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FGF23-klotho axis as predictive factors of fractures in type 2 diabetics with early chronic kidney disease

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ABSTRACT

Background: The aim of our study was to evaluate the relevance of FGF23-klotho axis in the predisposition for bone fractures in type 2 diabetic patients with early chronic kidney disease.
Methods: In a prospective study we included 126 type 2 diabetic patients with CKD stages 2–3 (from 2010 to 2017). We used descriptive statistics, ANOVA and chi-square test. Our population was divided into two groups according to the occurrence of a bone fracture event or not, and the groups were compared considering several biological and laboratorial parameters. We employed a multiple regression model to identify risk factors for bone fracture events and hazard ratios (HR) were calculated using a backward stepwise likelihood ratio (LR) Cox regression.
Results: Patients with a fracture event displayed higher levels of FGF-23, Phosphorus, PTH, TNF-α, OxLDL, HOMA-IR, calcium × phosphorus product and ACR and lower levels of Osteocalcin, α-Klotho, 25(OH)D3 and eGFR compared with patients without a fracture event ($p < 0.001$). The number of patients with a fracture event was higher than expected within inclining CKD stages ($\chi 2$, $p = 0.06$). The occurrence of fracture and the levels of TNF- α, klotho, 25(OH)D3 and OxLDL were found to predict patient entry into RRT ($p < 0.05$). Age, osteocalcin, α-Klotho and FGF-23 independently influenced the occurrence of bone fracture ($p < 0.05$).
Conclusions: α-Klotho and FGF-23 levels may have a good clinical use as biomarkers to predict the occurrence of fracture events.

1. Introduction

Chronic kidney disease (CKD) is a worldwide condition associated with high mortality and morbidity rates.[1] It is well established that abnormalities in mineral metabolism are apparently early in the course of CKD and play a major role in accelerating metabolic abnormalities associated with CKD.[1] One of the well-known consequences of CKD is predisposition to fragility fractures. Not only is the risk of fracture higher in the CKD population, but clinical outcomes are significantly worse.[2] This is a particularly critical health burden in patients with diabetes, who are at a higher risk for bone fracture than a non-diabetic person.[3,4]

Contrary to what should be expected, and despite their elevated bone mineral density (BMD), patients with Type 2 diabetes mellitus (T2DM), are at an increased risk of fracture. In 2017, a systematic review and meta-analysis of published data on the association between diabetes mellitus and fracture, showed the association between T2DM and increased risk of overall fracture.[5]

The primary function of bone is to maintain its structural integrity though a constant process of bone remodeling. Moreover, bone is also a key player in the regulation of mineral metabolism. More recently, bone was also recognized as an endocrine organ that releases two important hormones, fibroblast growth factor 23 (FGF-23) and undercarboxylated osteocalcin (Ocn).[6,7] FGF-23 regulates phosphate and vitamin D metabolism and Ocn is an essential in energy metabolism, and sexual reproduction.[8]

Both FGF-23 and Ocn levels are increased in CKD, making these proteins promising potential bio-markers for bone remodeling in CKD.[9,10] In addition, there are emerging data supporting that CKD patients with T2DM are at an increased risk for adynamic bone disease.[11]

In order to better prevent the occurrence of skeletal fracture it is important to better understand which could be the best biomarkers predisposing for bone fractures in populations at risk. In the present study, our main goal was to clarify the role of some bone related biochemical parameters and hormone levels in the predisposition for bone fractures in T2DM patients with CKD.

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2. Material and methods

2.1. Subjects

In an observational prospective study, we included 126 CKD patients with T2DM and 26 healthy individuals, recruited between 2010 and 2017 with a diagnosis of diabetic nephropathy (stages 2–3) in a stable clinical condition attending our outpatient clinic.

Institutional Ethics Committee approval (reference 207/2010) was obtained for this study.

The exclusion criteria were: Previous bone disease, age < 18 years or >75 years, uncontrolled hypertension (BP ≥ 140/90 mmHg), albumin-to-creatinine ratio (ACR) >500, estimated glomerular filtration rate (eGFR) ≤29 or >90 mL/min, type1 diabetes, known neoplastic or infectious diseases, non-diabetic renal disease (patients without previous history of diabetes, with diagnosis of glomerulopathies associated with other pathologies like systemic diseases, IgA nephropathy, kidney disease of unknown ethology, chronic interstitial nephritis, vasculitis, component complement 3 pathologies or renal hereditary diseases. Patients with parathyroid hormone (PTH) ≥ 350 pg/mL were excluded to avoid bias from anti-hyperparathyroidism medication that can affect Klotho and FGF-23 levels. The same happened with patients with phosphorus >5.5 and the phosphorus-chelating agents that affect the Klotho-FGF-23 axis. Patients undergoing therapy with vitamin D and vitamin D receptor activators and phosphate binders, as well as anticoagulant therapies (varfine, enoxaparin, clopidogrel, and platelet antiaggregant) were also excluded.

2.2. Follow-up

Patients returned on a regular basis (every 3 months) for in-person visits on Nephrology consultation. No patient was "lost to follow-up".

2.3. Blood measurements

Fasting samples were drawn from all subjects, and plasma was frozen at −80 °C in order to measure glomerular filtration rate (eGFR), phosphorus (P), calcium (Ca), osteocalcin (Ocn), hemoglobin (Hg), glycated hemoglobin (HbA1c), PTH, colesterol, albumine to creatinine ratio (ACR), insulin resistance degree (HOMA-IR), fibroblast growth factor-23 (FGF-23), 25(OH)D3 (vitamin D), oxidized low density lipoprotein (OxLDL), tumor necroses factor- alpha (TNF- α), and soluble α-Klotho (Klotho). FGF-23 levels were quantified using an enzyme-linked immunosorbent assay, Human FGF-23 (C-Term) (Cat.#60-6100 Immutopics Inc., San Clemente, CA, USA) and Human soluble α-Klotho ELISA kit (Code No. 27998, IBL – Immuno-Biological Laboratories Co., Ltd., Gunma, Japan), according to the manufacturer's instructions. The degree of insulin resistance was estimated using the homeostasis model assessment (HOMA-IR) described by Matthews et al.[12] We estimated the GFR according to the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) Equation.[13]

2.4. Outcomes

The primary outcome of this study was occurrence of bone fracture.

2.5. Statistical analyses

Statistical analysis was performed with SPSS17.0 for Windows. Descriptive statistics, Chi-square and logistic regression were used. to compare CKD patients with or without the occurrence of bone fracture event to a control healthy population, we used ANOVA and a post-hoc analysis with Scheffe test. Continuous variables were presented as mean ± standard error. A value of <0.05 was considered significant.

The hazard ratios (HR) were calculated using a backward stepwise likelihood ratio (LR) Cox regression for entry into renal replacement therapy (RRT). The logistic regression model was used to determine whether the variables were important predictive factors in the determination of occurrence of bone fracture event. Only the variables with a statistically significant relationship were introduced in a logistic regression model. The exponentials of the model parameters were the adjusted odds ratio (ORa) to other variables of the model. Prior to conducting the logistic regression and the Cox regression, subjects were categorized according to the 25th, 50th and 75th percentiles of FGF-23, Klotho, osteocalcin, phosphorus and oxLDL levels in the current study population were determined to exclude confounding factors that may influence the results. The subjects were categorized as follows: for FGF-23 group 1 (FGF-23 < 58), group 2 (FGF-23: 58–100) and group 3 (FGF-23 > 100); for Klotho group 1 (Klotho < 268), group 2 (Klotho: 268–440) and group 3 (Klotho > 440); for osteocalcin group 1 (Ocn < 16), group 2 (Ocn: 16–24) and group 3 (Ocn > 24); for phosphorus group 1 (phosphorus < 3.7), group 2 (phosphorus: 3.7–4.6) and group 3 (phosphorus > 4.6) and for oxLDL group 1 (oxLDL < 27), group 2 (oxLDL: 27–36) and group 3 (oxLDL > 36). CKD stages were defined by the eGFR (mL/min/1.73m^2), for Stage 2 (eGFR 60–89), for Stage 3a (eGFR 45–59), for Stage 3b (eGFR 30–44). The null hypothesis was rejected below the level of 5%.

3. Results

One hundred and twenty-six (126) consenting patients with type 2 diabetes mellitus (T2DM) and stage 2–3 of chronic kidney disease (CKD) were included in the study after confirming they did not meet any of the exclusion criteria. A negative control of 26 healthy individuals was also incorporated in the study. The mean age was 58.10 ± 6,66 [range 41–68] years, and 41.8% (64) were female.

Subjects were classified into three groups: CKD patients (and T2DM) with a fracture event, CKD patients (and T2DM) without a fracture event and a control population of healthy subjects. Table 1 displays patients' demographic and clinical parameters and a comparison between CKD patients (and T2DM) with a fracture event, CKD patients (and T2DM) without a fracture event and a control population of healthy subjects.

In Table 1 and Fig. 1, mean values of osteomineral parameters for CKD patients with T2DM, with and without the occurrence of bone fracture and for a control population of healthy individuals are listed.

CKD Patients with a fracture event displayed lower values for Osteocalcin ($p < 0.001$), α-Klotho ($p < 0.001$), and 25(OH)D3 ($p < 0.001$) when compared to both CKD patients without a fracture event and the control healthy subjects. Furthermore, CKD patients without a fracture event displayed lower α-Klotho ($p < 0.01$) when compared to control

Table 1
Patients demographic and clinical characteristics at baseline. Chronic kidney disease (CKD) patients with type 2 diabetics (T2DM), with and without the occurrence of bone fracture and a control population of healthy subjects.

Variable	Fracture (no) ($n = 100$)	Fracture (yes) ($n = 26$)	Control ($n = 27$)
Age (years)	56.99 ± 6.69	61.15 ± 6.60	59.30 ± 5.69
Osteocalcin (μg/L)	20.82 ± 0.67	8.88 ± 1.30 *	23.67 ± 1.10
α-Klotho (pg/mL)	379.08 ± 15.07	113.31 ± 13.89 *	490.67 ± 8.76 *
FGF-23 (RU/mL)	111.01 ± 13.41	304.63 ± 27.02 *	60.97 ± 1.70
25(OH)D3 (ng/mL)	22.73 ± 0.71	14.27 ± 1.06 *	25.87 ± 0.39
OxLDL (U/L)	36.81 ± 1.82	59.15 ± 4.44 *	29.65 ± 0.81
TNF-α (pg/mL)	7.55 ± 0.32	11.32 ± 0.90 *	5.90 ± 0.15
HOMA-IR	1.55 ± 0.16	3.04 ± 0.23 *	0.55 ± 0.01 **
Calcium × Phosphorus (mg/dL)	35.95 ± 0.91	43.56 ± 1.40 *	28.47 ± 1.38 *
ACR (μg/mg)	161.22 ± 1.10	261.18 ± 25.01*	114.70 ± 10.36
PTH (pg/mL)	100.35 ± 7.09	177.05 ± 16.34 *	73.30 ± 1.85
Phosphorus (mg/dL)	3.82 ± 0.08	4.52 ± 0.15 *	3.23 ± 0.14 *
Calcium (mg/dL)	9.52 ± 0.07	9.08 ± 0.50	8.97 ± 0.13*
eGFR (mL/min)	51.95 ± 1.66	41.02 ± 1.82 *	103.53 ± 1.37 *

Values are presented as mean ± standard deviation. *$p < .001$; **$p < .005$. All comparisons were made relative to CKD patients (and T2DM) without a fracture event.

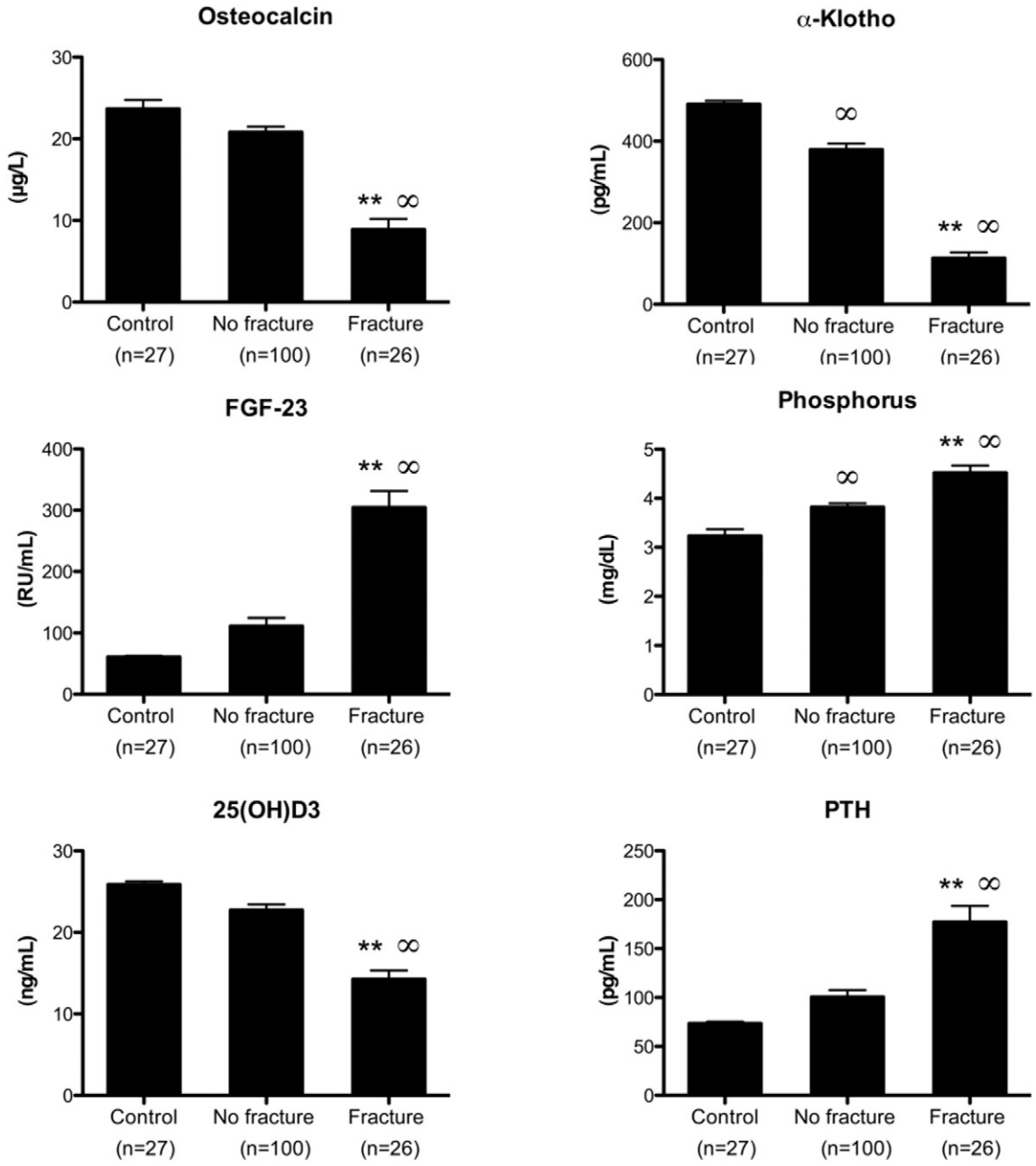

Fig. 1. Parameters of mineral metabolism in Chronic kidney disease (CKD) patients with type 2 diabetics (T2DM) with and without a fracture event. Results of post hoc analysis: ** $p < .001$ vs no fracture; ∝ $p < .01$ vs control.

healthy subjects. On the other hand, the levels of FGF-23 ($p < 0.001$), Phosphorus ($p < 0.001$) and PTH ($p < 0.001$) are increased in CKD patients with a fracture event. Moreover, the phosphorus levels of CKD patients without a fracture event are higher than those of the control population.

In Fig. 2, mean values of other parameter, such as inflammatory, oxidative stress, cardiovascular and kidney function markers are listed. TNF-α ($p < 0.001$), OxLDL ($p < 0.001$), HOMA-IR ($p < 0.001$), calcium × phosphorus product ($p < 0.001$) and ACR ($p < 0.001$) are increased in CKD patients with a fracture event. Both HOMA-IR ($p < 0.001$) and calcium × phosphorus product ($p < 0.001$) are also increased in the CKD patients without a fracture event when compared to the healthy control subjects. eGFR levels were lower in all CKD patients but even lower in CKD patients with a fracture event ($p < 0.01$).

Fig. 3 shows the occurrence of a fracture event according to the stage of renal disease. The patients were classified into three stages of CKD, according to the eGFR (mL/min/1.73 m^2): stage 2 (60–89), stage 3a (45–59) and stage 3b (30–44). The patients with a fracture event increase with inclining CKD stages ($\chi 2$, $p = 006$).

To evaluate the OR_a of osteomineral parameters for CKD patients with T2DM, that are predictors of bone fracture event, a model of logistic regression analyses was used. All comparisons were made relative to the reference value of each osteomineral parameter. The logistic regression analysis (Table 2) revealed that age, osteocalcin, α-Klotho and FGF-23 independently influenced the occurrence of bone fracture ($p < 0.05$).

Variables such as gender, age, phosphorus, PTH, Osteocalcin, FGF-23, Klotho, 25(OH)D3, OxLDL and the occurrence of fracture were analyzed using a multivariate Cox regression to identify independent risk factors of entry into RRT. The occurrence of fracture and the levels of TNF- α, klotho, 25(OH)D3 and OxLDL were found to predict patient entry into RRT ($p < 0.05$) (Table 3).

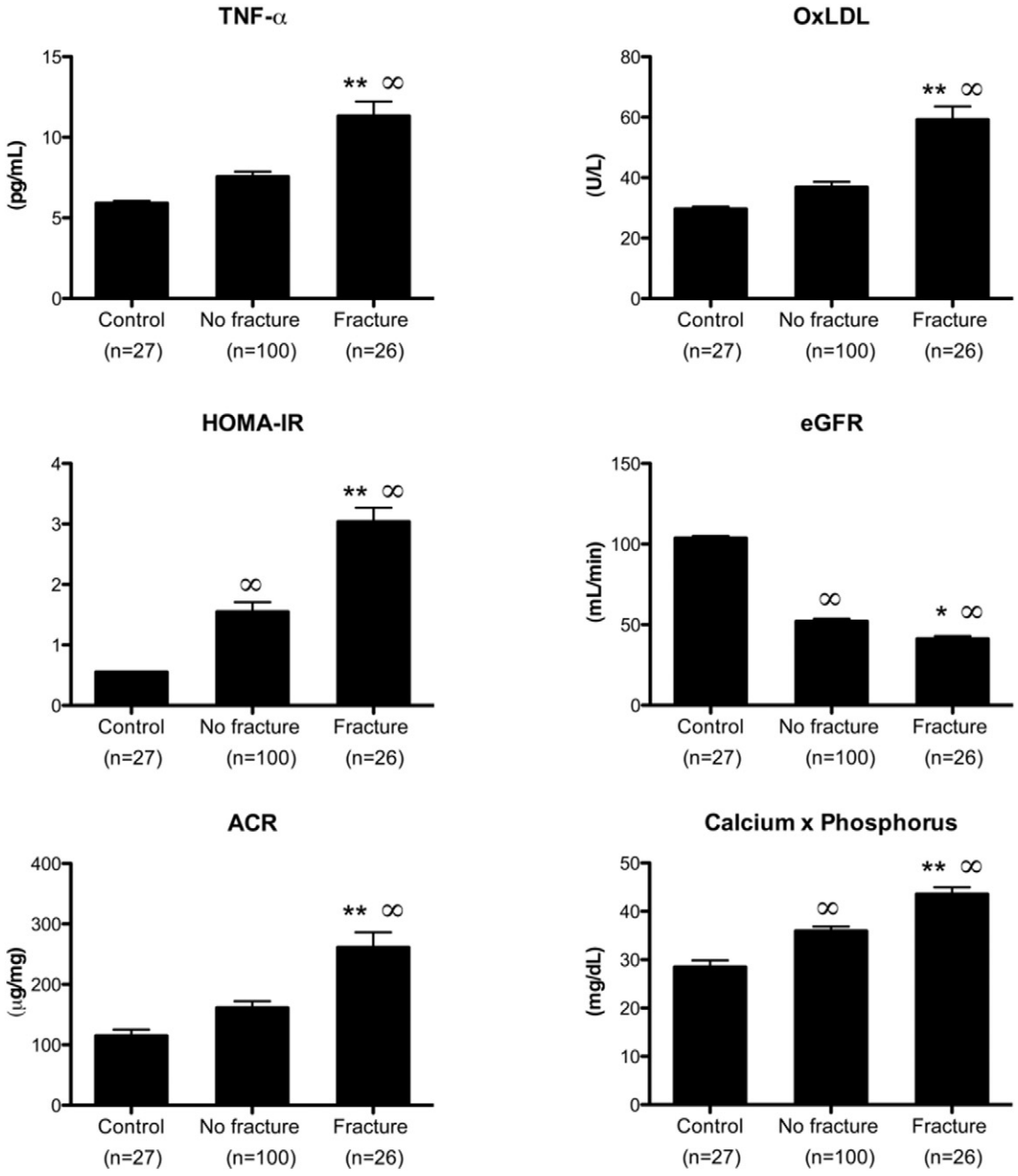

Fig. 2. Parameters in Chronic kidney disease (CKD) patients with type 2 diabetics (T2DM) with and without a fracture event. Results of post hoc analysis: ** $p < .001$ vs no fracture; ∝ $p < .01$ vs control.

4. Discussion and conclusion

As worldwide life expectancy continues to rise, the burden of fragility fractures is a persistently growing health problem. Bone fractures result in considerable morbidity, reduced lifetime and bigger health care costs; thus, it is urgent to find a good tool to identify patients with high fracture risk. The FRAX module identifies patients at risk of fracture considering the BMD, previous fracture, glucocorticoid use, and family history on the 10-year risk for fracture, as independent risk factors for fracture.[14] Identifying new biomarkers, such as hormones, for bone fragility would definitely improve this model as well as improve drug therapy to reduce fracture rates in high-risk populations. It is well established that diabetic CKD patients are at particularly high risk of bone fracture.[15] Both low eGFR and higher albuminuria are considered significant risk factors for fracture.[16] In this study, patients were classified into three stages of CKD, according to the eGFR (mL/min/1.73 m^2): stage 2 (60–89), stage 3a (45–59) and stage 3b (30–44). The number of patients with a fracture event was higher than expected within inclining CKD stages ($\chi 2$, $p = 0.06$). Moreover, ACR are also increased in CKD patients with a fracture event ($p < 0.001$).

In this study, we found that in a population of T2DM CKD patients (stage 2 and 3), age, osteocalcin, α-Klotho and FGF-23 levels independently influenced the occurrence of bone fracture. Interestingly, the association of higher FGF23 and lower Klotho levels with fracture occurrence is independent of eGFR.

Moreover, and using Cox analysis, the levels of TNF- α, klotho, 25 (OH)D3 and OxLDL and the occurrence of fracture were found to independently predict patient entry into RRT ($p < 0.05$).

These findings reinforce the idea that hormones and osteomineral parameters related to bone remodeling may be good candidates as biomarkers to predict the occurrence of fracture events.

Maintaining physiological phosphate balance is of crucial biological importance for bone health. Others and ourselves, have previously demonstrated that hyperphosphatemia is associated with greater

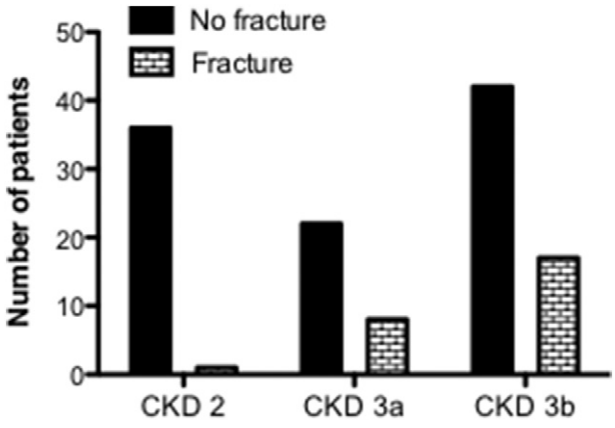

Fig. 3. The occurrence of a fracture event according to Chronic kidney disease (CKD) stage. (Pearson Chi-Square, $p = 006$). The patients were classified into three stages of CKD, according to the eGFR (mL/min/1.73 m^2): CKD 2 (60–89), CKD 3a (45–59) and CKD 3b (30–44).

cardiovascular mortality and morbidity even in patients in low to moderate stages of renal disease.[17–19] In the present study we showed that the levels of Phosphorus are specially increased in CKD patients with a fracture event ($p < 0.001$).

FGF-23 is a key player in the homeostasis of serum phosphate levels.[20] This hormone is secreted by osteocytes and osteoblasts in response to high phosphate levels, either caused by dietary phosphate intake or due to states of impaired renal excretion, such as chronic kidney disease.[21,22] FGF-23 stimulates urinary phosphate excretion by suppressing type IIa sodium phosphate co-transporter in the renal proximal tubule.[20] In the gut, FGF-23 reduces the efficiency of phosphate absorption by impairing production and accelerating degradation of the active form of vitamin D.[23] Recent data suggest that FGF-23 can also directly inhibit the expression and secretion of PTH but, by promoting 1,25(OH)2D3 deficiency it may also contribute to the development of secondary hyperparathyroidism.[24] Both PTH and vitamin D levels have been previously demonstrated to be informative for the definition of fracture risk in patients with CKD-mineral and bone disorders, being useful tools for the identification of CKD patients at high risk of fracture.[25,26] In agreement with this, our results showed that, on the one hand, FGF23 and PTH levels were increased in CKD patients with a fracture event ($p < 0.001$), on the other hand, CKD patients with a fracture event displayed lower values for 25(OH)D3 ($p < 0.001$) when compared to both CKD patients without a fracture event and the control healthy subjects. Excessive levels of FGF-23 are associated with bone disorders such as tumor-induced osteomalacia. In these pathological conditions, FGF-23-dependent phosphaturia and calcitriol deficiency leads to severe bone loss, as well as to bone pain and fractures.[27] Despite the numerous studies supporting that FGF-23 is a negative regulator of bone matrix mineralization both in vivo and in vitro[28,29], the relationship between FGF-23 elevation (in early CKD) and fracture risk is still a matter of debate due to conflicting results.[21,30–32] Some studies demonstrate an independent association between higher FGF-23 levels and greater fracture risk in elderly men[31,32]; whereas cross-sectional studies investigating the association of FGF-23 with bone mineral density have also showed contradictory results,[30,33,34] with the majority reporting no significant associations.[21,30,34] In the present study we observed a relationship between elevated FGF-23 levels and fracture risk in the studied population of T2DM patients with low to moderate CKD. This is in agreement with previous studies reporting that FGF-23 was also found to be a predictor of CKD progression in diabetic[35] and non-diabetic patients.[36] Interestingly, numerous data on pleotropic effects of FGF-23 is also emerging, namely the association of elevated FGF-23 levels with multiple cardiovascular risk factors[37–39] and mortality.[40] All together, this makes FGF-23 a potential biomarker of multiple disarrangements leading to negative patient outcome.

Table 2
Predictive factors of fracture event.

Variables	OR_a	95% CI	*p* values
Gender			
Male	Ref.		
Female	1.893	0.255–2.035	0.532
Age			
<65	Ref.		
≥65	2.206	1.07–4.50	0.044
CKD stages			
Stage 2 (60–89)	Ref.		
Stage 3a (45–59)	6.183	0.993–1.900	0.395
Stage 3b (30–44)	9.537	0.146–3.192	0.290
Phosphorus			
≤3.6	Ref.		
>3.6–4.6	0.332	0.613–8.783	0.510
>4.6	1.058	0.846–4.586	0.972
PTH			
<138	Ref.		
≥138	0.197	0.912–3.170	0.252
HOMA-IR			
<2	Ref.		
≥2	1.783	0.221–8.169	0.193
TNF-α			
<8	Ref.		
≥8	2.027	0.735–3.507	0.610
Osteocalcin			
<16	1.016	1.001–1.389	0.045
16–24	1.296	1.000–3.005	0.007
>24	Ref.		
α-Klotho			
<268	1.226	1.001–1.678	0.058
268–440	2.950	2.00–3.500	0.037
≥440	Ref.		
FGF-23			
<58	Ref.		
58–101	1.788	1.001–2.366	0.024
>101	3.853	2.020–7.428	0.015
25(OH)D3			
≥21	Ref.		
<21	0.203	0.702–2.427	0.201
OxLDL			
<27	Ref.		
27–36	1.032	0.351–3.021	0.138

Logistic regression model: OR_a adjusted odds ratio, *95% CI* confidence interval for the odds ratio, *Ref* category versus the one is making comparisons.

In the majority of cases, FGF-23 signal transduction requires a binding to FGF receptor and α–Klotho protein.[41] Soluble Klotho is a multi-function protein present in the blood, urine, and cerebrospinal fluid and plays important roles in anti-aging, energy metabolism, inhibition of Wnt signaling, anti-oxidation, ion transport, control of PTH and $1,25(OH)_2VD_3$ production, inhibition of insulin and antagonism of renin-angiotensin-aldosterone system. α–Klotho is stimulated by $1,25(OH)_2VD_3$ and inhibited by FGF-23 thereby creating an endocrine loop, as circulating Klotho can activate FGF-23-FGFR signaling.[42] In the majority of clinical studies CKD has been described has a state of FGF-23 resistance due to endocrine and renal Klotho deficiency.[43]

In this study, lower levels of Klotho seem to be an independent predictive factor for bone fracture, in T2DM patients with CKD. Interestingly, data suggesting that TNF- α is able to downregulate Klotho expression,[44] is also in line with our results, showing increased levels of the inflammatory cytokine TNF- α and decreased levels of Klotho in type 2 diabetic patients with CKD with a fracture event. Thus, it is not unreasonable to hypothesize that elevated Klotho levels may serve as an early biomarker

Table 3
Predictive factors for entry into renal function replacement technique.

Variables	Adjusted HR	95% CI	*p* values
Gender			
Female	Ref.		
Male	3.702	0.670–2.462	0.133
Age			
<65	Ref.		
≥65	0.385	0.048–3.067	0.385
ACR			
<95	Ref.		
95–235	2.133	0.126–36.041	0.600
>235	2.573	1.935–6.133	0.050
Phosphorus			
≤3.6	Ref.		
>3.6–4.6	0.590	0.031–1.192	0.725
>4.6	0.333	0.012–9.507	0.520
PTH			
<138	Ref.		
≥138	3.669	0.270–4.797	0.329
Fracture			
No	Ref.		
Yes	8.036	1.267–9.988	0.027
TNF-α			
<8	Ref.		
≥8	1.076	1.001–4.755	0.014
Osteocalcin			
<16	1.442	3.023–1.080	0.889
16–24	3.684	0.421–9.849	0.907
>24	Ref.		
α-Klotho			
<268	6.762	0.872–8.718	0.060
268–440	1.159	1.010–2.523	0.042
≥440	Ref.		
FGF-23			
<58	Ref.		
58–101	3.910	0.725–6.839	0.081
>101	0.485	0.203–1.847	0.776
25(OH)D3			
≥21	Ref.		
<21	2.184	1.824–3.125	0.009
OxLDL			
<27	Ref.		
27–36	1.183	1.081–2.758	0.040
>36	2.543	1.406–2.368	0.026

Multivariate Cox regression: *HR* hazard ratio. *95% CI* confidence interval for the HR. *Ref* category versus the one is making comparisons.

and a pathogenic contributor to chronic progression and complications in chronic kidney disease, such as bone fragility.

However, it is still controversial whether osteocalcin is a good marker of bone formation[45,46] and the fact that initially OC was believed to be only necessary to maintain the normal calcification and inhibit mineralization, more recently a much wide role of OC has been suggested in overall body metabolism, reproduction, cognition as well as regulating insulin production and secretion.[47] Our results show that, not only do low levels of OC independently influenced the risk for bone fracture in T2DM CKD patients, but patients with a bone fracture event also presented higher HOMA-IR values when compared to the ones without a fracture event, at least considering the population in this study. This is in line with previous results from Xuefei et al., showing that OC might improve glucose metabolism through increasing insulin secretion and improving insulin resistance in females with T2DM.[48]

Despite some limitations, namely the small size of the sample and the limited statistical power of these analyses, this study offers an added value by generating hypotheses. Nonetheless, further studies are required in order to confirm the observed associations.

In conclusion, the present study strongly suggests that a deregulation in mineral metabolism, reflected by low levels of Osteocalcin and Klotho and high levels of FGF-23, could be associated with an increased risk of bone fracture in T2DM patients with a diagnosis of mild to moderate CKD.

We believe the assessment of fracture risk can be better predicted by using a combination of biochemical markers of bone turnover - which are dynamic markers, and the BMD measurement - which provides a static picture of the skeleton, than by assessing either alone.

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Declaration of competing interest

The results presented in this paper have not been published previously in whole or part, except in abstract form.

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