renal transplant setting is challenging, because in some, if not the majority of transplanted patients, low bone volume is accompanied by other mineral bone disorders. Apart from bone loss, abnormal bone

quality, in terms of mineralization, cortical porosity and trabecular bone architecture seem to increase the risk of fractures. Nevertheless, the importance of impaired bone quality in the post-renal transplantation fracture risk is not well-defined[163].

Most studies assess bone loss after kidney transplantation, using DXA to measure bone mineral density. However, this technique gives no information on bone turnover and mineralization, for which we need a bone biopsy. Bone quality can be assessed by HR-pQCT at the distal radius and tibia, reporting the volumetric density of cortical and trabecular regions[99]. However, these techniques lack on giving information about bone turnover and mineralization, for which we need a bone biopsy.

It is postulated that fracture risk is four-fold higher in transplanted patients than the general population[19], and 34% higher than in dialysis patients in the first three to six months post-transplantation, and slowly decreases 1% each month thereafter[4]. It is estimated that 10 to 25% of renal transplanted patients will fracture over their follow-up[19,165]. Nevertheless, a recent meta-analysis failed to reach the same conclusions, based on the fact that the selected studies were heterogeneous[166]. A recent study from Belgium in 502 patients transplanted between 2006 and 2013 showed a fracture incidence of 14.2 fractures per 1000 person-years, with a median time to first fracture of 17 months[167]. The most relevant risk factors for fracture are diabetes and pancreas-kidney transplantation, BMI < 23, white race, age, female gender, immunosuppression (glucocorticoid dose and duration), abnormal PTH, and, probably, hypophosphatemia[4,82,148,164]. Furthermore, it is important to stress that the importance of impaired bone quality in the fracture risk post-renal transplantation is not well-defined[163].

The screening for fracture risk is possible through the FRAX, a tool developed by WHO in 2008, as stated previously. Unfortunately, this tool is not validated for patients younger than 40 years of age, and it is not validated for kidney-transplant recipients. Nevertheless, in 2014, Naylor and co-workers showed a positive prediction of fracture risk with FRAX in kidney-transplant recipients[168]. As in CKD patients, this tool doesn't adjust the risk for eGFR. As stated before, some investigators defend that the clinician should consider doubling the absolute fracture risk of fracture in patients with eGFR less than 45 mL/min[108,109]. TBS seems to help in predicting fractures after kidney

transplantation[169], and can be used to detect microarchitecture changes after renal transplantation[170].

VASCULAR CALCIFICATIONS

One of the pioneer studies into the impact of coronary artery calcification and mortality on renal transplanted recipients dates from 2010[171]. These authors studied 112 renal-transplanted patients with a median calcification score of 70.5 Agatston units, and no calcifications in 38 patients. They divided the population into two groups according to calcification score < or > 100 units, and found a difference in the mortality and cardiovascular event-free rates. Other studies followed[136], and the associations between Agatston score and cardiovascular event-free was maintained.

Another study performed in the transplant setting showed that bisphosphonate use was an independent risk for coronary artery calcification progression in kidney-transplanted patients[90].

It can be said that kidney transplantation slows, but does not halt, the progression of vascular calcifications[128], with some patients progressing their coronary artery calcification scores. In addition to traditional risk factors, dialysis vintage and secondary hyperparathyroidism seem to be non-traditional risks factors for this result.

It seems that not only the regression of a uremic milieu, but also CKD-MBD improvement has a positive influence on the calcification progression rate[128].

Nevertheless, to the best of our knowledge, there are no studies exploring the link between vitamin D deficiency or phosphorus levels or FGF23 levels or sclerostin levels and their potential role in vascular calcifications in renal-transplanted recipients. The relationship between vascular calcifications and metabolic bone disease is a field of interest, and the hypothesis of post-transplant bone loss and extra-osseous calcification in the transplant field remains to be proven.

Doubts

Diagnosis and correct treatment of MBD in post-transplant are required, as all subtypes of bone disease are believed to be linked to CV disease, morbidity and mortality, and the treatment is different depending on bone disease subtype. It would be important to have non-invasive tools for the diagnosis of the bone disease.

Furthermore, as this thesis states, the exact role of the new bone-derived hormones (FGF23 and sclerostin) and their association with bone biopsies and cardiovascular morbid-mortality in renal-transplanted patients is not known and is unexplored.

First, little is known about long-term MBD prevalence in kidney-transplanted patients (as double bone-biopsy studies in this population are lacking), and the effects of immunosuppression in this syndrome are still controversial; second, there are no studies correlating these new bone-derived markers with bone biopsy findings in transplantation; third, associations between FGF23 / sclerostin and vascular calcifications or cardiovascular events, taking into account ROD, in transplanted patients are absent. Further, in renal-transplanted recipients, with normal renal function, it has been proven that serum levels of FGF23 and sclerostin remain high. The pathophysiology of this event is not known, and how these osteocyte-derived hormones are regulated is still under debate. Calcitonin is gaining ground here[54,172]. With all these questions unresolved, no established clear and relevant therapeutic lines are recognized in MBD post-transplant, in order to improve bone health and to reduce CV morbidity and mortality.

Another important aspect is the cortical bone, which seems to be an important determinant of fractures, although not proven, and the evolution of cortical in terms of porosity or thickness in the post-transplant setting is not addressed.

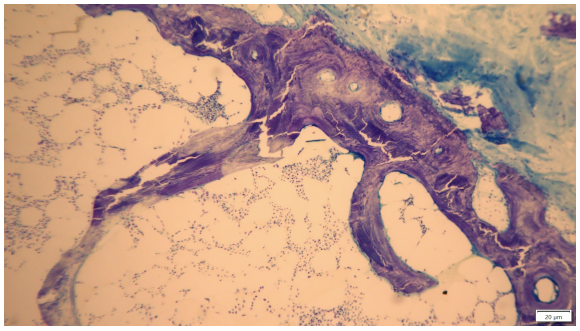


Figure 10 – Trabecularization of cortical bone

Our proposal aims to fill this knowledge gap, taking advantage of the fact that we are a reference invasive diagnostic European center in CKD-MBD. We aimed to characterize MBD phenotype pre- and post-renal transplantation, and to study the relationship of the new osteocyte-derived biomarkers of bone disease, FGF23 (and klotho) and sclerostin, with bone histologic changes, and CV events, taking into account the bone histology feature.

With this study, we have three primary aims:
1) To determine the prevalence, phenotype, and evolution of bone disease before and one year after transplantation;
2) To correlate FGF23 and klotho serum levels with bone histomorphometric parameters and CV disease;
3) To correlate serum sclerostin levels with bone histomorphometric parameters and CV disease.

The secondary aims:

- ✓ Correlations between bone biopsies, FGF23, and sclerostin *vs*. serum levels of PTH, calcitonin, alkaline phosphatase, BALP, 25-hydroxyvitamin D3, calcium, phosphorus, and magnesium;
- ✓ Subgroup analysis of the patients previously medicated with cinacalcet or vitamin D analogs or parathyroidectomised or even calcium doses regarding bone histologic features (turnover, mineralization, and volume);
- ✓ Correlation of bone histology and fractures and bone densitometry findings;
- ✓ Validation of Adragão vascular calcification score in a population of renal transplants;
- ✓ At the end of the study, and to learn the importance of transplantation in LVH, we choose a historical cohort, paired with a contemporary cohort in terms of age, gender, and dialysis vintage, and we compared the echocardiogram findings and evolution while on waiting list (historical cohort) *vs*. while submitted to renal transplantation (contemporary cohort). All patients included in the historical cohort have an ID number in order to ensure their anonymity.

PATIENTS AND METHODS

Study design and sample

This was a prospective observational cohort study of a convenience, consecutive sample on *de novo* renal-transplanted patients, admitted for isolated renal transplantation to our unit in Lisbon. *ClinicalTrials.gov* ID NCT02751099

RECRUITMENT OF THE SAMPLE STUDY

Considering the activity of kidney transplantation in our unit over the past years, prior to the beginning of this study (2013 n=55; 2014 n=53; 2015 n=62), and considering a 10% refusal, based on previous bone biopsy studies performed on end-stage CKD patients, we expected to perform a recruitment of 75 patients in a two-year time period.

CONSENT

The institutions' local ethics committees (from Nova Medical School and from Centro Hospitalar Universitário de Lisboa Central) approved this study and the National Committee for Data Protection approved the data collection and treatment.

Recruitment of patients was performed on the day the patient was called for transplant. The physician who called the patient was responsible for explaining the research project, and to ask for informed consent. The study characteristics were explained to the potential participants, and the patient information sheet was given to those who expressed an interest in the study. All patients could clarify doubts and all patients had at least 2 hours to decide whether to participate. Written consent was obtained from all participants prior to entering the protocol. Consent to take part in the study was recorded in each patient's notes and in the study records.

ELIGIBILITY CRITERIA

The exclusion criteria consisted of admission for double transplantation (liver-kidney or pancreas-kidney), age inferior to 18 or greater than 66 years old, patients with major cognitive impairment, inability to return for regular follow-up, and if patients did not undergo transplantation.

DATA COLLECTION

All patients included had an ID number in order to ensure their anonymity. The principal investigator was the person with the key between the ID number and name

of the patient. Because of the nature of the study, bias could occur. In order to avoid this, and regarding information bias (exposure or outcome misclassification), all the collected information was double-checked.

At inclusion, the following data were collected: age, gender, race, height, weight, CKD etiology, presence of diabetes, hypertension, hyperparathyroidism, hepatitis virus, ovulation status for woman (menopause or not), active medication pre-transplant and doses (with emphasis on vitamin D analogs, calcimimetics, and phosphate binders), history of parathyroidectomy, HLA phenotype and mismatches, donor data (age, gender, cause of death), and cold ischemia time. Evaluation of the last echocardiography performed in M mode and 2D, to assess left ventricular mass index (LVMI), calculated using the Devereux formula, indexed to body surface area, and to assess presence of valve calcifications was accomplished. Scoring of vascular calcifications through radiography of pelvis and hands by Teresa Adragão score[124] was made at inclusion. Laboratory analysis was performed before transplantation. A first bone biopsy was performed in the operating room, under general anesthesia, before engraftment.

Patients recruited were followed for a period of 1 year and the following data were recorded: immunosuppressive regimens and its cumulative doses, 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].

At the end of the first year, second laboratory analysis and bone biopsy were performed. A new echocardiogram and new scoring of vascular calcifications through radiography of the pelvis and hands were also performed. In addition, non-contrast computed tomography (CT) in a low-radiation exposure technique to characterize coronary artery calcification through Agatston score[173] and a dual-energy X-ray absorptiometry to measure bone mineral density were performed after 12 months of follow-up, within a week pre- or post-bone biopsy. DXA parameters included the evaluation of areal bone mineral density, Z-scores and T-scores of lumbar spine, femoral neck, and total femur.

Study Methods

As already mentioned, this study was designed to perform two bone biopsies on each patient (at day 0, in the operating room, under general anesthesia, and at nearly 12 months, using local anesthesia). At those two time points, biochemical analyses and radiography of pelvis and hands were performed. All patients who performed the second bone biopsy underwent a non-contrast CT in a low-radiation exposure technique, and coronary artery calcification was quantified using the Agatston method[173]. A DXA evaluation was also performed to measure areal bone mineral density, Z-scores, and T-scores at lumbar spine, femoral neck, and total femur. Osteoporosis was defined as a T score ≤ -2.5 in lumbar spine or femoral neck, or total femur, and osteopenia as between -1 and -2.4.

Biochemical analysis including routine hemogram, creatinine, urea, ionogram, uric acid, liver enzymes (AST; ALT), albumin, calcium, phosphorus, magnesium, and total alkaline phosphatase were performed using standard methods. Calcium serum levels were corrected for hypoalbuminemia (for each 1 g/dl for albumin decrease below 4 g/dl, 0.8 mg/dl will be added). Intact PTH levels were measured by immuno-chemiluminescence using a second-generation assay (Immulite 2000; Siemens Medical Solutions Diagnostics, Los Angeles, CA). Vitamin D [25(OH)D] levels were measured with RIA provided by IDS (Boldon, UK). BALP levels were measured using an enzyme immunoassay (EIA) utilizing a monoclonal anti-BALP antibody (MIcroVue BAP).

IMMUNOSUPPRESSION

Patients received induction immunosuppression with basiliximab or thymoglobulin, according to the immunologic risk. 500 mg of methylprednisolone was administered intraoperative and daily for two days, followed by maintenance of 20 mg of oral prednisolone (tapered through the year), tacrolimus (adjusted for levels of 8 – 12ng/mL for 3 months and 5 – 8 ng/mL thereafter) and mycophenolate mofetil (2g daily with dose adjustments and dose reduction through the year). In 5 patients, low doses of everolimus were added to low doses of tacrolimus, to minimize calcineurin inhibitors toxicity. Globally, patients were on steroid-based immunosuppression.

Aims

AIM 1: TO DETERMINE THE PREVALENCE, PHENOTYPE, AND EVOLUTION OF BONE HISTOLOGY BEFORE AND ONE YEAR AFTER TRANSPLANTATION

Transiliac bone biopsies were obtained from the anterior iliac crest using a 7G trocar (Osteobell T®), by manual puncture, in horizontal approach, under general anesthesia (1st biopsy) or with local anesthesia (2nd biopsy). In the case of the 2nd bone biopsy, tetracycline hydrochloride, 500mg, 12/12 hours, 3 days was given one month and one week before the biopsy.

Biopsy specimens of 4.5 mm in internal diameter by 1.0 – 1.5 cm in length were obtained and fixation in 70% alcohol followed by dehydration in 96% and 99.9% alcohol was performed. After the initial treatment, the specimens were cleared with xylene and embedded in methyl methacrylate. Serial decalcified 5 µm sections were obtained and stained with modified Masson-Goldner trichrome, Toluidine Blue, as well other routine staining: von Kossa (for osteoid evaluation), acid phosphatase (selective staining of osteoclasts), alkaline phosphatase (selective staining for osteoblasts), Perls (iron deposits) and solochrome azurine (aluminium deposits), for static histomorphometric parameters evaluation. Unstained 10 µm sections were prepared for fluorescent dynamic analysis in the 2nd biopsies. Each sample was composed of two cortices and a cylinder of trabecular bone.

Cortical bone was evaluated separately from trabecular bone. To characterize the former, cortical bone thickness and porosity were measured, and cortical porosity higher than 10% was considered abnormal, based on our findings (median values and interquartile range). This decision was made because of the missing reference values in the literature.

Trabecular bone was interpreted by bone remodeling and degree of cellular activation; efficacy of mineralization; quantification of possible metal deposits. We evaluated the trabecular bone for its volume (normal if bone volume/tissue volume – BV/TV ≥ 16%); bone remodeling and degree of cellular activation (normal if osteoblast surface/bone surface (ObS/BS) between 0.2 – 3.5%; and/or osteoclast surface/bone surface (OcS/BS) between 0.1 - 7.25%, plus analysis of bone formation rate (BFR) (normal if BFR/bone surface of 18-38 $\mu m^3/\mu m^2/year$[84,174,175] in the biopsies with dynamic evaluation) and mineral apposition rate; efficacy of mineralisation (abnormal if osteoid

thickness ≥ 12.5% plus no active osteoblasts in the mineralization front or mineralization lag time (MLT) > 100 days in the biopsies with dynamic evaluation)[176,177]. Osteoid volume/bone volume (OtV/BV) and osteoid surface/bone surface (OtS/BS) were obtained. In the second bone biopsy, mineralizing surface/bone surface (MS/BS), mineral apposition rate (MAR) and adjusted MAR (adjusted for OtS/BS), BFR/BS, and MLT were obtained. Bone histomorphometry was analyzed via a semi-automatic technique in the Osteomeasure software (Osteometrics, Atlanta, GA).

One observer evaluated all the bone biopsies. After this reading, the scores were classified into categorical variables, for clinical interpretation, according to TMV classification: T – low-turnover bone disease, high-turnover bone disease or normal turnover; M – mineralization defect or normal mineralization; V – low bone volume, normal bone volume, and high bone volume. Mixed lesions were identified if both high remodeling and abnormal mineralization were present. Renal osteodystrophy (ROD) was defined as abnormal turnover or mineralization. When static information was different from dynamic information, a mid-term of both was obtained after a second analysis of the bone specimens.

AIMS 2 AND 3: TO CORRELATE FGF23 AND KLOTHO SERUM LEVELS (AIM 2) AND SERUM SCLEROSTIN LEVELS (AIM 3) WITH BONE HISTOMORPHOMETRIC PARAMETERS AND CV DISEASE

We measured at the same time point as the bone biopsies [at day 0 (pre-transplant) and at nearly month 12] serum levels of FGF23, and its cofactor alpha-klotho, and sclerostin (and BALP), and correlated those levels with biopsy data on turnover, mineralization, and bone volume. For that propose, blood samples were collected: one 5mL tube containing ethylenediaminetetraacetic acid. (EDTA) for plasma and another 7mL tube, without anticoagulant, for serum. The blood samples were centrifuged at 3500 rpm for 10 minutes and stored at -80°C for further analysis in the immunology lab of CEDOC, Nova Medical School.

FGF23 was measured using a second-generation enzyme-linked immunosorbent assay (ELISA) kit that 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 two 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. The normal values for these hormones are given in Table 2.

	Normal values
PTH	14.8 to 83.1 pg/mL
Vitamin D	4.8 to 52.8 ng/mL
BALP	
Women premenopausal	11.6 to 29.6 U/L
Women postmenopausal	14.2 to 42.7 U/L
Men	15 – 41.3 U/L
Sclerostin	
Women premenopausal	0.45±0.15 ng/mL
Women postmenopausal	0.51±0.14 ng/mL
Men	0.59±0.13 ng/mL
FGF23	≤ 180 RU/mL
α-klotho	845±330 pg/mL

Table 2 – Normal values for the different bone-related measurements

Echocardiogram, performed in M mode and 2D, to assess both valve calcifications and LVH (calculated using Devereux formula, indexed to body surface area) was performed at the time of the 2nd bone biopsy, and was compared with the one performed pre-transplantation (all patients on the waiting list have to undergo echocardiography on a yearly basis). LVH was defined as a LVMI > 95 g/m^2 in women and >115 g/m^2 in men. At inclusion and on the same day as the 2nd bone biopsy, radiography of pelvis and hands was made for scoring of vascular calcifications by Teresa Adragão score[124]. Also, a non-contrast, low-radiation exposure technique, cardiac CT was performed after 12 months of transplantation to quantify coronary artery calcification score using the Agatston method[173], with the exception of three patients who underwent prior angioplasty. CV events recorded were analyzed and we studied possible correlations between those variables.

From these findings, we tried to discover potential new risk factors for vascular calcifications in renal transplant patients and potential pathophysiological mechanisms for this phenomenon.

SECONDARY AIMS:

- ✓ Correlations between bone biopsies, FGF23, and sclerostin vs. serum levels of PTH, calcitonin, alkaline phosphatase, BALP, 25-hydroxyvitamin D3, calcium, phosphorus, and magnesium;
- ✓ Subgroup analysis of patients previously medicated with cinacalcet or Vitamin D analogs or even calcium doses regarding bone histology features (turnover, mineralization, and volume);
- ✓ Correlation of bone histology and fractures;
- ✓ Correlation of bone histology and bone densitometry findings;
- ✓ Validation of Adragão vascular calcifications score in a population of renal transplants;
- ✓ At the end of the study, and in order to learn about the importance of transplantation in LVH, we chose a historical cohort, paired with the contemporary cohort in terms of age, gender, and dialysis vintage, and we compared the echocardiogram findings and evolution while on waiting list (historical cohort) vs. while submitted to renal transplantation (contemporary cohort). All patients included in the historical cohort had an ID number in order to ensure their anonymity. The principal investigator held the key between the ID number and name of the patient.

Timetable

	Day 0	3rd month	12 – 15 months
Bone biopsy	X		X
Objective examination*	X	X	X
Basic biochemical analysis**	X	X	X
Plain X-ray (hands and pelvis)	X		X
Echocardiography			X
Klotho | FGF23 | Sclerostin | bAP	X		X
Cardiac CT scan			X
Osteodensitometry			X

Table 3 – Timetable of the study

* - Weight, blood pressure. ** According to page 50

Statistical analysis

Continuous variables were expressed as median (interquartile range). Categorical variables were expressed as frequencies.

The prospective evaluation assessed the differences between bone biopsy data, bone-related biochemical parameters (such as FGF23, klotho, and sclerostin), vascular calcification score, and echocardiographic findings, using Wilcoxon matched-pair test (if comparing continuous variables) or paired McNemar's test (if comparing categorical variables).

At each time-point (baseline and 1-year after transplant) a cross-sectional analysis was performed, applying Mann-Whiney test, Fisher exact test, Kruskal-Wallis test or ordered logistic regression. Outcome variables were the bone histomorphometry (turnover, mineralization, volume) and cardiovascular abnormalities (Adragão score, Agatston score, presence of valve calcifications, and LVH). Predictors' variables were FGF23 and sclerostin, and other bone-related variables. Cut-off values for the outcomes studied were made based on ROC curves, using Youden's index.

Correlations between bone-related biochemical parameters at the two time-points were assessed with Spearman correlation test (in case of linear correlations) and comparisons of the low levels vs. high levels of the different bone-related biochemical variables was performed by Mann-Whitney test, as we divided the interest variable according to its median value or according to its clinical significance. Evaluation of serum levels of those parameters, demographic data, and bone densitometry data was assessed by Kruskal-Wallis rank test or Mann-Whitney test or Fisher exact test, depending on the demographic variable.

The score of vascular calcification was divided into two groups of severity and comparisons between the groups were performed using Fisher exact test or Mann-Whitney test. Presence / absence of valve calcifications were studied using Mann-Whitney test or Fisher exact test. A multivariate analysis (logistic regression analysis) was also performed to study independent predictors of vascular calcification severity or valve calcifications besides age and diabetes, including plausible predictor variables that revealed to be statistically related to the outcome in the univariate model.

The different degrees of severity of coronary calcifications were evaluated using ordered logistic regression, and multivariate ordered logistic regression was performed to detect possible risk factors for coronary artery calcification. In this analysis, the outcome variable was the three levels of severity of coronary calcification percentiles, and we included plausible predictor variables that had a $p \leq 0.1$ in the univariate model. The final model evaluated the relationship between high bone turnover and severity of coronary calcifications, adjusted for potential confounders that could theoretically interact with both turnover and calcifications (previous time on dialysis, estimated glomerular filtration rate by EPI, sclerostin baseline values, BALP 1-year after transplant, and bone volume at baseline).

Correlations between bone biopsy data (cortical bone or trabecular bone data) and demographic or laboratory data were obtained using Mann-Whitney test, Fisher exact test or Kruskal-Wallis rank test, or linear regression analysis, depending on outcome variable studied. Logistic regression for multivariate analysis was also performed to investigate the relationship between turnover and PTH or BALP, adjusting for potential confounders that could theoretically interact with both traditional serum markers and high or low turnover (as age, dialysis vintage, race, diabetes, phosphate, sclerostin, FGF23 or klotho). Logistic regression for multivariate analysis was performed to investigate the relationship between mineralization and phosphate, adjusting for plausible predictor variables that had a significant association with mineralization in the univariate model (age, BMI, and BALP).

A survival analysis to study mortality and CV events was performed using Cox proportional hazards model. We evaluated the relationship between high sclerostin serum levels and mortality, adjusted for potential confounders that could theoretically interact with both variables, such as age, gender and hypertension.

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

RESULTS

During the recruitment period (November 1st, 2015 and February 28th, 2018; 28 months) 151 patients were submitted to renal transplantation at our center. We excluded 50 patients, as patients would be submitted to a pancreas-kidney (37 patients) or liver-kidney (7 patients) transplant or were over 66 years old (6 patients). Of the remaining 101 patients, 14 refused to enter the study and 3 were not included due to logistic problems.

We included 84 patients at baseline and performed a bone biopsy in all. At the end of 12 months, 69 patients performed a second bone biopsy, as we had 15 patients who left the study: 6 refused to perform a 2nd biopsy, 5 had primary non-function of the kidney graft, and 4 patients died.

Of these numbers, we had 82 first bone biopsies, instead of 84, as 2 fragments didn't show bone tissue. One of these 2 patients did perform a second bone biopsy after 12 months (for clinical reasons). Flow chart of the study is presented in Figure 11.

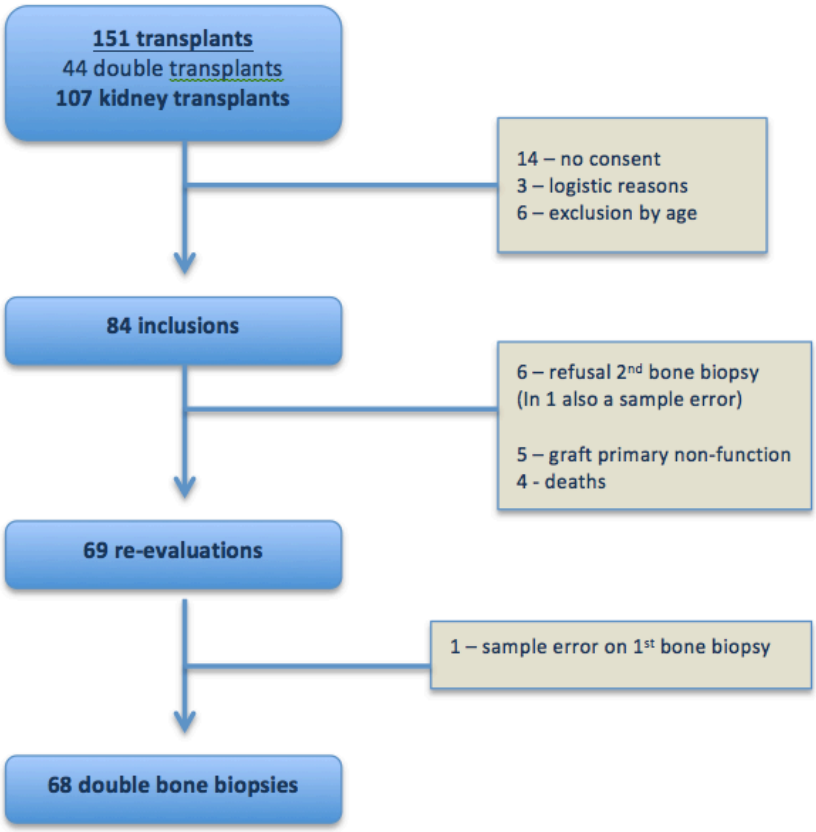


Figure 11 – Flow chart of the study

Time 0 study – ESRD patients

Eighty-four patients entered in the study. The demographic characterization of the population is shown in Table 4.

Demographic characterization	
Age (Years)	53.5 (40.5 – 61.5)
Gender M : F (n,[%])	59 (70.2) : 25 (29.8)
Caucasian race (n, [%])	65 (77.4)
PD (previous or current) : HD (n, [%])	11 (13.1) : 80 (95.2)
Dialysis vintage (months)	55.0 (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 (19.0)
Hypertensive nephrosclerosis	15 (17.9)
ADPKD	13 (15.5)
Diabetic nephropathy (type 1 & 2)	7 (8.3)
Alport disease	2 (2.4)
Glomerulonephrities	
Chronic glomerulonephritis	7 (8.3)
IgAN | 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)

Table 4 – Characterization of the population in baseline

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

This was a middle-aged population, mainly male and Caucasian patients. Of the 25 women, 15 were in menopause. The median dialysis vintage was nearly 5 years. The majority of patients were hypertensive, only 12% were diabetic; 70% had hyperparathyroidism, and 7 patients were submitted to a parathyroidectomy prior to the renal transplant. The median body mass index (BMI) was 24.4 (22.7 – 27.7) Kg/m^2.

Cardiovascular disease was present in 25% of the population. Most patients had an ischemic event [coronary (n=7), cerebrovascular (n=4), intestinal (n=1), retinal (n=1)], followed by 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).

At the time of the transplant, most patients (n=75, 89.3%) were prescribed bone-related pills, with 29% of the population under calcimimetic therapy, as Table 5 shows.

Medication prescribed	
Hypertension-related medication (n, [%])	59 (70.2)
ACEi | ARB II	22 (26.2)
Beta-blockers	44 (52.4)
Calcium channel blockers	28 (33.3)
Alpha-agonists	12 (14.3)
Bone-related medication (n, [%])	75 (89.3)
Phosphate binders	30 (35.7)
Cholecalciferol	26 (30.9)
Vitamin D analogs | Calcitriol	55 (65.5)
Calcimimetics	24 (28.6)
Intravenous iron (n, [%])	54 (64.3)
Erythropoiesis stimulants (n, [%])	68 (81)
Statins (n, [%])	34 (41.5)

Table 5 – Medication at time of transplant

ACEi – angiotensin-converting-enzyme inhibitors; ARB II – Angiotensin II receptor blockers

METABOLIC EVALUATION

The baseline laboratory results are shown in Tables 6 and 7.

The results found in the general laboratory evaluation at baseline were within the expected range, and median values were in accordance with KDIGO guidelines for anemia and CKD-MBD management. Most of the bone-related parameters were within the KDIGO-suggested values in terms of calcium, phosphorus, and PTH. 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 klotho had lower than normal values. Although median vitamin D levels were below 30 ng/mL, the lowest value we want to achieve, not all patients were supplemented with the native hormone. The patients supplemented with the native hormone (30.9% of the population) had a median value of 27.9 (19.8 – 38.1) ng/mL, which was higher than the values for those not supplemented [19.8 (11.5 – 30.4) ng/mL, p=0.008]. In our population, patients with insufficiency / deficiency of the hormone were not demographically different from patients with normal values of the hormone.

General laboratory evaluation at baseline	
Hemoglobin (g/dL)	11.6 (10.8 – 12.6)
Platelets (x1000/ µL)	210.0 (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)	139.0 (137.0 – 141.0)
Potassium (mEq/L)	5.0 (4.5 – 5.6)
Chloride (mEq/L)	101.0 (99.0 – 103.0)
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)

Table 6 – Laboratory evaluation at baseline

Median and IQ range is given.

Except for calcitonin, whose values were higher in male patients than in female (p<0.001), none of these bone-related parameters differed according to gender. BALP

tended to be lower (p=0.02) and FGF23 tended to be higher (p=0.02) in Caucasian persons than in other races. Older patients had higher levels of sclerostin (p<0.001). Patients taking cinacalcet had higher PTH values (821.0 vs. 450.0 pg/mL, p=0.005), higher FGF23 values (6617.8 vs. 3630.7 RU/mL, p=0.033), and lower sclerostin values (1.8 vs. 2.2 ng/mL, p=0.045). The only difference found in laboratory levels in patients submitted to parathyroidectomy was lower levels of PTH (368.6 vs. 528.7 pg/mL, p<0.001).

Bone-related laboratory evaluation at baseline	
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 – 853.0)
Sclerostin (ng/mL)	1.9 (1.2 – 2.8)

Table 7 – Laboratory evaluation of bone-related parameters at baseline

Median and IQ range is given.

PTH – parathyroid hormone; FGF23 – fibroblast growth factor 23

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.

IMAGING EXAMS EVALUATION

The imaging exams results (echocardiographic results and X-ray scores using radiography of hands and pelvis) are presented in Table 8. Adragão score was low in this population (median score of 1).

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 8 – Vascular and valve calcifications evaluation

LV – left ventricular

The majority of our patients presented a low score, as Table 9 shows.

Adragão score	
Score 0 (n, [%])	36 (43.4)
Score 1 (n, [%])	16 (19.3)
Score 2 (n, [%])	18 (21.7)
Score 3 (n, [%])	7 (8.4)
Score 4 (n, [%])	2 (2.4)
Score 5 (n, [%])	1 (1.2)
Score 6 (n, [%])	2 (2.4)
Score 8 (n, [%])	1 (1.2)

Table 9 – Vascular calcification by Adragão score

Analysing 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, as Table 10 shows. For that reason, and to better present and understand the results, we divided Adragão score into 2 groups (1st group – scores 0 and 1; 2nd group – scores ≥2).

	Adragão score			
	Score 0	Score 1	Score 2	Score 3
Age (years)	48.5	47.0	56.5	61.5
Male gender (%)	66.7	68.7	72.2	78.6
Dialysis vintage (months)	49.0	60.5	53.0	69.5
Diabetes (%)	5.6	0.0	22.2	28.6
BMI (Kg/m^2)	23.5	24.2	27.2	26.7

Table 10 – Baseline characteristics according to Adragão score

BMI – body mass index

HISTOLOGIC EVALUATION

From the 84 bone biopsies, we achieved 81 complete readings, as 2 fragments did not show bone tissue and 1 fragment only had cortical bone. ROD was present in many of our patients, specifically in 52 (64.2%), as 29 had normal turnover, volume, and mineralization. The median measured results of the bone biopsies are presented in Table 11.

In addition to histomorphometric indexes, we also looked at iron deposition [positive in 17 patients (20.5%)] and aluminium (3 patients, mild and old deposits). We transformed the measurements into categorical variables in terms of turnover, volume, and mineralization.

Histomorphometric bone parameters	
Cortical bone	
Porosity (%) >10% (n, [%])	7.8 (5.15 – 11.9) 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 (µm)	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)

Table 11 – Histomorphometric results of cortical and trabecular bone

Median and IQ range

Cortical bone

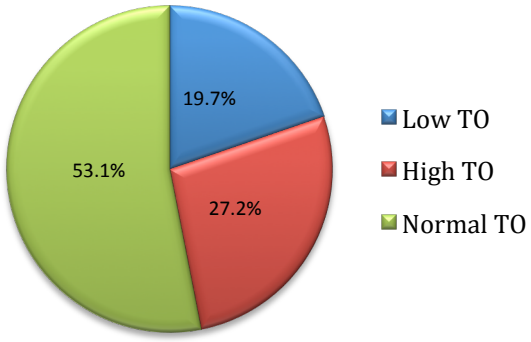
Thickness was not associated with any parameter of trabecular bone. Patients presenting with extreme PTH levels (< 150 pg/mL or > 800 pg/mL) had higher cortical thickness (869.3 µm vs. 698.5 µm, p=0.044), and this observation was also true for patients with hypercalcemia (p=0.016).

Trabecular bone

It is interesting to see that the 7 patients submitted to parathyroidectomy had lower levels of PTH (66.0 vs. 477.0 pg/mL, p<0.001), but no other differences regarding the other bone-related hormones or minerals, nor even regarding histomorphometric bone parameters, although there was a trend toward lower cellular activity.

- ✓ Remodeling / turnover

Most patients had normal remodeling parameters (n=43), and 38 had remodeling changes, as presented in Graphic 1. Nearly 20% of the population presented low bone turnover (n=16): low turnover with normal volume in 7 patients; low turnover and low volume (adynamic bone disease) in 8 patients; or as osteomalacia in 1. High bone turnover was presented in 22 patients, in the form of hyperparathyroid bone disease in 19 patients (twelve with normal volume; seven with low volume), and as mixed renal osteodystrophy in three patients.



Graphic 1 – Bone turnover at baseline

Comparing the three different remodeling categories, when applying Kruskal-Wallis rank test, 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). 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). Normal turnover was

associated with shorter dialysis vintage (p=0.009) and higher sclerostin levels (p=0.007). We found no differences regarding FGF23 or klotho levels, as demonstrated in Table 12. Concerning medication, the non-use of sevelamer was associated with normal turnover (p=0.018).

	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)	1075.0(528.6–3620.0)	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
Severe score VC (%)	50.0	35.7	36.4	0.590
Valve calcification (%)	37.5	12.5	31.8	0.072

Table 12 – Predictors of different bone turnover diseases

Statistical analysis: Kruskal-Wallis test (continuous variables) or Fisher exact test (categorical variables). 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; VC – vascular calcifications

Comparing low turnover with no-low turnover bone disease, in univariate analysis, age (p=0.002), diabetes (p=0.022), and PTH levels (p=0.009) were found to be

independent risk factors. In a multivariate analysis with those 3 parameters, 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 univariate analysis, age (p=0.019), menopause status (p=0.023), race (p=0.005), dialysis vintage (p=0.007), PTH (p<0.001), BALP (p<0.001), sclerostin (p<0.001), klotho (p=0.045), and phosphorus (p=0.028) had significant associations. 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 Table 13.

	High bone turnover		
	OR	95% CI	p-value
Age (years)	0.99	0.93 – 1.06	0.872
Dialysis vintage (months)	1.21*	1.02 – 1.43	0.026
Caucasian race (%)	0.17	0.03 – 0.89	0.034
BALP (U/L)	1.48**	1.02 – 2.15	0.038
Phosphate (mg/mL)	2.48	1.38 – 4.47	0.002
Sclerostin (ng/mL)	0.86***	0.78 – 0.96	0.006

Table 13 – Multivariate analysis for independent predictors of high bone turnover

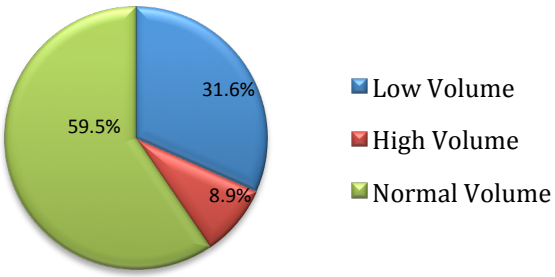
Statistical analysis: logistic regression test. BALP – bone alkaline phosphatase

(*For each increase in 10 months; ** for each increase in 10 units; *** for each decrease in 0.1 unit)

- ✓ Volume

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. 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.

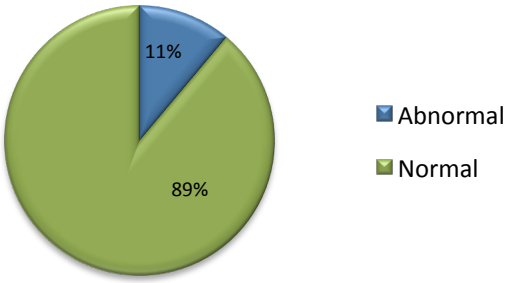
High bone volume was present in 7 patients. Those were younger (p=0.008) than those with normal or low bone volume. Volume categories are shown in Graphic 2.



Graphic 2 – Bone volume at baseline

- ✓ Mineralization

Mineralization was abnormal in nine patients, as shown in Graphic 3. Only one patient presented osteomalacia (low bone turnover and normal volume) and three patients presented mixed renal osteodystrophy (high bone turnover and normal volume). Five additional patients had abnormal mineralization with normal bone turnover and normal volume (n=4) or low volume (n=1).



Graphic 3 – Bone mineralization at baseline

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) vs. 31.7 (25.2 – 38.9), 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 Table 14 shows. Our cut-off values for this association were phosphate levels below 3.3 mg/dL, and BALP above 43.7 U/L.

	Mineralization defects		
	OR	95% CI	p-value
Age (years)	0.94	0.86 – 1.02	0.120
BMI (Kg/m^2)	1.39	0.98 – 1.96	0.062
BALP (U/L)	1.05	1.02 – 1.08	0.002
Phosphate (mg/dL)	0.25	0.08 – 0.79	0.018

Table 14 – Logistic regression for independent risk factors for abnormal mineralization

Statistical analysis: logistic regression test.

BMI – body mass index; BALP – bone alkaline phosphatase;

CARDIOVASCULAR ASSESSMENT

We looked for the associations between vascular calcifications and respective score, valve calcifications and LVMI, and bone-related biochemical parameters or bone-related histomorphometric parameters.

Vascular calcifications

In univariate analysis, both sclerostin and FGF23 related with the severity of Adragão score (p=0.006 and p=0.009), and were associated with the severity of vascular calcifications, in opposition to the other mineral-related parameters (such as PTH, BALP, Klotho, vitamin D, calcium, or magnesium) or bone histomorphometric parameters, as Table 15 shows.

Although not statistically significant, valve calcifications were present mostly in the group of patients with most severe vascular calcifications. Medication did not have an impact on vascular calcification score, although there was a trend toward calcium-based binders being associated with the score severity.

	Mild score of vascular calcifications (n=52)	Severe score of vascular calcifications (n=31)	p-value
Adragão score	0 (0 – 1)	2 (2 – 3)	-
Valve calcifications (%)	18.0	32.0	0.143
Male gender (%)	67.3	74.2	0.342
Age (years)	**47.5 (35.5 – 58.5)**	**60.0 (50.0 – 63.0)**	**0.001**
Caucasian race (%)	69.2	90.3	0.069
BMI (Kg/m^2)	**23.9 (22.1 – 25.5)**	**27.0 (23.9 – 29.0)**	**0.003**
Diabetes (%)	**3.8**	**25.8**	**0.005**
Hypertension (%)	84.6	90.3	0.350
Dialysis vintage (M)	55.0 (34.5 – 81.5)	57.0 (44.0 – 89.0)	0.127
HD with/out PD (%)	92.3	100.0	0.147
Only PD (%)	17.3	6.4	0.140
Parathyroidectomy (%)	5.8	12.9	0.232
Sclerostin (ng/mL)	**1.8 (1.2 – 2.6)**	**2.6 (1.7 – 2.9)**	**0.006**
FGF23 (RU/mL)	**999.5 (464.0 – 5020.0)**	**3295.6 (1497.0 – 11737.0)**	**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)	21.0 (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)	4.0 (3.1 – 4.9)	4.7 (3.5 – 5.6)	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

Table 15 – Severity of vascular calcifications and associated factors

Statistical analysis: Mann-Whitney test or Fisher exact test; BMI – body mass index; M – months; HD – hemodialysis; PD – peritoneal dialysis; FGF23 – fibroblast growth factor 23; PTH – parathyroid hormone; BALP – bone alkaline phosphatase; BV / TV – bone volume / tissue volume; TV – tissue volume; OcS / BS – osteoclast surface / bone surface; ObS / BS - osteoblast surface / bone surface.

In a multivariate model (logistic regression test), sclerostin loses significance; FGF23 (p=0.005) appears as an important independent factor for severity of vascular calcifications, namely values above 436.7 RU/mL, as does age (p=0.009), BMI (p=0.020), and presence of diabetes (p=0.011) (model: p<0.001; ROC curve 0.84), as shown in Table 16.

	Severity of Adragão Score		
	OR	95% CI	p-value
Age	2.06*	1.12 – 3.80	0.021
Diabetes	26.9	2.25 – 322.5	0.009
BMI	1.20	1.00 – 1.44	0.041
FGF23	1.17**	1.04 – 1.31	0.007
Sclerostin	1.37	0.69 – 2.73	0.365

Table 16 – Multivariate analysis for independent predictors of severity in Adragão score

Statistical analysis: logistic regression test. BMI – body mass index; FGF23 – fibroblast growth factor 23 (*For each increase in 10 years; ** for each increase in 1000 units)

LVMI or LVH

Concerning echocardiographic evaluation, we found no relationship between LVMI or presence of LVH and bone-derived data (laboratory and histologic). The only positive finding, although not statistically significant, was that LVMI tended to be higher with the highest values of FGF23, as shown in Table 17.

	1st tertile LVMI (n=21)	2nd tertile LVMI (n=18)	3rd tertile LVMI (n=19)
LVMI g/m^2	84.0±13.4	110.0.8±7	151.0±26.0
FGF23 RU/mL	5138.6	5112.3	6113.9

Table 17 – Left ventricular mass index and median FGF23 serum levels

Statistical analysis: Kruskal-Wallis test. LVMI – Left ventricular mass index; FGF23 – fibroblast growth factor 23

Valve calcifications

The presence of valve calcifications was studied as well, as shown in Table 18.

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 turnover was associated with absence of valve calcifications (p=0.020).

	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 (39.0 – 60.0)**	**62.0 (53.0 – 64.0)**	**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)	55.0 (3.09 – 82.0)	53.0 (43.0 – 84.0)	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 (%)	**20.0 (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

Table 18 – Valve calcifications and associated variables

Statistical analysis: Mann-Whiney test or Fisher exact test. BMI – body mass index; HD – hemodialysis; PD – peritoneal dialysis; 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

In multivariate analysis, adjusting for age and diabetes, the only independent variable associated with valve calcifications was adynamic bone [OR=7.9 (1.25 – 49.57), p=0.028]. If instead of adynamic bone disease, we introduce normal turnover, patients with a normal turnover have reduced odds of having the disorder (OR 0.3, p=0.041). In this case, diabetes also emerged associated with calcifications (OR of 6.6, p=0.016).

Follow-up assessment in CV events and mortality

We followed these patients over the first year after transplantation. 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 respectively, two further patients died (cardiovascular event and opportunistic infection). During this time period, three patients presented four cardiovascular events (acute myocardial infarction, congestive heart failure, and arrhythmia).

We found an association between CV events and FGF23, dividing the variable into thirds (p=0.031), as shown in Table 19.

	FGF23 thirds			
	1st 463.7	2nd 1716.3	3rd 8444	p-value
Adragão Score	0.5 (0.0 – 1.0)	1.0 (0.0 – 3.0)	1.5 (0.0 – 2.0)	0.153
CV events (%)	0.0	0.0	100.0	0.031

Table 19 – FGF23 and cardiovascular events

Statistical analysis: Kruskal-Wallis test. FGF23 – fibroblast growth factor 23; CV – cardiovascular.

Patients with cardiovascular events presented a trend toward higher levels of FGF23 (p=0.050), higher levels of magnesium (p=0.051), and longer dialysis vintage (p=0.063), as shown in Table 20.

Nevertheless, we found no predictor of cardiovascular event in a survival analysis.

	No CV event (n=80)	CV event (n=4)	p-value
Age (years)	53.5 (40.0 – 62.0)	67.0 (41.0 – 69.0)	0.453
Male gender (%)	71.6	100.0	0.379
Caucasian race (%)	77.0	100.0	1.000
BMI (Kg/m^2)	24.3 (22.7 – 27.6)	23.9 (22.7 – 25.9)	0.709
Diabetes (%)	12.2	0.0	0.685
Hypertension (%)	85.1	100.0	0.626
Dialysis vintage (months)	54.5 (42.0 – 82.0)	89.0 (83.0 – 98.0)	0.063
HD with/out PD (%)	94.6	100.0	0.850
PD (%)	13.5	0.0	0.655
Parathyroidectomy (%)	8.1	33.3	0.252
Valve calcifications (%)	23.9	0.0	0.451
Adragão score	0.0 (0.0 – 1.0)	1.0 (0.0 – 1.0)	0.253
PTH (pg/mL)	475.2 (279.3 – 800.1)	307.4 (66.3 – 403.6)	0.148
Klotho (pg/mL)	552.3 (351.0 –846.0)	571.0 (530.0 – 583.5)	0.950
FGF23 (RU/mL)	**1647.1 (561.2 – 6220.9)**	**8592.0 (6215.6 – 13417.4)**	**0.050**
Sclerostin (ng/mL)	1.9 (1.3 – 2.7)	2.9 (1.9 – 3.8)	0.148
BALP (U/L)	33.5 (24.6 – 46.2)	26.8 (25.8 – 30.0)	0.258
Vitamin D (ng/mL)	20.8 (15.0 – 34.1)	43.0 (20.4 – 66.8)	0.100
Magnesium (mg/dL)	**2.3 (2.0 – 2.5)**	**2.5 (2.4 – 4.3)**	**0.051**
Calcium (mg/dL)	9.3 (8.7 – 9.6)	10.0 (9.6 – 10.2)	0.075
Phosphate (mg/dL)	4.2 (3.3 – 5.1)	4.9 (4.0 – 5.1)	0.461
Cortical porosity (%)	7.8 (5.0 – 11.6)	4.8 (4.3 – 5.3)	0.194
BV/TV (%)	19.1 (15.7 – 24.5)	14.4 (9.1 – 20.7)	0.188
OcS/BS (%)	1.5 (0.3 – 2.8)	0.7 (0.0 – 1.1)	0.213
ObS/BS (%)	2.6 (0.8 – 5.2)	0.6 (0.0 – 7.8)	0.402

Table 20 – Characterization of the population according to CV events

Statistical analysis: Mann-Whiney test and Fisher exact test. BMI – body mass index; HD – hemodialysis; PD – peritoneal dialysis; PTH – parathyroid hormone; FGF23 – fibroblast growth factor 23; BALP – bone alkaline phosphatase; BV / TV – bone volume / tissue volume; OcS / BS – osteoclast surface / bone surface; ObS / BS – osteoblast surface / bone surface.

In a survival analysis, patients that died had significantly higher levels of sclerostin (HR=3.24, p=0.041), as Table 21 shows. This association maintained (HR 4.5, p=0.038) when adjusting for age, gender, and hypertension.

	Patient survival (n=80)	Death (n=4)	p-value
Age (years)	53.0 (40.0 – 61.0)	62.0 (53.5 – 65.0)	0.108
Male gender (%)	68.7	100.0	0.236
Caucasian race (%)	76.2	100.0	0.593
BMI (Kg/m^2)	24.3 (22.4 – 27.7)	26.2 (23.7 – 29.2)	0.401
Diabetes (%)	12.5	0.0	0.596
Hypertension (%)	86.2	100.0	0.564
Dialysis vintage (months)	55.0 (42.0 – 83.5)	70.0 (53.5 – 143.5)	0.219
HD with/out PD (%)	95.0	100.0	0.820
PD (%)	12.5	25.0	0.436
Parathyroidectomy (%)	7.5	25.0	0.299
Valve calcifications (%)	23.4	25.0	0.665
Adragão score	1.0 (0.0 – 2.0)	1.5 (0.5 – 4.0)	0.394
PTH (pg/mL)	468.2 (259.9 – 774.4)	269.7 (110.8 – 438.3)	0.180
Klotho (pg/mL)	564.0 (367.2 – 853.0)	493.9 (356.1 – 1232.1)	0.875
FGF23 (RU/mL)	1716.3 (609.8 – 6218.3)	1661.5 (442.3 – 8390.2)	0.737
Sclerostin (ng/mL)	**1.9 (1.2 – 2.7)**	**3.3 (2.4 – 3.8)**	**0.038**
BALP (U/L)	34.3 (26.6 – 46.5)	27.4 (21.6 – 42.2)	0.450
Vitamin D (ng/mL)	21.1 (15.3 – 34.0)	31.1 (19.1 – 44.1)	0.334
Magnesium (mg/dL)	2.3 (2.0 – 2.5)	2.5 (2.0 – 2.8)	0.401
Calcium (mg/dL)	9.3 (8.7 – 9.7)	9.2 (8.8 – 9.5)	0.760
Phosphate (mg/dL)	4.2 (3.3 – 5.1)	3.8 (2.8 – 4.7)	0.556
Cortical porosity (%)	7.8 (4.9 – 11.2)	13.0 (8.1 – 18.7)	0.123
BV/TV (%)	19.0 (15.0 – 24.0)	24.8 (15.5 – 28.3)	0.412
OcS/BS (%)	**1.5 (0.3 – 2.5)**	**0.1 (0.0 – 0.4)**	**0.059**
ObS/BS (%)	2.3 (0.8 – 5.5)	1.3 (0.3 – 3.7)	0.484
Adynamic bone disease (%	9.0	33.3	0.271
Abnormal mineralization (%)	10.3	33.3	0.301

Table 21 – Characterization of the population according to survival

Statistical analysis: Mann-Whiney test and Fisher exact test. BMI – body mass index; HD – hemodialysis; PD – peritoneal dialysis; PTH – parathyroid hormone; FGF23 – fibroblast growth factor 23; BALP – bone alkaline phosphatase; BV / TV – bone volume / tissue volume; OcS / BS – osteoclast surface / bone surface; ObS / BS – osteoblast surface / bone surface.

SUPPLEMENTARY DATA

Metabolic evaluation

We performed an exploratory data analysis of each bone-related variable by dividing each target variable into two groups according to its median levels (Group 1 – below the median value; Group 2 – above the median value) and looking for associations with other bone-related variables. The associations are shown in Tables 22.1; 22.2, and 22.3.

We obtained linear correlations with PTH and phosphorus (p=0.003), and PTH and FGF23 (p=0.004). Dividing PTH into two groups, associations were obtained with phosphorus, magnesium, FGF23, and klotho.

Related variables	Group 1	Group 2	p-value
	Calcium		
	8.7 (8.3 – 9.0)	9.6 (9.4 – 10.2)	
Phosphate (mg/dL)	4.2 (3.4 – 5.0)	4.1 (3.2 – 5.1)	0.811
Magnesium (mg/dL)	2.1 (2.0 – 2.5)	2.3 (2.1 – 2.5)	0.119
FGF23 (RU/mL)	**864.3 (463.9 – 2927.6)**	**3295.6 (1114.2 – 7487.0)**	**0.004**
Klotho (pg/mL)	637.0 (467.2 – 952.0)	530.0 (243.3 – 838.0)	0.144
PTH (pg/mL)	490.6 (240.5 – 800.1)	390.9 (232.3 – 696.1)	0.479
BALP (U/L)	35.4 (27.1 – 44.3)	31.3 (23.8 – 46.8)	0.549
Vitamin D (ng/mL)	20.2 (16.1 – 34.1)	21.1 (15.5 – 33.9)	0.910
Sclerostin (ng/mL)	1.9 (1.2 – 2.7)	2.1 (1.2 – 2.9)	0.493

Table 22.1 – Associations between bone-related variables

Statistical analysis: Mann-Whitney test. Median and IQ range values are presented. PTH – parathyroid hormone; FGF23 – fibroblast growth factor 23; BALP – bone alkaline phosphatase.

Sclerostin was inversely associated with PTH (p=0.036), meaning that the group with lower values of sclerostin had higher values of PTH; calcitonin groups were associated with significant different PTH levels (p=0.02).

We found no associations involving vitamin D.

Related variables	Group 1	Group 2	p-value
	PTH		
	237.5 (150.0 – 339.0)	742.3 (531.0 – 946.0)	
Phosphate (mg/dL)	**3.8 (2.9 – 4.7)**	**4.7 (3.5 – 5.6)**	**0.005**
Calcium (mg/dL)	9.4 (8.9 – 9.9)	9.1 (8.7 – 9.6)	0.268
Magnesium (mg/dL)	**2.1 (2.0 – 2.3)**	**2.3 (2.1 – 2.6)**	**0.037**
FGF23 (RU/mL)	**1162.0 (555.4 – 3854.8)**	**2987.0 (853.3 – 8807.6)**	**0.016**
Klotho (pg/mL)	**500.4 (334.7 – 760.0)**	**701.7 (467.2 – 1041.0)**	**0.045**
BALP (U/L)	31.7 (25.8 – 39.0)	35.8 (27.1 – 48.0)	0.263
Vitamin D (ng/mL)	21.0 (15.8 – 33.9)	21.1 (15.5 – 35.3)	0.876
Sclerostin (ng/mL)	2.1 (1.3 – 3.9)	1.8 (1.2 – 2.7)	0.229
	BALP		
	24.6 (20.1 – 27.9)	44.7 (35.9 – 61.8)	
Phosphate (mg/dL)	4.4 (3.3 – 5.1)	4.0 (3.3 – 5.1)	0.576
Calcium (mg/dL)	9.4 (8.7 – 9.6)	9.1 (8.7 – 9.9)	0.752
Magnesium (mg/dL)	2.3 (2.1 – 2.5)	2.1 (2.0 – 2.5)	0.064
FGF23 (RU/mL)	**2859.5 (864.3 – 14837)**	**1179.2 (463.9 – 3385.2)**	**0.011**
Klotho (pg/mL)	**530.0 (333.5 – 718.0)**	**703.5 (467.2 – 952.0)**	**0.059**
PTH (pg/mL)	403.6 (307.4 – 591.4)	490.0 (232.3 – 805.9)	0.328
Vitamin D (ng/mL)	20.0 (12.4 – 33.8)	23.0 (16.1 – 35.9)	0.284
Sclerostin (ng/mL)	2.2 (1.2 – 2.9)	1.9 (1.2 – 2.6)	0.141
	Sclerostin		
	1.2 (1.1 – 1.3)	2.7 (2.2 – 2.9)	
Calcium (mg/dL)	9.3 (8.8 – 9.4)	9.4 (8.7 – 9.9)	0.230
Phosphate (mg/dL)	4.0 (3.3 – 5.1)	4.2 (3.3 – 5.1)	0.892
Magnesium (mg/dL)	2.2 (2.1 – 2.5)	2.3 (2.0 – 2.5)	0.803
FGF23 (RU/mL)	1115.3 (552.0 – 6281.6)	2229.4 (798.4 – 6215.6)	0.312
Klotho (pg/mL)	637.0 (394.0 – 1147.0)	522.2 (351.0 – 809.7)	0.190
PTH (pg/mL)	**490.0 (339.0 – 940.0)**	**411.0 (193.6 – 646.8)**	**0.036**
BALP (U/L)	37.1 (26.8 – 61.8)	32.9 (25.8 – 38.7)	0.075
Vitamin D (ng/mL)	20.0 (13.2 – 29.9)	22.2 (16.7 – 38.1)	0.164

Table 22.2 – Associations between bone-related variables

Statistical analysis: Mann-Whitney test. Median and IQ range values are presented. PTH – parathyroid hormone; FGF23 – fibroblast growth factor 23; BALP – bone alkaline phosphatase.

Related variables	Group 1	Group 2	p-value
	Klotho		
	367.7 (201.7 – 479.8)	853.0 (731.0– 1381.0)	
Calcium (mg/dL)	9.3 (8.9 – 9.6)	9.3 (8.7 – 9.9)	0.569
Phosphate (mg/dL)	3.9 (3.2 – 5.0)	4.3 (3.4 – 5.2)	0.295
Magnesium (mg/dL)	2.3 (2.1 – 2.5)	2.2 (2.0 – 2.5)	0.914
FGF23 (RU/mL)	1728.3 (543.7 – 5245.3)	1679.7 (757.1 – 7215.6)	0.681
PTH (pg/mL)	374.7 (193.6 – 693.8)	482.4 (334.9 – 890.6)	0.100
BALP (U/L)	**32.2 (23.8 – 36.8)**	**38.7 (27.1 – 60.4)**	**0.009**
Vitamin D (ng/mL)	20.1 (16.7 – 33.8)	23.2 (15.0 – 34.1)	0.502
Sclerostin (ng/mL)	2.2 (1.3 – 2.9)	1.8 (1.2 – 2.6)	0.068
	FGF23		
	599.4 (417.3 – 1014.2)	6218.3 (3295.6 – 12000.0)	
Phosphate (mg/dL)	**3.6 (2.9 – 4.7)**	**4.8 (3.7 – 5.7)**	**0.001**
Calcium (mg/dL)	**9.0 (8.7 – 9.4)**	**9.5 (9.0 – 10.2)**	**0.004**
Magnesium (mg/dL)	**2.1 (2.0 – 2.4)**	**2.3 (2.1 – 2.5)**	**0.019**
Klotho (pg/mL)	586.2 (384.7 – 846.0)	554.1 (363.5 – 860.0)	0.792
PTH (pg/mL)	367.7 (209.4 – 653.2)	520.6 (307.4 – 822.6)	0.068
BALP (U/L)	**36.5 (27.3 – 52.5)**	**30.9 (20.7 – 39.0)**	**0.011**
Vitamin D (ng/mL)	23.2 (16.1 – 35.3)	20.1 (15.5 – 33.9)	0.378
Sclerostin (ng/mL)	**1.7 (1.2 – 2.2)**	**2.5 (1.3 – 2.9)**	**0.015**
	Phosphorus		
	3.2 (2.6 – 3.6)	5.0 (4.7 – 6.0)	
Calcium (mg/dL)	9.3 (8.9 – 9.9)	9.3 (8.7 – 9.5)	0.311
Magnesium (mg/dL)	**2.1 (2.0 – 2.3)**	**2.4 (2.2 – 2.7)**	**<0.001**
FGF23 (RU/mL)	**765.8 (428.9 – 3171.0)**	**3391.2 (1437.7 – 8191.6)**	**<0.001**
Klotho (pg/mL)	532.5 (366.0 – 785.9)	595 (367.2 – 979.0)	0.444
PTH (pg/mL)	**367.1 (202.8 – 587.7)**	**520.6 (322.1 – 901.8)**	**0.016**
BALP (U/L)	35.8 (27.2 – 49.9)	32.4 (25.2 – 44.7)	0.318
Vitamin D (ng/mL)	20.7 (16.1 – 36.4)	23.0 (12.3 – 34.0)	0.654
Sclerostin (ng/mL)	1.9 (1.2 – 2.7)	2.2 (1.3 – 2.9)	0.307

Table 22.3 – Associations between bone-related variables

Statistical analysis: Mann-Whitney test. Median and IQ range values are presented. PTH – parathyroid hormone; FGF23 – fibroblast growth factor 23; BALP – bone alkaline phosphatase.

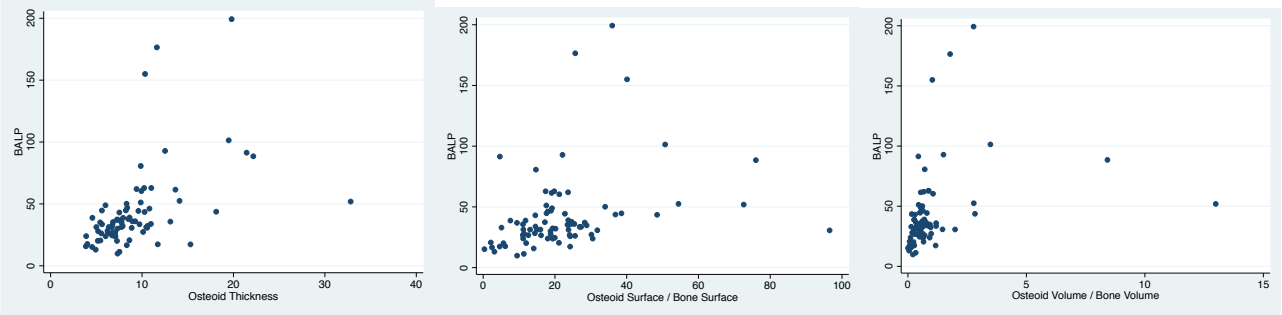
FGF23 groups had different phosphorus (p=0.001), calcium (p=0.004), magnesium (p=0.019), and sclerostin (p=0.015) levels, and were inversely associated with BALP (p=0.01), as in this case the group with higher values of FGF23 had lower values of BALP.

Klotho groups did not have a relation with FGF23 levels and had significant different BALP levels (p=0.009). BALP was associated with klotho (p=0.059), and inversely with FGF23 (p=0.011).

Histologic evaluation

Cortical bone

Patients with cortical porosity >10% were unlikely to be on PD (p=0.037) or on cinacalcet (p=0.035) and had higher levels of BALP (37.4 U/L vs. 31.3 U/L, p=0.007). Cortical porosity >10% was associated with osteoid volume / bone volume in trabecular bone (4.3 % vs. 2.5%, p=0.021), but we noticed that the group of patients with the highest levels of BALP had the highest % of osteoid surface / bone surface (r=0.3, p=0.004) and osteoid thickness (r=0.5, p<0.001), or even osteoid volume / bone volume or per tissue volume (r=0.3, p=0.001), as shown below in Graphic 4. Curiously, patients with extreme PTH levels (< 150 pg/mL or > 800 pg/mL) presented higher cortical porosity (9% vs. 6.8%, p=0.017).

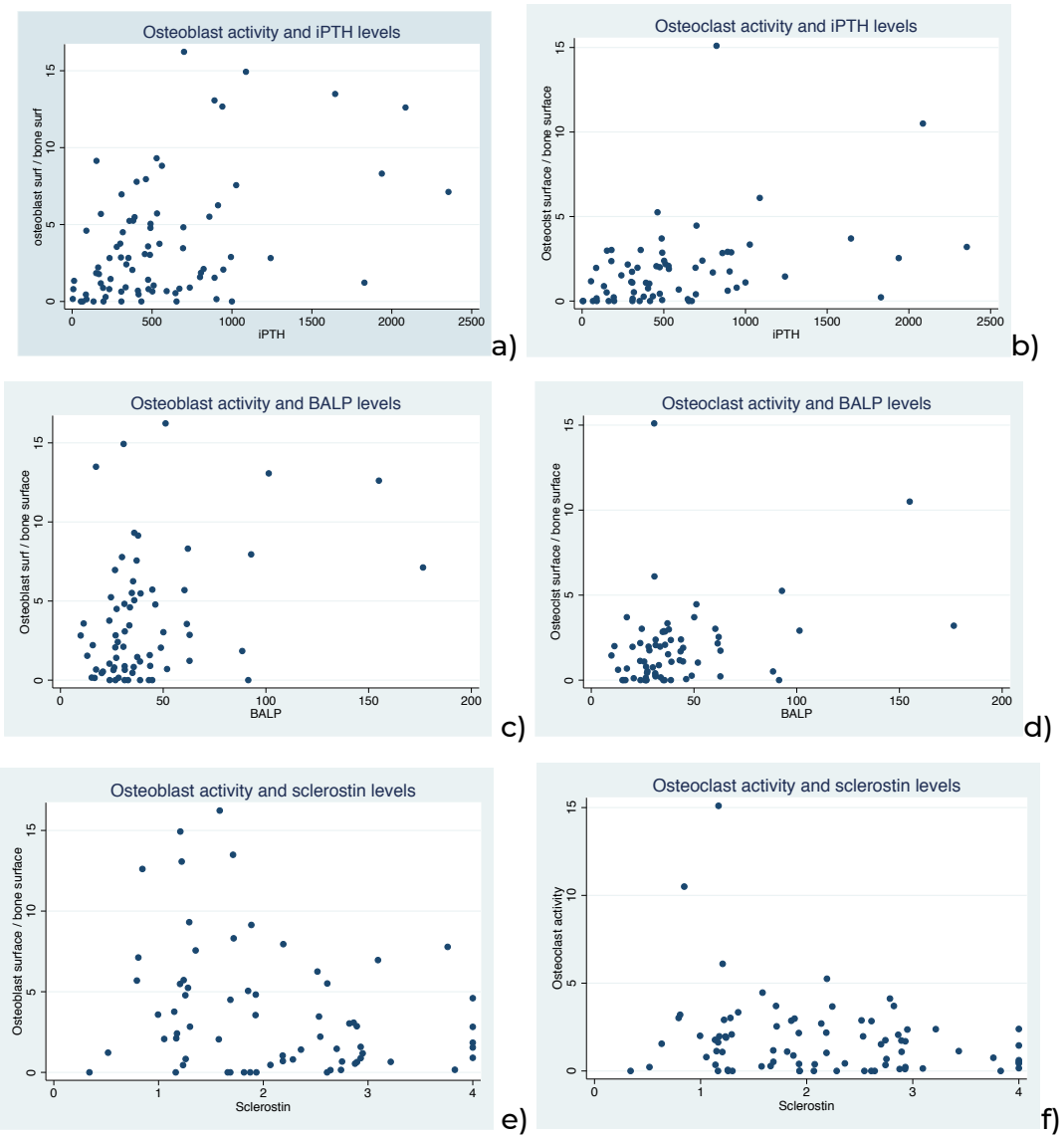


Graphic 4 – Correlations between BALP values and osteoid measurements in bone biopsies

Trabecular bone

We also analyzed which biochemical factors could be related with osteoblast or osteoclast activity. We found that PTH (both osteoblast and osteoclast surface: r=0.4, p<0.001), BALP (osteoblast surface: r=0.4, p=0.002; osteoclast surface: r=0.2, p=0.034)

and phosphorus (osteoblast surface: r=0.3, p=0.020; osteoclast surface: r=0.2, p=0.025) were correlated with both activities. An inverse/ negative correlation was found with sclerostin (osteoblast surface: r=-0.3, p=0.019; osteoclast surface: r=-0.2, p=0.039). Osteoblast activity was also correlated with klotho levels (r=0.3, p=0.016); osteoclast activity was also negatively correlated with calcium levels (r=-0.3, p=0.017). A summary is shown in Graphic 5.



Graphic 5 – Correlations between osteoclast and osteoblast surfaces (per bone surface) and bone-derived hormones:

a) Osteoblast surface and PTH; b) Osteoclast surface and PTH; c) Osteoblast surface and BALP; d) Osteoclast surface and BALP; e) Osteoblast surface and sclerostin; f) Osteoclast surface and sclerostin

Evolution of laboratory parameters at 3 months of follow-up

After 3 months, most laboratory parameters were already different: hemoglobin levels rose, as did sodium, chloride, and calcium levels. As expected, urea, creatinine, potassium, phosphorus, PTH, total alkaline phosphatase and calcitonin decreased. This information is shown in Tables 23 and 24.

General laboratory evaluation at baseline and after 3 months			
	Baseline	**3 months**	**p-value**
Hemoglobin (g/dL)	11.6 (10.8 – 12.6)	12.3 (11.7 – 13.7)	<0.001
Platelets (x1000/ µL)	210.0 (181.5 – 258.5)	205.0 (171.0 – 254.0)	0.449
Urea (mg/dL)	101.0 (65.5 – 133.5)	62.0 (49.0 – 78.0)	<0.001
Creatinine (mg/dL)	8.1 (5.7 – 10.7)	1.5 (1.2 – 1.8)	<0.001
Sodium (mEq/L)	139.0 (137.0 – 141.0)	141.0 (140.0 – 142.0)	<0.001
Potassium (mEq/L)	5.0 (4.5 – 5.6)	4.5 (4.2 – 4.9)	0.003
Chloride (mEq/L)	101.0 (99.0 – 103.0)	107.0 (104.0 – 109.0)	<0.001

Table 23 – Laboratory evaluation at baseline and after 3 months

Statistical analysis: Wilcoxon matched-pairs test. Median and IQ range values are given.

Bone-related laboratory evaluation at baseline and after 3 months			
	Baseline	**3 months**	**p-value**
Alkaline phosphatase (U/L)	83.0 (62.0 – 109.5)	60.0 (53.0 – 81.0)	<0.001
Calcium (mg/dL)	9.3 (8.7 – 9.6)	9.8 (9.3 – 10.3)	<0.001
Phosphorus (mg/dL)	4.1 (3.3 – 5.1)	2.9 (2.3 – 3.6)	<0.001
Magnesium (mg/dL)	2.3 (2.0 – 2.5)	1.6 (1.5 – 1.8)	<0.001
Calcitonin (ng/dL)	3.4 (2.0– 10.7)	2.0 (2.0 – 3.0)	<0.001
Vitamin D (ng/mL)	21.0 (15.7 – 34.1)	17.3 (13.2 – 21.7)	0.002
PTH (pg/mL)	458.1(237.3– 742.4)	153.2(103.4 – 255.2)	<0.001

Table 24 – Bone-related laboratory evaluation at baseline and after 3 months

Statistical analysis: Wilcoxon matched-pairs test. Median and IQ range values are given. PTH – parathyroid hormone.

Interestingly, levels of magnesium and vitamin D also decreased, as shown in Table 24.

Comparisons before transplantation and 1-year after transplantation: the evolution

At the end of the 12 months, we had 69 patients that agreed to continue in the study, as demonstrated in Figure 10. Demographic characterization is provided in Table 25.

Demographic characterization	
Age (years)	53.0 (41.0 – 62.0)
Gender M : F (n, [%])	48 (69.6) : 21 (30.4)
Caucasian race (n, [%])	53 (76.8)
BMI at transplant (Kg/m^2)	24.5 (22.7 – 27.8)
eGFR by CKD-EPI 1-year after transplant	53.0 (37.3 – 69.0) mL/min/1.73m^2
PD (previous or current) : HD (n, [%])	9 (13.0) : 65 (94.2)
Dialysis vintage (months – median, IQ range)	55.0 (42.0 – 84.0)
Hypertension at transplant (n, [%])	59 (85.5)
Diabetes | PTDM (n, [%])	9 (13.0) | 11 (15.9)
Hyperparathyroidism at transplant (n, [%])	50 (72.5)
Parathyroidectomy prior to transplant (n, %)	6 (8.7)
HIV, HBV, HCV (n, [%])	2 (2.9) : 0 : 3 (4.3)
Living kidney donor (n, [%])	10.1
Etiology of renal disease (n, [%])	
Unknown	13 (18.8)
Hypertensive nephrosclerosis	11 (15.9)
ADPKD	11 (15.9)
Diabetic nephropathy (type 1 & 2)	6 (8.9)
Alport disease	2 (2.9)
Glomerulonephritis	
Chronic glomerulonephritis	5 (7.2)
IgAN | Mensangial proliferation	6 (8.8) | 1 (1.4)
HIVAN	1 (1.4)
FSGS	1 (1.4)
Membranous nephropathy	2 (2.9)
Lupus nephritis	1 (1.4)
Vasculitis	
Pauci-immune | Goodpasture	2 (2.9) | 1 (1.4)
Lithiasis	3 (4.4)
CAKUT	3 (4.4)

Table 25 – Demographic and relevant medical history of the population

IQ range – interquartile range; M – male; F – female; BMI – body mass index; eGFR – estimated glomerular filtration rate; PD – peritoneal dialysis; HD – hemodialysis; PTDM – post-transplant diabetes mellitus; HIV – human immunodeficiency virus; HBV – hepatitis B virus; HCV – hepatitis C virus; ADPKD – autosomal polycystic kidney disease; IgAN – IgA nephropathy; HIVAN – HIV-associated nephropathy; FSGS – focal segmental glomerulosclerosis; CAKUT – congenital anomalies of the kidney and urinary tract.

These 69 patients were middle-aged, mostly Caucasian and male, with a median dialysis vintage of almost 5 years. Six patients underwent parathyroidectomy prior to the transplantation. The median BMI was 24.6 (22.0 – 27.8). The median prednisolone cumulative dose was 3580.0 (3257.5 – 4072.5) mg, but the median cumulative steroid dose (prednisolone + methylprednisolone) was 5692.5 (5260.0 – 7250.0) mg.

Five patients were treated with low doses of everolimus and low doses of tacrolimus, in an attempt to minimize calcineurin inhibitors toxicity. None of the patients had been prescribed anti-osteoporotic drugs during the post-transplant period. Of the 16 patients who were on cinacalcet, seven retained the drug post transplantation (10.1%). Cholecalciferol supplementation was implemented in 24 patients (34.8%), and calcitriol in 7 patients (10.1%).

In addition to repeating the evaluations performed at baseline (bone biopsy, laboratory evaluation, echocardiographic evaluation, and vascular calcifications characterization by Adragão score), those patients were also submitted to a bone densitometry and CT of coronary arteries.

METABOLIC EVALUATION

Tables 26 and 27 show the differences in the 69 patients pre-transplant and 1-year after renal transplantation.

Starting with laboratory evaluation, as expected, we found a rise in hemoglobin (slightly more than observed at the end of the 3rd month), and in uric acid values, and a decrease in levels of urea, creatinine, sodium, potassium, and chloride. Total alkaline phosphatase, after an initial decrease in the first 3 months, rose again and was not different from baseline. Albumin was similar at the two time points. Calcium and alpha-klotho rose significantly, and phosphorus, magnesium, calcitonin, PTH, BALP, FGF23, and sclerostin decreased significantly.

General laboratory evaluation at baseline and 1-year after transplantation			
	Baseline	**12 months**	**p-value**
Hemoglobin (g/dL)	**11.5 (10.9 – 12.6)**	**12.9 (12.2 – 14.3)**	**<0.001**
Platelets (x1000/ µL)	206.0 (181.0 – 255.0)	228.0 (176.0 – 256)	0.605
Glucose (mg/dL)	88.0 (79.0 – 102.0)	92.0 (81.0 – 103.0)	0.248
Urea (mg/dL)	**104.0 (66.0 – 138.0)**	**60.0 (44.0 – 78.0)**	**<0.001**
Creatinine (mg/dL)	**8.2 (5.7 – 10.6)**	**1.4 (1.1 – 1.8)**	**<0.001**
Uric acid (mg/dL)	**5.1 (3.5 – 7.0)**	**6.4 (5.6 – 7.1)**	**<0.001**
Sodium (mEq/L)	**139.0 (137.0 – 141.0)**	**141.0 (140.0 – 142.0)**	**<0.001**
Potassium (mEq/L)	**5.0 (4.5 – 5.6)**	**4.5 (4.2 – 4.9)**	**<0.001**
Chloride (mEq/L)	**101.0 (99.0 – 103.0)**	**106.0 (105.0– 108.0)**	**<0.001**
ALP (U/L)	83.0 (61.0 – 103.0)	78.0 (57.0 – 119.0)	0.859
Albumin (g/dL)	4.2 (4.0 – 4.5)	4.3 (4.1 – 4.5)	0.509
T. cholesterol (mg/dL)	187.0 (154.0 – 219.0)	181.0 (159.0– 212.0)	0.601

Table 26 – Laboratory evaluation at baseline and 1 year after transplantation

Statistical analysis: Wilcoxon matched-pairs test. Median and IQ range values are given. ALP – alkaline phosphatase; T. cholesterol – total cholesterol

The majority of patients (79.7%) had low levels of vitamin D (<30 ng/mL). Vitamin D levels were similar at both time points and irrespective of oral supplementation with cholecalciferol, but the 5 patients under a calcineurin inhibitor minimization scheme had higher levels of this hormone (p=0.025). We found that patients under cholecalciferol supplementation had the highest levels of klotho (70.8% of patients under supplementation were within the higher levels of klotho vs. 40% of patients with no supplementation, p=0.014). Klotho levels had no association with vitamin D levels.

Likewise, patients with the highest levels of klotho (p=0.006) and PTH (p=0.006) were on cinacalcet (7 patients). Cumulative steroid dose was different in the FGF23 groups: the group with the higher levels of the hormone had a higher cumulative dose [6885 mg (5332.5 – 7557.5), vs. 5313.7 mg (5060 – 6457.5), p=0.002]. No other relation between cumulative steroid dose and serum bone-related parameters were observed.

Bone-related laboratory evaluation at baseline and 1-year after transplantation			
	Baseline	**12 months**	**p-value**
Calcium (mg/dL)	**9.3 (8.7 – 9.6)**	**9.8 (9.3 – 10.4)**	**<0.001**
Phosphorus (mg/dL)	**4.2 (3.3 – 5.1)**	**3.1 (2.8 – 3.5)**	**<0.001**
Magnesium (mg/dL)	**2.3 (2.1 – 2.5)**	**1.7 (1.6 – 1.8)**	**<0.001**
Calcitonin (ng/dL)	**3.3 (2.0 – 10.5)**	**2.0 (2.0 – 4.9)**	**<0.001**
Vitamin D (ng/mL) Hypovitaminosis D	20.2 (15.0 – 30.4) 73.9%	22.5 (14.3 – 29.0) 79.7%	0.881 0.371
PTH (pg/mL)	**475.0 (301.0 – 748.7)**	**135.0 (90.1 – 232.7)**	**<0.001**
BALP (U/L)	**33.8 (26.7 – 44.7)**	**23.0 (17.2 – 35.2)**	**0.001**
FGF23 (RU/mL)	**1806.5 (613.7 – 6281.6)**	**135.2 (101.1 – 168.5)**	**<0.001**
Klotho (pg/mL)	**571.0 (363.5 – 846.0)**	**945.2 (485.0 – 2044.2)**	**<0.001**
Sclerostin (ng/mL)	**1.9 (1.3 – 2.7)**	**0.7 (0.5 – 1.0)**	**<0.001**

Table 27 – Bone-related laboratory evaluation at baseline and 1 year after transplantation

Statistical analysis: Wilcoxon matched-pairs test or paired McNemar's test. Median and IQ range values are given.

It is interesting to note that in all these 69 patients, both sclerostin and FGF23 serum levels had decreased, with a median percentage reduction of 62.0% and 91.1% [delta value of -1.1 (-1.7 to -0.7) and -1656.5 (-6156.6 to -537.7)] respectively. Nevertheless, PTH, BALP and klotho expressed some behaviour variability, as shown in Table 28.

Variables	% of variability	Median Delta value
Sclerostin (ng/mL)	100% decreased values	-1.1 (-1.7 to -0.7)
FGF23 (RU/mL)	100% decreased values	-1656.5 (-6156.6 to -537.7)
PTH (pg/mL)	89.9% decreased values	-373.6 (-631.4 to -159.4)
BALP (U/L)	68.1% decreased values	-17.6 (-27.8 to -9.3)
Klotho (pg/mL)	65.2% increased values	992.4 (279.8 to 1932.2)

Table 28 – Variability and delta values of some bone-related parameters

The median percentage reduction in PTH was 70.5%, which contrasted with the median percentage increase of 11.2% of the patients in whom PTH did not decrease (10.1% of the population, 7 patients). Some exploratory data can be given: the three

exceptions for the decrease in PTH levels were dialysis vintage (median of 83 months in those without decreased PTH values vs. median of 54 months in those whose PTH levels had decreased, p=0.037), parathyroidectomy before transplantation (42.9% vs. 4.8%, p=0.012), and low PTH levels before transplantation (the 7 patients had PTH median levels of 66.3 pg/mL vs. 488.5 pg/mL, p<0.001), which remained an important factor after excluding the 3 parathyroidectomized patients. Having no reduction in PTH levels after the transplant had no impact on bone histomorphometry, but 42.9% of those patients (3 of the 7 patients) had higher Agatston calcification scores than patients whose PTH levels had decreased; even so, this was without statistical significance (p=0.078).

In 22 (31.9%) of our patients, BALP levels did not decrease. These patients had a median percentage increase of 31.4%, which was also in contrast with the median percentage reduction of 46.8% in patients whose BALP levels did decrease. Patients whose BALP levels did not decrease had lower BALP levels at baseline (26.2 U/L vs. 35.9U/L, p<0.001), higher calcium levels at baseline and after transplantation (9.6 mg/dL vs. 9.1 mg/dL at baseline; 10.2 mg/dL vs. 9.6 mg/dL for T1 levels, p=0.020), higher FGF23 serum levels (6884.3 RU/mL vs. 1497.5 RU/mL, p<0.001), as well as lower klotho levels (492.9 pg/mL vs. 703.5 pg/mL, p=0.036), both at baseline. Contrary to PTH, the maintenance of high levels of BALP had influence on bone activity. Considering turnover, patients whose BALP levels did not decrease did not have as many cases of low bone-turnover after transplantation (27.3.%) as those whose BALP levels decreased (55.3%, p=0.027). This was in line with the association found between the absence of decreasing levels and ObS/BS (2.8% vs. 1.7%, p=0.044) and OcS/BS (0.7% vs. 0.3%, p=0.037), BFR/BS (27.9 $\mu m^3/\mu m^2$/day vs. 14.7 $\mu m^3/\mu m^2$/day, p=0.034) and BFR/TV (12.6 vs. 3.9, p=0.033). Although it did not associate with presence / absence of abnormal mineralization as a yes or no question, it did associate with OtV/BV (4.6% vs. 3.9%, p=0.034) and osteoid thickness (12.5 μm vs. 8.7 μm, p=0.004), and mineralization surface (8.2 vs. 2.4, p=0.011). We did not find any association with vascular calcifications.

Also, in 24 (34.8%) patients klotho levels did not increase. These patients had higher levels of klotho at baseline (844.0 pg/mL vs. 494.7 pg/mL, p<0.001) and lower levels of calcium at baseline (8.8 mg/dL vs. 9.4 mg/dL, p=0.002), without any other characteristic features. This observation did not associate with bone histology or

vascular calcifications. The percentage reduction was 47.2% (contrasting with the percentage increase of 188.5% in those whose alpha-klotho levels did increase).

HISTOLOGIC EVALUATION

From the 69 double bone biopsies, we achieved 137 complete readings, as 1 fragment in the 1st biopsy showed no bone tissue; nonetheless that patient did perform a second bone biopsy after 12 months, for clinical reasons. In the second bone biopsies, it was possible to analyze the dynamic parameters of the trabecular bone, as shown in Table 29.

Histomorphometric bone parameters			
	Baseline	**12 months**	**p-value**
Cortical bone			
Porosity (%)	7.4 (4.9 – 10.6)	5.9 (3.8 – 9.8)	0.094
Porosity >10% (n, [%])	23 (33.3)	18 (26.1)	0.297
Thickness (µm)	**737.9 (552.7– 973.9)**	**629.2 (403.5– 849.2)**	**0.006**
Trabecular bone			
Bone volume / Tissue volume (%)	18.8 (14.3 – 24.3)	19.3 (15.8 – 24.8)	0.339
Osteoid surface / Bone volume (%)	3.2 (1.7 – 4.9)	4.2 (2 – 5.8)	0.660
Osteoid thickness (µm)	**7.8 (6.7 – 10.3)**	**9.1 (6.8 – 12.6)**	**0.005**
Osteoid volume / Bone volume (%)	3.2 (1.7 – 4.9)	4.2 (2.0 – 5.8)	0.261
Mineralized bone volume / TV (%)	18.3 (13.7 – 23.0)	18.4 (14.7 – 23.6)	0.389
Osteoblast surface / BS (%)	**2.3 (0.7 – 5.5)**	**1.9 (1.1 – 3.2)**	**0.030**
Osteoclast surface / BS (%)	**1.3 (0.2 – 2.5)**	**0.4 (0 – 0.9)**	**<0.001**
Adj mineral apposition rate (µm/d)	-	0.3 (0.1 – 0.4)	-
Bone formation rate (μm^3 /μm^2 /year)	-	21.4 (4.7 – 32.2)	-
Mineralisation lag time (days)	-	40.3 (25.5 – 85.0)	-

Table 29 – Static and dynamic parameters of the bone biopsies

Statistical analysis: Wilcoxon matched-pairs test or symmetry test. Median and IQ range values are given. TV – tissue volume; BS – bone surface; Adj mineral apposition rate - Adjusted mineral apposition rate
Normal values: cortical porosity < 10%; BV/TV ≥ 16%; ObS/BS of 0.2% to 3.5% OcS/BS of 0.1% to 7.25%; BFR/BS of 18-38 $\mu m^3/\mu m^2$/year; osteoid thickness < 12.5%; MLT < 100 days.

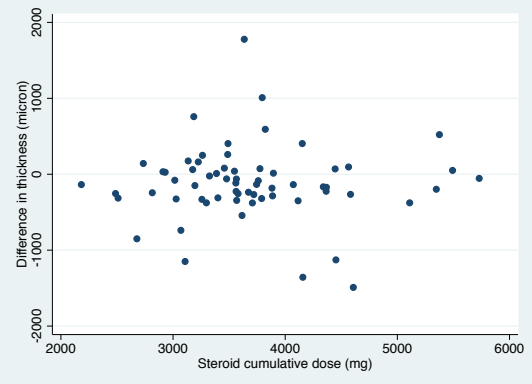
In the second bone biopsy, positive Perls staining was found in 23% of the fragments (16 bone biopsies), and aluminium in two bone fragments (2.9%). ROD was present in many of our patients, specifically in 47 (68.1%), with no differences compared to

baseline (p=0.084; McNemar's test for symmetry). Overall, we found that histological findings improved, as patients with high bone turnover, low bone volume, abnormal mineralization, or bone porosity >10% decreased. In the first bone biopsy, 21 patients (30.9%) had a normal biopsy, and this number rose to 22 patients (31.9%) in the second biopsy.

Contrary to what is written in the literature, we observed no loss of bone volume, even finding a slight increase. We observed a significant reduction in both osteoblast and osteoclast activity. Osteoid thickness increased after 12 months of transplantation.

Cortical bone

In our patients, cortical thickness decreased 1-year after transplant, with no correlation with cumulative steroid cumulative dose (p=0.891), as shown in Graphic 6.



Graphic 6 – Correlations between reduction in thickness and steroid cumulative dose

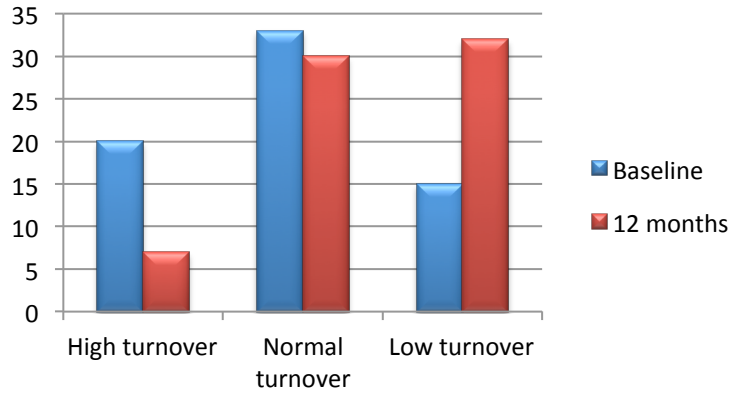
As at baseline, thickness was not associated with any parameter of trabecular bone. In transplant (and in dialysis), patients with hypercalcemia (calcium > 10.2 mg/dL) had higher cortical thickness than those without hypercalcemia (p=0.021). Additionally, transplanted patients presented significant lower cortical thickness in the presence of hypophosphatemia (p=0.005; phosphate <2.5 mg/dL), abnormal low levels of klotho (p=0.005; klotho < 500 pg/mL), or abnormal high levels of sclerostin (p=0.016; sclerostin > 0.6 ng/mL).

A non-significant trend toward lower cortical bone porosity was observed. Abnormal PTH high levels (> 150 pg/mL) or hypercalcemia (calcium > 10.2 mg/dL) were related to an increased porosity (p=0.025, p=0.012, respectively). In transplant patients we found no association between cortical porosity and trabecular results, namely osteoid measurements or bone formation rate.

Trabecular bone

- ✓ Remodeling

Less than half of the patients had normal remodeling parameters in both biopsies (n=33 vs. n=31, p=0.590), but we observed a large decrease in the number of patients with high-remodeling disease (20 vs. 7 patients, p<0.001), and a significant increase in patients with low-remodeling disease (15 vs. 31 patients, p=0.001), as demonstrated in Graph 7.



Graphic 7 – Differences in bone remodeling pre and after transplantation

In the 1st biopsy, 15 patients presented with low bone turnover: 7 with adynamic bone disease (low bone volume and low turnover) and 1 patient with osteomalacia. In the 2nd biopsy, low bone turnover was present in 44.9% of the population (n=31) in the form of adynamic bone disease in 10 patients (those with low volume), or as osteomalacia in 3 patients. The remaining 18 patients had normal volume and mineralization. There was no significant change in the development of adynamic bone disease (p=0.405) or osteomalacia (p=0.157).

On the other hand, in the 1st biopsy, 20 patients had hyperparathyroid bone disease [with normal volume (n=14) or low volume (n=6)], including 2 patients with mixed renal osteodystrophy. In the 2nd biopsy, high bone turnover was present only in 7 patients, the majority with normal volume (n=6), with no cases of mixed renal osteodystrophy. There was a significant decrease in terms of hyperparathyroid bone disease (p<0.001).

Despite these results, the changes within each remodeling category, even in the normal turnover status, were many, as Figure 12 and Table 30 show.

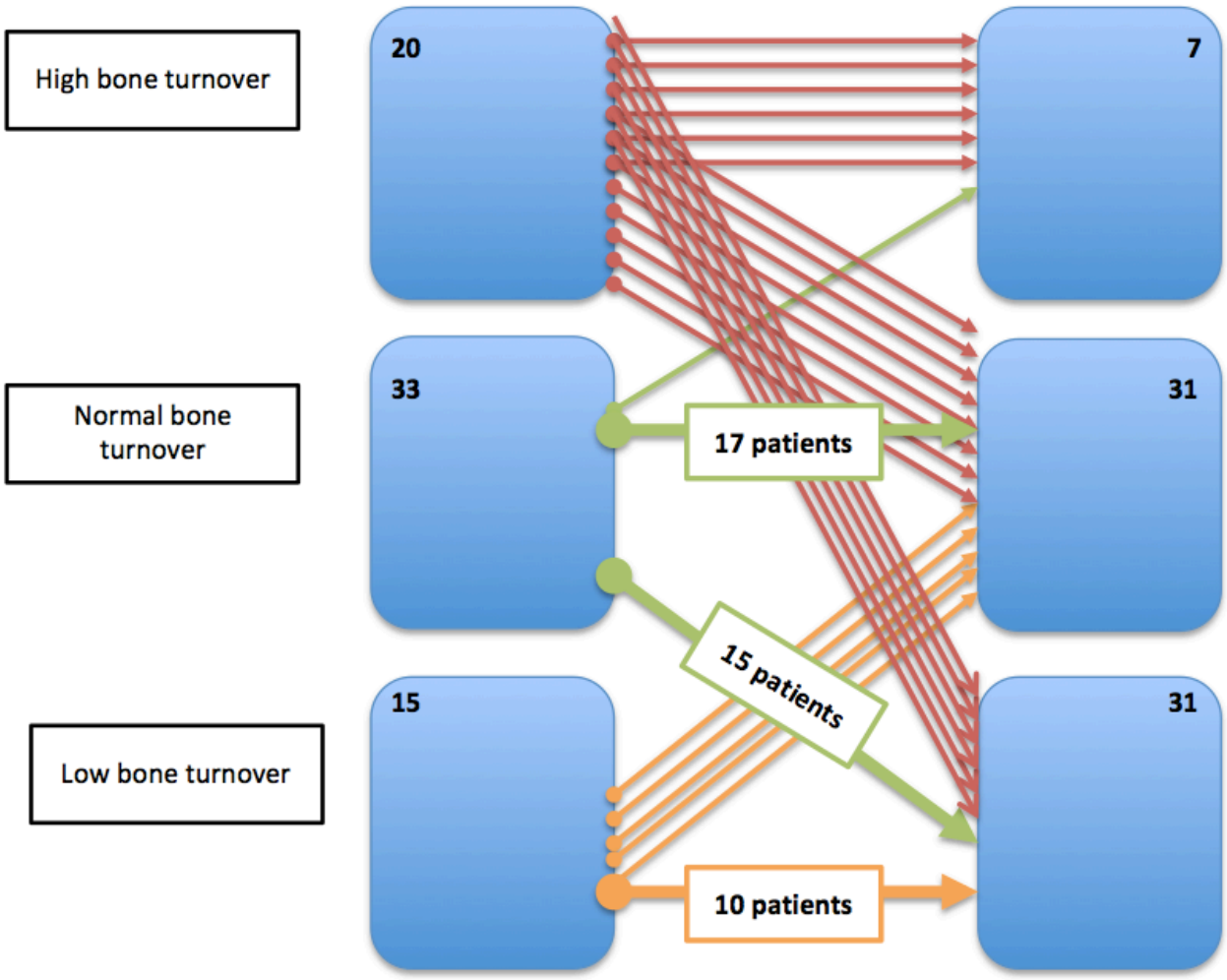


Figure 12 – Category changes in terms of bone remodeling

Reflecting only 68 bone biopsies

	Baseline	12 months		
		High TO	Normal TO	Low TO
High TO	20	6	8	6
Normal TO	33	1	17	15
Low TO	15	-	5	10
	Total cases*	7	31	31

Table 30 – Category changes in terms of bone remodeling

Reflecting only 68 bone biopsies performed at the baseline (1 additional bone biopsy performed at 1-year after transplantation had no trabecular tissue in the 1st biopsy).

TO – turnover.

In this study, 33 (48.5%) patients maintained their original turnover; 29 (42.1%) decreased their turnover, and 6 (15.8%) increased their turnover, as shown in Figure 12. The majority of patients whose biopsies showed an increase in bone remodeling presented with low turnover at baseline (p=0.001). Although these are only six patients, we provide some data: these patients had lower cumulative prednisolone dose (3150.0 mg vs. 3755.0 mg, p=0.042) and 50% of those were under everolimus (vs. 3.2% of patients that did not have an increase in bone turnover and were under everolimus, p=0.004). Also, these patients had lower PTH levels at baseline (179.4 pg/mL vs. 481.1 pg/mL, p=0.032), had higher calcium levels at baseline (10.3 mg/dL vs. 9.3 mg/dL, p=0.011), with a significant difference in the percentage of change of vitamin D (-95.1% vs. 7.5%, p=0.016) and without significant difference in the percentage of change of PTH after 1-year (p=0.067). Patients whose turnover went from low to normal also had lower alpha-klotho levels at baseline (218.5 vs. 743.5 pg/mL, p=0.037), and different percentage of change of alpha-klotho (205.2% vs. 742.8%, p=0.037).

	Increasing bone turnover from baseline		
	OR	95% CI	p-value
T0 Calcium (mg/dL)	**13.6**	**1.41 – 130.7**	**0.012**
Delta vitamin D (ng/mL)	1.08	0.96 – 1.22	0.144
Everolimus (%)	**117.8**	**2.83 – 130.7**	**0.012**
Prednisolone dose (mg)	0.99	0.99 – 1.00	0.143

Table 31 – Logistic regression for independent risk factors for an increase in bone turnover

Statistical analysis: Logistic regression test. T0 – baseline.

In multivariate analysis, the most important factors for increasing bone turnover were everolimus (p=0.012) and calcium levels at baseline (p=0.012) in a model that included steroid dose and vitamin D differences (model: p<0.0001, ROC curve 0.98). Nevertheless, as it is related to only six patients, the data is only explanatory.

We notice that the twenty-nine patients who had experienced decreases in bone remodeling categories had higher alpha-klotho levels after 1-year of transplantation (1288.6 pg/mL vs. 675.8 pg/mL, p=0.025); greater increases in alpha-klotho, compared to baseline [Delta alpha-klotho of 895.5 pg/mL (155.4 to 1932.2) for those with reduction in turnover vs. 14.1 (-375.7 to 710.7) for the remaining, p=0.014], and a

different percentage increase in alpha-klotho (174.3% vs. 4.7%). We noted a difference in the levels of sclerostin 1-year after transplantation (0.5 ng/mL for those who decreased bone turnover vs. 0.8 ng/mL compared to those who had not, p=0.029), and a percentage reduction of sclerostin (68.5% vs.58.5%), as shown in Table 32.

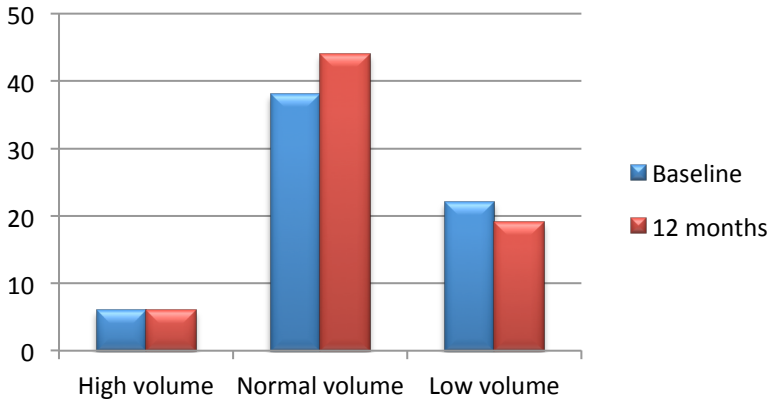
	Reduced bone turnover	Maintained or increased bone volume	p-value
T0 alpha-klotho (pg/mL)	500.4 (334.7 – 860.0)	701.7 (401.7 – 842.0)	0.199
T1 alpha-klotho (pg/mL)	**1288.6 (619.0 – 2466.0)**	**675.8(453.3 – 1269.0)**	**0.025**
Delta alpha-klotho	**895.5 (155.4 to 1932.2)**	**14.1(-375.7 to 710.7)**	**0.015**
% reduction of sclerostin	**68.5 (56.4 – 77.3)**	**58.5 (50.5 – 65.0)**	**0.004**
T1 Sclerostin (ng/mL)	**0.5 (0.3 – 0.9)**	**0.8 (0.6 – 1.0)**	**0.029**

Table 32 – Alpha-klotho and sclerostin levels and reductions in bone remodeling

Statistical analysis: Mann-Whitney test. T0 – baseline; T1 – at 1-year

✓ Volume

The number of patients with normal or high bone volume increased (from 44 to 50 patients, p=0.512) and the number of patients with low bone volume decreased, as shown in Graphic 8.



Graphic 8 – Differences in bone volume pre and after transplantation

We found that 12 out of 22 patients (54.5%) with low volume at baseline normalized volume and only 26.5% of patients maintained low bone volume. Few patients with normal baseline volume changed the type of volume: only 17.5% decreased bone volume and 10% fell into the high-volume category. Half of the 6 patients with high

baseline volume normalized bone volume; 2 patients did not change volume type and 1 abruptly decreased the volume, as shown in Table 33.

	Baseline	12 months		
		High volume	Normal volume	Low volume
High volume	**6**	2	3	1
Normal volume	**40**	4	29	7
Low volume	**22**	-	12	10
	Total cases	**6**	**44**	**18**

Table 33 – Category changes in terms of bone volume

Figure 13 shows in a schematic form the changes in volume pre and 1-year post-transplant.

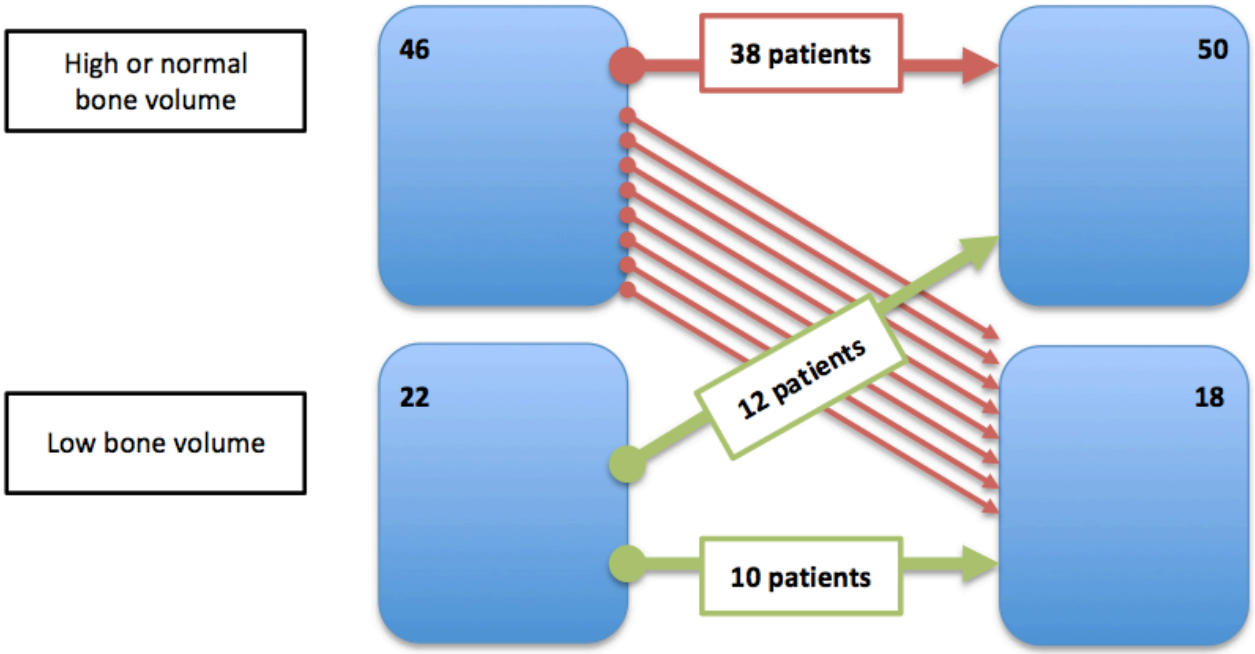


Figure 13 – Changes in volume after transplantation

We notice that patients whose bone volume decreased after transplant were the ones with the highest BMI at baseline [28.1 (26.4 – 29.4) Kg/m^2 vs. 24.2 (22.4 – 27.3) Kg/m^2, p=0.007], highest levels at sclerostin at baseline [2.5 (2.2 – 4) ng/mL vs. 1.7 (1.2 – 2.7) ng/mL, p=0.006], and the ones with a significant decrease in sclerostin values from baseline [-2.2 (-3.1 - -1.2) vs. -1.1 (-1.6 – 0.7), p=0.004]. No association with cumulative steroid doses was observed.

	Reduced bone volume	Maintained bone volume	p-value
T0 Sclerostin (ng/mL)	**2.5 (2.1 – 4.0)**	**1.7 (1.2 – 2.7)**	**0.006**
T1 Sclerostin (ng/mL)	0.8 (0.5 – 1.0)	0.7 (0.4 – 1.0)	0.667
Delta Sclerostin	**-2.2 (-3.1 – -1.2)**	**-1.0 (-1.6 – -0.7)**	**0.004**

Table 34 – Delta values of sclerostin and reductions in bone volume

Statistical analysis: Mann-Whitney test. T0 – baseline; T1 – at 1-year

At baseline, 8 had isolated low bone volume, since the only bone abnormality was the reduction in bone volume. After 1-year, 6 of those patients normalized volume, and 2, although low volume, presented additional low turnover, as shown in Table 35.

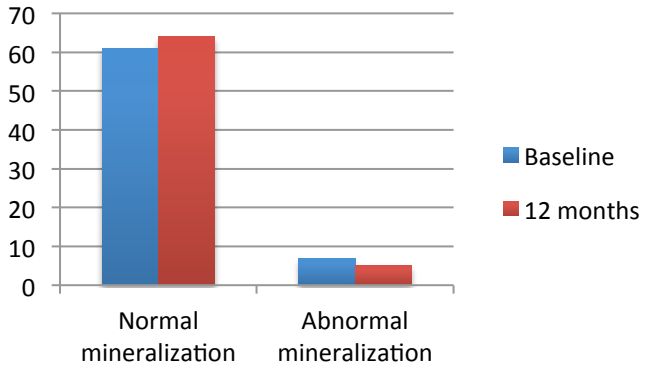
	Baseline	12 months	
	Low volume	Normal volume	Adynamic bone disease
Isolated low volume	**8**	6	2

Table 35 – Evolution of osteoporotic patients by bone biopsy after 12 months of transplant

In our population, 6 additional patients developed isolated low bone volume after renal transplant; 4 of them already had low volume at baseline, but also had changes in bone remodeling (both low and high turnover), which had normalized after transplantation; 2 additional patients decreased their bone volume.

- ✓ Mineralization

Very few patients had abnormal mineralization: seven in the 1st bone biopsy (one osteomalacia, two mixed ROD, three with normal volume and remodeling, and one with low volume and normal remodeling), and this number decreased to five in the 2nd bone biopsy (3 osteomalacia, 2 with normal volume and remodeling), without statistical significance (p=0.479), as shown in Graphic 9. There were no differences in mixed ROD (p=0.157) or osteomalacia (p=0.157) between the two points.



Graphic 9 – Differences in bone mineralization pre and after transplantation

Of the seven patients classified as having abnormal mineralization, five (71.4%) had normalized mineralization. However, three additional patients (4.9%) moved from the normal group, leaving a total of five patients with this condition (Figure 14).

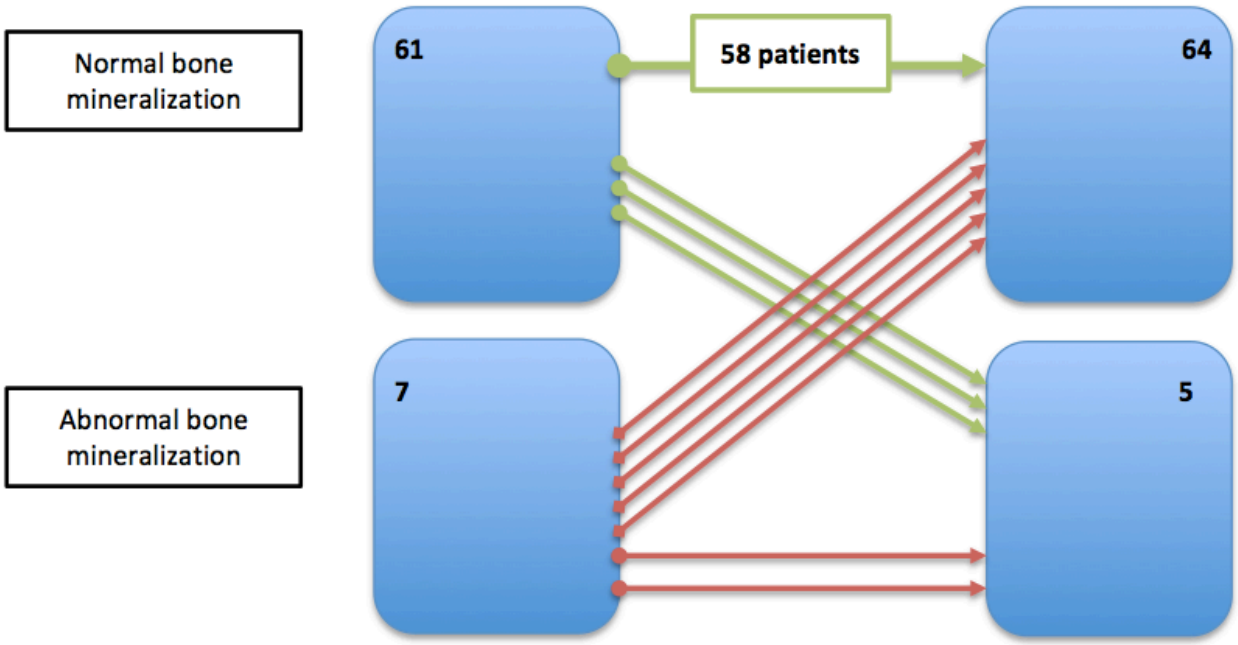


Figure 14 – Category changes in terms of bone mineralization

Reflecting only 68 bone biopsies (not 69).

The only factor associated with the reduced mineralization was the delta value of BALP: median 7.3 (4.4 – 23.5) U/L higher at 12 months vs. -12.4 (-21.0 – 1.4) U/L in normal mineralization cases, as shown in Table 36.

	Reduced mineralization	Maintained mineralization status	p-value
T0 BALP (U/L)	20.1 (17.0 – 63.0)	34.3 (26.8 – 44.7)	0.464
T1 BALP (U/L)	43.6 (21.7 – 70.2)	23 (16.9 – 34.7)	0.165
Delta BALP	**7.3 (4.4 – 23.5)**	**-12.96 (-21 to 1.43)**	**0.004**

Table 36 – Delta values of BALP and reductions in bone mineralization

Statistical analysis: Mann-Whiney test. BALP – bone alkaline phosphatase; T0 – baseline; T1 – at 1-year

When analysing which bone-related factors could be associated with abnormal mineralization in T1 (independent of the evolution from baseline), we found no associations with a yes or no question (having or not abnormal mineralization), other than having mineralization defects at baseline (p=0.046).

IMAGING EXAMS EVALUATION

The major echocardiographic findings were not different from those at baseline. The exception was the shortening fraction, which increased significantly. Likewise, the vascular calcification score did not change, as Table 37 shows.

Imaging exams			
	Baseline	**12 months**	**p-value**
Echocardiographic findings			
LVMI (g/m^2)	107.0 (91.5 – 140.5)	108.5 (98.0 – 138.0)	0.091
IV septal thickness (mm)	11.0 (10.0 – 12.0)	11.0 (9.0 – 12.0)	0.492
LVH (n, [%])	29 (42.0)	26 (39.4)	1.000
Valve calcifications (n, %)	15 (21.7)	16 (23.5)	0.781
LV fractional shortening (%)	**40.0 (35.0 – 43.0)**	**43.0 (37.0 – 47.0)**	**0.015**
Vascular calcification score (Adragão score)			
Hands score	0 (0 – 2)	1 (0 – 2)	0.589
Pelvis score	0 (0 – 1)	0 (0 – 1)	0.873
Total score	1 (0 – 2)	1 (0 – 2)	0.196
Low score (0; 1) (%)	63.8	56.5	0.165
High score (≥2) (%)	36.2	43.5	

Table 37 – Differences in imaging results comparing baseline to 1-year of follow-up

Statistical analysis: Wilcoxon matched pairs or McNemar's test for symmetry.

LVMI – left ventricular mass index; IV septal thickness – interventricular septal thickness; LVH – left ventricular hypertrophy

Time 1 study – Renal transplanted patients at the end of the 1st year

In addition to the laboratory evaluation, bone biopsy, X-ray of hands and pelvis and echocardiographic evaluation, these patients were submitted to new exams that were not performed at baseline: a CT scan and a bone densitometry.

IMAGING EXAMS EVALUATION

Similarly to what we did at baseline, we divided Adragão score into 2 groups of severity: absent or mild calcification (score 0 & 1) and moderate to severe calcification (scores ≥2).

Adragão score	
Score 0 (n, [%])	26 (38.2)
Score 1 (n, [%])	13 (19.2)
Score 2 (n, [%])	19 (27.9)
Score 3 (n, [%])	4 (5.9)
Score 4 (n, [%])	4 (5.9)
Score 5 (n, [%])	1 (1.5)
Score 6 (n, [%])	1 (1.5)

Table 38 – Vascular calcification score by Adragão

Vascular calcification severity score, divided by the two groups, was associated with age, diabetes, and FGF23 at 1-year after transplantation, as Table 39 shows. Also, having both low vitamin D (< 15 ng/mL) and phosphate levels (< 2.5 mg/dL) was associated to a higher score of vascular calcification. Compared to baseline, BMI and sclerostin lost the association with severity of vascular calcification.

Concerning bone histomorphometry, neither cortical porosity nor the type of remodeling (high – normal – low) or BFR were associated with vascular calcification score, although we found a trend toward a lower BFR to be associated with a more severe score of vascular calcifications, as shown in Table 39.

Nevertheless, in multivariate analysis, only age and diabetes were predictors for severity of vascular calcifications, as shown in Table 40.

	Mild vascular calcification score (n=39)	Severe vascular calcification score (n=30)	p-value
Adragão score	0.0 (0.0– 1.0)	2.0 (2.0 – 3.0)	-
Valve calcifications(%)	15.4	34.5	0.061
Male gender (%)	69.2	70.0	0.579
Age (years)	**44 (35 – 57)**	**58.5 (53 – 63)**	**<0.001**
BMI (Kg/m^2)	24.5 (21.6 – 28.1)	24.9 (23.9 – 26.1)	0.446
Diabetes (%)	**5.1**	**23.3**	**0.031**
PTDM (%)	15.4	16.7	0.570
T1 Sclerostin (ng/mL)	0.7 (0.5 – 0.9)	0.8 (0.5 – 1.2)	0.361
T1 FGF23 (RU/mL)	**118.2 (87.2 – 161.4)**	**142.6 (120.4 – 177.3)**	**0.039**
T1Alpha-klotho (pg/mL)	1151.7 (519.2 – 2044.2)	779.7 (372.5– 2182.2)	0.442
T1 PTH (pg/mL)	130.0 (86.5 – 232.7)	135.8 (101.1 – 237.7)	0.707
T1 BALP (U/L)	26.6 (15.7 – 37.0)	21.6 (17.2 – 34.7)	0.594
T1 Phosphate (mg/dL)	3.1 (2.7 – 3.4)	3.2 (2.9 – 3.6)	0.154
T1 Calcium (mg/dL)	9.9 (9.3 – 10.5)	9.6 (9.3 – 10.3)	0.658
T1 Magnesium (mg/dL)	1.7 (1.5 – 1.0)	1.7 (1.6 – 1.9)	0.414
T1 Vitamin D (ng/mL)	24.4 (13.7 – 29.5)	21.7 (14.3 – 28.3)	0.799
Low Vit D and Pi (%)	**0**	**13.3**	**0.032**
BFR/BS (µm^3/µm^2/year)	25.5 (6.9 – 36.8)	10.7 (4.4 – 24.2)	0.062

Table 39 – Risk factors for severity of calcification scores

Statistical analysis: Mann-Whitney test or Fisher exact test. BMI – body mass index; PTDM – post-transplant diabetes mellitus; FGF23 – fibroblast growth factor 23; PTH – parathyroid hormone; BALP – bone alkaline phosphatase; Vit D – vitamin D; Pi – phosphate; BFR / BS – bone formation rate / bone surface. T1 – at 1-year

	Severity of Adragão Score		
	OR	95% CI	p-value
Age	2.70*	1.52 – 4.81	0.001
Diabetes	9.46	1.19 – 75.2	0.034
T1 FGF23	1.00	0.99 – 1.00	0.020

Table 40 – Multivariate analysis for independent predictors of severity in Adragão score

Statistical analysis: logistic regression test FGF23 – fibroblast growth factor 23; T1 – at 1-year

(*For each increase in 10 years)

We also looked at valve calcification, in a similar way to how we did at baseline. These results are presented in Table 41.

	Absence of valve calcification (n=52)	Presence of valve calcification (n=16)	p-value
Adragão score	**1 (0 – 2)**	**2 (1 - 2)**	**0.023**
Age (years)	**48.5 (37 – 57.5)**	**61 (56 – 63.5)**	**0.001**
Male gender (%)	73.1	56.2	0.167
Caucasian race (%)	75.0	81.2	1.000
BMI (Kg/m^2)	24.6 (22.0 – 26.6)	25.6 (24.0 – 28.6)	0.272
Diabetes (%)	**5.7**	**37.5**	**0.004**
PTDM (%)	**9.6**	**37.5**	**0.016**
Hypertension (%)	84.6	87.5	0.567
Dialysis vintage (M)	55.0 (40.0 – 83.0)	63.5 (42.5 – 83.5)	0.474
HD with/out PD (%)	92.3	100.0	0.332
PD (%)	15.4	6.2	0.319
Parathyroidectomy (%)	5.7	12.5	0.335
Cortical porosity (%)	5.8 (4.0 – 9.5)	6.2 (3.5 – 10.6)	0.988
BV/TV (%)	19.3 (15.8 – 24.2)	19.3 (13.5 – 26.3)	0.700
Mineralized volume /TV(%)	18.2 (15.4 – 22.8%)	18.0 (12.2 – 24.9)	0.644
Normal turnover (%)	44.2	43.7	0.602
Adynamic bone disease (%)	15.4	12.5	0.567
OcS/BS (%)	0.3 (0.0 – 1.0)	0.5 (0.1 – 0.7)	0.609
ObS/BS (%)	1.9 (1.1 – 3.2)	2.1 (0.9 – 3.4)	0.954
BFR/BS (µm^3/µm^2/year)	21.4 (4.6 – 33.2)	21.1 (8.0 – 45.5)	0.465
Sclerostin (ng/mL)	0.7 (0.5 – 0.9)	0.8 (0.5 – 1.1)	0.588
FGF23 (RU/mL)	135.6 (94.7 – 172.9)	123.3 (102.9 – 154.9)	0.756
Alpha-klotho (pg/mL)	1000.1(469.4 – 2155.7)	761.3 (578.7 – 1219.2)	0.355
PTH (pg/mL)	138.5 (90.0 – 238.9)	121.5 (85.0 – 191.2)	0.553
BALP (U/L)	22.2 (16.5 – 34.8)	27.6 (19.2 – 36.2)	0.529
Vitamin D (ng/mL)	23.1 (14.7 – 29.3)	20.5 (14.2 – 27.4)	0.613
Magnesium (mg/dL)	1.7 (1.6 – 1.8)	1.6 (1.4 – 1.8)	0.142
Calcium (mg/dL)	9.8 (9.3 – 10.4)	9.6 (9.2 – 10.3)	0.633
Phosphate (mg/dL)	3.1 (2.8 – 3.5)	3.3 (2.6 – 3.6)	0.638

Table 41 – Potential predictors of valve calcification

Statistical analysis: Mann-Whiney test and Fisher exact test. BMI – body mass index; PTDM – post-transplant diabetes mellitus; M – months; HD – hemodialysis; PD – peritoneal dialysis; BV/TV – bone volume / tissue volume; TV – tissue volume; OcS/BS – osteoclast surface / bone surface; ObS/BS - osteoblast surface / bone surface; BFR / BS – bone formation rate / bone surface; FGF23 – fibroblast growth factor 23; PTH – parathyroid hormone; BALP – bone alkaline phosphatase.

Curiously, neither dialysis vintage nor hypertension associated with valve calcifications, and we found no associations with bone-related molecules or with bone-related measurements. The only positive associations were with age and diabetes (prior to transplant or acquired post-transplantation). Also, at this time point, Adragão score had a significant association with presence of valve calcifications.

Coronary artery calcifications

A CT scan was performed to quantify coronary artery calcification using the Agatston score. More than half of the patients had an absolute score quantified as mild. However, when the absolute score is adjusted for age, gender and race, the different percentiles of severity were homogeneous in the population: one-third of the patients had mild coronary artery calcification (n=22, 33.3%); moderate coronary artery calcification (n=24, 36.4%), and less than a third of the population had severe coronary artery calcification (n=20, 30.3%), as shown in Table 42.

Coronary Artery Calcium Score	
Absolute values	
Median Agatston score	48.5 (0.0 – 535.0)
≤100 (n, [%])	39 (56.5)
101 – 1000 (n, [%])	20 (29.0)
> 1000 (n, [%])	10 (14.5)
Adjusted percentile	
Median Percentile	82.0 (0.0 – 93.0)
≤50 (n, [%])	22 (31.9)
51 - 90 (n, [%])	24 (34.8)
>90 (n, [%])	23 (33.3)

Table 42 – Coronary artery calcium score at 12 months after transplantation

We investigated the risk factors for artery coronary calcification using an ordered logistic regression and found different predictors according to the absolute value of

Agatston score vs. adjusted percentile of coronary calcification, as shown in Tables 43 and 44. Nevertheless, as the more important value in clinical practice is the percentile where the patients are included, we performed an additional multivariate analysis in the study of the predictors of different percentiles in those patients.

In the absolute score divided into three levels of severity (as presented in Table 42), we found that older age, male gender, diabetes, and longer dialysis vintage were significantly different according to the severity of Agatston coronary calcification score, as well as high PTH and high FGF23. Also, low bone volume was associated with the severity of the coronary calcifications (Table 43). Diabetes or post-transplant diabetes mellitus did not correlate with severity of Agatston score' neither did phosphate nor sclerostin.

	Agatston Score ≤100 (n=39)	Agatston Score 101 – 1000 (n=20)	Agatston Score > 1000 (n=10)	p-value
Agatston value	2 (0 – 18)	517 (207 – 645.5)	1615 (1580 –2090)	-
Valve calcifications (%)	**10.3**	**35.0**	**55.6**	**0.002**
Vascular calcifications	**0 (0 – 2)**	**2 (1 – 3)**	**2 (1 – 2)**	**<0.001**
Age (years)	**45 (35 – 57)**	**56.5 (43 – 61.5)**	**63.5 (57 – 65)**	**<0.001**
Male gender (%)	**59.0**	**80.0**	**90.0**	**0.028**
Dialysis vintage (M)	**49 (24 – 64)**	**63.5 (47.5 – 93)**	**83 (44 – 89)**	**0.012**
Thymoglobulin use (%)	**41.0**	**65.0**	**70.0**	**0.037**
T1 PTH (pg/mL)	**130 (85.9 –179.3)**	**131.2 (95 – 241)**	**202 (115 – 477)**	**0.007**
T1 FGF23 (RU/mL)	**122 (88.5 – 156)**	**133.5 (109.2 –169)**	**205 (136 – 299.8)**	**0.053**
T1 eGFR (ml/min/1.73m^2)	56.4 (39.8 – 72)	46.9 (31.3 – 66.5)	48.3 (34.3 –67.8)	0.299
T0 Cortical porosity (%)	7.1 (4.6 – 10.2)	8.2 (4.9 – 10.9)	8.9 (5.3 – 10.6)	0.842
T0 Low bone volume (%)	**17.9**	**40.0**	**70.0**	**0.002**
T0 BV/TV (%)	**22.4 (16.3 –27.3)**	**18 (13.9 – 22)**	**14.3 (10.8 –15.8)**	**<0.001**
T0 MBV/TV (%)	**20.7 (16.2 –23.6)**	**16.7 (12.4 – 21.7)**	**17.9 (11.3 –24.8)**	**<0.001**

Table 43 – Predictors for Agatston coronary artery calcium score

Statistical analysis: ordered logistic regression. M – months; PTH – parathyroid hormone; FGF23 – fibroblast growth factor 23; eGFR – estimated glomerular filtration rate; BV/TV – bone volume / tissue volume; MBV/TV – mineralized bone volume / tissue volume; T0 – baseline; T1 – at 1-year

We compared the three levels of severity of the percentiles of coronary calcification.

	Agatston Percentile ≤50% (n=22)	Agatston Percentile 51 - 90 (n=24)	Agatston Percentile >90 (n=13)	p-value
Agatston percentile	0 (0 – 0)	84 (74 – 87.5)	97.5 (94 - 99)	-
Age (years)	42.5 (33.0 – 50.0)	59.5 (49.5 – 63.0)	57.0 (42.0 – 62.0)	-
Male gender (%)	63.6	66.7	78.3	-
Caucasian race (%)	63.6	79.2	87.0	-
Valve calcifications	**9.1**	**20.8**	**40.9**	**0.014**
Vascular calcifications	**0 (0 – 1)**	**2 (1 – 2.5)**	**1 (0 – 2)**	**0.019**
PD | Only HD (%)	27.3 | 86.4	4.2 | 95.8	8.7 | 100.0	0.058
Dialysis vintage (M)	**51.5 (24 – 64)**	**48.5 (43 – 70.5)**	**81 (55 – 98)**	**0.010**
T1 eGFR (ml/min/1.73m^2)	57.1 (42.6 – 84.4)	46.5 (31.2 – 64.8)	55.6 (38.6 – 70.0)	0.747
T0 PTH (pg/mL)	468.2 (308.0 – 671.3)	529.9 (290.1 – 774.4)	454.8 (240.5 –912.7)	0.361
T0 Calcium (mg/mL)	9.0 (8.7 – 9.5)	9.3 (8.6 – 9.6)	9 (8.4 – 9.4)	0.056
T0 Phosphorus (mg/dL)	3.9 (3.2 – 4.9)	4.1 (3.3 – 5.0)	4.7 (3.4 – 5.6)	0.156
T0 Magnesium (mg/dL)	2.2 (2.1 – 2.4)	2.1 (2.0 – 2.4)	2.3 (2.1 – 2.6)	0.242
T0 BALP (U/L)	32.1 (23.9 – 43.0)	34.3 (26.7 – 43.6)	35.4 (26.8 – 50.1)	0.484
T0 Sclerostin (ng/mL)	**1.7 (1.2 – 2.2)**	**2.1 (1.3 – 2.9)**	**2.1 (1.7 – 2.9)**	**0.026**
T0 FGF23 (RU/mL)	1402.7 (463.9 – 6220.9)	1575.4 (599.4 – 3673.6)	3854.8 (798.4 – 8807.6)	0.101
T1 PTH (pg/mL)	**122.3 (84.9 – 179.3)**	**128 (88.3 – 181)**	**150.8 (111.7 – 268)**	**0.033**
T1 Calcium (mg/mL)	**9.65 (9.2 – 9.9)**	**9.6 (9.3 – 10)**	**10.3 (9.6 – 10.8)**	**0.042**
T1 Phosphorus (mg/dL)	3.1 (2.9 – 3.5)	3.1 (2.9 – 3.4)	3 (2.3 – 3.9)	0.837
T1 Magnesium (mg/dL)	1.7 (1.6 – 1.8)	1.6 (1.4 – 1.8)	1.7 (1.6 – 1.8)	0.411
T1 BALP (U/L)	**18.2 (13.3 – 30.8)**	**26.4 (19.7 – 36.4)**	**28.6 (18.4 – 64.7)**	**0.009**
T1 Sclerostin (ng/mL)	0.6 (0.4 – 0.8)	0.7 (0.5 – 0.9)	0.8 (0.5 – 1.2)	0.121
T1 FGF23 (RU/mL)	119.3 (88.5 – 143.2)	123.6 (96 – 164.1)	153.4 (126.1 –260.2)	0.161
T0 BV/TV (%)	22.4 (16.3 – 27.3)	18 (13.9 – 22)	14.3 (10.8 – 15.8)	0.072
T1 Porosity (%)	**4.4 (3.5 – 7.5)**	**5.3 (3.9 – 10.6)**	**7.9 (4.9 – 12.9)**	**0.013**
T1 BV/TV (%)	20.0 (15.0 – 24.8)	19.0 (17.1 – 23.4)	19.2 (13.5 – 26.0)	0.622
T1 OtV/BV	**4 (1.9 – 5.2)**	**3.9 (1.5 – 4.9)**	**5.1 (2.4 – 11.2)**	**0.032**
BFR/BS (µm^3/µm^2/year)	**14.7 (2.5 – 25.5)**	**17.7 (4.7 – 37.2)**	**25.2 (7.1 – 45.5)**	**0.013**
T1 High turnover (%)	4.5	4.2	21.7	0.053

Table 44 – Predictors for the percentile of Agatston coronary artery calcium score

Statistical analysis: ordered logistic regression. PD – peritoneal dialysis; HD – hemodialysis; M – months; eGFR – estimated glomerular filtration rate; PTH – parathyroid hormone; BALP – bone alkaline phosphatase; FGF23 – fibroblast growth factor 23; BV/TV – bone volume / tissue volume; OtV/BV – Osteoid volume / Bone volume; BFR / BS – bone formation rate / bone surface; T0 – baseline; T1 – at 1-year

Comparing the three levels of severity of the percentiles of coronary calcifications, (already adjusted for age, gender, and race), we observed that longer time on dialysis, high sclerostin at baseline, high serum levels of calcium, BALP and PTH 1-year after transplant, higher osteoid volume/bone volume (OtV/BV), and higher cortical porosity were associated with calcification severity, as shown in Table 44. In addition, BFR/BS was associated with the severity of the percentiles of coronary calcifications.

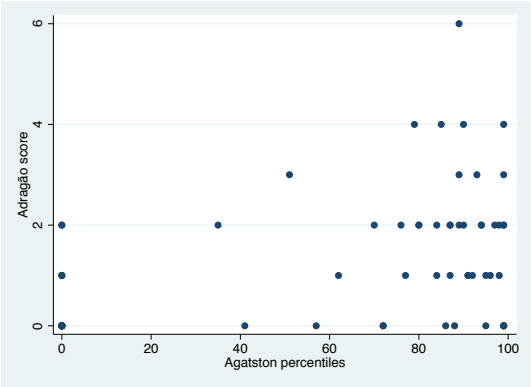
In multivariate analysis, dialysis vintage (p=0.001), baseline sclerostin levels (p=0.006), baseline low bone volume (p=0.016), and high bone turnover at 1-year after transplant (p=0.040) were the main predictors of coronary calcification percentiles, as shown in Table 45.

	Agatston percentiles		
	OR	95% CI	p-value
Dialysis vintage (months)	**1.26***	**1.09 – 1.45**	**0.001**
T0 Sclerostin (ng/mL)	**2.61**	**1.38 – 4.92**	**0.006**
T1 BALP (U/L)	**1.38**	**1.04 – 1.90**	**0.050**
eGFR (mL/min/1.73m^2)	0.95*	0.74 – 1.21	0.668
T0 BV/TV (%)	**0.90**	**0.83 – 0.97**	**0.016**
T1 High bone turnover	**10.4**	**1.18 – 92.6**	**0.040**

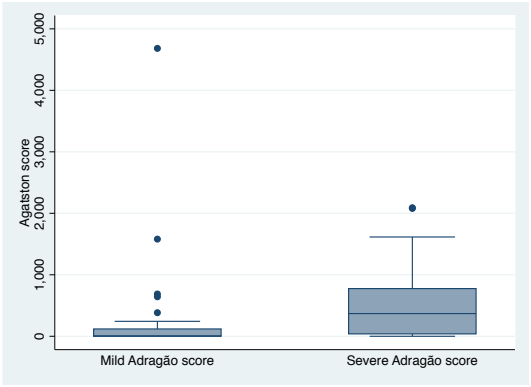
Table 45 – Ordered logistic regression for independent associations with Agatston percentiles

Statistical analysis: ordered logistic regression. BALP – bone alkaline phosphatase; eGFR – estimated glomerular filtration rate; BV/TV – Bone Volume / Tissue Volume; T0 – baseline; T1 – at 1-year (*For each increase in 10 units)

In both analyses, vascular calcifications, obtained by Adragão score, and valve calcifications correlated well with the increased severity of coronary calcifications, as demonstrated in Graphics 10 and 11.



Graphic 10 – Correlations between Adragão score and percentiles of coronary artery calcifications



Graphic 11 – Associations between Adragão score and Agatston score

Statistical analysis: Mann-Whiney test.

Bone densitometry evaluation

Bone densitometry was performed in 67 patients, and median values of lumbar spine, total femoral and femoral neck are presented in Table 46. According to this exam, 18 patients (26.9%) were classified as having osteoporosis, as the T-scores of one or more of the scanned bone sites were ≤ -2.5; 33 (49.0%) as having osteopenia, as T-scores of one or more of the scanned bone sites were between -1 and -2.5.

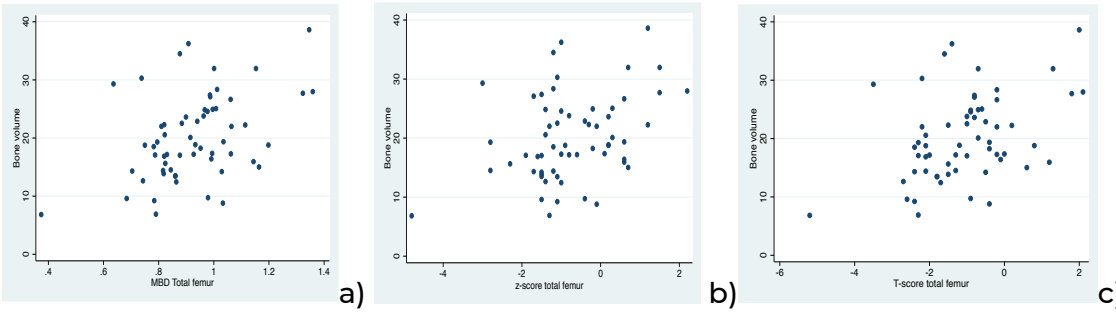
Only 16 patients had a normal DXA exam. Patients with osteoporosis were Caucasian (p=0.02), had a lower BMI (p<0.001), and had a higher percentile of coronary calcifications (p=0.04) than patients without this diagnosis obtained by DXA.

Bone densitometry	
Lumbar spine	
Bone Mineral Density	1.1 (1.0 – 1.2)
T-score	-1.1 (-1.9 to -0.1)
Z-score	-0.8 (-1.8 to 0.4)
Femoral neck	
Bone Mineral Density	0.9 (0.77 to 0.96)
T-score	-1.4 (-2.2 to -0.6)
Z-score	-0.7 (-1.5 to 0.0)
Total femur	
Bone Mineral Density	0.9 (0.8 to 1.0)
T-score	-1.0 (-2.1 to -0.4)
Z-score	-1.0 (-1.45 to 0.2)
FRAX risk	
Osteoporotic fracture | hip fracture	3.5% (2.2 – 6.2)| 0.8% (0.2 – 2.7)

Table 46 – Bone densitometry performed 12 months after transplantation

Median and IQ range

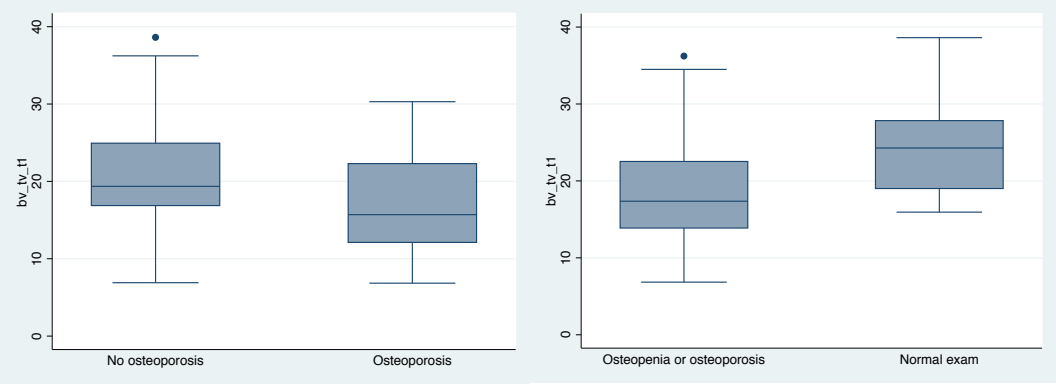
Overall, bone volume measured in the bone biopsies correlated well with densitometry findings. Correlations with total femur mineral bone density ($p<0.001$), its Z-scores ($p=0.001$), and its T-scores ($p=0.005$); with lumbar spine mineral bone density ($p=0.007$), its Z-scores ($p=0.007$), and its T-scores ($p=0.006$), and with femoral neck mineral bone density ($p=0.003$), and its Z-scores ($p=0.004$) had statistical significance. The DXA parameters that correlated better with the bone volume measured in the bone biopsies were the mineral bone density and T-scores of total femur (both with ROC curve of 92.0%) and of femoral neck (both with ROC curve of 93.0%). One of the correlations is demonstrated in Graphic 12.



a) b) c)

Graphic 12 – Correlations between bone volume obtained by a bone biopsy and DXA results in total femur: a) bone mineral density, b) Z-score; c) T-score

Further, we observed a difference in bone volume obtained by a bone biopsy when classifying patients by presence or absence of osteoporosis via DXA (osteoporosis: BV/TV 17.3%; no osteoporosis: BV/TV 21.0%, p=0.054) or when classifying patients by presence of a normal DXA exam (normal exam: BV/TV 24.3%; osteoporosis or osteopenia: BV/TV 18.6%, p=0.003), as Graphic 13 shows.



Graphic 13 – Differences in bone volume (BV/TV) by presence of osteoporosis or by presence of a normal exam

Statistical analysis: Mann-Whiney test.

Analysing patients by the diagnosis of osteoporosis obtained by DXA (in 18 patients) and by bone volume obtained by histomorphometric measurements, 9 out of 18 patients (in whom DXA revealed osteoporosis) effectively had low volume (BV / TV < 16%) at the bone biopsy, as Table 47 shows. The sensitivity of the DXA exam to detect low bone volume compared to the gold standard (a bone biopsy) was 47.0% and the specificity of the test was 81.2%. The positive predictive value was 50.0% and the negative predictive value (NPV) was 80.0%.

	Volume BV/TV		
T-score	**<16%**	**≥ 16%**	**Total**
≤ - 2.5	9	9	18
> - 2.5	10	39	49
Total	19	48	67

Table 47 – Osteoporosis detected by a bone biopsy and by DXA scan

Sixteen patients had a normal DXA exam (T-score > -1), and all of them had a BV/TV ≥ 16% in the bone biopsy. This underlines the previous results showing the specificity and NPV of the DXA exam.

As expected, other bone disorders related to remodeling or mineralization had no correlation with DXA findings. For instance, three out of those nine patients with osteoporosis on DXA and low bone volume obtained by the bone biopsy had low bone turnover and three also had high bone turnover, leaving the remaining three patients with low bone volume with no other associated bone disorders. Two out of the 16 patients with normal DXA exam and normal volume in a bone biopsy had abnormal mineralization (although normal volume), and 9 had low bone turnover (although normal volume); these numbers include the 2 patients with osteomalacia.

We found correlations between the results of DXA imaging and both alpha-klotho and sclerostin serum levels. The group of patients with higher alpha-klotho levels also had higher values of bone mineral density (0.89 vs. 0.78, p=0.007), and significantly different T-scores in femoral neck (-1.1 vs. -1.9, p=0.009), with no significant differences in its Z-scores. These associations were maintained after adjusting for age and for renal function, as shown in Table 48.

	Femoral neck – bone mineral density		
	Coefficient	IC 95%	p-value
Alpha-klotho (pg/mL)	0.088	0.006 – 0.17	0.035
Age (years)	-0.001	-0.005 – 0.002	0.497
eGFR (mL/min/1.73m^2)	0.0003	-0.001 – 0.002	0.656

Table 48 – Multivariate analysis for the association between mineral bone density of femoral neck and alpha-klotho low or high levels

Statistical analysis: linear regression. eGFR – estimated glomerular filtration rate.

Patients with a normal DXA exam were mostly in the group presenting the highest levels of alpha-klotho (p=0.034). Nevertheless, after adjusting for age, this association was lost.

Looking at sclerostin, people with higher levels of sclerostin had higher bone mineral density (1.02 vs. 1.12, p=0.017), T-scores (-1.65 vs. -0.7, p=0.017) and Z-scores (-1.3 vs. 0,

p=0.005) at spine, and higher bone mineral density (0.86 vs. 0.97, p=0.032), T-scores (-1.5 vs. -0.7, p=0.028), and higher Z-scores (-1.2 vs. -0.25, p=0.007) at total femur. All those association were maintained, after adjusting for age and for renal function, as shown in Table 49.

	Lumbar spine – Bone mineral density		
	Coefficient	**IC 95%**	**p-value**
Sclerostin (ng/mL)	0.127	0.02 – 0.24	0.024
Age (Years)	-0.0008	-0.005 – 0.004	0.748
eGFR (mL/min/1.73m^2)	0.001	-0.001 – 0.003	0.368
	Total femur – Bone mineral density		
	Coefficient	**IC 95%**	**p-value**
Sclerostin (ng/mL)	0.11	0.01 – 0.20	0.024
Age (Years)	-0.003	-0.007 – 0.001	0.177
eGFR (mL/min/1.73m^2)	0.001	-0.002 – 0.002	0.892

Table 49 – Multivariate analysis for the associations between mineral bone density of lumbar spine or total femur and low or high levels of sclerostin

Statistical analysis: linear regression. eGFR – estimated glomerular filtration rate.

Overall, we found no correlation between the values obtained by DXA (as continuous variable) and coronary calcifications scores or percentiles obtained by cardiac CT. Nevertheless, osteoporosis diagnosis obtained by DXA was more frequent in patients with higher calcium percentiles (p=0.037).

Also, we found no associations between bone-related therapy, namely vitamin D supplementation or cinacalcet prescription, or even with renal function and DXA findings.

Some of these results were published in the form of three original articles [178-180] that are available upon request.

METABOLIC EVALUATION

As at baseline, we tried to study possible associations between metabolic markers, bone disorders, and cardiovascular abnormalities, such as extra-osseous calcifications and high LVMI at the end of the first year.

At this stage, we looked again at the potential correlations between bone-related biochemical parameters, to see if the associations revealed at baseline were the same after the transplant. For this metabolic evaluation after 1 year, we divided the variables of interest into two groups according to the value of interest (the value above or below the normal with good renal function); see tables 50.1, 50.2 and 50.3. In this case, patients with PTH above 150 pg/mL or BALP above 40 U/L were considered as having secondary hyperparathyroidism; patients with vitamin D below 15 ng/mL as having hypovitaminosis D; patients with phosphate levels below 2.5 mg/dL as having hypophosphatemia, and patients with calcium levels above 10.2 mg/dL as having hypercalcemia. FGF23 and sclerostin were divided according to their normal upper limit (180 RU/mL and 0.6 ng/mL, respectively) and alpha-klotho by its lower limit (500 pg/mL).

Eleven patients (15.9%) had hypophosphatemia. Patients with hypophosphatemia post-transplantation had longer dialysis vintage. This was associated with higher PTH and calcium levels and lower sclerostin levels. Hypophosphatemic patients had more osteoid comparing with others (osteoid thickness; osteoid surface/bone surface; osteoid volume/bone volume or osteoid volume/tissue volume).

Hypercalcemia was present in 33.3% (n=23) of the patients. These patients had a longer dialysis vintage and had higher PTH and a trend toward higher FGF23 levels. Hypercalcemia was also associated to hypophosphatemia. We did not find any associations between hypercalcemia and bone biopsy findings.

The twenty-seven patients (39.1%) with PTH > 150 pg/mL showed no difference in demographic characteristics. As at baseline, a higher or lower level of PTH was not associated with differences in BALP or alpha-klotho levels. We found a difference in sclerostin levels: lower levels of sclerostin in patients with the highest values of PTH, and a difference in FGF23 levels, as these levels were higher in the group of patients with PTH above 150 pg/mL (similar to baseline). At this time point, the group of patients with higher PTH presented higher levels of calcium (which was not present at baseline) and lower levels of phosphate (which is the inverse of the association observed in baseline), with no differences in magnesium. Also, those patients with high PTH levels had statistically significant lower vitamin D levels. The only bone parameters that differed were osteoid surface/bone surface and osteoid volume/bone

volume, the percentages of which were higher in the group of patients with higher PTH levels (see Table 50.1 and 51). We found no association with the dynamic values, or with other static measurements, such as osteoblast or osteoclast surface.

Related variables	Group 1	Group 2	p-value
	Phosphorus		
	2.3 (2.1 – 2.3)	3.3 (3.0 – 3.6)	
Dialysis vintage (M)	**96.0 (44.0 – 168.0)**	**54.0 (41.0 – 82.0)**	**0.028**
Calcium (mg/dL)	**10.7 (10.3 – 10.9)**	**9.6 (9.3 – 10.1)**	**0.003**
Magnesium (mg/dL)	1.7 (1.4 – 1.9)	1.7 (1.6 – 1.8)	0.896
FGF23 (RU/mL)	154.7 (116.0 – 200.0)	133.5 (94.7 – 168.5)	0.333
Alpha-klotho (pg/mL)	1172.2 (831.0 –2528.2)	779.7 (457.2 – 2044.2)	0.116
PTH (pg/mL)	**241.5 (232.7 – 381.5)**	**120.5 (86.4 – 159.7)**	**<0.001**
BALP (U/L)	32.1 (13.9 – 53.1)	22.9 (17.4 – 34.5)	0.555
Vitamin D (ng/mL)	20.7 (14.0 – 29.6)	23.1 (15.4 – 29.0)	0.718
Sclerostin (ng/mL)	**0.4 (0.3 – 0.6)**	**0.8 (0.6 – 1.0)**	**0.009**
	Calcium		
	9.5 (9.2 – 9.8)	10.7 (10.4 – 11.0)	
Dialysis vintage (M)	**48.5 (36.0 – 67.0)**	**81.0 (49.0 – 96.0)**	**0.020**
Phosphate (mg/dL)	**3.4 (3.0 – 3.7)**	**2.6 (2.3 – 3.1)**	**<0.001**
Magnesium (mg/dL)	1.7 (1.6 – 1.8)	1.7 (1.5 – 1.8)	0.829
FGF23 (RU/mL)	124.8 (91.0 – 159.7)	150.0 (118.2 – 192.2)	0.067
Alpha-klotho (pg/mL)	957.2 (485.0 – 2055.2)	831.0 (453.3 – 1551.2)	0.620
PTH (pg/mL)	**120.5 (85.9 – 171.7)**	**164.9 (115.1 – 253.9)**	**0.040**
BALP (U/L)	22.2 (16.1 – 33.4)	32.1 (18.3 – 41.3)	0.136
Vitamin D (ng/mL)	21.1 (13.7 – 28.2)	24.6 (16.8 – 30.4)	0.192
Sclerostin (ng/mL)	0.8 (0.5 – 1.1)	0.6 (0.4 – 0.8)	0.239

Table 50.1 – Associations between bone-related variables

Statistical analysis: Mann-Whiney test. Median and IQ range values are presented. PTH – parathyroid hormone; FGF23 – fibroblast growth factor 23; BALP – bone alkaline phosphatase

Fewer patients had BALP levels above the normal range (if comparing with PTH). Those were fourteen patients (20.3%), and 6 of them (42.9%) had also PTH levels above the normal range. Again, demographic characteristics were similar between the two groups of BALP. We found no association between higher BALP levels and the metabolic analysis.

Related variables	Group 1	Group 2	p-value
	PTH		
	103.3 (78.0 – 129.9)	241.5 (182.9 – 277.6)	
Phosphate (mg/dL)	**3.3 (3.0 – 3.6)**	**2.7 (2.3 – 3.2)**	**0.002**
Calcium (mg/dL)	**9.6 (9.2 – 10.0)**	**10.1 (9.6 – 10.8)**	**0.004**
Magnesium (mg/dL)	1.7 (1.5 – 1.8)	1.7 (1.6 – 1.9)	0.420
FGF23 (RU/mL)	**115.4 (88.5 – 143.2)**	**168.5 (131.8 – 200.0)**	**<0.001**
Alpha-klotho (pg/mL)	779.7 (481.7 – 1737.2)	1266.2 (552.5–2335.2)	0.214
BALP (U/L)	22.9 (17.2 – 34.5)	24.7 (16.1 – 37.7)	0.676
Vitamin D (ng/mL)	**27.0 (18.8 – 29.6)**	**18.7 (13.7 – 24.6)**	**0.021**
Sclerostin (ng/mL)	**0.8 (0.5 – 1.1)**	**0.6 (0.3 – 0.9)**	**0.023**
	BALP		
	21.0 (15.7 – 28.6)	59.3 (49.7 – 74.4)	
Phosphate (mg/dL)	3.1 (2.9 – 3.5)	3.1 (2.3 – 3.9)	0.742
Calcium (mg/dL)	9.7 (9.3 – 10.3)	10.0 (9.5 – 10.9)	0.181
Magnesium (mg/dL)	1.7 (1.6 – 1.8)	1.7 (1.4 – 1.9)	0.545
FGF23 (RU/mL)	136.0 (94.7 – 169.7)	123.6 (104.7 – 161.4)	0.777
Alpha-klotho (pg/mL)	785.2 (399.2 – 2055.2)	1289.6 (774.2–1934.2)	0.107
PTH (pg/mL)	122.0 (86.5 – 23.7)	147.6 (130.5 – 232.7)	0.317
Vitamin D (ng/mL)	22.3 (15.4 – 28.2)	23.5 (13.0 – 30.0)	0.964
Sclerostin (ng/mL)	0.7 (0.5 – 0.9)	0.8 (0.5 – 1.1)	0.596
	FGF23		
	99.2 (86.4 – 117.3)	168.5 (147.7 – 260.2)	
Dialysis vintage (M)	50.0 (36.0 – 82.0)	82.0 (53.0 – 96.0)	0.065
Phosphate (mg/dL)	3.1 (2.8 – 3.6)	3.1 (2.7 – 3.5)	0.958
Calcium (mg/dL)	9.6 (9.3 – 10.4)	10.0 (9.6 – 10.7)	0.220
Magnesium (mg/dL)	1.7 (1.5 – 1.8)	1.7 (1.6 – 1.8)	0.314
Alpha-klotho (pg/mL)	831.0 (481.7 – 2044.2)	1148.6 (552.5–2335.2)	0.363
PTH (pg/mL)	**129.9 (86.5 – 171.7)**	**272.5 (115.1 – 476.9)**	**0.004**
BALP (U/L)	23.0 (17.4 – 37.0)	22.1 (15.7 – 35.2)	0.665
Vitamin D (ng/mL)	24.6 (15.4 – 30.0)	19.5 (13.7 – 27.5)	0.122
Sclerostin (ng/mL)	0.7 (0.5 – 0.9)	0.7 (0.4 – 1.2)	0.834

Table 50.2 – Associations between bone-related variables

Statistical analysis: Mann-Whiney test. Median and IQ range values are presented. PTH – parathyroid hormone; FGF23 – fibroblast growth factor 23; BALP – bone alkaline phosphatase.

Concerning FGF23, fourteen patients (20.3%) maintained levels above normal. There was a trend toward longer dialysis vintage in the group of patients with FGF23 > 180 RU/mL No other demographic associations were found. Also, no associations were found with metabolic evaluation (with the exception of PTH) or histomorphometric evaluation when comparing the two groups of patients (those with normal FGF23 levels and those with above the normal FGF23 levels).

Eighteen patients (26.1%) had low levels of alpha-klotho. As at baseline, having low alpha-klotho levels did not associate with demographic differences. Low levels of alpha-klotho were associated with higher levels of sclerostin and lower levels of BALP and calcitonin, with no differences in FGF23, PTH, vitamin D, calcium, phosphate, or magnesium, as shown in Table 50.2. In bone analysis, we found that a deficiency in alpha-klotho was associated with a lower cellular activity (seen particularly in osteoclasts) and with a lower osteoid thickness, as Table 51 shows.

More than half the patients had sclerostin levels higher than 0.6 ng/mL (59.4%, n=41). Sclerostin maintained its significant association with age, as those with high levels of sclerostin were older. The group of patients who had higher levels of the molecule had lower levels of PTH (as at baseline), lower levels of alpha-klotho, higher levels of vitamin D, and higher phosphate levels. This molecule associated with a decrease in all histomorphometric osteoid values (osteoid thickness; osteoid surface/bone surface; osteoid volume/bone volume or osteoid volume/tissue volume), but not with other bone measurements, such as bone volume, mineralized bone volume, or any dynamic parameters.

Hypovitaminosis D was present in eighteen patients (26.1% of patients). Vitamin D insufficiency was associated with higher levels of BALP and a trend toward higher levels of PTH, lower levels of sclerostin, with no differences in minerals (calcium, phosphate, or magnesium), or in other bone-derived markers, such as FGF23 or alpha-klotho. The deficiency of this hormone did not associate with any bone parameter.

Related variables	Group 1	Group 2	p-value
	Sclerostin		
	0.4 (0.3 – 0.5)	0.4 (0.3 – 0.5)	
Age (years)	**41.5 (32.5 – 56.0)**	**57.0 (49.0 – 63.0)**	**<0.001**
Calcium (mg/dL)	9.9 (9.6 – 10.6)	9.6 (9.2 – 10.3)	0.077
Phosphate (mg/dL)	**2.9 (2.4 – 3.4)**	**3.3 (3.0 – 3.6)**	**0.021**
Magnesium (mg/dL)	1.7 (1.6 – 1.8)	1.7 (1.5 – 1.8)	0.756
FGF23 (RU/mL)	122.7 (86.8 – 179.1)	135.2 (113.6 – 159.7)	0.549
Alpha-klotho (pg/mL)	**1267.6 (888.1 – 2232.2)**	**638.3 (380.0 – 1555.2)**	**0.004**
PTH (pg/mL)	**165.7 (115.5 – 247.7)**	**120.1 (86.4 – 150.8)**	**0.022**
BALP (U/L)	23.3 (17.9 – 35.6)	23.0 (16.9 – 35.2)	0.797
Vitamin D (ng/mL)	**18.1 (13.4 – 25.8)**	**27.0 (19.2 – 29.5)**	**0.035**
	Alpha-klotho		
	304.2 (262.5 – 399.2)	1269.0 (774.2 –2335.2)	
Calcium (mg/dL)	9.6 (9.1 – 10.3)	9.8 (9.3 – 10.5)	0.431
Phosphate (mg/dL)	3.2 (2.9 – 3.6)	3.1 (2.6 – 3.5)	0.244
Magnesium (mg/dL)	1.6 (1.6 – 1.8)	1.7 (1.6 – 1.8)	0.827
FGF23 (RU/mL)	139.8 (127.3 – 159.1)	125.0 (91.0 – 169.8)	0.232
PTH (pg/mL)	122.3 (80.4 – 164.9)	136.6 (101.1 – 238.8)	0.224
BALP (U/L)	**18.6 (15.7 – 23.0)**	**27.4 (17.7 – 41.3)**	**0.020**
Vitamin D (ng/mL)	27.3 (18.8 – 29.5)	21.1 (13.7 – 29.0)	0.131
Sclerostin (ng/mL)	**0.8 (0.6 – 1.1)**	**0.5 (0.4 – 0.9)**	**0.040**
Calcitonin (ng/dL)	**2 (2.0 – 2.7)**	**3.1 (2.0 – 5.3)**	**0.004**
	Vitamin D		
	12.7 (12.1 – 13.9)	27.1 (21.1 – 30.0)	
Calcium (mg/dL)	9.7 (9.5 – 10.1)	9.8 (9.2 – 10.5)	0.748
Phosphate (mg/dL)	3.1 (2.5 – 3.6)	3.1 (2.8 – 3.5)	0.934
Magnesium (mg/dL)	1.6 (1.6 – 1.8)	1.7 (1.5 – 1.8)	0.834
FGF23 (RU/mL)	129.0 (87.2 – 197.7)	135.2 (104.7 – 168.5)	0.632
Alpha-klotho (pg/mL)	1220.6 (638.3 – 2129.2)	924.0 (399.2 – 1737.2)	0.268
PTH (pg/mL)	159.7 (102.0 – 267.9)	119.7 (85.9 – 148.2)	0.080
BALP (U/L)	**34.1 (19.9 – 50.7)**	**22.0 (16.1 – 32.3)**	**0.036**
Sclerostin (ng/mL)	**0.5 (0.3 – 0.9)**	**0.8 (0.6 – 1.1)**	**0.027**

Table 50.3 – Associations between bone-related variables

Statistical analysis: Mann-Whiney test. Median and IQ range values are presented. PTH – parathyroid hormone; FGF23 – fibroblast growth factor 23; BALP – bone alkaline phosphatase.

Although we found no association between BALP levels and the metabolic analysis, we did find associations with dynamic values, as shown in Table 51.

Related variables	Group 1	Group 2	p-value
	PTH		
OtV/BV (%)	3.9 (1.4 – 5.1)	4.8 (3.0 – 9.2)	0.012
OtS/BS (%)	14.4 (7.4 – 23.0)	24.4 (17.8 – 34.3)	<0.001
Cortical porosity (%)	5.2 (3.5 – 7.9)	7.5 (4.4 – 12.9)	0.025
	BALP		
ObS/BS (%)	1.8 (0.9 – 3.2)	2.6 (1.4 – 4.2)	0.303
OcS/BS (%)	0.3 (0 – 0.7)	1.0 (0.6 – 1.3)	0.002
OtV/TV (%)	0.8 (0.3 – 1.3)	1.1 (0.6 – 1.8)	0.037
Ot Thickness (µm)	8.7 (6.6 – 11.9)	13.0 (9.8 – 14.0)	0.002
BFR/BS ($\mu m^3/\mu m^2/y$)	13.4 (4.6 – 27.7)	36.2 (21.4 – 60.7)	0.009
Cortical porosity (%)	5.6 (3.6 – 8.7)	8.5 (5.3 – 11.1)	0.054
	Alpha-klotho		
ObS/BS (%)	1.6 (0.8 – 2.5)	2.3 (1.1 – 3.6)	0.214
OcS/BS (%)	0.2 (0 – 0.4)	0.5 (0.1 – 1.1)	0.044
Ot Thickness (µm)	6.9 (5.7 – 8.9)	9.8 (7.4 – 12.7)	0.005
	Sclerostin		
OtV / TV (%)	1.0 (0.5 – 1.4)	0.6 (0.2 – 1.3)	0.044
OtV / BV (%)	4.6 (3.7 – 6.4)	3.6 (1.2 – 5.5)	0.030
OtS / BS (%)	22.1 (17.8 – 32.3)	17.2 (7.5 – 25.1)	0.023
Ot Thickness (µm)	11.1 (8.8 – 12.7)	7.5 (6.1 – 11.7)	0.016
Cortical porosity (%)	5.2 (2.5 – 8.5)	6.7 (4.3 – 11.9)	0.058
	Calcium		
Cortical Thickness (µm)	554.3 (388.4 –784.9)	771.6 (586.5 -1001.0)	0.021
Cortical porosity (%)	4.5 (3.6 – 8.4)	7.5 (5.3 – 12.8)	0.012
	Phosphorus		
OtV / TV (%)	1.6 (1.1 – 3.1)	0.7 (0.3 – 1.2)	<0.001
OtV / BV (%)	8.2 (4.4 – 12.2)	3.9 (1.7 – 5.2)	<0.001
OtS / BS (%)	33.7 (24.0 – 43.1)	17.8 (10.6 – 24.5)	<0.001
Ot Thickness (µm)	12.6 (11.9 – 16.1)	8.9 (6.6 – 11.7)	0.005

Table 51 – Associations between bone-related variables and bone biopsy findings

Statistical analysis: Mann-Whiney test. Median and IQ range values are presented. PTH – parathyroid hormone; FGF23 – fibroblast growth factor 23; BALP – bone alkaline phosphatase; OtV – osteoid volume; BV – bone volume; TV – tissue volume; OtS/BS – osteoid surface / bone surface; Ot – osteoid; ObS/BS – osteoblast surface / bone surface; OcS/BS – osteoclast surface/bone surface; BFR/BS – bone formation rate / bone surface

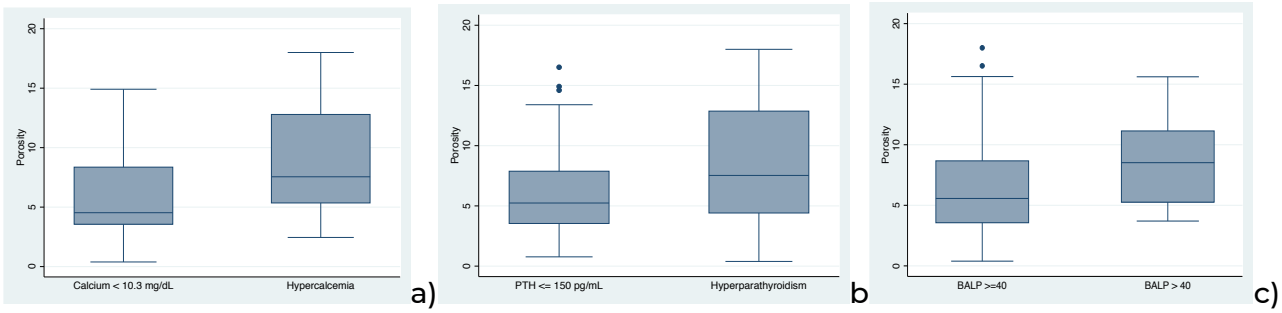
We found higher bone formation rate in the group of patients with higher BALP levels, and with static findings, as the group of patients with high BALP levels also had higher osteoclast surface, and a trend toward higher osteoblast surface, although this was without statistical significance. Notably, osteoid measurements were also associated with BALP levels.

HISTOLOGIC EVALUATION

As shown in Table 51, some deviations in bone-derived biomarkers had significant associations with histological bone findings.

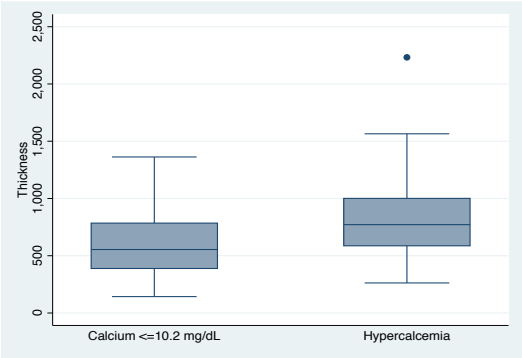
Cortical bone

Patients with high PTH levels or high calcium levels or high BALP levels had higher cortical porosity, as shown in Graphic 14.



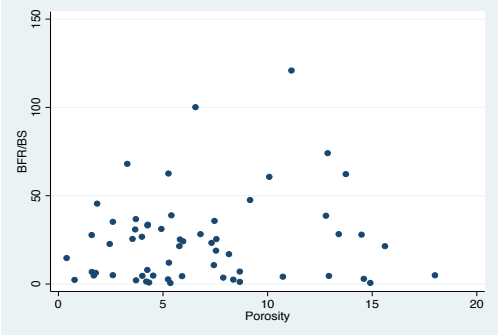
Graphic 14 – Associations between high levels of calcium (a), PTH (b) and BALP (c) and cortical porosity.

It was curious to note that the only biochemical parameter that had an association with cortical thickness was calcium (p=0.021), and patients with hypercalcemia had higher cortical thickness than the remaining ones, as exposed in Graphic 15.



Graphic 15 – Associations between levels of calcium and cortical thickness.

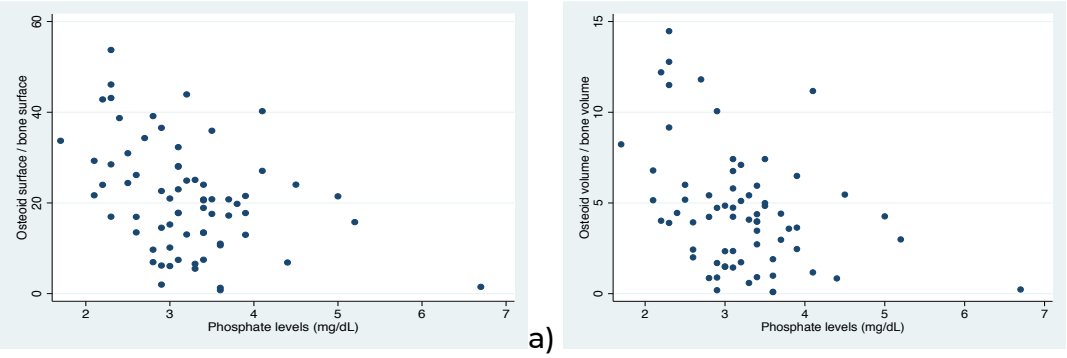
Despite these results, we did not find any association between cortical findings (thickness and porosity) and trabecular results. The non-significant correlation between BFR/BS and porosity is shown in Graphic 16.



Graphic 16 – Correlations between BFR/BS and cortical porosity.

Trabecular bone

As Table 51 shows, vitamin D levels correlated negatively with osteoid surface/bone surface (r=-0.2, p=0.033), and with osteoid mineralization lag time (r=0.3, p=0.03). Phosphorus correlated negatively with osteoid volume/bone volume (r=-0.4, p=0.001), and osteoid surface/bone surface (r=-0.4, p=0.002), as shown in Graphic 17. Likewise, hypophosphatemic patients had more osteoid than other patients (osteoid thickness; osteoid surface/bone surface; osteoid volume/bone volume and osteoid volume/tissue volume). Four patients (5.8%) had both vitamin D and phosphate low levels. Those patients showed no increased percentage of mineralization defects.

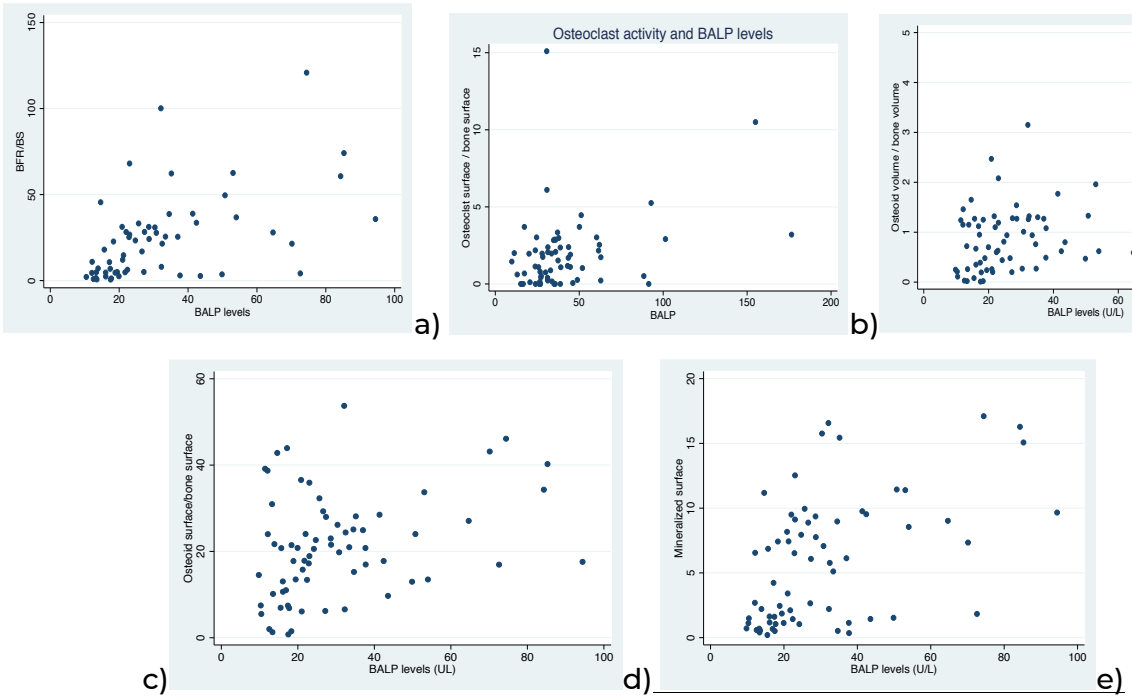


Graphic 17 – Correlations between phosphate levels and: a) osteoid surface/bone surface; b) osteoid volume/bone volume

Calcium correlated with osteoid volume/bone volume (r=-0.3, p=0.014), and with osteoid surface/bone surface (r=-0.3, p=0.020), osteoid thickness (r=0.3, p=0.008), mineralized surface (r=0.5, p<0.001), mineralized osteoid (r=0.3, p=0.012), and adjusted MAR (r=0.4, p=0.006). Sclerostin levels above normal were associated with a decrease in all histomorphometric osteoid values (osteoid thickness; osteoid surface/bone surface; osteoid volume/bone volume or osteoid volume/tissue volume). Conversely, a deficiency in alpha-klotho was associated with a lower osteoid thickness.

The group of patients with PTH levels above 150 pg/mL had higher percentages of osteoid surface/bone surface and osteoid volume/bone volume. Osteoid measurements were also superior in patients with higher BALP levels. Unlike in PTH, this group of patients with BALP levels above the normal range had higher bone formation rate, higher osteoclast surface, and a trend toward higher osteoblast surface, although this was without statistical significance. Conversely, a deficiency in alpha-klotho was associated with a lower cellular activity (seen particularly in osteoclasts).

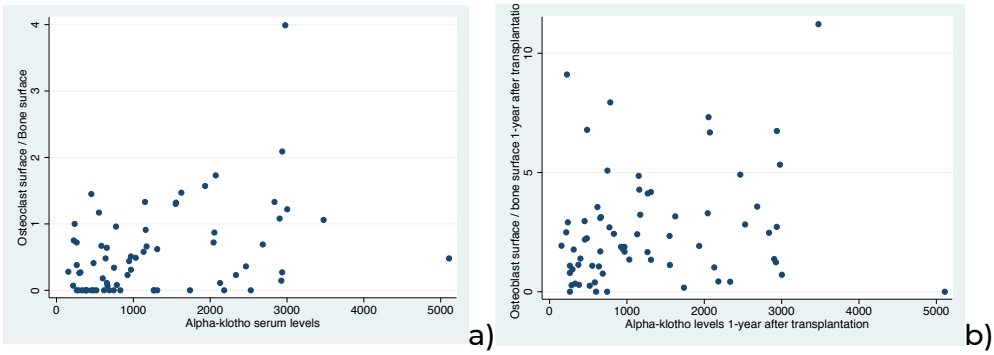
Looking at the bone-related markers as continuous variables (instead of divided into groups based on the normal values), it is interesting to note that the only biochemical parameter that correlated to BFR/BS was BALP levels (r=0.52, p<0.001), and not PTH, as Graph 18 illustrates.



Graphic 18 – a) Correlation between BALP serum levels and a) BFR/BS; b) osteoclast surface/bone surface); c) osteoid volume/bone volume; d) osteoid surface/bone surface; e) mineralized surface.

BALP – bone alkaline phosphatase; BFR/BS – bone formation rate / bone surface

BALP correlated with osteoclast surface (r=0.4, p=0.003), OtV/BV (r=0.5, p<0.001), OtS/BS (r=0.3, p=0.007), osteoid thickness (r=0.3, p=0.008), mineralized surface/bone surface (r=0.5, p<0.001), mineralized osteoid (r=0.3, p=0.012), and adjusted MAR (r=0.4, p=0.006). The other marker that correlated with cellular activity was alpha-klotho. Alpha-klotho serum levels correlated with osteoclast surface/bone surface (r=0.4, p=0.001), as Graphic 19 shows. We would expect to find an association between osteoblast surface/bone surface and alpha-klotho levels, but we did not find that association.



Graphic 19 – Correlation between alpha-klotho serum levels and a) osteoclast surface / bone surface and b) osteoblast surface / bone surface 1-year after transplantation

We found no correlations between osteoblast activity and the different bone-related biochemical evaluation.

- ✓ Volume

We found no associations between bone volume data measured in the 2nd bone fragments (BV/TV) and biochemical / serum bone-related parameters, nor even demographic characteristics, such as age, gender, BMI, or therapeutic drugs. Nevertheless, we found a trend of association between low bone volume and Caucasian race compared to other races, and a trend between low bone volume and menopause status.

- ✓ Remodeling

Looking at the different categories of bone remodeling after transplantation, we obtained different predictors for remodeling categories compared to the findings at baseline, as shown in Table 52. At this stage demographic characteristics did not seem to be relevant. BALP maintained as an important factor, but not PTH, and we found that deficiency in vitamin D was prevalent in high turnover disease.

Studying low bone turnover in comparison with normal or high bone turnover, neither age nor PTH levels were associated with low bone turnover, as it was at baseline. We found white race (p=0.029), low BALP (17.6 U/L, vs. 32.1 U/L p<0.001) levels, high FGF23 levels (146.6 RU/mL vs. 122.2 RU/mL, p=0.028), high vitamin D levels (25.9 ng/mL vs. 20.3 ng/mL, p=0.019), and lower eGFR (46.2 mL/min/1.73m^2 vs. 59.9 mL/min/1.73m^2, p=0.016) to be potential predictors of low bone turnover (vs. normal-high bone turnover). Interestingly, low bone turnover at baseline was not significantly associated with low bone turnover after 1-year of transplant (p=0.076).

These results were supported when applying a logistic regression for low vs. normal-high bone turnover: low BALP levels, low eGFR, high vitamin levels were predictors of low bone turnover, adjusting for age and race, as presented in Table 53.

	Low turnover (n=31)	Normal turnover (n=31)	High turnover (n=7)	p-value
ObS/BS	1.3 (0.4 – 2.3)	2.9 (1.4 – 4.2)	4.2 (2.3 – 5.1)	-
OcS/BS	0.09 (0.0 – 0.3)	0.7 (0.4 – 1.2)	0.9 (0 – 1.3)	-
BFR/BS	4.6 (2.4 – 7.1)	28.1 (24.2 – 33.6)	68.0 (49.5 – 100.1)	-
Age (years)	53.5 (43.5 – 62.5)	53.5 (34.0 – 62.0)	48.0 (41.0 – 60.0)	0.302
Male gender	20 (62.5%)	24 (80.0%)	4 (57.1%)	0.436
Caucasian race (%)	87.5	66.7	71.4	0.071
Dialysis vintage (Month)	55.5 (45.0 – 86.0)	55.0 (36-0 – 83.0)	48.0 (33.0 – 98.0)	0.563
PTDM	3 (9.4%)	5 (16.7%)	3 (42.7%)	0.070
T1 BMI (Kg/m^2)	25.4 (22.5 – 30.6)	24.3 (22.2 – 25.6)	24.2 (20.1 – 28.1)	0.536
T1 PTH (pg/mL) PTH > 150 pg/mL	130.2 (82.6 – 245.9) 11 (34.4%)	132.4 (101.1 – 190.3) 12 (40.0%)	150.7 (122.0 – 232.7) 4 (57.1%)	- 0.338
T1 BALP (U/L) BALP > 40 U/L	**17.2 (13.4 – 21.7)** **3 (9.4%)**	**29.5 (22.9 – 37.0)** **6 (20.0%)**	**54.0 (32.1 – 84.3)** **5 (71.4%)**	**-** **0.004**
T1 FGF23 (RU/mL) FGF23 > 180 RU/mL	146.6 (113.5 – 172.9) 7 (21.9%)	120.2 (86.4 – 150.0) 5 (16.7%)	126.1 (102.2 – 192.2) 2 (28.6%)	- 0.934
T1 Alpha-klotho (pg/mL) Klotho < 500 (pg/mL)	764.7 (376.2 – 2258.7) 11 (34.4%)	1000.1 (552.5 – 2044.2) 7 (23.2%)	1160.0 (748.3 – 1551.2) 0	- 0.079
T1 Sclerostin (ng/mL) Sclerostin > 0.6 ng/mL	0.7 (0.4 – 1.0) 20 (62.5%)	0.7 (0.5 – 0.9) 18 (60.0%)	0.6 (0.5 – 1.0) 3 (42.9%)	- 0.482
T1 Calcium (mg/dL) Calcium > 10.2 mg/dL	9.6 (9.2 – 10.4) 11 (34.4%)	9.7 (9.4 – 10.3) 9 (30.0%)	10.1 (9.9 – 10.7) 3 (42.9%)	- 0.978
T1 Phosphate (mg/dL) Phosphate < 2.5 mg/dL	3.1 (2.8 – 3.6) 4 (12.5%)	3.1 (2.8 – 3.5) 5 (16.7%)	3.4 (2.3 – 3.4) 2 (28.6%)	- 0.360
T1 Vitamin D (ng/mL) Vitamin D < 15 ng/mL	**25.9 (19.5 – 30.5)** **4 (12.5%)**	**20.3 (13.9 – 27.5)** **10 (33.3%)**	**14.3 (13.0 – 25.5)** **4 (57.1%)**	**-** **0.008**

Table 52 – Predictors of different bone turnover categories

Statistical analysis: Kruskal-Wallis test and Fisher exact test. ObS/BS – osteoblast surface / bone surface; OcS/BS – osteoclast surface / bone surface; PD – peritoneal dialysis; BMI – body mass index; PTH – parathyroid hormone; BALP – bone alkaline phosphatase; FGF23 – fibroblast growth factor 23; VC – vascular calcifications; T0 – baseline; T1 – at 1-year

	Low bone turnover		
	OR	95% CI	p-value
T1 vitamin D (ng/mL)	2.60*	1.12 – 6.05	0.026
T1 BALP (U/L)	0.51*	0.32 – 0.82	0.005
T1 eGFR (mL/min/1.73m^2)	0.74*	0.57 – 0.97	0.032
T0 Low bone turnover	1.55	0.30 – 28.4	0.544

Table 53 – Logistic regression for independent associations with low bone turnover

Statistical analysis: Logistic regression test. BALP – bone alkaline phosphatase; eGFR – estimated glomerular filtration rate; T0 – baseline; T1 – at 1-year

(*For each increase in 10 units)

High bone turnover (vs. normal-low) was associated with high BALP levels at T1 (54.0 U/L vs. 22.2 U/L, p=0.016) and with high bone turnover at baseline (p=0.002), both in univariate and multivariate analysis, adjusted for age and GFR, as presented in Table 54.

	High bone turnover		
	OR	95% CI	p-value
T1 BALP (U/L)	1.85*	1.17 – 2.93	0.009
T0 High bone turnover	21.1	1.64 – 272.0	0.019

Table 54 – Logistic regression for independent associations with high bone turnover

Statistical analysis: Logistic regression test BALP – bone alkaline phosphatase; T0 – baseline; T1 – at 1-year

(*For each increase in 10 units)

In our model, values of vitamin D above 18.8 ng/L and of BALP below 11.4 U/L could be associated with low bone turnover, whereas high bone turnover can be suspected in patients whose BALP levels are above 43.6 U/L.

Cardiac comparisons between a historical cohort and the contemporary cohort

A separate analysis aiming to compare the contemporary cohort with a historical cohort in terms of cardiac geometry was performed in the form of a mix case-control analysis. At the end of the first year of transplantation, our patients (cases) were paired for age, sex, dialysis vintage, presence of diabetes, and hypertension with a dialysis population listed for kidney transplantation (controls) on a 1 to 1 basis. We compared LVMI and presence of LVH in both populations: in the case cohort at baseline and after 1-year of transplant; in the control cohort, we evaluated the last echocardiogram performed while on the waiting list and the one performed the year before. Different operators performed the echocardiograms.

We found no statistical difference between the demographic characteristics of both populations, in line with our expectations. Although LVMI was similar in both populations, we found a significant difference in the presence of LVH if we added 12 months of dialysis in a patient that otherwise could be submitted to transplant, as shown in Table 55.

	Transplant cohort	Waiting-list cohort	p-value
Age (years)	53.0 (41.0 – 62.0)	48 (39 – 55)	0.166
Male gender (%)	69.6%	53.3%	0.181
Dialysis vintage (months)	55.0 (42.0 – 84.0)	50 (36 – 74)	0.201
LVMI (g/m^2) at baseline	107 (91.5 – 140.5)	104 (92– 155)	0.934
LVH (%) at baseline	42%	45%	0.163
LVMI (g/m^2) at the end	108.5 (98-138)	120 (108 – 138)	0.258
LVH (%) at the end	**39.4%**	**60%**	**0.005**
Delta LVMI	8.5 (-16 – 28.5)	16 (-14 – 30)	0.728

Table 55 – Comparisons of two cohorts: transplanted cohort and the dialysis (on waiting list) cohort

Statistical analysis: Mann-Whiney test and Fisher exact test. LVMI – left ventricular mass index; LVH – left ventricular hypertrophy

This analysis was published in the form of letter in the Portuguese Journal of Nephrology and Hypertension[181].

DISCUSSION

This work is divided into four main sections: 1) the evaluation of mineral, bone, and cardiovascular condition of 84 uremic patients listed and called for renal transplantation; 2) the evolution of mineral, bone, and cardiovascular conditions pre and 1-year after transplantation in 69 patients who continued in the study; 3) the evaluation of extra imaging exams (DXA and coronary CT) and the associations of those with mineral and bone metabolism; 4) the evaluation of mineral, bone, and cardiovascular condition at 1-year post-transplantation.

The bone biopsies were performed with disposable 4.5 mm internal diameter trephines instead of the classical 7.5 mm inner diameter Meunier / Bordier trephine. A recent study proved that evaluation of bone biopsies with smaller needles is comparable and sufficient for a correct ROD diagnosis[182].

1) Mineral, bone, and cardiovascular condition of 84 uremic patients

In the first section of the study, we found that in a population of patients listed for kidney transplantation, baseline biochemical evaluation was in line with KDIGO guidelines for anemia and CKD-MBD management. Despite these findings, ROD was present in more than half of the patients, if we add remodeling abnormalities (in 38 patients, 46.9%) to mineralization defects (in 9 patients, 11.1%). Additionally, 25 patients (29.8%) had low volume. Low turnover was associated with low PTH levels only in 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. Isolated low volume was associated with high sclerostin levels, and abnormal mineralization with low phosphorus 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 (and sclerostin in univariate analysis), as did age, BMI, and diabetes. The presence of valve calcifications was associated with low volume, a lower mineralized bone volume and with adynamic bone disease. The presence of normal turnover was associated with absence of valve calcifications. In multivariate analysis, adynamic bone disease was associated with valve calcifications and normal turnover was associated with absence of valve calcifications. In our population, higher levels of sclerostin led to a higher HR of death.

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 a comparison of the three types of turnover (low-normal-high), we found that a high level of sclerostin was associated with low turnover, similar to the result of other studies in pre-dialysis patients[60]. 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. Likewise, PTH levels were not associated with low bone turnover in multivariate analysis. Sclerostin, a glycoprotein product of the *SOST* gene in mature osteocytes[23] and a negative regulator of bone metabolism[48], 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[56] thus the production of sclerostin is increased in CKD patients. The sources are not only bone cells (especially osteocytes, but also osteoclast precursors)[51], as we learn that sclerostin mRNA in bone is not increased in aged and CKD patients[183], but the source can also be the vascular cells[51], since increased sclerostin expression has been demonstrated during vascular smooth muscle cell calcification in an animal model[62]. Indeed, sclerostin serves as an inhibitor of bone formation[7,23,48] and this protein was the only parameter to predict isolated low bone volume in our patients.

Some studies hypothesize that sclerostin may negatively influence mineralization via regulation of FGF23[48] and alteration of vitamin D synthesis in proximal tubular cells[53]. We did not observe that. We could expect to find an association between high levels of FGF23 and mineralization defect, as FGF23 could inhibit bone mineralization through vitamin D inactivation[15]. Nevertheless, in our patients admitted for renal transplantation, abnormal mineralization was existent in the presence of low FGF23 levels, low phosphate levels and high BALP levels, with no association with vitamin D levels. 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. We also found that BALP was correlated with osteoid production, and as BALP is produced by osteoblasts, this correlation could be expected. As demonstrated by others, BALP was a better tool than PTH to differentiate high from low or normal bone turnover[121]. According to our study, low

levels of PTH could not predict low bone turnover when adjusting for age, and similarly, could not predict high bone turnover when adjusted for age, race, and dialysis vintage. 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[121].

In order to perform the majority of its functions, FGF23 needs to bind to its receptors, and needs a co-factor, the alpha-klotho protein, which acts as a co-receptor[15]. None of those factors were associated with LVMI or with LVH, contrary to the findings of studies with larger populations[24]. Perhaps the fact that we only had 84 patients enrolled influenced this lack of association. Even so, FGF23 was identified as an independent risk factor for higher severity for vascular calcification score in this population, which presented, overall, a low Adragão score. A study by Scialla and colleagues, 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[184]. We should acknowledge that our patients are different from those analyzed in the reported study since all have CKD stage 5 and the median levels of FGF23 were nearly 13 times higher than the levels of the patients studied by Scialla and co-workers. Furthermore, our patients with cardiovascular events in a 12-month period had higher levels of FGF23 than the remaining ones, meaning that this hormone is a powerful marker in predicting cardiovascular risk, a finding which is aligned with other reports[25].

Although it has been demonstrated that in a population of prevalent hemodialysis patients, Adragão score is related to worst outcome if ≥3, 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.

As expected, age, diabetes, and dialysis vintage were important factors for calcifications. Curious was the fact that phosphorus wasn't a risk factor for calcification, probably because the nephrology community is focused on controlling this risk factor. A trend toward higher vascular calcification score with the use of calcium carbonate was present [71.4% of the patients that used calcium carbonate

had a severe Adragão score (≥2) vs. 28.7% of patients that did not use the drug]. Bone histomorphometric analysis did not correlate with Adragão score, but we could correlate the baseline bone morphological findings with valve calcifications. We know that in addition to patients' genetics, mechanical stress, inflammation, or even endocarditis, abnormalities of mineral and bone metabolism are important risk factors for valve calcifications[130]. Baseline bone morphological findings, such as turnover status, bone volume, and mineralized bone volume were associated with valve calcifications. Adynamic bone disease, which combines low volume with low turnover, was associated with higher odds for the disorder. We found no association between the therapy and presence of valve calcifications. Other studies also highlighted the importance of mineral and bone metabolism for valve calcifications[26], and the importance of low bone volume to coronary calcifications[80]. In a recent Portuguese study, the authors found that higher mineralized bone volume was associated to a lower plain X-ray vascular calcification[28]. We had a similar finding in univariate analysis, concerning valve calcifications.

Although sclerostin was not a strong predictor of severity of vascular calcifications, in our population of patients admitted for renal transplantation, this protein 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 of sclerostin are associated with better survival in hemodialysis patients[49,50], suggesting a protective role through inhibition of vascular calcifications[49,63], while other studies have found an association between high levels and CV mortality in dialysis patients[54], and association with the degree of vascular calcifications[61]. A phase III randomized study to evaluate efficacy and safety of romosozumab, a sclerostin inhibitor, in men with osteoporosis, revealed a difference in cardiovascular serious adverse events, with more cases in the treatment group[67], indicating the protective role of the molecule.

Cortical bone has not been well evaluated in ESRD patients, as trabecular bone is better related with metabolic abnormalities in those patients. Some authors point out that porosity is associated to high bone turnover, and we found that extremes of PTH were related to cortical porosity, as well BALP high levels. We showed that cortical porosity >10% was related with osteoid volume.

It is interesting to note that patients submitted to parathyroidectomy had lower levels of PTH, but no other differences regarding the other bone-related hormones, proteins or minerals, nor even regarding histomorphometric bone parameters. We believe that the fact that some patients who undergo parathyroidectomy have no differences in most of the parameters, other than an immediate reduction in PTH levels, is because bone cellular and mineral metabolism are long-time answers to achieving a new equilibrium.

We had a surprise in the metabolic evaluation at baseline. We found that the associations between bone-related variables were not mutual, meaning that, after dividing a variable of interest by its median and studying the association with a bone-related laboratory parameter, presented as a continuous variable, the associations were not in two-way directions. This can be related to the fact that we did not divide the biomarker by an abnormal value, but instead by its median. As an example, the two groups of sclerostin (lowest levels vs. highest levels) were associated with PTH, so patients with greater inhibition of bone formation had lower values of PTH. But the group of patients with the highest values of PTH did not necessarily have significant differences in sclerostin values. In this line of thought, sclerostin could contribute to low turnover osteodystrophy, as it is associated with low PTH. Similarly, patients within the group of high FGF23 values had the highest values of sclerostin and patients with low FGF23 values had the lowest values of sclerostin. Nevertheless, the two groups of sclerostin divided by the median value did not have differences in FGF23 serum levels. Additionally, high PTH levels correlated with high FGF23 levels, and high FGF23 levels with high sclerostin levels. A second surprise was the fact that alpha-klotho had no relation to FGF23 levels in the pre-transplant setting (but also 1-year after transplantation).

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.

2) Evolution of mineral, bone, and cardiovascular conditions

The second section of our study was dedicated to evaluate the progression of metabolic, bone and cardiovascular assessments at both time periods (baseline and 1-year after transplant).

The major findings of the second part of our study are summarized in the present paragraph. Comparing the evolution of biochemical parameters, we found that laboratory evaluation 1-year after transplant to be as expected, considering the significant improvement in renal function, with a median eGFR of 53 mL/min/1.73m^2, calculated by the CKD-EPI formula[185]. Calcium and alpha-klotho rose significantly; phosphorus, magnesium, calcitonin, PTH, BALP, FGF23, and sclerostin decreased significantly. We did not presume an increase in uric acid values, as serum creatinine decreased significantly. Nevertheless, uric acid is lowered by dialysis and most of those patients were prescribed diuretics. Despite these results, ROD was present in 68.1% of our patients 1-year after transplantation, but overall, we believe that histological results improved. One important finding was that, in our population, volume did not change pre- and post-transplant, was stable at the two evaluations, and mineralization was also not statistically different at the two time points. Considering bone remodeling, a significant increase in patients with low remodeling disease occurred (15 to 31 patients), and low bone turnover was present in 43.5% of the population, in the form of adynamic bone disease in 10 patients (if we define it as low bone turnover plus low bone volume), or as osteomalacia in 3 patients. On the other hand, high bone turnover significantly decreased and hyperparathyroid bone disease was present only in 7 patients after transplantation. Neither echocardiographic findings nor vascular calcification score was different at the two points of the study. Nonetheless, 12 months is a short time period to observe major differences in the main echocardiographic findings. Even so, we found that LVH is reduced after 1-year of transplantation if comparing to the waiting list population, reinforcing the benefits of transplantation in CKD patients.

The histomorphometric changes after transplantation were positive, showing an evolution to a healthier bone. Although cortical thickness had decreased, there was no difference in the porosity % between the two studied periods. Overall, trabecular bone dynamics were similar to those published in the recent literature[160-162], although different reference ranges for turnover were applied (Salusky, composite parameters,

and Malluche respectively), as we still lack from agreement in the turnover diagnostic cut-offs. Although a similar approach (double bone biopsy) was used in the aforementioned studies, we included an extra osseous calcification analysis, which has not been studied by other authors. In addition, the Belgian and Finnish studies included only Caucasian patients, a high number of males, and a high number of diabetic patients. Although our population characteristics were closer to those of the Brazilian cohort, they only addressed living-donor recipients and excluded those with low PTH levels and low bone turnover diagnosed by a bone biopsy prior to entry into the study.

We observed a significant reduction in the number of patients with high turnover bone disease and a significant increase in the number of patients with low turnover bone disease, with stable numbers of normal turnover. The reduction in bone remodeling was associated with a greater increase in the levels of alpha-klotho and higher levels of alpha-klotho at 1-year after transplantation (and the opposite, the increase in bone turnover, showed inverse correlations – association with a lower level of alpha-klotho at baseline, and a more discrete increase of the hormone after 1-year), but not with PTH or BALP levels, as expected. In fact, PTH did not have an influence on trabecular bone dynamics. In addition, changes in BALP were not associated with turnover deviations, but we noted that the development of abnormal mineralization post-transplantation occurred in patients with a greater increase in levels of BALP in comparison to baseline. A recent study showed that alkaline phosphatase levels could predict mineralization defects in a pediatric population[186]. In our population, BALP levels in T1 associated with OtS/BS, OtV/BV, osteoid thickness, mineralized surface, and mineralized osteoid and as this marker is produced by osteoblasts during bone formation[43], inactivating pyrophosphate, which inhibits mineralization[45], and osteopontin, which is a calcification inhibitor[42], we can suspect some resistance to BALP actions at the bone after transplantation. Actually, after transplantation, osteoblast cells can become dysfunctional and have a lower ALP expression[187], and increase their own apoptosis, which contributes to bone disorders in these patients[153]. It is curious that FGF23 supresses the activity of tissue non-specific alkaline phosphatase, decreasing the degradation of pyrophosphate, and thus decreasing phosphorus levels[42], but this hormone had no relation with osteoid measurements in bone. Although low bone turnover increased significantly, only 3 patients presented with osteomalacia, and only 10 patients presented with low turnover with low volume

(adynamic bone disease). Even so, it is important to note that the presence of adynamic bone disease (defined as low turnover and low volume) did not increase with the transplant, which is a good result, as we saw an association between adynamic bone disease and valve calcifications at baseline. It looked like, at a clinical level, having or maintaining a high bone turnover after the transplant is more important than having a low bone turnover after, as this was associated with the severity of coronary calcifications. The introduction of vitamin D analogs (if calcium levels allow) or calcimimetics in those patients to halt bone turnover seems to be protective[137].

Contrary to what is reported in the literature, we observed no loss of bone volume, even with a slight increase of bone volume, as the number of patients with normal or high bone volume increased from 44 to 50. It should be noted that we found no relationship between the cumulative dose of steroids and bone volume or loss of bone volume. The decrease in bone volume was associated with the highest levels of sclerostin at baseline, which is explained by the fact that sclerostin is an inhibitor of bone formation[7,23,48]. However, these results should be interpreted with caution, as decreased bone volume occurred in only eight patients.

There are other relevant findings that we can discuss in this section of the work, for instance, the dynamics of PTH, BALP and alpha-klotho. Ten % of our patients did not have reduced PTH levels compared to baseline; 31.9% did not have reduced BALP levels compared to the baseline, and 34.8% did not have increased alpha-klotho levels compared to the baseline. The first observation was associated with longer dialysis vintage, and low PTH levels pre-transplant. We could not expect that patients with normal serum levels of PTH at the transplant decrease its levels further. We should acknowledge that PTH median levels were above the normal (135 pg/mL), but the optimal range of PTH level in renal transplanted patients is unknown[19]. Maintenance of serum PTH high levels, although there is normal renal function, is observed in nearly 25% of transplanted patients[83], and this is known as tertiary hyperparathyroidism. One of the reasons for disturbed PTH levels in the transplant setting is that parathyroid cells have a long lifespan of approximately 20 years[19], and monoclonal glandular hyperplasia can be occurring[82]. Other factors include longer dialysis vintage (as in our population), along with high serum levels of phosphorus and PTH pre-transplant (we did not find this, even excluding patients submitted to

parathyroidectomy), and low levels of 25 hydroxyvitamin D_3 and 1,25 di-hydroxyvitamin D_3. Medication, such as cinacalcet use, had no role in the evolution of PTH levels in our population. The decrease in BALP levels was also expected (as was the decrease in PTH). Patients in whom this wasn't achieved had higher BFR/BS, higher BFR/TV, higher bone cellular activity, and consequently increased osteoid, since the new bone had no time to mineralize, due to the high turnover rate. This event did not have relation with changes in PTH levels.

Turning to the decrease in bone turnover after transplantation, Komala demonstrated that osteocyte-specific klotho loss results in increased bone formation and increased bone volume, enhancing osteoblast activity[188]. So, it seems that, at a cellular level, this would be expected to happen: high levels of alpha-klotho would result in less osteoblast activity, decreasing bone remodeling. Nevertheless, we found no association between alpha-klotho levels and osteoblast activity. We also found no correlations between osteoblasts surface and steroid cumulative dose or levels of the different bone-related molecules studied. Nevertheless, patients who did have an increase in bone remodeling presented with lower steroid cumulative dose, highlighting the importance of glucocorticoids in the activity of bone cells. Additionally, and based on an exploratory analysis, we saw that patients receiving everolimus also had increased bone turnover, which is in line with the belief that mTOR inhibitors are sparing bone immunosuppressive agent[19,149]. It should be noted that increasing bone remodeling happened mostly in patients with low turnover at baseline, which normalized their turnover.

Regarding vascular calcifications, we found that transplanted patients did not show a progression in vascular calcifications score obtained by radiography of hands and pelvis, and did not show a progression in valve calcifications. The fact that transplant could slow the progression of calcifications has been suggested by other studies[128]. As we did not have a control population, we cannot conclude that renal transplantation halts the evolution of vascular calcifications.

3) Imaging exams, including DXA and coronary CT and the associations with mineral and bone metabolism

In addition to radiography of pelvis and hands, at the end of the first year of transplantation, these patients were evaluated based on CT scans of coronary arteries and bone densitometry.

Concerning radiographic studies, we found that age, gender, dialysis vintage, FGF23, sclerostin, cortical porosity, low bone volume, and bone turnover were the most reliable predictors of calcifications in this population of renal transplanted patients, when addressing Adragão score and valve calcifications. Again, and similar to what we found in the pre-transplant setting, age and diabetes were important determinants for vascular calcifications in the post-transplant period. We found no associations between vitamin D or sclerostin and Adragão score. As in the pre-transplant period, bone histology was not associated with Adragão vascular calcification score.

In this population we found that both absolute scores and Agatston percentiles correlated well with the vascular calcification score and with the presence of valve calcifications. Therefore, Adragão score, a simple and inexpensive tool, seems to be a good tool for calcification screening.

Concerning the results of coronary artery scores, dialysis vintage, sclerostin, low bone volume, and bone turnover were the most reliable predictors of coronary calcifications, as expected[128]. Surprisingly, we found different associations when analysing percentiles of coronary calcifications. Please note that the percentiles are adjusted to age, gender, and race, and so, probably are the gold-standard measurement in these patients. In this second analysis, the risk factors considered promoters of coronary calcifications percentiles, in univariate analysis, were PTH, BALP, and calcium at the end of first year of transplantation, and sclerostin levels at baseline. In this case, FGF23 levels were no different according to the increase in severity of percentiles of calcification. We would expect to see more associations with bone-related hormones at baseline and Agatston percentile severity, as we suppose that coronary calcifications did not change significantly over a 12 month-period.

Patients with higher cortical porosity at 1-year of transplant (patients with the highest values of BALP, and the ones with greater osteoid volume) also presented higher percentiles of coronary calcification in univariate analysis but not in multivariate analysis, where only trabecular features were possible determinants of its severity (low bone volume and high bone turnover). This highlights the importance of trabecular bone in extra-osseous calcifications in comparison with cortical bone, which is less metabolically active, and probably plays a more robust role in fracture prevention, which is relevant in these patients[167].

In multivariate analysis, high bone turnover, and BALP at 1-year post-transplantation were some of the predictors of higher percentiles of coronary calcifications. Also, low bone volume was correlated with calcification severity 1-year after the transplant. It is of utmost importance to identify these patients at risk for calcifications in order to implement cardiovascular protective measures to safeguard them from an early cardiovascular event. Performing bone densitometry one year after the transplant can rule out volume abnormalities, in patients with a normal exam, as we demonstrated. This would lead us to recognize patients who could benefit from more invasive studies (such as bone biopsies), especially those with higher calcification scores, before starting antiresorptive or osteoformer therapies. These therapies could also be effective in patients that don't normalize bone volume with the transplant.

As already stated, sclerostin is a soluble Wnt pathway antagonist and a negative regulator of bone metabolism[48]. Wnt signalling has been involved in vascular calcifications, and increased sclerostin expression has been demonstrated during vascular smooth muscle cell calcification in an animal model[23,62]. As stated before, in CKD patients, different studies have reached different conclusions. Some studies have demonstrated that sclerostin high levels are associated with better survival in hemodialysis patients[49,50], suggesting a protective role through inhibition of vascular calcifications[49,63], while other studies have found an association between high levels and CV mortality in dialysis patients[54], justified by the propensity for vascular calcifications via low-bone-turnover disease[48], leading investigators to speculate that a U-shaped dose effect could be the cause of these findings[23]. A recent study performed in end-stage renal disease patients showed that sclerostin was associated with the degree of vascular calcifications[61]. Nevertheless, the role of sclerostin in CV health is very important to clarify as a sclerostin antibody is being evaluated for

osteoporosis treatment in post-menopausal women[64]. We can conclude that not only the transplantation environment, but also the period on dialysis is determinant for severity of calcifications, as dialysis vintage and some serum values at baseline (such as sclerostin) were found to be predictors for the outcome.

Not only the transplantation environment (i.e., the presence of high bone turnover), but also the period on dialysis determines the severity of calcifications: dialysis vintage, sclerostin serum values and bone volume at baseline were found to be predictors of severe calcifications. Preventing cardiovascular events through the timely identification of patients who would benefit from anti-osteoporotic/bone remodeling control drugs should be considered in a prospective study.

The observations with DXA and the relationship between DXA findings and bone biopsy measurements were very interesting, as we are able to demonstrate that DXA, overall, reflects bone volume. We must recognize that, although this is a prospective study, as patients were followed for a period of 12 months since transplantation, this report could be seen as a cross-sectional report in month 12 after the renal transplant. Also, we know that a bone biopsy is a snapshot at a given moment, and like any biopsy, can have sampling errors.

We found that low bone volume, obtained by a bone biopsy, was present in 28.4% (19 patients) of our population. Only 9 of these had osteoporosis in DXA examination. Attending to these results, we found that in this population DXA exam had a good specificity and a good NPV. The exam was useful in ruling out low bone volume and is useful in selecting patients that would not need osteoporosis treatment. The screening for fracture risk is possible through the Fracture Risk Assessment Tool, but unfortunately we could not investigate this tool, as only 1 patient fractured during the course of the 12 months in which we followed these patients.

In our patients, we found no relation between bone volume assessed by histomorphometry, and age. We found a trend towards lower bone volume in Caucasian race. Cumulative steroid doses did not correlate with bone volume. We can suppose that as steroid doses were similar in all patients, and as the sample size is small, the differences in bone volume were not relevant. Nevertheless, we cannot forget that our patients with the lowest steroids doses were the ones in whom bone

turnover decreased, which is in line with the known effects of steroids: direct inhibition of osteoblasts function; impair osteoblastogenesis; increase osteoblasts and osteocytes apoptosis, and increase osteoclastogenesis[147, 19,148].

In line with other studies, we show that DXA lacks sensitivity and specificity in giving information on bone turnover and mineralization, for which we need a bone biopsy. A less invasive way than bone biopsy to study bone quality is microindentation. This handheld system is a technique that measures bone material quality in adults, assessing the bone material strength index, and it seems to be a fast, painless, and easy way to assess bone mechanical properties[189] in CKD or renal transplanted patients. It would be interesting to study this technique as a tool to predict fractures or to study its association with extra-osseous calcifications or even cardiovascular events in CKD and renal transplanted patients.

One interesting finding was that imaging from total femur was the site that best correlated with bone volume assessed by bone biopsy, compared to hip or lumbar spine. However in a recent consensus, those latter 2 skeletal sites (hip and lumbar spine) were named as the best for the evaluation of bone mineral density in CKD patients[101].

Curious was the fact that alpha-klotho and sclerostin did correlate with DXA findings, but not with bone volume measured by histomorphometry. The Wnt/β-catenin pathway is important for bone formation: once activated, bone is formed; once inhibited, bone formation is halted. Genetic mutation on the Wnt/β-catenin pathway leads to premature coronary disease and severe osteoporosis, providing evidence of the importance of the Wnt signalling in the bone-vessels axis[51]. Klotho acts as an antagonist of Wnt/β-catenin pathway activation through interactions with extracellular activators of the pathway[190]. Sclerostin is a direct inhibitor of the pathway[49,50]. We can suppose that patients with the highest mineral bone density have increased levels of those hormones as a feedback loop.

This study reinforces that bone biopsy is the only method that quantifies and evaluates bone mineralization, making it possible to distinguish osteoporosis from hyperabsorption and osteoporosis from deficient bone formation. However, this is an expensive, invasive, and one-shot procedure, and is performed in only a few centers

worldwide[4,82,117], due to the expertise needed. The future could combine newer imaging techniques with newer laboratory biomarkers towards a virtual bone biopsy[122].

4) Mineral, bone, and cardiovascular condition at 1-year post-transplantation

At 1-year post-transplantation we aimed to address the associations between bone biopsy features, laboratory evaluation, and calcification assessments.

Going back to laboratory associations, after transplantation PTH revealed its known actions in mineral metabolism, as we associated the two distinct groups of PTH levels with increasing calcium and decreasing phosphorus levels. Higher PTH levels were associated with lower levels of vitamin D and sclerostin. PTH also maintained the correlation with FGF23 (as in the baseline), and conversely, FGF23 maintained the correlation with PTH. Both hormones are positive stimulus for their secretion.

We learned that FGF23 is a phosphaturic hormone, a suppressor of vitamin D, and has some non-consensual effect on PTH, and that calcium is a stimulus for its secretion[13-15,191]. Indeed, in our ESRD patients, FGF23 was associated with phosphorus, as well as with calcium levels. Nevertheless, in the post-transplant period, these two associations and the association with sclerostin levels were lost, as FGF23 abnormal high levels only maintained the link with PTH levels. Could it be that PTH is a stronger stimulus for FGF23 secretion than phosphorus itself, in a normal renal function scenario? Also, looking at the two groups of phosphorus (abnormal low levels vs. the remaining) there was no association with FGF23, only with calcium, with PTH (low levels of phosphorus related with high calcium and high PTH levels), and with sclerostin (low levels of phosphate were associated with low levels of sclerostin). Both in the pre- and post-transplant period, the group of patients with the lower levels of sclerostin had higher levels of PTH. Our results are contrary to the results of other authors, who showed that high levels of sclerostin are accompanied by high levels of PTH[7], but are in line with the theory that PTH is a regulator of the Wnt/ beta-catenin pathway in bone[192]. Sclerostin high levels were associated with high levels of vitamin D and low levels of alpha-klotho. Sclerostin seems to have the opposite effect on mineral metabolism, compared to PTH, as it is associated with high levels of phosphorus and a trend toward low levels of calcium was found. High levels of

calcium, in their turn, are associated with more secretion of PTH and FGF23, and this reinforces the fact that calcium is a stimulator of FGF23[191].

It is important to discuss our findings related to cortical bone. Both high levels of PTH and BALP (in a less expressive way) were associated with high porosity. Hypercalcemia was another association found. In the light to these findings, we would expect to catch a correlation between bone formation rate and cortical porosity or an association between the different remodeling categories and cortical porosity. That correlation wasn't achieved, but the number of patients presented in this study is not prominent. We learn that cortical loss occurs in ESRD patients, and we found that thickness was higher in hypercalcemic patients[193]. We should remember that dialysis patients usually have hypocalcemia.

Looking at trabecular bone 1-year post-transplantation, PTH and BALP correlated with osteoblast surface and osteoclast surface (per bone surface) in a significant way. Additionally, BALP correlated with BFR/BS, and so it seems that this hormone has more sensitivity for bone remodeling than PTH, as pointed out before. BALP was correlated with osteoid measurements (higher BALP levels meaning more osteoid). This association was also found very recently in another report[186]. We think this is related with the speed of new bone formation. On the contrary, sclerostin, a bone inhibitor, had an inverse association with osteoid measurements. Looking at remodeling categories, we found that BALP was associated with high turnover bone disease in both univariate and multivariate analysis, similar to the pre transplant period, and that high levels of vitamin D were associated with low bone turnover disease. Indeed, vitamin D is an inhibitor of PTH.

As expected, hypophosphatemia was associated with osteoid[177], and hypovitaminosis D with mineralization lag time, another important parameter in the diagnostic approach of mineralization defects[177]. This was also reported previously in another study[187].

LIMITATIONS

We acknowledge that this is an observational, small, unicentric study, with only 81 complete bone biopsies at baseline and with only 68 complete double bone biopsy readings, and as this is an observational study, associations do not mean a cause-

effect relationship. Also, patients were prescribed different bone-related pills that could have confounded our results.

Only the second bone biopsies have dynamic evaluations; the first biopsies could not benefit from tetracycline labelling as patients were recruited on the day they were admitted at the hospital, meaning that turnover could have been misclassified in the first bone biopsies[174]. It is the opinion of the authors that we can categorize remodeling in the majority of the biopsies that have only static evaluations. Although there is a study that defends that static parameters are unable to replace dynamic evaluations[174], in our evaluation, the categorization of the second bone biopsies did not differ significantly in both static and dynamic evaluations. Likewise, we should remember that depending on the reference (Malluche vs. Salusky vs. Novel-Catin vs. Racker) used, bone turnover can actually differ, and turnover can be classified as low bone turnover or as normal bone turnover, depending in which reference range we chose to use.[175,194].

It should be noted that we classified more than 70% of the patients has having secondary hyperparathyroidism, based in the fact that those were receiving either vitamin D analogs or calcimimetics or both at the time of transplantation. Although this fact, the median values of PTH at baseline were 475.0 (301.0 – 748.7) pg/mL, which are aligned with most of the studies. Other limitation in generalization of the study is that as this was a population listed for kidney transplantation, meaning that these are the healthiest patients among ESRD patients, the results cannot be generalized for all ESRD patients.

As we stored at -80 °C blood samples for non-routine analysis (BALP, FGF23, alpha-klotho, and sclerostin), alpha-klotho values can be inexact, as lower results can be obtained in stored versus in fresh serum samples[195,196]. Finally, the study would be more complete if we had measured, in association to PTH and BALP, other bone markers of bone formation, such as osteocalcin or PINP or other bone markers of bone reabsorption, such as pyridinoline, C-telopeptides of type I collagen or even TRAPS5b. Nevertheless, some of those mediators are dependent on the kidney function, have a low stability, and the sensitivity and sensibility are low.

CONCLUSIONS

With the current study we could determine the phenotypic evolution of CKD-MBD in transplanted patients with a good functioning graft, and the more important risk factors for extra-osseous calcifications. This prospective double bone-biopsy analysis is one of the biggest studies performed in this area, only supplanted by a very recent publication that enrolled 97 patients[197], and is the only that included the evaluation of vascular and valve calcifications, beyond prospective laboratory and histomorphometric bone analysis. Furthermore, only one study looked prospectively at the cortical bone[161], as we did, and besides reporting its changes, we could find associations between porosity or thickness and biochemical analysis.

We determine that bone disease is frequent in the healthiest ESRD patients and in renal transplanted patients. Nearly 20% of the population admitted to this study presented low bone turnover and nearly 28% high bone turnover. After transplantation, low remodeling bone disease became the major deviation, and we confirmed that mineralization defects were rare in both periods, and bone volume stable compared to the pre transplant period.

Although our investigation could not find a single marker for characterization of TMV, we confirmed that PTH in dialysis patients is not a good marker for turnover deviations, and BALP (as well as sclerostin) can give more accurate information on turnover. We now know that in dialysis patients high BALP levels (and not only low phosphate levels) are associated with mineralization defects, and these two markers were associated with osteoid measurements in transplanted patients, but did not reach statistical significance for mineralization abnormalities after transplantation. Sclerostin was associated with low bone volume in dialysis patients.

Another important issue was the role of some bone-related molecules and the evolution of TMV categories after the transplant: high BALP levels were associated with the development of new mineralization defects; high sclerostin levels were associated with loss of bone volume; and alpha-klotho was associated with a significant decrease in bone remodeling. Concerning volume in transplanted patients, our study showed a correlation between bone volume measured by a bone biopsy and bone mineral density and T-scores obtained by DXA.

We also learned that bone volume (and not only turnover) is extremely important for cardiovascular health in both dialysis and transplant: low bone volume (BV/TV) was associated with extra-osseous calcifications in both populations, as was lower mineralized bone and adynamic bone disease (defined as low turnover plus low bone volume) in dialysis patients. With this study, we learned that baseline features of uremic patients are extremely important for cardiovascular health, and we would like to highlight not only volume, but also high sclerostin levels, as those were associated with the severity of coronary calcifications after transplantation.

This new knowledge can change our clinical practice:

1. The value of BALP is highlighted and the state of the art in the follow-up of these patients must now include other available bone markers in our daily practice besides PTH, as measurements of BALP.
2. As bone histomorphometry (low bone volume, high bone turnover) and bone-related hormones (FGF23, sclerostin, BALP) were found to have an association with severity of vascular calcifications and valve calcifications, our threshold for a bone biopsy is lower, especially in the case of a fast progression of vascular calcifications in young patients, as targeted treatment of ROD can impact outcomes.
3. We underline the importance of monitoring bone volume in the dialysis setting and the importance of a timely diagnosis of high bone turnover in the transplant setting to minimize the progression of vascular calcifications.

Few studies have looked at cortical bone and, side by side with trabecular bone, cortical bone should be explored and the associations with extra-osseous calcifications confirmed. Further studies with more patients or data aggregation are needed to confirm these observations.

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REFERENCES

1. Keith DS, Nichols GA, Gullion CM, Brown JB, Smith DH. Longitudinal follow-up and outcomes among a population with chronic kidney disease in a large managed care organization. Arch Intern Med 2004;164:659-63.
2. Sarnak MJ, Coronado BE, Greene T, et al. Cardiovascular disease risk factors in chronic renal insufficiency. Clin Nephrol 2002;57:327-35.
3. Kumar S, Bogle R, Banerjee D. Why do young people with chronic kidney disease die early? World J Nephrol 2014;3:143-55.
4. Alshayeb HM, Josephson MA, Sprague SM. CKD-mineral and bone disorder management in kidney transplant recipients. Am J Kidney Dis 2013;61:310-25.
5. Evenepoel P, Rodriguez M, Ketteler M. Laboratory abnormalities in CKD-MBD: markers, predictors, or mediators of disease? Semin Nephrol 2014;34:151-63.
6. Moe S, Drueke T, Cunningham J, et al. Definition, evaluation, and classification of renal osteodystrophy: a position statement from Kidney Disease: Improving Global Outcomes (KDIGO). Kidney Int 2006;69:1945-53.
7. Vervloet MG, Massy ZA, Brandenburg VM, et al. Bone: a new endocrine organ at the heart of chronic kidney disease and mineral and bone disorders. Lancet Diabetes Endocrinol 2014;2:427-36.
8. Ball AM, Gillen DL, Sherrard D, et al. Risk of hip fracture among dialysis and renal transplant recipients. JAMA 2002;288:3014-8.
9. Daya NR, Voskertchian A, Schneider AL, et al. Kidney Function and Fracture Risk: The Atherosclerosis Risk in Communities (ARIC) Study. Am J Kidney Dis 2016;67:218-26.
10. Jamal SA, West SL, Miller PD. Fracture risk assessment in patients with chronic kidney disease. Osteoporos Int 2012;23:1191-8.
11. Fried LF, Biggs ML, Shlipak MG, et al. Association of kidney function with incident hip fracture in older adults. J Am Soc Nephrol 2007;18:282-6.
12. Fuller DS, Dluzniewski PJ, Cooper K, Bradbury BD, Robinson BM, Tentori F. Combinations of mineral and bone disorder markers and risk of death and hospitalizations in the international Dialysis Outcomes and Practice Patterns Study. Clin Kidney J 2020;13:1056-62.
13. Seeherunvong W, Wolf M. Tertiary excess of fibroblast growth factor 23 and hypophosphatemia following kidney transplantation. Pediatr Transplant 2011;15:37-46.

14. Wolf M, Molnar MZ, Amaral AP, et al. Elevated fibroblast growth factor 23 is a risk factor for kidney transplant loss and mortality. J Am Soc Nephrol 2011;22:956-66.
15. Liu S, Quarles LD. How fibroblast growth factor 23 works. J Am Soc Nephrol 2007;18:1637-47.
16. Dhayat NA, Ackermann D, Pruijm M, et al. Fibroblast growth factor 23 and markers of mineral metabolism in individuals with preserved renal function. Kidney Int 2016;90:648-57.
17. Di Marco GS. Off-target effects and adverse outcomes of fibroblast growth factor 23 in chronic kidney disease. Port J Nephrol Hypert 2018;32:57-63.
18. John GB, Cheng CY, Kuro-o M. Role of Klotho in aging, phosphate metabolism, and CKD. Am J Kidney Dis 2011;58:127-34.
19. Evenepoel P. Recovery versus persistence of disordered mineral metabolism in kidney transplant recipients. Semin Nephrol 2013;33:191-203.
20. Westerberg PA, Linde T, Wikstrom B, Ljunggren O, Stridsberg M, Larsson TE. Regulation of fibroblast growth factor-23 in chronic kidney disease. Nephrol Dial Transplant 2007;22:3202-7.
21. Gutierrez O, Isakova T, Rhee E, et al. Fibroblast growth factor-23 mitigates hyperphosphatemia but accentuates calcitriol deficiency in chronic kidney disease. J Am Soc Nephrol 2005;16:2205-15.
22. Larsson TE. The role of FGF-23 in CKD-MBD and cardiovascular disease: friend or foe? Nephrol Dial Transplant 2010;25:1376-81.
23. Jean G, Chazot C. Sclerostin in CKD-MBD: one more paradoxical bone protein? Nephrol Dial Transplant 2013;28:2932-5.
24. Faul C, Amaral AP, Oskouei B, et al. FGF23 induces left ventricular hypertrophy. J Clin Invest 2011;121:4393-408.
25. Gutierrez OM, Mannstadt M, Isakova T, et al. Fibroblast growth factor 23 and mortality among patients undergoing hemodialysis. N Engl J Med 2008;359:584-92.
26. Haring R, Enserro D, Xanthakis V, et al. Plasma Fibroblast Growth Factor 23: Clinical Correlates and Association With Cardiovascular Disease and Mortality in the Framingham Heart Study. J Am Heart Assoc 2016;5.
27. Jongbloed F, Galassi A, Cozzolino M, et al. Clinical significance of FGF-23 measurement in dialysis patients. Clin Nephrol 2011;76:201-9.
28. Jorge C. Vitamin D – new insights into an old molecule. Port J Nephrol Hypert 2019;33:155-62.

29. Hollis BW, Wagner CL. Normal serum vitamin D levels. N Engl J Med 2005;352:515-6; author reply -6.
30. Lambert PW, Stern PH, Avioli RC, et al. Evidence for extrarenal production of 1 alpha ,25-dihydroxyvitamin D in man. J Clin Invest 1982;69:722-5.
31. Zittermann A, Pilz S. Vitamin D and Cardiovascular Disease: An Update. Anticancer Res 2019;39:4627-35.
32. Holick MF. Vitamin D deficiency. N Engl J Med 2007;357:266-81.
33. Cannata-Andia JB, Gomez Alonso C. Vitamin D deficiency: a neglected aspect of disturbed calcium metabolism in renal failure. Nephrol Dial Transplant 2002;17:1875-8.
34. Kidney Disease: Improving Global Outcomes CKDMBDUWG. 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 (2011) 2017;7:1-59.
35. Matias PJ, Ferreira C, Jorge C, et al. 25-Hydroxyvitamin D3, arterial calcifications and cardiovascular risk markers in haemodialysis patients. Nephrol Dial Transplant 2009;24:611-8.
36. Ferreira AC, Matias P, Jorge C, et al. Vitamin D, inflammation and malnutrition in prevalent haemodialysis patients – is there a link? Port J Nephrol Hypert 2008;22:305-12.
37. Matias PJ, Jorge C, Ferreira C, et al. Cholecalciferol supplementation in hemodialysis patients: effects on mineral metabolism, inflammation, and cardiac dimension parameters. Clin J Am Soc Nephrol 2010;5:905-11.
38. Pereira L, Meng C, Marques D, Frazao JM. Old and new calcimimetics for treatment of secondary hyperparathyroidism: impact on biochemical and relevant clinical outcomes. Clin Kidney J 2018;11:80-8.
39. Goodman WG, Salusky IB, Juppner H. New lessons from old assays: parathyroid hormone (PTH), its receptors, and the potential biological relevance of PTH fragments. Nephrol Dial Transplant 2002;17:1731-6.
40. Gao P, Scheibel S, D'Amour P, et al. Development of a novel immunoradiometric assay exclusively for biologically active whole parathyroid hormone 1-84: implications for improvement of accurate assessment of parathyroid function. J Bone Miner Res 2001;16:605-14.
41. Zaher DM, El-Gamal MI, Omar HA, et al. Recent advances with alkaline phosphatase isoenzymes and their inhibitors. Arch Pharm (Weinheim) 2020;353:e2000011.

42. Nizet A, Cavalier E, Stenvinkel P, Haarhaus M, Magnusson P. Bone alkaline phosphatase: An important biomarker in chronic kidney disease - mineral and bone disorder. Clin Chim Acta 2020;501:198-206.
43. Bover J, Urena-Torres P, Cozzolino M, Rodriguez-Garcia M, Gomez-Alonso C. The Non-invasive Diagnosis of Bone Disorders in CKD. Calcif Tissue Int 2021;108:512-27.
44. Magnusson P, Larsson L, Magnusson M, Davie MW, Sharp CA. Isoforms of bone alkaline phosphatase: characterization and origin in human trabecular and cortical bone. J Bone Miner Res 1999;14:1926-33.
45. Vervloet MG, Brandenburg VM, CKD-MBD working group of the ERA-EDTA. Circulating markers of bone turnover. J Nephrol 2017;30:663-70.
46. Drechsler C, Verduijn M, Pilz S, et al. Bone alkaline phosphatase and mortality in dialysis patients. Clin J Am Soc Nephrol 2011;6:1752-9.
47. Maruyama Y, Taniguchi M, Kazama JJ, et al. A higher serum alkaline phosphatase is associated with the incidence of hip fracture and mortality among patients receiving hemodialysis in Japan. Nephrol Dial Transplant 2014;29:1532-8.
48. Moyses RM, Schiavi SC. Sclerostin, Osteocytes, and Chronic Kidney Disease - Mineral Bone Disorder. Semin Dial 2015;28:578-86.
49. Drechsler C, Evenepoel P, Vervloet MG, et al. High levels of circulating sclerostin are associated with better cardiovascular survival in incident dialysis patients: results from the NECOSAD study. Nephrol Dial Transplant 2015;30:288-93.
50. Viaene L, Behets GJ, Claes K, et al. Sclerostin: another bone-related protein related to all-cause mortality in haemodialysis? Nephrol Dial Transplant 2013;28:3024-30.
51. Brandenburg VM, D'Haese P, Deck A, et al. From skeletal to cardiovascular disease in 12 steps-the evolution of sclerostin as a major player in CKD-MBD. Pediatr Nephrol 2016;31:195-206.
52. Brandenburg VM, Kramann R, Koos R, et al. Relationship between sclerostin and cardiovascular calcification in hemodialysis patients: a cross-sectional study. BMC Nephrol 2013;14:219.
53. Ryan ZC, Ketha H, McNulty MS, et al. Sclerostin alters serum vitamin D metabolite and fibroblast growth factor 23 concentrations and the urinary excretion of calcium. Proc Natl Acad Sci U S A 2013;110:6199-204.
54. Goncalves FL, Elias RM, dos Reis LM, et al. Serum sclerostin is an independent predictor of mortality in hemodialysis patients. BMC Nephrol 2014;15:190.

55. Gooi JH, Pompolo S, Karsdal MA, et al. Calcitonin impairs the anabolic effect of PTH in young rats and stimulates expression of sclerostin by osteocytes. Bone 2010;46:1486-97.
56. Cejka D, Marculescu R, Kozakowski N, et al. Renal elimination of sclerostin increases with declining kidney function. J Clin Endocrinol Metab 2014;99:248-55.
57. Bonani M, Rodriguez D, Fehr T, et al. Sclerostin blood levels before and after kidney transplantation. Kidney Blood Press Res 2014;39:230-9.
58. de Oliveira RB, Graciolli FG, dos Reis LM, et al. Disturbances of Wnt/beta-catenin pathway and energy metabolism in early CKD: effect of phosphate binders. Nephrol Dial Transplant 2013;28:2510-7.
59. Cejka D, Herberth J, Branscum AJ, et al. Sclerostin and Dickkopf-1 in renal osteodystrophy. Clin J Am Soc Nephrol 2011;6:877-82.
60. Neto R, Pereira L, Magalhães J, et al. Sclerostin and dkk1 circulating levels associate with low bone turnover in patients with chronic kidney disease stages 3 and 4. Clinical Kidney Journal 2021;14:2401-08.
61. Qureshi AR, Olauson H, Witasp A, et al. Increased circulating sclerostin levels in end-stage renal disease predict biopsy-verified vascular medial calcification and coronary artery calcification. Kidney Int 2015;88:1356-64.
62. Zhu D, Mackenzie NC, Millan JL, Farquharson C, MacRae VE. The appearance and modulation of osteocyte marker expression during calcification of vascular smooth muscle cells. PLoS One 2011;6:e19595.
63. Claes KJ, Viaene L, Heye S, Meijers B, d'Haese P, Evenepoel P. Sclerostin: Another vascular calcification inhibitor? J Clin Endocrinol Metab 2013;98:3221-8.
64. McClung MR, Grauer A, Boonen S, et al. Romosozumab in postmenopausal women with low bone mineral density. N Engl J Med 2014;370:412-20.
65. Padhi D, Jang G, Stouch B, Fang L, Posvar E. Single-dose, placebo-controlled, randomized study of AMG 785, a sclerostin monoclonal antibody. J Bone Miner Res 2011;26:19-26.
66. Saag KG, Petersen J, Brandi ML, et al. Romosozumab or Alendronate for Fracture Prevention in Women with Osteoporosis. N Engl J Med 2017;377:1417-27.
67. Lewiecki EM, Blicharski T, Goemaere S, et al. A Phase III Randomized Placebo-Controlled Trial to Evaluate Efficacy and Safety of Romosozumab in Men With Osteoporosis. J Clin Endocrinol Metab 2018;103:3183-93.

68. Hoff AO, Catala-Lehnen P, Thomas PM, et al. Increased bone mass is an unexpected phenotype associated with deletion of the calcitonin gene. J Clin Invest 2002;110:1849-57.
69. Malluche HH, Faugere MC, Ritz E, Caillens G, Wildberger D. Endogenous calcitonin does not protect against hyperparathyroid bone disease in renal failure. Miner Electrolyte Metab 1986;12:113-8.
70. Giachelli CM. Vascular calcification mechanisms. J Am Soc Nephrol 2004;15:2959-64.
71. Tentori F, Blayney MJ, Albert JM, et al. Mortality risk for dialysis patients with different levels of serum calcium, phosphorus, and PTH: the Dialysis Outcomes and Practice Patterns Study (DOPPS). Am J Kidney Dis 2008;52:519-30.
72. Block GA, Klassen PS, Lazarus JM, Ofsthun N, Lowrie EG, Chertow GM. Mineral metabolism, mortality, and morbidity in maintenance hemodialysis. J Am Soc Nephrol 2004;15:2208-18.
73. McGovern AP, de Lusignan S, van Vlymen J, et al. Serum phosphate as a risk factor for cardiovascular events in people with and without chronic kidney disease: a large community based cohort study. PLoS One 2013;8:e74996.
74. Sakaguchi Y, Fujii N, Shoji T, et al. Magnesium modifies the cardiovascular mortality risk associated with hyperphosphatemia in patients undergoing hemodialysis: a cohort study. PLoS One 2014;9:e116273.
75. Ben-Dov IZ, Galitzer H, Lavi-Moshayoff V, et al. The parathyroid is a target organ for FGF23 in rats. J Clin Invest 2007;117:4003-8.
76. Moorthi RN, Moe SM. CKD-mineral and bone disorder: core curriculum 2011. Am J Kidney Dis 2011;58:1022-36.
77. Bonewald LF. The amazing osteocyte. J Bone Miner Res 2011;26:229-38.
78. Ott SM. Bone histomorphometry in renal osteodystrophy. Semin Nephrol 2009;29:122-32.
79. Carvalho C, Magalhaes J, Pereira L, Simoes-Silva L, Castro-Ferreira I, Frazao JM. Evolution of bone disease after kidney transplantation: A prospective histomorphometric analysis of trabecular and cortical bone. Nephrology (Carlton) 2016;21:55-61.
80. Adragao T, Herberth J, Monier-Faugere MC, et al. Low bone volume--a risk factor for coronary calcifications in hemodialysis patients. Clin J Am Soc Nephrol 2009;4:450-5.

81. Ferreira AC, Cohen-Solal M, D'Haese PC, Ferreira A, European Renal Osteodystrophy (EUROD), an initiative of the CKD-MBD working group of the ERA-EDTA. The Role of Bone Biopsy in the Management of CKD-MBD. Calcified Tissue International 2021;108:528-38.
82. Kalantar-Zadeh K, Molnar MZ, Kovesdy CP, Mucsi I, Bunnapradist S. Management of mineral and bone disorder after kidney transplantation. Curr Opin Nephrol Hypertens 2012;21:389-403.
83. Malluche HH, Monier-Faugere MC, Herberth J. Bone disease after renal transplantation. Nat Rev Nephrol 2010;6:32-40.
84. Malluche HH, Mawad HW, Monier-Faugere MC. Renal osteodystrophy in the first decade of the new millennium: analysis of 630 bone biopsies in black and white patients. J Bone Miner Res 2011;26:1368-76.
85. Lindsay R, Krege JH, Marin F, Jin L, Stepan JJ. Teriparatide for osteoporosis: importance of the full course. Osteoporos Int 2016;27:2395-410.
86. Consensus development conference: diagnosis, prophylaxis, and treatment of osteoporosis. Am J Med 1993;94:646-50.
87. Lippuner K. The future of osteoporosis treatment - a research update. Swiss Med Wkly 2012;142:w13624.
88. Sambrook P, Cooper C. Osteoporosis. Lancet 2006;367:2010-8.
89. Tella SH, Gallagher JC. Biological agents in management of osteoporosis. Eur J Clin Pharmacol 2014;70:1291-301.
90. Pacifici R. Estrogen, cytokines, and pathogenesis of postmenopausal osteoporosis. J Bone Miner Res 1996;11:1043-51.
91. Klawansky S, Komaroff E, Cavanaugh PF, Jr., et al. Relationship between age, renal function and bone mineral density in the US population. Osteoporos Int 2003;14:570-6.
92. Nickolas TL, McMahon DJ, Shane E. Relationship between moderate to severe kidney disease and hip fracture in the United States. J Am Soc Nephrol 2006;17:3223-32.
93. Tentori F, McCullough K, Kilpatrick RD, et al. High rates of death and hospitalization follow bone fracture among hemodialysis patients. Kidney Int 2014;85:166-73.
94. Pimentel A, Urena-Torres P, Bover J, Luis Fernandez-Martin J, Cohen-Solal M. Bone Fragility Fractures in CKD Patients. Calcif Tissue Int 2021;108:539-50.

95. Matias PJ, Laranjinha I, Azevedo A, et al. Bone fracture risk factors in prevalent hemodialysis patients. J Bone Miner Metab 2020;38:205-12.
96. Anderson GL, Limacher M, Assaf AR, et al. Effects of conjugated equine estrogen in postmenopausal women with hysterectomy: the Women's Health Initiative randomized controlled trial. JAMA 2004;291:1701-12.
97. Maraka S, Kennel KA. Bisphosphonates for the prevention and treatment of osteoporosis. BMJ 2015;351:h3783.
98. Black DM, Geiger EJ, Eastell R, et al. Atypical Femur Fracture Risk versus Fragility Fracture Prevention with Bisphosphonates. N Engl J Med 2020;383:743-53.
99. Bouquegneau A, Salam S, Delanaye P, Eastell R, Khwaja A. Bone Disease after Kidney Transplantation. Clin J Am Soc Nephrol 2016;11:1282-96.
100. Jamal SA, Ljunggren O, Stehman-Breen C, et al. Effects of denosumab on fracture and bone mineral density by level of kidney function. J Bone Miner Res 2011;26:1829-35.
101. Evenepoel P, Cunningham J, Ferrari S, et al. European Consensus Statement on the diagnosis and management of osteoporosis in chronic kidney disease stages G4-G5D. Nephrol Dial Transplant 2021;36:42-59.
102. Jamal S, Cheung AM, West S, Lok C. Bone mineral density by DXA and HR pQCT can discriminate fracture status in men and women with stages 3 to 5 chronic kidney disease. Osteoporos Int 2012;23:2805-13.
103. Naylor KL, Garg AX, Zou G, et al. Comparison of fracture risk prediction among individuals with reduced and normal kidney function. Clin J Am Soc Nephrol 2015;10:646-53.
104. Yenchek RH, Ix JH, Shlipak MG, et al. Bone mineral density and fracture risk in older individuals with CKD. Clin J Am Soc Nephrol 2012;7:1130-6.
105. Iimori S, Mori Y, Akita W, et al. Diagnostic usefulness of bone mineral density and biochemical markers of bone turnover in predicting fracture in CKD stage 5D patients--a single-center cohort study. Nephrol Dial Transplant 2012;27:345-51.
106. West SL, Lok CE, Langsetmo L, et al. Bone mineral density predicts fractures in chronic kidney disease. J Bone Miner Res 2015;30:913-9.
107. Ketteler M, Block GA, Evenepoel P, et al. Executive summary of the 2017 KDIGO Chronic Kidney Disease-Mineral and Bone Disorder (CKD-MBD) Guideline Update: what's changed and why it matters. Kidney Int 2017;92:26-36.

108. Nitsch D, Mylne A, Roderick PJ, Smeeth L, Hubbard R, Fletcher A. Chronic kidney disease and hip fracture-related mortality in older people in the UK. Nephrol Dial Transplant 2009;24:1539-44.
109. Kanis JA, Hans D, Cooper C, et al. Interpretation and use of FRAX in clinical practice. Osteoporos Int 2011;22:2395-411.
110. Whitlock RH, Leslie WD, Shaw J, et al. The Fracture Risk Assessment Tool (FRAX(R)) predicts fracture risk in patients with chronic kidney disease. Kidney Int 2019;95:447-54.
111. Moe SM, Nickolas TL. Fractures in Patients with CKD: Time for Action. Clin J Am Soc Nephrol 2016;11:1929-31.
112. Fishbane S, Hazzan AD, Jhaveri KD, Ma L, Lacson E, Jr. Bone Parameters and Risk of Hip and Femur Fractures in Patients on Hemodialysis. Clin J Am Soc Nephrol 2016;11:1063-72.
113. Mittalhenkle A, Gillen DL, Stehman-Breen CO. Increased risk of mortality associated with hip fracture in the dialysis population. Am J Kidney Dis 2004;44:672-9.
114. Silva BC, Leslie WD, Resch H, et al. Trabecular bone score: a noninvasive analytical method based upon the DXA image. J Bone Miner Res 2014;29:518-30.
115. Aleksova J, Kurniawan S, Elder GJ. The trabecular bone score is associated with bone mineral density, markers of bone turnover and prevalent fracture in patients with end stage kidney disease. Osteoporos Int 2018;29:1447-55.
116. Rampersad C, Whitlock RH, Leslie WD, et al. Trabecular bone score in patients with chronic kidney disease. Osteoporos Int 2020;31:1905-12.
117. Wesseling-Perry K, Bacchetta J. CKD-MBD after kidney transplantation. Pediatr Nephrol 2011;26:2143-51.
118. Covic A, Voroneanu L, Apetrii M. PTH and/or Bone Histology: Are We Still Waiting for a Verdict From the CKD-MBD Grand Jury? Am J Kidney Dis 2016;67:535-8.
119. Barreto FC, Barreto DV, Moyses RM, et al. K/DOQI-recommended intact PTH levels do not prevent low-turnover bone disease in hemodialysis patients. Kidney Int 2008;73:771-7.
120. Lamb EJ, Delaney MP. Does PTH offer additive value to ALP measurement in assessing CKD-MBD? Perit Dial Int 2014;34:687-91.
121. Sprague SM, Bellorin-Font E, Jorgetti V, et al. Diagnostic Accuracy of Bone Turnover Markers and Bone Histology in Patients With CKD Treated by Dialysis. Am J Kidney Dis 2016;67:559-66.

122. Diamond T, Elder GJ. Is there a practical role for bone biopsy in chronic kidney disease? Nephrology (Carlton) 2017;22 Suppl 2:22-6.
123. Adragao T, Frazao JM. Cardiovascular risk in dialysis patients: an X-ray vision on vascular calcifications. Kidney Int 2008;74:1505-7.
124. Adragao T, Pires A, Lucas C, et al. A simple vascular calcification score predicts cardiovascular risk in haemodialysis patients. Nephrol Dial Transplant 2004;19:1480-8.
125. Anaya P, Blomquist GA, Davenport DL, Monier-Faugere MC, Sorrell VL, Malluche HH. Coronary artery calcification in CKD-5D patients is tied to adverse cardiac function and increased mortality. Clin Nephrol 2016;86 (2016):291-302.
126. Hwang IC, Park HE, Kim HL, et al. Systemic Inflammation Is Associated With Coronary Artery Calcification and All-Cause Mortality in Chronic Kidney Disease. Circ J 2016;80:1644-52.
127. McClelland RL, Chung H, Detrano R, Post W, Kronmal RA. Distribution of coronary artery calcium by race, gender, and age: results from the Multi-Ethnic Study of Atherosclerosis (MESA). Circulation 2006;113:30-7.
128. Cianciolo G, Capelli I, Angelini ML, et al. Importance of vascular calcification in kidney transplant recipients. Am J Nephrol 2014;39:418-26.
129. Moshar S, Bayesh S, Mohsenikia M, Najibpour R. The Association of Calcium-Phosphorus Product With the Severity of Cardiac Valves Failure in Patients Under Chronic Hemodialysis. Cardiol Res 2016;7:80-3.
130. Urena-Torres P, D'Marco L, Raggi P, et al. Valvular heart disease and calcification in CKD: more common than appreciated. Nephrol Dial Transplant 2020;35:2046-53.
131. Xiao Y, Peng C, Huang W, et al. Circulating fibroblast growth factor 23 is associated with angiographic severity and extent of coronary artery disease. PLoS One 2013;8:e72545.
132. Wolf M. Update on fibroblast growth factor 23 in chronic kidney disease. Kidney Int 2012;82:737-47.
133. Udell JA, Morrow DA, Jarolim P, et al. Fibroblast growth factor-23, cardiovascular prognosis, and benefit of angiotensin-converting enzyme inhibition in stable ischemic heart disease. J Am Coll Cardiol 2014;63:2421-8.
134. Malluche HH, Blomquist G, Monier-Faugere MC, Cantor TL, Davenport DL. High Parathyroid Hormone Level and Osteoporosis Predict Progression of Coronary Artery Calcification in Patients on Dialysis. J Am Soc Nephrol 2015;26:2534-44.

135. Pencak P, Czerwienska B, Ficek R, et al. Calcification of coronary arteries and abdominal aorta in relation to traditional and novel risk factors of atherosclerosis in hemodialysis patients. BMC Nephrol 2013;14:10.
136. Stompor T. Coronary artery calcification in chronic kidney disease: An update. World J Cardiol 2014;6:115-29.
137. Torregrosa J-V, Ferreira AC, Cucchiari D, Ferreira A. Bone Mineral Disease After Kidney Transplantation. Calcified Tissue International 2021;108:551-60.
138. Ferreira AC, Ferreira A. Mineral and bone disease in renal transplantation. Port J Nephrol Hypert 2021;33:111-3.
139. Kanaan N, Claes K, Devogelaer JP, et al. Fibroblast growth factor-23 and parathyroid hormone are associated with post-transplant bone mineral density loss. Clin J Am Soc Nephrol 2010;5:1887-92.
140. Filler G, Liu D, Sharma AP, Grimmer J. Are fibroblast growth factor 23 concentrations in renal transplant patients different from non-transplanted chronic kidney disease patients? Pediatr Transplant 2012;16:73-7.
141. Malyszko J, Koc-Zorawska E, Matuszkiewicz-Rowinska J, Malyszko J. FGF23 and Klotho in relation to markers of endothelial dysfunction in kidney transplant recipients. Transplant Proc 2014;46:2647-50.
142. Baia LC, Humalda JK, Vervloet MG, et al. Fibroblast growth factor 23 and cardiovascular mortality after kidney transplantation. Clin J Am Soc Nephrol 2013;8:1968-78.
143. Borchhardt K, Sulzbacher I, Benesch T, Fodinger M, Sunder-Plassmann G, Haas M. Low-turnover bone disease in hypercalcemic hyperparathyroidism after kidney transplantation. Am J Transplant 2007;7:2515-21.
144. Evenepoel P, Naesens M, Claes K, Kuypers D, Vanrenterghem Y. Tertiary 'hyperphosphatoninism' accentuates hypophosphatemia and suppresses calcitriol levels in renal transplant recipients. Am J Transplant 2007;7:1193-200.
145. Evenepoel P, Meijers BK, de Jonge H, et al. Recovery of hyperphosphatoninism and renal phosphorus wasting one year after successful renal transplantation. Clin J Am Soc Nephrol 2008;3:1829-36.
146. van der Plas WY, Gomes Neto AW, Berger SP, et al. Association of time-updated plasma calcium and phosphate with graft and patient outcomes after kidney transplantation. Am J Transplant 2021;21:2437-47.
147. Canalis E, Mazziotti G, Giustina A, Bilezikian JP. Glucocorticoid-induced osteoporosis: pathophysiology and therapy. Osteoporos Int 2007;18:1319-28.

148. Buckley L, Humphrey MB. Glucocorticoid-Induced Osteoporosis. N Engl J Med 2018;379:2547-56.
149. Cianciolo G, Galassi A, Capelli I, Angelini ML, La Manna G, Cozzolino M. Vitamin D in Kidney Transplant Recipients: Mechanisms and Therapy. Am J Nephrol 2016;43:397-407.
150. Martins L, Queiros J, Ferreira A, et al. Renal osteodystrophy: histologic evaluation after renal transplantation. Transplant Proc 2000;32:2599-601.
151. Carlini RG, Rojas E, Weisinger JR, et al. Bone disease in patients with long-term renal transplantation and normal renal function. Am J Kidney Dis 2000;36:160-6.
152. Montalban C, de Francisco AL, Marinoso ML, et al. Bone disease in long-term adult kidney transplant patients with normal renal function. Kidney Int Suppl 2003:S129-32.
153. Rojas E, Carlini RG, Clesca P, et al. The pathogenesis of osteodystrophy after renal transplantation as detected by early alterations in bone remodeling. Kidney Int 2003;63:1915-23.
154. Cruz EA, Lugon JR, Jorgetti V, Draibe SA, Carvalho AB. Histologic evolution of bone disease 6 months after successful kidney transplantation. Am J Kidney Dis 2004;44:747-56.
155. Abdallah KA, Jorgetti V, Pereira RC, et al. Improvement of adynamic bone disease after renal transplantation. Braz J Med Biol Res 2006;39:31-41.
156. Lehmann G, Ott U, Stein G, Steiner T, Wolf G. Renal osteodystrophy after successful renal transplantation: a histomorphometric analysis in 57 patients. Transplant Proc 2007;39:3153-8.
157. Cueto-Manzano AM, Konel S, Hutchison AJ, et al. Bone loss in long-term renal transplantation: histopathology and densitometry analysis. Kidney Int 1999;55:2021-9.
158. Monier-Faugere MC, Mawad H, Qi Q, Friedler RM, Malluche HH. High prevalence of low bone turnover and occurrence of osteomalacia after kidney transplantation. J Am Soc Nephrol 2000;11:1093-9.
159. Julian BA, Laskow DA, Dubovsky J, Dubovsky EV, Curtis JJ, Quarles LD. Rapid loss of vertebral mineral density after renal transplantation. N Engl J Med 1991;325:544-50.
160. Evenepoel P, Behets GJ, Viaene L, D'Haese PC. Bone histomorphometry in de novo renal transplant recipients indicates a further decline in bone resorption 1 year posttransplantation. Kidney Int 2017;91:469-76.

161. Marques IDB, Araujo M, Graciolli FG, et al. A Randomized Trial of Zoledronic Acid to Prevent Bone Loss in the First Year after Kidney Transplantation. J Am Soc Nephrol 2019;30:355-65.
162. Keronen S, Martola L, Finne P, Burton IS, Kroger H, Honkanen E. Changes in Bone Histomorphometry after Kidney Transplantation. Clin J Am Soc Nephrol 2019;14:894-903.
163. Drueke TB, Evenepoel P. The Bone after Kidney Transplantation. Clin J Am Soc Nephrol 2019;14:795-7.
164. Bellorin-Font E, Rojas E, Carlini RG, Suniaga O, Weisinger JR. Bone remodeling after renal transplantation. Kidney Int Suppl 2003:S125-8.
165. Naylor KL, Li AH, Lam NN, Hodsman AB, Jamal SA, Garg AX. Fracture risk in kidney transplant recipients: a systematic review. Transplantation 2013;95:1461-70.
166. Sidibe A, Auguste D, Desbiens LC, et al. Fracture Risk in Dialysis and Kidney Transplanted Patients: A Systematic Review. JBMR Plus 2019;3:45-55.
167. Evenepoel P, Claes K, Meijers B, et al. Bone mineral density, bone turnover markers, and incident fractures in de novo kidney transplant recipients. Kidney Int 2019;95:1461-70.
168. Naylor KL, Leslie WD, Hodsman AB, Rush DN, Garg AX. FRAX predicts fracture risk in kidney transplant recipients. Transplantation 2014;97:940-5.
169. Naylor KL, Prior J, Garg AX, et al. Trabecular Bone Score and Incident Fragility Fracture Risk in Adults with Reduced Kidney Function. Clin J Am Soc Nephrol 2016;11:2032-40.
170. Chandran M, Hao Y, Kwee AK, et al. Addressing bone quality and bone density after renal transplantation: A prospective evaluation of the evolution of trabecular bone score and bone mineral density over the first 5 years following renal transplantation in Asian patients. Clin Transplant 2019;33:e13671.
171. Roe P, Wolfe M, Joffe M, Rosas SE. Inflammation, coronary artery calcification and cardiovascular events in incident renal transplant recipients. Atherosclerosis 2010;212:589-94.
172. Felsenfeld AJ, Levine BS. Calcitonin, the forgotten hormone: does it deserve to be forgotten? Clin Kidney J 2015;8:180-7.
173. Agatston AS, Janowitz WR, Hildner FJ, Zusmer NR, Viamonte M, Jr., Detrano R. Quantification of coronary artery calcium using ultrafast computed tomography. J Am Coll Cardiol 1990;15:827-32.

174. Salam S, Gallagher O, Hughes D, Khwaja A, Eastell R. The role of static bone histomorphometry in diagnosing renal osteodystrophy. Bone 2021;142:115689.
175. Malluche HH, Monier-Faugere MC. Renal osteodystrophy: what's in a name? Presentation of a clinically useful new model to interpret bone histologic findings. Clin Nephrol 2006;65:235-42.
176. Dempster DW, Compston JE, Drezner MK, et al. Standardized nomenclature, symbols, and units for bone histomorphometry: a 2012 update of the report of the ASBMR Histomorphometry Nomenclature Committee. J Bone Miner Res 2013;28:2-17.
177. Bhan A, Qiu S, Rao SD. Bone histomorphometry in the evaluation of osteomalacia. Bone Rep 2018;8:125-34.
178. Ferreira AC, Mendes M, Silva C, et al. Bone densitometry versus bone histomorphometry in renal transplanted patients: a cross-sectional study. Transpl Int 2021;34:1065-73.
179. Ferreira AC, Cotovio P, Aires I, et al. The Role of Bone Volume, FGF23 and Sclerostin in Calcifications and Mortality; a Cohort Study in CKD Stage 5 Patients. Calcif Tissue Int 2022;110:215-24.
180. Ferreira AC, Mendes M, Silva C, et al. Improvement of mineral and bone disorders after renal transplantation. Transplantation 2022; online ahead of print.
181. Ferreira AC, Ferreira A. The importance of the first year of kidney transplantation in the presence of left ventricular hypertrophy. Port J Nephrol Hypert 2021;35:270-1.
182. Novel-Catin E, Pelletier S, Fouque D, et al. Quantitative histomorphometric analysis of halved iliac crest bone biopsies yield comparable ROD diagnosis as full 7.5mm wide samples. Bone 2020;138:115460.
183. Roforth MM, Fujita K, McGregor UI, et al. Effects of age on bone mRNA levels of sclerostin and other genes relevant to bone metabolism in humans. Bone 2014;59:1-6.
184. Scialla JJ, Lau WL, Reilly MP, et al. Fibroblast growth factor 23 is not associated with and does not induce arterial calcification. Kidney Int 2013;83:1159-68.
185. Levey AS, Stevens LA, Schmid CH, et al. A new equation to estimate glomerular filtration rate. Ann Intern Med 2009;150:604-12.
186. Soeiro EMD, Castro L, Menezes R, et al. Association of parathormone and alkaline phosphatase with bone turnover and mineralization in children with CKD on dialysis: effect of age, gender, and race. Pediatr Nephrol 2020;35:1297-305.

187. Neves CL, dos Reis LM, Batista DG, et al. Persistence of bone and mineral disorders 2 years after successful kidney transplantation. Transplantation 2013;96:290-6.
188. Komaba H, Kaludjerovic J, Hu DZ, et al. Klotho expression in osteocytes regulates bone metabolism and controls bone formation. Kidney Int 2017;92:599-611.
189. Ovejero Crespo D. Microindentation: A New Technique for Bone Quality Assessment. Adv Ther 2020;37:47-54.
190. Liu H, Fergusson MM, Castilho RM, et al. Augmented Wnt signaling in a mammalian model of accelerated aging. Science 2007;317:803-6.
191. Rodriguez-Ortiz ME, Lopez I, Munoz-Castaneda JR, et al. Calcium deficiency reduces circulating levels of FGF23. J Am Soc Nephrol 2012;23:1190-7.
192. Kulkarni NH, Halladay DL, Miles RR, et al. Effects of parathyroid hormone on Wnt signaling pathway in bone. J Cell Biochem 2005;95:1178-90.
193. Nickolas TL, Stein EM, Dworakowski E, et al. Rapid cortical bone loss in patients with chronic kidney disease. J Bone Miner Res 2013;28:1811-20.
194. Salusky IB, Coburn JW, Brill J, et al. Bone disease in pediatric patients undergoing dialysis with CAPD or CCPD. Kidney Int 1988;33:975-82.
195. Barker SL, Pastor J, Carranza D, et al. The demonstration of αKlotho deficiency in human chronic kidney disease with a novel synthetic antibody. Nephrol Dial Transplant 2015;30:223-33.
196. Neyra JA, Moe OW, Pastor J, et al. Performance of soluble Klotho assays in clinical samples of kidney disease. Clinical Kidney Journal 2019;13:235-44.
197. Jørgensen H, Behets G, Bammens B, et al. Natural History of Bone Disease Following Kidney Transplantation. Journal of the American Society of Nephrology 2022;33:638-52.

SUMMARY

This study was designed to study the evolution of three pillars of CKD-MBD in patients with ESRD submitted to renal transplantation. Each patient performed two bone biopsies (at day 0, in the operating room, under general anesthesia, and at nearly 12 months, using local anesthesia). At those two time points, biochemical analyses and radiography of pelvis and hands were performed. All patients who performed the second bone biopsy underwent a non-contrast CT in a low-radiation exposure technique, and coronary artery calcification was quantified using the Agatston method[173]. A DXA evaluation was also performed to measure areal bone mineral density, Z-scores, and T-scores at lumbar spine, femoral neck, and total femur.

Aims and results

In summary, and going through all the study objectives

1) To determine the prevalence, phenotype, and evolution of bone disease before and one year after transplantation.

This study showed us that ROD is usual both in dialysis and in the transplant period. At the 1st bone biopsy, ROD was present in 64.2% of the population; at the 2nd bone biopsy, in 68.1% of the patients. We observed a dynamic evolution of the TMV classification: turnover tended to decrease (high remodeling disease fell from 20 to 7 patients, and low remodeling disease rose from 15 to 32 patients); mineralization was stabilized (abnormal mineralization was present in 7 patients and this number fell to 5 patients); as was volume (low bone volume was present in 22 patients at baseline, and this number declined to 18 patients after 12 months of transplantation).

We found markers for the dynamic changes of TMV post-transplantation: alpha-klotho was a determinant in the reduction of turnover post-transplantation; low PTH, high calcium, increases in vitamin D, reduced steroids dose and everolimus were associated with increasing bone turnover; high levels of sclerostin pre-transplant were the main factor for decreasing bone volume; and BALP was a key-marker for abnormal mineralization.

2) To correlate FGF23 and klotho serum levels with bone histomorphometric parameters and CV disease.

In this study, FGF23 was correlated with severity of vascular calcifications, measured using Adragão score, both in the ESRD period and in the transplant period. Additionally, this hormone presented as an independent predictor of coronary calcifications, but this importance was lost when dividing the patients according to the percentiles' severity of coronary calcification. Moreover, although not statistically significant, left ventricular mass

index showed a trend toward association with FGF23, as higher LVMI values were present in patients with higher values of FGF23. Interestingly, patients with cardiovascular events in the 12-month period after renal transplantation presented higher levels of FGF23, but this was not achieved in a survival analysis. Relationships between FGF23 and bone parameters were observed between the low levels of this hormone and osteomalacia in the ESRD period but only in the univariate analysis, and not in the transplant setting.

We observed no correlations between alpha-klotho serum levels and CV disease. Turning to bone parameters, alpha-klotho serum levels were one of the variables that correlated with osteoblast activity in the ESRD period, along with PTH, BALP, phosphorus, and sclerostin. Nevertheless, in the transplant period, this association was not achieved, but alpha-klotho did correlate with osteoclast activity. Also, we could observe in comparing pre-transplant and post-transplant bone biopsies that patients that had moved to a lower bone-remodeling category than the initial one had greater increases in alpha-klotho, compared to baseline.

3) To correlate serum sclerostin levels with the bone histomorphometric parameters and CV disease

Similarly to FGF23, sclerostin was also associated with CV disease. In the ESRD period, sclerostin was associated with severity of vascular calcifications, obtained by Adragão score. Nevertheless, when applying a multivariable model, this association was lost. Looking at calcifications (Adragão score or valve calcifications) 1-year post-transplantation, we did not observe this association. Nevertheless, using Agatston score and its percentiles, sclerostin associated with severity of both. We could observe that patients that died during the first 12 months after transplantation had higher values of sclerostin. This should be confirmed, as there were few events to make the association meaningful.

In bone histomorphometry, sclerostin was revealed to be correlated with bone turnover in the baseline period, shown to be a predictor of high bone turnover in a negative association (lower levels predict more remodeling). Also, in the ESRD period, sclerostin was correlated with both osteoblast and osteoclast activity, in a negative or inverse association. Comparing that period with the post-transplant one, we notice that patients whose bone volume had decreased after transplant were the ones with the highest levels at sclerostin at baseline, and the ones with a significant decrease in sclerostin values from baseline to 12 months after transplant.

The secondary aims:

- ✓ Correlations between bone biopsies, FGF23, and sclerostin *vs*. serum levels of PTH, calcitonin, alkaline phosphatase, 25-hydroxyvitamin D3, calcium, phosphorus, and magnesium.

Calcitonin did not reach any significant association with the histomorphometric findings, neither calcium, magnesium, nor vitamin D. Bone alkaline phosphatase proved to be an important mediator of bone turnover, especially high bone turnover and mineralization in the ESRD period. After transplantation, BALP maintained its importance in mediating bone turnover, correlating with BFR/BS, with low levels in low bone turnover and high levels in high bone turnover. Phosphate also seems to have a role in high bone turnover in ESRD patients but not in transplanted patients, maybe because its levels are low in the transplant setting. PTH, the hormone that we measure in clinical practice to assess bone turnover, loses significance in multivariate models in ESRD patients and did not show any importance in transplant patients. There were no associations with bone volume and those mediators. Mineralization defects only correlated with BALP and phosphate at baseline, and we had significant associations with the different osteoid measurements and phosphate, BALP, PTH, sclerostin, and alpha-klotho at 1-year after transplantation.

- ✓ Subgroup analysis of the patients previously medicated with cinacalcet or Vitamin D analogs or parathyroidectomised or even calcium doses regarding bone histologic features (turnover, mineralization, and volume).

Parathyroidectomy (performed in 7 patients in the pre-transplant period) did not influence histomorphometric parameters (although there was a trend toward lower cellular activity) or cardiovascular health. The only relation with parathyroidectomy was the level of PTH (66 vs. 477 pg/mL, $p<0.001$).

Patients taking cinacalcet had higher PTH values (821 vs. 450 pg/mL, $p=0.005$), higher FGF23 values (6617.8 vs. 3630.7 RU/mL, $p=0.03$), and lower sclerostin values (1.8 vs. 2.2 ng/mL, $p=0.04$) at baseline. We found no relation with medication and histology findings (in terms of TMV) or with vascular or valve calcifications. No relationships were found with vitamin D analogs or calcium doses and TMV.

- ✓ Correlation of bone histology and fractures and bone densitometry findings.

With this study, we show that DXA measurements correlated well with bone volume obtained by a bone biopsy. We also demonstrated that the exam has a high specificity and a high NPV. Consequently, if a patient has a normal DXA exam, we can assume that the patient is not eligible for osteoporosis treatment. If patient is classified as having osteoporosis, we must confirm the diagnosis with a bone biopsy prior to any directed treatment, with the advantage that it will also allow us to exclude low or high bone turnover or even osteomalacia.

We could not evaluate fractures in this population, as only one patient fractured. This was a Caucasian, 61-year-old woman in menopause, with 55 months on hemodialysis, treated with

tacrolimus and mycophenolate and a cumulative prednisolone dose of 5730 mg. Her BMI was 20.5 g/m^2, which maintained stable through the study. Her bone volume (BV/TV) measured by a bone biopsy was 10.0% at baseline and 9.6% after 1-year and the cortical porosity of 11.2% had increased to 16.5% at the end of the study. She had low bone turnover in both assessments and normal mineralization. In DXA evaluation, osteoporosis was found. Her Adragão score was 1, Agatston score was 187 and the percentile was 91.

- ✓ Validation of Adragão vascular calcifications score in a population of renal transplant patients.

We assume we can validate Adragão score as a surrogate marker of poor vascular health. We found that valve calcifications and both absolute Agatston scores and adjusted percentiles (the gold standard) of coronary calcifications, were well related with Adragão scores. Therefore, Adragão score, which is simple and inexpensive, seems to be a good tool for calcification screening. The ordered variable was correlated with the percentiles of coronary calcification (Graphic 17), and the severity of Adragão score also correlated with the absolute Agatston score ($p<0.001$), as shown in Graphic 18, and respective percentile ($p=0.017$).

- ✓ At the end of the study, and in order to assess the importance of transplantation in LVH, we chose a historical cohort, paired with the contemporary cohort in terms of age, gender, and dialysis vintage, and we compared the echocardiogram findings and evolution while on waiting list (historical cohort) *vs.* while submitted to renal transplantation (contemporary cohort). All patients included in the historical cohort had an ID number in order to ensure their anonymity.

We found no statistical difference between the demographic characteristics of both populations, in line with our expectations. Although LVMI was similar in both populations and the delta value between the 1-year periods was alike, we had a significant difference in the presence of LVH if we add 12 months of dialysis in a patient that otherwise could be submitted to transplant.

RESUMO

Este estudo foi desenhado para se avaliar a evolução dos três pilares da doença mineral e óssea associada à doença renal em doentes com DRC estadio 5 submetidos a transplante renal. Cada doente efetuou 2 biopsias ósseas (no dia 0, no bloco operatório, sob anestesia geral aquando do transplante e após 12 meses, sob anestesia local). Simultaneamente às biopsias ósseas, foram efetuadas análise laboratoriais e radiografias convencionais das mãos e bacia. Todos os doentes que efetuaram a segunda biopsia óssea, foram avaliados por TC não contrastada de baixa radiação para quantificação de calcificação coronária usando o método de Agatston[173]. Estes doentes efetuaram ainda densitometria óssea para avaliação de densidade mineral óssea areal, Z-scores e T-scores na coluna lombar, colo do fémur e fémur total.

Objetivos e resultados

1) Determinar a prevalência, fenótipo e evolução da doença óssea antes e 1 ano após a transplantação renal

Neste estudo demostrámos que a osteodistrofia renal (ODR) é frequente tanto em doentes dialisados e em lista para transplante como após a transplantação renal. Na 1ª biopsia óssea, a ODR estava presente em 64.2% da população, na 2ª biopsia em 68.1% dos doentes. Observámos uma evolução dinâmica da classificação de TMV: a remodelação tendeu a diminuir (a doença de alta remodelação desceu de 20 para 7 doentes, a baixa remodelação aumentou de 15 para 31 doentes); a mineralização manteve-se estável (alterações de mineralização eram inicialmente observáveis em 7 doentes e, após a transplantação, em 5 doentes), assim como o volume (o baixo volume ósseo desceu de 22 para 18 doentes).

Estas alterações associaram-se a marcadores de doença mineral e óssea. O alfa-klotho surgiu como eventual determinante da redução da remodelação óssea após a transplantação (enquanto que a PTH baixa, o cálcio elevado, o aumento da vitamina D, o uso de doses mais baixas de corticoides e o uso de everolimus associaram-se ao aumento da remodelação óssea). Os níveis elevados de esclerostina na data do transplante foram determinantes de redução de volume ósseo e os níveis elevados de fosfatase alcalina óssea (FAb) os determinantes de alterações da mineralização.

2) Correlacionar os valores de FGF23 e alfa-klotho com os achados de histomorfometria óssea e doença cardiovascular

Os níveis de FGF23 correlacionaram-se com a severidade das calcificações vasculares tanto no período em diálise, como após a transplantação renal, se avaliadas pela escala Adragão. Esta molécula apresentou-se ainda como um preditor independente do score de calcificação coronária, mas não se associou ao percentil de severidade de calcificação. Apesar de não ter

sido obtido significância estatística, valores mais elevados de FGF23 tendencialmente associaram-se a índice da massa do ventrículo esquerdo mais elevado. Foi igualmente interessante o facto de se verificar que os doentes com eventos cardiovasculares ao longo do primeiro ano de transplantação renal apresentaram valores de FGf23 mais elevados à data do transplante. No entanto, este facto não foi comprovado usando uma análise de sobrevida. Relativamente aos dados das biopsias ósseas, verificamos que valores baixos desta molécula se associaram à osteomalacia em doentes em diálise, mas apenas na análise univariada, não se observando qualquer associação entre os valores da molécula e os resultados das biopsias ósseas nos doentes transplantados.

Não observámos qualquer correlação entre os níveis de alfa-klotho e doença cardiovascular. No que diz respeito aos resultados das biopsias ósseas, descobrimos que os níveis séricos de alfa-klotho se correlacionaram com a atividade ostoblástica no período de diálise (tal como a PTH, FAb, fósforo e esclerostina). No entanto, após a transplantação renal, os níveis de alfa-klotho apenas se correlacionaram com a atividade osteoclástica. Um outro dado relevante foi o de associarmos o aumento dos níveis de alfa-klotho à redução da remodelação óssea, após a transplantação.

3) Correlacionar os valores de esclerostina com os achados de histomorfometria óssea e doença cardiovascular

Tal como com o FGF23, também a esclerostina se associou a doença cardiovascular. No período em diálise, os níveis de esclerostina associaram-se à severidade de calcificações vasculares obtidas pela classificação Adragão, em análise univariada. No período após o transplante renal, não observámos esta associação (nem para calcificação vascular, nem para calcificação valvular). No entanto, encontrámos uma associação entre os níveis de esclerostina na data do transplante e a severidade das calcificações coronárias usando a classificação Agatston e os seus percentis de severidade. Observámos ainda que os doentes que faleceram ao longo do primeiro ano de transplante renal tinham níveis mais elevados de esclerostina à data do transplante. No entanto, este dado deverá ser confirmado com estudos maiores, uma vez que o número de eventos foi reduzido.

Quanto à histomorfometria óssea, à data de entrada no estudo, os níveis de esclerostina correlacionaram-se com a remodelação óssea (níveis mais baixos a associarem-se com maior remodelação). Também foram demostradas correlações negativas entre níveis da molécula e a atividade osteoclástica e osteoblástica. Verificámos ainda que os doentes onde o volume ósseo se reduziu ao longo do primeiro ano de transplante foram os que apresentavam os níveis mais elevados da molécula à data de entrada no estudo.

Os objetivos secundários:

- ✓ Correlações entre os resultados das biopsias ósseas, FGF23 e esclerostina com os níveis séricos de PTH, calcitonina, fosfatase alcalina, 25-hidroxivitamina D3, fósforo e magnésio

Os níveis de calcitonina não demonstraram ter qualquer associação com os achados da histomorfometria óssea, nem os valores de cálcio, magnésio ou vitamina D. A FAb provou ser um marcador de remodelação óssea (de elevada remodelação), bem como de alterações de mineralização no período dialítico. Após a transplantação, a FAb correlacionou-se com BFR/BS, obtendo-se níveis baixos de FAb nos casos de baixa remodelação e níveis elevados nos casos de elevada remodelação. O fósforo também pareceu ter um papel na remodelação óssea durante o período dialítico, mas não após a transplantação renal, talvez porque os níveis de fósforo após o transplante são, na generalidade, baixos. Os níveis de PTH, o que usamos na nossa prática clínica diária para aceder á remodelação óssea, perdeu importância no modelo de análise multivariada efetuada no período dialítico e não revelou ter qualquer importância após o transplante. Não obtivemos associações entre o volume ósseo e estes marcadores escolhidos. Os defeitos de mineralização correlacionaram-se com os níveis de FAb e fósforo à data de entrada no estudo, e obtivemos associações entre os valores de osteóide obtidos pela análise das biopsias ósseas e os níveis séricos de fósforo, FAb, PTH, esclerostina e alfa-klotho após o transplante.

- ✓ Análise de subgrupos de doentes medicados com cinacalecet ou análogos de vitamina D à data de entrada no estudo, bem como de paratiroidectomizados e avaliação de doses de cálcio e seu efeito em termos de TMV

A paratiroidectomia foi efetuada em 7 doentes, no período pré transplante, e não mostrou ter influência nem nos parâmetros histomorfométricos avaliadas (apesar de uma tendência para menor atividade celular) nem na avaliação cardiovascular. A única relação encontrada foi com os níveis de PTH (66 vs. 477 pg/mL, $p<0.001$).

Os doentes sob cinacalcet apresentaram níveis mais elevados de PTH (821 vs. 450 pg/mL, $p=0.005$) e de FGF23 (6617.8 vs. 3630.7 RU/mL, $p=0.03$) e níveis mais baixos de esclerostina (1.8 vs. 2.2 ng/mL, $p=0.04$) à data de entrada no estudo. Não encontramos associações entre o uso desta medicação e os achados histológicos (TMV) ou calcificações vasculares ou valvulares. Não encontramos associações entre o uso de análogos de vitamina D, cálcio (e doses) e a classificação TMV.

- ✓ Correlações entre histologia óssea e fraturas e achados de densitometria

Com este estudo demostramos que as medidas obtidas pela densitometria se correlacionaram com o volume ósseo obtido por biopsia óssea. Demostrámos que este exame

é específico e com valor preditivo negativo para o diagnóstico de osteoporose. Neste sentido, um doente com um exame densitométrico normal provavelmente não é elegível para tratamento de osteoporose, enquanto que se apresentar um exame que demonstre osteoporose ou osteopénia, deveremos confirmar o diagnóstico com uma biopsia óssea antes de qualquer tratamento dirigido, com a vantagem de que a biopsia permitir-nos-á excluir alterações de remodelação e/ou de mineralização.

Nesta população de doentes, não pudemos avaliar as fraturas, uma vez que tivemos apenas um evento. O evento ocorreu numa doente de caucasiana de 61 anos, com tempo prévio em diálise de 55 meses, tratada com tacrolimus, micofenolato de mofetil e prednisolona, com dose cumulativa de 5730mg. O IMC era de 20.5 g/ m^2, que se manteve estável ao longo do estudo. O volume ósseo medido na primeira biopsia óssea foi de 10.0% e de 9.6% após o primeiro ano de transplante. A porosidade cortical foi de 11.2% e aumentou para 16.5% ao longo do estudo. Ambas as biopsias ósseas revelaram doença de baixa remodelação, com normal mineralização. O score Adragão foi de 1, o de Agatston de 187, correspondendo ao percentil 91.

- ✓ Validação do score de calcificação vascular de Adragão, na população de doentes transplantados renais

Assumimos que podemos validar o score Adragão como um marcador de doença vascular. Os scores de Adragão correlacionaram-se tanto com as calcificações valvulares, como com os scores de calcificação coronária de Agatston e respetivos percentis. Assim, este score simples e barato, pareceu-nos uma boa ferramenta para avaliação de calcificação estra-óssea.

- ✓ Para aceder à importância da transplantação em termos de geometria cardíaca (hipertrofia ventricular esquerda - HVE), fizemos um estudo de caso-controlo, sendo os casos os doentes ativos do estudo e os controlos uma população de doentes em lista para transplante renal na nossa Unidade, que foram emparelhados em ternos de tempo em diálise, idade, sexo, presença de diabetes e hipertensão arterial e comparámos os achados ecocardiográficos de ambas as populações. Aos doentes escolhidos para controlo foi dado um número de identificação de forma a assegurar o seu anonimato.

Não encontrámos diferenças estatisticamente significativas entre os dados demográficos de ambas as populações, em linha com as nossas expectativas. Apesar do índice de massa ventricular esquerda ter sido similar em ambas as populações e a diferença de valores entre o último ecocardiograma efetuado e o anterior ser semelhante, verificamos uma diferença estatisticamente significativa na presença de HVE nos doentes que se mantiveram em lista ativa de transplante em comparação com os, entretanto, transplantados.

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