Bone metabolism parameters in haemodialysis patients with chronic kidney disease and in patients after kidney transplantation

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Short title: Bone metabolism parameters in HD and Tx patients

Abstract

Chronic kidney disease adversely affects the structure and metabolism of bone tissue, which may be a result of disturbed biochemical processes in adipose tissue. Renal replacement therapy is a life-saving therapy but it does not restore all metabolic functions and sometimes even escalates some disturbances. The study included 126 subjects: 47 haemodialysis patients (HD), 56 patients after renal transplantation (Tx) and 23 healthy controls (K). Bone density at the femoral neck (FN) and lumbar spine (LS), as well as body composition (adipose tissue content and lean body mass) were measured in each patient using the DXA method. In addition, serum concentrations of glucose, calcium, phosphorus, parathormone, FGF23, Klotho, osteocalcin, leptin, adiponectin and 1,25-dihydroxyvitamin D_3 were measured. We observed significantly higher concentrations of leptin, FGF23 and Klotho proteins in the HD patients (77.2 ± 48.1 ng/ml, 54.7 ± 12.4 pg/ml, 420.6 ± 303.8 ng/ml, respectively) and the Tx group (33.2 ± 26.5 ng/ml; 179.8 ± 383.9 pg/ml; 585.4 ± 565.7, respectively) compared to the control group (24.4 ± 24.6 ng/ml, 43.3 ± 37.3 pg/ml, 280.5 ± 376.0 ng/ml). Significantly lower bone density at FN was observed in the HD and Tx patients in comparison to the controls and in the HD patients compared to the Tx group. There were no significant differences in body mass composition between the studied groups. The results of this study indicate that both haemodialysis and transplantation are associated with increased serum concentrations of leptin, FGF23 and Klotho proteins, as well as lower bone density at femoral neck.

Key words: body composition, bone metabolism parameters, bone mineral density, kidney failure

Introduction

Chronic kidney disease (CKD) negatively influences the quality of life of patients. Adverse changes affect many systems and tissues, including cardiovascular, bone, and hormonal, and escalate with the progression of the disease (Nasuto *et al*. 2016). According to previous studies, disturbances in calcium and phosphate have been proven to cause vascular sclerosis, rapid atherosclerosis progression and an increased risk of cardiovascular death in CKD patients (Bansal *et al*. 2017, Webster *et al*. 2017). Initial changes can be noticed in the early stages of CKD. The progression of the disease enhances these disorders and renal replacement therapy does not correct them (Maréchal *et al*. 2012). Recent studies indicate strong relationships between bone and fat metabolism. Adipokines released by adipose tissue, such as leptin and adiponectin, besides regulating appetite and carbohydrate metabolism, also influence bone tissue metabolism. On the other hand, proteins, such as osteocalcin, which are involved in bone tissue metabolim, play an important role in the regulation of carbohydrate metabolism (Bonnet 2017). Leptin is a hormone produced by adipocytes. Its multidirectional activity includes regulation of satiety by influencing the appetite, regulation of glycemic homeostasis, and insulin sensitivity. In CKD, the concentration of leptin increases with GFR (glomerular filtration rate) deterioration (Pedone *et al*. 2015). In haemodialysis patients, leptin was shown to be related to arterial stiffness and bone turnover (Kuo *et al*. 2018, Zoccali *et al*. 2004).

Adiponectin is also released by adipose tissue. It correlates negatively with nutritional state, reduces insulin resistance and shows the anti-inflammatory properties (Liu and Liu 2014). In hemodialysis males, adiponectin was associated with decreased bone density (Okuno *et al*. 2012). Rhee *et al*. showed that high adiponectin levels in haemodialysed patients are associated with a higher risk of death (Rhee *et al*. 2015). Osteocalcin is a protein produced by osteoblasts and its synthesis is stimulated by vitamin D. It has affinity for

hydroxyapatite and is involved in mineralisation of bone tissue (Baumgrass *et al*. 1997). As recently shown, osteocalcin has been associated with insulin resistance (Vervloet *et al*. 2014). Experimental studies in animals have demonstrated its positive influence on reducing of glycaemia (Ferron *et al*. 2008).

FGF23 (fibroblast growth factor 23), similar to osteocalcin, is produced by osteoblasts. It is bound to the receptor for FGF (FGFR) with the αKlotho protein. The αKlotho/FGF23 complex blocks the expression of NaPi2a and NaPi2c sodium-phosphate cotransporter in the proximal tubules and thereby inhibits reabsorption of phosphates (Courbebaisse and Lanske 2018). In CKD, the concentration of FGF23 increases with GFR deterioration as a response to hyperphosphatemia (Isakova *et al*. 2011). FGF23 levels correlated with inflammatory markers such as CRP and IL-6 (Munoz Mendoza *et al*. 2012). Elevated FGF23 levels are a risk factor for the progression of renal disease and death due to cardiovascular events, independently of serum phosphate levels (Gutiérrez *et al*. 2008). The synthesis of FGF23 is stimulated by vitamin D, while FGF23 inhibits the synthesis of vitamin D (Liu *et al*. 2006). Vitamin D is activated by renal 1α-hydroxylase and is responsible for maintaining calcium-phosphate homeostasis by increasing calcium and phosphate absorption. Patients with CKD are affected by vitamin D deficiency, which has even been reported after successful kidney transplantation (Ewers *et al*. 2008). Low levels of vitamin D in haemodialysed patients were reported to be associated with obesity and metabolic syndrome (Ahmadi *et al*. 2016). Also, Klotho and FGF23 proteins closely related to calcium-phosphate metabolism may be associated with insulin resistance (Kanbay *et al*. 2017).

Proteins, such as osteocalcin, leptin and adiponectin, play a dual role, being markers of bone metabolism as well as carbohydrate metabolism. Currently, their importance in kidney diseases is not fully understood.

The aim of this study was to examine the concentrations of adipokines, osteocalcin, Klotho and FGF23 in CKD patients on haemodialysis and patients after kidney transplantation, as well as to investigate their relationship with other metabolic parameters, body composition and bone density.

Material and methods

The study included 126 subjects: 47 CKD patients (13 female and 34 male) treated with haemodialysis (HD), 56 patients (23 female and 33 male) after kidney transplantation (Tx), and 23 healthy subjects (13 female and 10 male) as the control group (K). Inclusion criteria for the control group were: aged over 18 years, glomerular filtration (GFR) above 60 ml/min/1.73 m^2, lack of chronic inflammatory diseases (lupus or rheumatoid arthritis) and osteoporosis. For the HD group, the inclusion criteria were: aged over 18 years, receiving renal replacement therapy for at least 12 months and less than 5 years at qualification, treatment with constant doses of the active vitamin D (alfadiol), (four patients received additionally cinacalcet), calcium preparations and heparin, and on haemodialysis for the last 3 months before inclusion, and Kt/V above 1.2. All HD patients with diabetes were treated with insulin. The inclusion criteria for renal transplant patients were: kidney transplantation at least 2 years prior to qualification, stable doses of immunosuppressive drugs over the last 3 months, standard immunosuppression with calcineurin inhibitor, mycophenolate mofetil, corticosteroids. Patients after renal transplantation with diagnosed diabetes (11 subjects) were treated with insulin (4 subjects) or with sulphonylurea derivatives (7 subjects). The Subjects diagnosed with cancer and osteoporosis before entering the study or treated with heparin or anticoagulants were excluded from the study. All participants provided written informed consent. This study was approved by the Bioethical Commission at the Pomeranian Medical

University and was performed in accordance with the guidelines proposed in Declaration of Helsinki (2000) of the World Medical Association.

The etiology of CKD in HD patients was as follows: diabetes mellitus (DM) 9 subjects, hypertension (HA) 8, autosomal dominant polycystic kidney disease (ADPKD) 2, glomerulonephritis (GN) 7, and other 21. In the kidney graft recipient group, etiology was: DM 5 subjects, HA 11, ADPKD 6, GN 16, and other 18.

Densitometric examination of bone density at the femoral neck (FN) and lumbar spine (LS) regions, as well as of body composition was performed using dual X-ray absorptiometry (DXA; Lunar Prodigy Advance, GE Healthcare). Measurements of bone density (BMD; g/cm^2) were expressed as a T-score according to the WHO guidelines, and body composition measurements as mass of body fat or lean tissue in kilograms (kg).

Fasting blood samples were collected from the peripheral vein in the K and Tx groups for biochemical analysis (using Sarstedt S-Monovette tubes with clotting activator). In the HD patients, blood samples were collected before a haemodialysis procedure.

Standard biochemical parameters were assessed by spectrophotometry using an Architect 800 biochemistry analyser (Abbott Diagnostics, USA). eGFR was estimated using the CKD-EPI equation. Serum concentrations of 1,25-dihydroxyvitamin D_3, leptin, adiponectin, osteocalcin, FGF23 and Klotho protein examined in the study were determined by the ELISA method using commercially available kits.

Statistical analysis

Since distributions of most biochemical parameters were significantly different from the normal distribution ($p<0.05$; Shapiro-Wilk test), non-parametric tests were used for statistical analysis of quantitative variables. The Mann-Whitney test was used for comparisons between two groups and Spearman's rank correlation coefficient for assessment

of associations between variables within groups. $P<0.05$ without correction for multiple tests was considered as statistically significant.

Results

Characteristics of the studied groups, showing selected biochemical parameters of lipid, carbohydrate and calcium-phosphate metabolism, as well as bone density and body composition, are presented in Table 1. There were no significant differences between the three groups as regards age and sex.

Serum concentrations of fasting glucose, total cholesterol, triglycerides, phosphorus, and leptin were significantly higher in the HD patients compared to the control group, while glucose, phosphorus, and leptin concentrations were higher in the HD patients compared to the Tx group. HD and Tx patients had significantly higher serum concentrations of PTH (parathormone), osteocalcin, FGF23 and Klotho protein compared to the control group. The Tx patients had significantly higher concentrations of 1,25-dihydroxyvitamin D_3 compared to the controls and the HD patients (Table 1).

Results of densitometric examination indicated significantly lower FN bone density in HD and Tx patients compared to the controls and in HD patients compared to the Tx group. There were no statistically significant differences in LS bone density, and fatty tissue and lean mass between the studied groups (Table 1).

In the control group, Klotho protein concentrations showed a significant positive correlation with age and PTH levels and a significant negative correlation with GFR, fatty and lean body mass. 1,25-dihydroxyvitamin D_3 concentrations presented a significant positive correlation with adiponectin concentration. PTH concentrations showed a significant positive correlation with age and Klotho protein concentrations and a significant negative correlation with GFR and fatty tissue mass.

In the HD patients, Klotho protein concentrations were significantly positively correlated with FGF23 and significantly negatively correlated with the leptin concentrations. 1,25-dihydroxyvitamin D_3 concentrations significantly negatively correlated with FN and LS bone density and the PTH concentrations. The PTH concentrations were significantly positively correlated with lean body mass FN and LS bone density and significantly negatively correlated with adiponectin concentrations. Osteocalcin concentrations showed a negative correlation with eGFR and a positive correlation with LS bone density. Adiponectin negatively correlated with lean body mass. Leptin positively correlated with BMI and fat body mass (Table 2).

In the kidney recipients (Tx group), we observed a negative correlation between Klotho protein and leptin concentrations and a positive correlation with the adiponectin concentrations. The FGF23 concentrations were positively correlated only with osteocalcin concentrations. The PTH concentrations were negatively correlated with e-GFR and FN bone density, but positively with BMI. Concentrations of leptin showed a significant positive correlation with BMI and body fat, and negative correlation with the adiponectin concentrations. Adiponectin concentrations were significantly negatively correlated with lean body mass (Table 3).

Discussion

In this study, we examined the bone metabolism parameters in haemodialysis patients with chronic kidney disease and in patients after kidney transplantation. We assessed serum concentrations of adipokines, osteocalcin, Klotho and FGF23, and investigated their correlation with other metabolic parameters, body composition and bone density.

Previous studies have indicated that bone density decreases with the progression of CKD (West *et al*. 2015). Unfortunately, kidney transplantation does not significantly restore

normal bone tissue metabolism (Pérez-Sáez *et al*. 2017). It has been shown that bone density is influenced, not only by agents that are directly involved in the regulation of calcium-phosphate homeostasis, but also by adipokines produced by adipose tissue, such as leptin and adiponectin. Previous studies reported that high concentrations of adiponectin were negatively correlated with bone density (Naot *et al*. 2017). Recent animal studies confirmed the ability of adiponectin to reduce Klotho secretion by renal tubular cells and, therefore, confirmed the influence of adiponectin on the regulation of bone mass (Rutkowski *et al*. 2017). Leptin can directly stimulate bone cells to form bone tissue (Cornish *et al*. 2002). However, by acting on the hypothalamus and activating the sympathetic system, leptin can also inhibit bone formation (Hamrick and Ferrari 2008).

In our study, only osteocalcin concentrations correlated positively with bone density in the LS spine segment in the HD group. Canoz *et al*. reported that 70% of 100 eligible renal transplant patients showed reduced bone density in the FN and LS regions (Canoz *et al*. 2015). We observed that the FN bone density differed significantly between the studied groups. It was lower in the HD patients than in the Tx group. In turn, bone density in the LS region did not show significant differences between the studied groups.

The increase in GFR after kidney transplantation did not reduce concentrations of FGF23 and Klotho, which were highest in this group. FGF23 is a phosphaturic hormone and, in CKD patients, its concentration increases with decrease in GFR (Gutierrez *et al*. 2005). High FGF23 levels have been shown to be associated with higher mortality in CKD, HD and Tx patients (Wolf 2012, Wolf *et al*. 2011). Patients with a renal transplant often present parathyroid hormone dysfunction associated with persistent secondary hyperparathyroidism. The lack of restoration of normal function in parathyroid cells can be explained by their long life (about 20 years), which leads to persistently higher FGF23 concentrations (Parfitt 1997). Klotho protein concentrations were highest in the Tx group. Liu *et al*. showed that Klotho

levels were lower in patients with advanced renal disease compared to those with higher GFR. Cox regression analysis revealed that low Klotho protein concentrations were an independent risk factor for the progression of renal disease (Liu *et al*. 2018). In the study by Tan *et al*. of patients just before and a few months after a kidney transplantation, it was observed that Klotho concentrations decreased during the first week after a transplantation and then increased. The reasons for this phenomenon are currently unknown. Tan *et al*. showed that Klotho concentrations were not related to the reduction of immunosuppression or to biochemical blood parameters, and, therefore, suggested non-renal related causes of the increased Klotho concentrations (Tan *et al*. 2017). In our study, we also found the Klotho concentrations to be highest in patients after kidney transplantation. Analysis of relationship between Klotho and adipose tissue showed that, in the control group, Klotho levels negatively correlated with adipose tissue mass and lean body mass. These correlations were not observed in other studied groups. However, in the HD and Tx groups, the Klotho concentrations correlated negatively with leptin, while in the Tx group there was a positive correlation with adiponectin.

We did not demonstrate correlation of FGF23 with adipose tissue or adipokines. Montford *et al*., in a study conducted in dialyzed patients, confirmed the association of higher FGF23 concentrations with lower BMI and dyslipidemia (Montford *et al*. 2013). However, in a study conducted in subjects without kidney disease, FGF23 was elevated in obese subjects (Hu at al. 2018).

We observed a negative correlation of adiponectin concentrations with lean body mass in both of the studied groups. A study by Rhee *et al*. demonstrated that high concentrations of adiponectin were associated with a higher mortality rate in haemodialysis patients (Rhee *et al*. 2015). It was noticed that the survival of CKD patients, including those subjected to haemodialysis, is positively related to the lean body mass (Kalantar-Zadeh *et al*. 2017). It

seems likely that the negative correlation between adiponectin and lean body mass that was reported in our study is associated with this issue.

Unfortunately our study has some limitations - fasting blood samples were collected from the peripheral vein in the K and Tx groups for biochemical analysis, whereas in the HD patients, blood samples were collected before a haemodialysis procedure. Another limitation of this exploratory study was analysis of multiple correlations between parameters without corrections for multiple statistical tests. The statistical power of the study was too low to detect significant associations after e.g. Bonferroni correction. Therefore, further studies with greater numbers of subjects should be performed to verify the results of the current study.

The results of this study indicate that both haemodialysis and transplantation is associated with the increase of leptin, FGF23 and Klotho proteins serum concentrations as well as lower bone density at femoral neck.

Conflict of interest

The authors have no conflicts of interest to declare.

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Table 1. Characteristics of the studied groups presenting selected biochemical parameters of lipid, carbohydrate, and calcium-phosphate metabolism, as well as bone density, and body composition.

Parametes	**K mean±SD**	**HD mean±SD**	**Tx mean±SD**	**HD vs K**	**Tx vs K**	**Tx vs HD**
	n=23	n=47	n=56		**p***	
Age (years)	53.8± 13.2	59.1±14.5	55.0±12.4	0.09	0.74	0.07
GFR (ml/min/1.73 m^2)	91.1±17.1	-	56.6±22.8	<0.001	<0.001	<0.001
Glucose (mmol/l)	5.35±1.17	6.20±1.77	5.53±1.08	0.01	0.13	0.04
Cholesterol (mg/dl)	182.9±23.7	200.8±30.8	189.8±35.3	0.02	0.39	0.13
Triacylglycerols (mg/dl)	163.9±35.7	186.8±55.4	174.1±41.8	0.02	0.30	0.06
Calcium (mmol/l)	2.34±0.26	2.26±0.18	2.29±0.14	0.30	0.90	0.06
Phosphorus (mmol/l)	1.55±0.20	1.91±0.40	1.51±0.22	<0.001	0.27	<0.001
Leptin (ng/ml)	24.4±24.6	77.2±48.1	33.2±26.5	<0.001	0.12	<0.001
Adiponectin (ug/ml)	11.9±6.4	15.7±9.5	15.7±8.3	0.25	0.07	0.61
Parathyroid hormone (pg/ml)	48.8±30.7	423.1±271.3	100.9±77.0	<0.001	<0.001	<0.001
Osteocalcin (ng/ml)	6.3±1.8	39.8±15.2	15.7±7.8	<0.001	<0.001	<0.001
FGF23 (pg/ml)	43.3±37.3	54.7±12.4	179.8±383.9	0.04	<0.001	<0.001
Klotho protein (ng/ml)	280.5±376.0	420.6±303.8	585.4±565.7	0.007	0.01	0.51
1,25-$(OH)_2$ Vitamin D_3 (pg/ml)	49.8±47.8	53.6±41.8	72.9±51.9	0.73	0.01	0.046
BMI (kg/m^2)	26.6±4.1	26.8±4.7	26.8±4.0	0.92	0.88	0.96
Lean mass (kg)	48.2±11.3	50.6±10.0	49.6±11.6	0.29	0.53	0.56
Body fat (kg)	27.7±6.8	24.4±10.0	24.9±7.4	0.30	0.12	0.87
FN T-score	-0.09±1.14	-1.80±0.92	-1.19±1.10	<0.001	<0.001	0.006
LS T-score	0.02±1.40	-0.79±1.87	-0.56±1.65	0.08	0.12	0.64

Reference ranges for standard laboratory measurements: Glucose <5.50 (mmol/l); Cholesterol <200 (mg/dl); Triacylglycerols <150 (mg/dl); Calcium 2.15-2.50 (mmol/l); Phosphorus 0.74-1.52 (mmol/l); Parathyroid hormone 15.0-68.3 (pg/ml)

* Mann-Whitney test

FN - femoral neck

LS - lumbar spine

Table 2. Correlations of selected parameters in the group of hemodialyzed patients.

Parameter	Klotho	FGF23	Osteocalcin	1,25(OH)$_2$Vit. D$_3$	PTH	Adiponectin	Leptin
				R_S			
Age	0.06	-0.15	-0.03	-0.28	-0.12	-0.10	-0.22
GFR	-0.21	-0.15	**-0.35**	-0.02	-0.13	-0.10	0.09
BMI	-0.03	0.04	0.22	-0.02	0.12	-0.12	**0.44**
FAT	-0.02	0.05	0.28	-0.07	0.09	-0.21	**0.50**
LEAN	0.01	-0.004	0.04	-0.24	**0.57**	**-0.53**	0.23
FN T-score	0.08	-0.10	0.29	**-0.39**	**0.42**	-0.13	0.00
LS T-score	-0.02	-0.15	**0.30**	**-0.57**	**0.38**	-0.26	0.09
Leptin	**-0.30**	-0.24	0.01	0.22	0.18	-0.16	
Adiponectin	0.11	0.10	0.02	0.11	**-0.47**		
PTH	-0.13	-0.09	0.04	**-0.32**			
1,25(OH)$_2$Vit. D$_3$	-0.06	0.04	-0.13				
Osteocalcin	0.20	0.18					
FGF23	**0.31**						

the statistically significant values have been bolded ($p<0.05$)
R_s - Spearman rank correlation coefficient
FN - femoral neck
LS - lumbar spine

Table 3. Correlations between selected parameters in the group of patients after kidney transplantation.

Parameter	Klotho	FGF23	Osteocalcin	1,25(OH)$_2$Vit. D$_3$	PTH	Adiponectin	Leptin
				R_S			
Age	0.12	-0.004	-0.18	0.04	0.16	0.25	0.06
GFR	-0.07	-0.09	-0.21	0.07	**-0.27**	-0.25	-0.03
BMI	-0.05	-0.11	-0.17	0.07	**0.32**	-0.06	**0.30**
FAT	0.03	-0.04	-0.15	0.10	**0.31**	-0.03	**0.54**
LEAN	-0.21	-0.14	-0.15	-0.06	0.08	**-0.33**	-0.19
FN T-score	-0.08	0.08	0.11	-0.003	**-0.34**	-0.22	-0.07
LS T-score	-0.08	-0.08	-0.08	-0.02	-0.01	-0.17	0.07
Leptin	**-0.39**	0.14	0.13	0.04	0.16	**-0.30**	
Adiponectin	**0.43**	0.08	-0.22	0.11	0.04		
PTH	0.21	0.02	0.23	-0.06			
1,25(OH)$_2$Vit. D$_3$	0.19	0.16	-0.14				
Osteocalcin	-0.17	**0.29**					
FGF23	-0.04						

the statistically significant values have been bolded ($p<0.05$)
R_s - Spearman rank correlation coefficient
FN - femoral neck
LS - lumbar spine