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Original Articles

Secreted Klotho and FGF23 in chronic kidney disease Stage 1 to 5: a sequence suggested from a cross-sectional study

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Abstract

Background. Klotho and fibroblast growth factor 23 (FGF23) are key regulators of mineral metabolism in renal insufficiency. FGF23 levels have been shown to increase early in chronic kidney disease (CKD); however, the corresponding soluble Klotho levels at the different CKD stages are not known.

Methods. Soluble Klotho, FGF23, parathyroid hormone (PTH), 1,25-dihydroxy vitamin D_3 (1,25D) and other parameters of mineral metabolism were measured in an observational cross-sectional study in 87 patients. Locally weighted scatter plot smoothing function of these parameters were plotted versus estimated glomerular filtration rate (eGFR) to illustrate the pattern of the relationship. Linear and non-linear regression analyses were performed to estimate changes in mineral metabolism parameters per 1 mL/min/1.73 m^2 decline.

Results. In CKD 1–5, Klotho and 1,25D linearly decreased, whereas both FGF23 and PTH showed a baseline at early CKD stages and then a curvilinear increase. Crude mean Klotho level declined by 4.8 pg/mL (95% CI 3.5–6.2 pg/mL, $P < 0.0001$) and 1,25D levels by 0.30 ng/L (95% CI 0.18–0.41 ng/L, $P < 0.0001$) as GFR declined by 1 mL/min/1.73 m^2. After adjustment for age, gender, serum 25-hydroxyvitamin D levels and concomitant medications (calcium, supplemental vitamin D and calcitriol), we estimated that the mean Klotho change was 3.2 pg/mL (95% CI 1.2–5.2 pg/mL, $P = 0.0019$) for each 1 mL/min/1.73 m^2 GFR change. FGF23 departed from the baseline at an eGFR of 47 mL/min/1.73 m^2 (95% CI 39–56 mL/min/1.73 m^2), whereas PTH departed at an eGFR of 34 mL/min/1.73 m^2 (95% CI 19–50 mL/min/1.73 m^2).

Conclusions. Soluble Klotho and 1,25D levels decrease and FGF23 levels increase at early CKD stages, whereas PTH levels increase at more advanced CKD stages.

Keywords: chronic kidney disease (CKD); fibroblast growth factor 23 (FGF23); Klotho; parathyroid hormone (PTH); 1,25-dihydroxyvitamin D_3 (1,25D)

Introduction

Klotho, expressed in the kidney [1, 2], parathyroid glands [3, 4] and the choroid plexus [5], is a single transmembrane protein whose extracellular domain is cleaved by the α-secretases ADAM 10 and 17 [6] to generate large amounts of soluble Klotho into blood, urine and cerebrospinal fluid [7, 8]. Soluble Klotho activates ion channels TRPV5 and 6 in the nephron and the intestine [9, 10], and regulates the sodium-phosphate co-transporters Type-2a and c (NaPiIIa and c) independently of fibroblast growth factor 23 (FGF23) [8].

Transmembrane Klotho acts as an important co-factor for FGF23. FGF23, a phosphaturic hormone produced by osteocytes, binds with only modest affinity to the family of FGF receptors (mainly Type 1, 3 and 4), whereas *in vivo* Klotho is required for FGF23-mediated receptor activation (mainly FGFR1c): it forms a complex with the fibroblast growth factor receptor (FGFR), thereby increasing its affinity for FGF23 [11, 12]. Thus, FGF23 and Klotho synergize to regulate phosphate homeostasis [13] by promoting renal phosphate excretion: they do so via reduction in the number of NaPiIIa and NaPiIIc phosphate co-transporters in the proximal tubule and reduction in intestinal phosphate absorption, the latter following a decreased renal synthesis of 1,25-dihydroxy vitamin D_3 (1,25D) [14].

Rodent studies indicate that soluble Klotho levels in urine and blood are highly correlated with renal Klotho expression [15]. Studies in patients with chronic kidney disease (CKD) or acute kidney injury indicate a decrease in Klotho expression with decreasing GFR [16]; however, these studies encompass a very limited number of patients and Klotho levels were measured only semi-quantitatively by western blots performed in concentrated urine samples.

Cross-sectional studies have shown that the curvilinear slope of FGF23 versus estimated glomerular filtration rate (eGFR) ascends at CKD Stage 2–3, whereas that of parathyroid hormone (PTH) versus eGFR ascends at CKD

Stage 3 [17, 18]. However, at which CKD stage the fall of serum Klotho occurs in patients with CKD remains a matter of debate because a reliable assay for soluble Klotho has not been available until recently [19, 20], and data on the expression, function and regulatory mechanisms of soluble Klotho are scarce.

Our cross-sectional study is the first systematic determination of serum levels of Klotho, FGF23, PTH, 1,25D and other parameters of mineral metabolism performed in a cohort of patients with chronic renal insufficiency at CKD Stages 1–5. We examined the pattern of the respective changes in the mentioned parameters over the entire range of CKD stages; the question is relevant as Klotho or FGF23 may turn out to be important markers of early kidney disease, of its progression as well as of its prognosis, besides the fact that both might become future therapeutic targets [21].

Materials and methods

Study participants and procedures

Eighty-seven patients at different stages of CKD not affected by polycystic kidney disease nor having undergone previous kidney transplantation, aged 18–84 years, were enrolled in the study. CKD patients were classified according to eGFR; CKD Stage 1 (≥90 mL/min/1.73 m^2), CKD Stage 2 (60–89 mL/min/1.73 m^2), CKD Stage 3 (30–59 mL/min/1.73 m^2), CKD Stage 4 (15–29 mL/min/1.73 m^2) and CKD Stage 5 [<15 mL/min/1.73 m2 or dialysis (5D)]. Twenty-one healthy volunteers, aged 37–62 years, without a medical history of renal disease, and screened negative for microhaematuria or microalbuminuria, served as control group.

Sitting blood pressure was measured by a nurse, a blood sample was drawn and a spot urine sample (second fasting morning urine, after voiding the first urine of the day prior to the visit to the clinics) was collected between 8 a.m. and 10 a.m. Haemodialysis patients were analysed at their steady-state condition: after the long interval, blood was taken immediately after placing the dialysis needle and thus before start of dialysis treatment. Residual renal function in CKD5D patients was calculated according to guidelines for the measurement of renal function [22] applying the formula of Daugirdas [23]. Briefly, the mean of urea and creatinine clearance, determined from 48-h urine collections and normalized to 1.73 m^2, were calculated. In anuric patients, the value was set to zero.

The serum and urine aliquots were stored at −80°C. Blood was analysed for Klotho, FGF23, PTH, phosphate, ionized calcium, creatinine, 25-hydroxyvitamin D (25D) and 1,25D. Spot urine was analysed for phosphate and creatinine.

The study was conducted according to the Declaration of Helsinki and the guidelines of Good Clinical Practice (GCP), and was approved by the local Ethics Committee. All patients gave written, informed consent.

Analytical methods

A novel enzyme-linked immunosorbent assay (ELISA) method detecting human soluble Klotho has been developed first by establishing a monoclonal antibody with strong affinity for human Klotho protein, recognizing with high selectivity the tertiary protein structure of its extracellular domain (Immuno-Biological Laboratories Co., Ltd., Japan). The established protein detection method has been subsequently tested comparing serum Klotho levels of healthy human volunteers with a human case where the Klotho gene carries a mutation that hinders the expression of Klotho in the test subject. The results of the analysis indicated that the ELISA system can specifically detect and measure the circulating serum Klotho levels in humans [20]. We further validated the assay in patients affected by autosomal-dominant polycystic kidney disease: low serum Klotho levels were found to constrain the phosphaturic effect of FGF23 and to correlate inversely with cyst growth and kidney volume [19]. The respective intra-assay and intra-subject coefficient of variations of Klotho were 2.6 ± 1.1 and 5.6 ± 2.1%, respectively, in the present study.

The levels of carboxy-terminal FGF23 (second generation, Immutopics Inc., San Clemente, CA, USA) and intact PTH (second generation, Biomerica Inc., Newport Beach, CA, USA) were measured in serum by ELISA according to the manufacturer's protocol. As previously published, we and others have shown that intact FGF23 and C-terminal FGF23 levels closely correlate in early and late CKD stages [14,18,24,25].

Serum 25D and 1,25D have been determined using the radioimmunoassay-kits from Diasorin (Stillwater, MN, USA) and Immunodiagnostic Systems (Fountain Hills, AZ, USA), respectively. Phosphate concentrations were measured in serum and urine using standard methods. Creatinine in serum and urine was assayed by the isotope dilution mass spectrometry (IDMS) traceable modified Jaffé method. The glomerular filtration rate was estimated by using the CKD Epidemiology Collaboration (CKD-EPI) equation [26]. Phosphate and creatinine (IDMS-traceable modified Jaffé method) concentrations were measured in serum and urine. The ratio of the maximum rate of tubular phosphate reabsorption to the glomerular filtration rate (TmP/GFR) was calculated as follows:

$$\text{TmP/GFR (mmol/L)} = P_P - U_P \times \frac{P_{Crea}}{U_{Crea}}$$

where P_P, U_P, P_{Crea} and U_{Crea} refer to the plasma and urinary concentration of phosphate and creatinine, respectively [27]. TmP/GFR allows us to estimate the net renal phosphate transport and is referred to as the theoretical renal phosphate threshold [28]. This corresponds to the theoretical lower limit of plasma phosphate below which all filtered phosphate would be absorbed (normal range 0.80–1.35 mmol/L).

Statistical analysis

Locally weighted scatter plot smoothing (LOWESS) function of Klotho, FGF23, PTH, 1,25D and serum phosphate versus eGFR were fitted by the default function of the STATA version 11.2 software (STAT Corp., College Station, TX, USA) with a bandwidth of 0.8. LOWESS is a modelling method designed to address situations in which the classical linear regression procedures do not perform well [29]. At each point in the data set, a low-degree polynomial is fitted to a subset of the data, with explanatory variable values near the point whose response is being estimated. LOWESS combines linear least squares regression with the flexibility of non-linear regression and does not require to specify a global function of any form to fit a model. LOWESS allows determination of a relationship without having a specified global function of any form to fit a model, whereas linear models 'force' the line to fit the a priori model, e.g. quadratic function.

Based on the LOWESS shape, a linear model was fitted for Klotho and 1,25D and the crude mean change for each 1 mL/min/1.73 m^2 eGFR was estimated. Vitamin D increases Klotho expression *in vivo* in mice [30] and *in vitro* in a variety of cell lines [31]. Therefore, the Klotho-related data were adjusted by fitting an a priori model containing the following variables: vitamin D supplementation, 1,25D treatment, calcium administration (calcium acetate, calcium supplement), calcium-free phosphate binder (sevelamer, lanthanum) and serum 25D levels. Thus, the estimated mean changes in Klotho and 1,25D were adjusted for treatment with vitamin D compounds and 25D levels, covariates that potentially influence these profiles over eGFR. The association between FGF23 and PTH with eGFR was not linear and thus we fitted a segmented model consisting of smoothly fitted baseline and quadratic function to estimate the departure point of the curve from the baseline.

In an additional analysis, differences among the CKD stages and healthy volunteers were compared by one-way analysis of variance. When the difference was significant, statistical comparisons were done by using Dunnett's *post hoc* test with the healthy volunteers as a reference group. Spearman's rank correlation coefficient was calculated to measure the statistical dependence between the two variables.

All P values were two-sided for the comparison between the groups and values <0.05 were considered as statistically significant. Statistical analyses were performed using SAS statistical software, version 9.2. (SAS Institute Inc., Cary, NC, USA).

Results

Patients were studied at the outpatient clinic of the Division of Nephrology at the University Hospital of Zurich, Switzerland, from March 2010 to May 2011. Table 1

Table 1. Characteristics of patients with CKD and healthy volunteers (HV)

	CKD 1 ($n = 17$)	CKD 2 ($n = 19$)	CKD 3 ($n = 11$)	CKD 4 ($n = 20$)	CKD 5 ($n = 20$)	HV ($n = 21$)
Age, years	41 ± 14	40 ± 12	57 ± 15	64 ± 12	61 ± 17	48 ± 8
Sex, no. (%)						
Female	7 (41)	12 (63)	6 (55)	9 (45)	8 (40)	9 (43)
Male	10 (59)	7 (37)	5 (45)	11 (55)	12 (60)	12 (57)
Body mass index, kg/ m^2	28 ± 6	23 ± 5	28 ± 4	28 ± 6	24 ± 6	24 ± 2
Creatinine, mg/dL	0.8 ± 0.2	1.0 ± 0.2	1.5 ± 0.3	2.5 ± 0.7	6.6 ± 2.8	0.9 ± 0.1
eGFR, mL/min/1.73 m^2	104.1 ± 11.3	81.1 ± 16.5	42.6 ± 6.0	25.3 ± 5.8	5.7 ± 4.9	91.2 ± 14.1
Blood pressure, mmHg						
Systolic	139 ± 13	130 ± 13	144 ± 21	145 ± 19	138 ± 26	129 ± 15
Diastolic	83 ± 10	84 ± 8	78 ± 14	79 ± 12	69 ± 21	80 ± 13
Urinary protein excretion						
Protein-to-creatinine ratio, g/mmol	0.04 (0.01, 0.06)	0.01 (0.01, 0.04)	0.03 (0.01, 0.06)	0.02 (0.01, 0.07)	NA	0.02 (0.01, 0.02)
Medication, no. (%)						
25-Hydroxy-vitamin supplement	7 (41)	7 (37)	9 (82)	13 (65)	8 (40)	0
1,25-Dihydroxy vitamin D_3 treatment	0 (0)	1 (5)	1 (9)	5 (25)	4 (20)	0
Calcium-free phosphate binder	0 (0)	0 (0)	0 (0)	2 (10)	9 (45)	0
Calcium administration	4 (24)	5 (26)	1 (9)	5 (25)	10 (50)	0
Bicarbonate supplement	0 (0)	0 (0)	0 (0)	4 (20)	1 (5)	0
Diuretics	1 (6)	2 (11)	4 (36)	14 (70)	6 (30)	0
Prednisone	3 (18)	6 (32)	4 (36)	2 (10)	3 (15)	0

eGFR, estimated glomerular filtration rate. Values are means ± standard deviation and numbers (percentage).

shows the characteristics of 87 CKD patients and 21 healthy volunteers. The patients were classified into CKD stages according to the CKD EPI equation: 19% of the participants belonged to CKD Stage 1, 22% to Stage 2, 13% to Stage 3, 23% to Stage 4 and 23% to Stage 5 (18 of 20 patients on chronic haemodialysis treatment, median dialysis vintage 1.4 years). Causes of nephropathies in CKD patients were: hypertensive nephropathy ($n = 20$, 10% biopsy confirmed), IgA-nephropathy ($n = 12$, 83% biopsy confirmed), diabetic nephropathy ($n = 6$, 33% biopsy confirmed), focal segmental glomerulosclerosis ($n = 10$, 100% biopsy confirmed), lupus nephritis ($n = 5$, 80% biopsy confirmed), other glomerulonephritides ($n = 13$, 46% biopsy confirmed), other kidney diseases ($n = 16$, 81% biopsy proven) and CKD of unknown aetiology ($n = 5$). Supplemental Table 1 displays disease classifications according to the CKD stages. In the CKD 4 and 5 strata, diabetic and hypertensive patients were enriched. The frequency of 1,25D and phosphate binder treatment increased with advancing CKD stages. At any CKD stage, at least 40% of the patients were supplemented with nutritional vitamin D (Table 1). None of the patients were currently or had been treated in the past with bisphosphonates.

Relationship between Klotho, GFR and parameters of mineral metabolism

Klotho, 1,25D, FGF23 and PTH levels were plotted versus eGFR and the respective relationships analysed by the LOWESS function, a statistical technique without assumption on the shape of the relationship.

Figure 1A reveals that serum Klotho and eGFR were associated linearly. Klotho levels continuously declined with progressive degree of severity of renal insufficiency; this fitted a linear model ($r^2 = 0.41$, $P < 0.0001$) and it was estimated that the crude mean Klotho level declined by 4.8 pg/mL (95% CI 3.5–6.2 pg/mL, $P < 0.0001$) as GFR declined by 1 mL/min. After adjustment for age, gender, serum 25D levels, and concomitant medications (calcium, supplemental vitamin D and calcitriol), we estimated that the mean Klotho change was 3.2 pg/mL (95% CI 1.2–5.2 pg/mL, $P = 0.0019$) for each 1 mL/min GFR change. We noted that many patients had low 25D levels. To rule out for the possibility that low Klotho levels might just reflect vitamin D deficiency, we separately estimated Klotho changes in subjects with serum 25D levels of 20 µg/L and above ($n = 37$): crude and adjusted mean Klotho level declined by 4.9 pg/mL (95% CI 3.0–6.8 pg/mL, $P < 0.0001$) and by 3.6 pg/mL (95% CI 0.4–6.8 pg/mL, $P = 0.03$), respectively, as GFR declined by 1 mL/min. We obtained similar results when separately analysing subjects without vitamin D supplementation ($n = 43$): crude and adjusted mean Klotho level declined by 4.7 pg/mL (95% CI 2.8–6.5 pg/mL, $P < 0.0001$) and by 3.7 pg/mL (95% CI 0.9–6.5 pg/mL, $P = 0.01$), respectively, as GFR declined by 1 mL/min.

We assessed the association of Klotho with other parameters of mineral metabolism (Table 2). In the univariate analysis, Klotho was associated with serum calcium, phosphate, 1,25D, FGF23 and PTH. However, these associations were attenuated after adjusting for age, gender and eGFR. Age and eGFR remained independently associated with Klotho, underlining the importance of both factors when interpreting serum Klotho levels.

The serum Klotho levels among 17 haemodialysis patients meeting the criteria for secondary hyperparathyroidism (PTH >65 ng/mL, serum phosphate >1.1 mmol/L, serum calcium < 2.6 mmol/L and calcitriol treatment<1

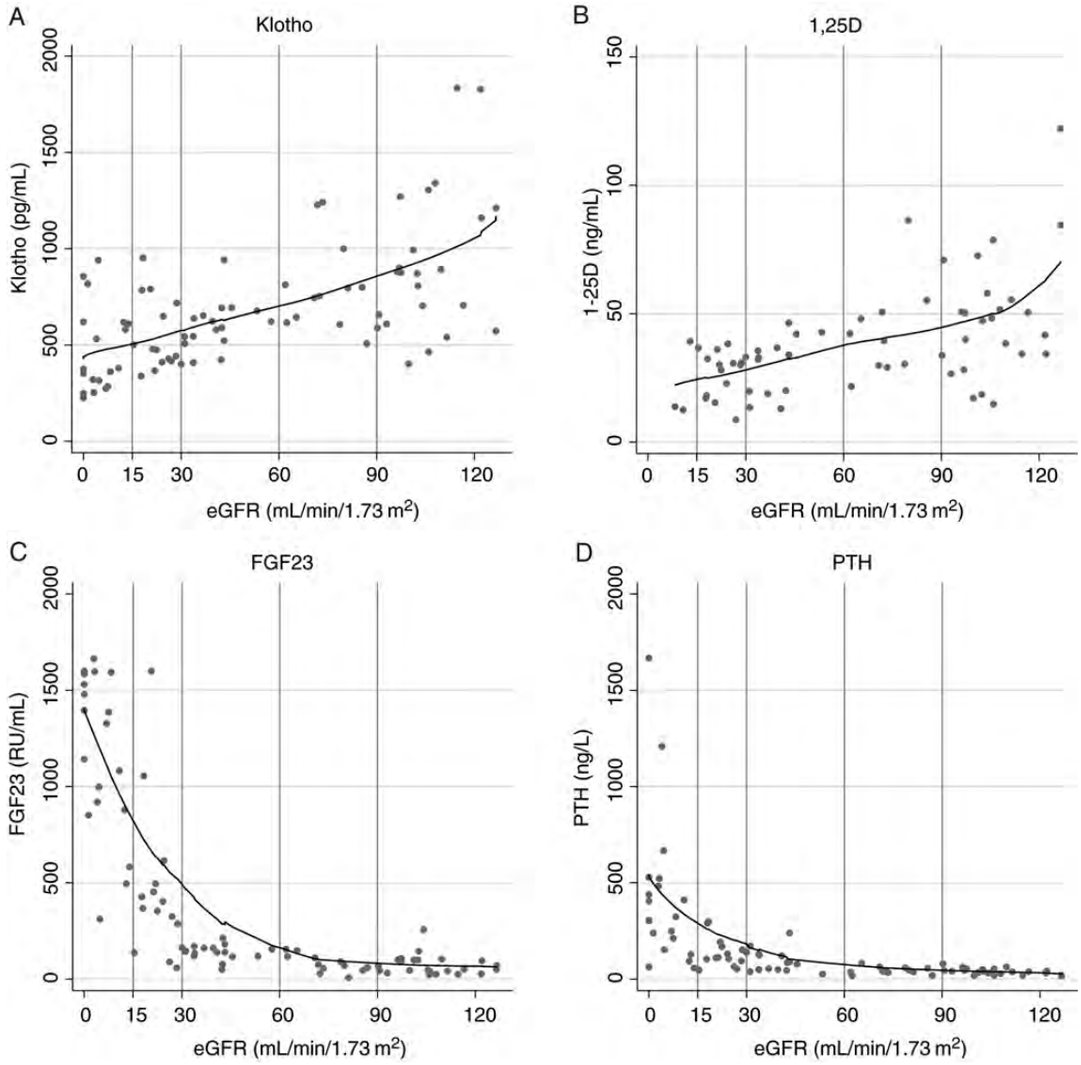

Fig. 1. Scatter plot graphs of with LOWESS lines of (A) Klotho, (B) 1,25-dihydroxy-vitamin D_3 (1,25D), (C) carboxy-terminal fibroblast growth factor 23 (FGF23) and (D) intact parathyroid hormone (PTH) versus estimated glomerular filtration rate (eGFR) in CKD patients. Each symbol represents one patient.

µg/week [32, 33]) were 463.4 ± 237.0 pg/mL (median 375.0 pg/mL, IQR 273.7–618.9 pg/mL).

Relationship between serum levels of 1,25D, FGF23 and PTH with eGFR

Figure 1B confirms that 1,25D and eGFR are linearly associated and 1,25D levels decline with progressive GFR loss justifying the fitting of a linear model. We estimated that the crude mean 1,25D levels decrease by 0.30 ng/L (95% CI 0.18–0.41 ng/L, $P < 0.0001$) as eGFR declines by 1 mL/min. The adjustment for age, gender, serum 25D levels and the concomitant medications did only marginally change the estimated 1,25D slope: 0.30 ng/L per 1 mL eGFR (0.10–0.49 ng/L per 1 mL eGFR; $P = 0.0036$).

Based on the visual inspection of the LOWESS plots, FGF23 and PTH were not linearly associated with eGFR. Thus, we fitted a segmented, non-linear model that consists of two segments connected in a smooth fashion, and estimated the departure point of the curve from the baseline. The iterative optimization converges after six (FGF23 versus eGFR) and seven (PTH versus eGFR) iterations, respectively. We estimated that the FGF23 curve departs from the baseline (81 RU/mL) at a GFR of 47 mL/min (95% CI 39–56 mL/min), whereas the PTH curve departs from the baseline (58 ng/mL), at an eGFR of 34 mL/min (95% CI 19–50 mL/min). However, it is noteworthy that, in their respective relationship to eGFR, FGF23 and PTH both showed a baseline at approximately CKD Stages 1–3 for PTH and 1–2 for FGF23 and an exponential increase at higher respective CKD stages in the LOWESS plots (Figure 1C and D), whereas the segmented model estimated a later departure from the baseline indicating a Type 2 error, pointing to the fact that the

Table 2. Association of Klotho levels with other serum parameters of mineral metabolism in CKD patients: β estimates and (P-values) of the univariate and multivariate regression analyses

Regression models	Calcium	Phosphate	1,25-dihydroxy vitamin D_3	FGF23[a]	PTH [a]
Univariate					
Klotho[a]	−0.3 (< 0.0001)	−0.2 (0.01)	0.003 (<0.001)	−0.2 (<0.001)	−0.2 (<0.001)
Multivariate					
Klotho[a]	−0.02 (0.9)	−0.01 (0.2)	0.001 (0.5)	−0.002 (1.0)	0.03 (0.7)
Age (years)	−0.004 (0.01)	−0.004 (0.01)	0.001 (0.01)	−0.003 (0.01)	−0.004 (0.01)
Gender (male = 1)	0.01 (0.03)	0.03 (0.04)	0.01 (0.7)	0.01 (0.7)	0.009 (0.8)
eGFR[b]	0.001 (0.02)	0.001 (0.03)	0.001 (0.03)	0.002 (0.06)	0.002 (0.03)

[a]Log transformed.
[b]Estimated GFR according to the CKD-EPI formula.

Table 3. Parameters of phosphate metabolism in patients with CKD and healthy volunteers (HV)

Parameter	CKD 1 (n = 17)	CKD 2 (n = 19)	CKD 3 (n = 11)	CKD 4 (n = 20)	CKD 5 (n = 20)	HV (n = 21)
Serum						
Klotho, pg/mL	964.3 ± 398.8	820.2 ± 283.4	638.1 ± 128.7	539.7 ± 165.1	460.2 ± 222.8	1078.6 ± 1810.2
Q1/Median/Q3	703.2/880.1/1159.8	616.2/749.3/999.1	423.9/622.6/684.9	415.1/490.2/640.8	282.2/368.3/612.9	428.7/600.3/861.5
FGF23, RU/mL	89.9 ± 54.3	65.1 ± 41.7	137.7 ± 45.9	377.2 ± 370.3	1200.6 ± 416.4	24.8 ± 13.7
Q1/median/Q3	51.1/95.5/101.4	38.2/50.8/82.9	50.8/140.1/161.3	141.6/307.0/434.0	910.0/1356.7/1586.4	18.3/24.2/32.4
PTH, ng/mL	43.0 ± 17.0	40.4 ± 16.6	87.4 ± 63.8	129.1 ± 71.2	422.8 ± 392.7	55.6 ± 25.4
Q1/median/Q3	32.7/42.3/52.8	31.2/39.6/45.9	25.9/76.6/88.1	87.2/119.4/156.7	196.9/314.2/492.4	36.4/46.2/67.1
25-Hydroxy-vitamin D, μg/L	15.1 ± 8.9	20.4 ± 8.2	17.6 ± 6.0	21.9 ± 12.5	25.9 ± 12.4	21.6 ± 9.1
Q1/median/Q3	8.5/15.4/20.8	14.7/20.9/24.0	7.9/19.3/19.8	12.7/18.8/27.3	16.7/26.2/34.1	15.7/18.5/25.6
1,25-Dihydroxy-vitamin D_3, ng/L	48.5 ± 25.9	45.7 ± 19.6	31.7 ± 12.7	27.12 ± 8.7	21.8 ± 15.0	65.4 ± 24.4
Q1/median/Q3	34.3/41.6/55.4	29.8/48.0/51.7	13.0/35.4/42.3	19.3/30.4/33.0	13.2/13.8/26.5	48.9/61.6/79.4
Phosphate, mmol/L	0.96 ± 0.17	1.02 ± 0.24	1.01 ± 0.22	1.15 ± 0.21	1.69 ± 0.48	1.00 ± 0.19
Q1/median/Q3	0.84/0.96/1.08	0.85/1.02/1.24	0.62/1.03/1.11	1.04/1.09/1.30	1.33/1.58/1.99	0.86/0.97/1.14
Ionized calcium, mmol/L	1.20 ± 0.05	1.24 ± 0.09	1.23 ± 0.05	1.22 ± 0.06	1.19 ± 0.08	NA
Q1/median/Q3	1.18/1.20/1.21	1.20/1.22/1.25	1.18/1.22/1.24	1.17/1.21/1.25	1.14/1.17/1.22	
Calcium, mmol/L	2.32 ± 0.13	2.32 ± 0.13	2.39 ± 0.16	2.36 ± 0.14	2.33 ± 0.17	2.22 ± 0.09
Q1/median/Q3	2.26/2.32/2.40	2.22/2.33/2.41	2.28/2.36/2.44	2.25/2.34/2.47	2.22/2.30/2.41	2.18/2.24/2.28
Spot urine						
Phosphate, mmol/L	12.8 ± 5.8	16.5 ± 12.9	9.7 ± 4.6	12.5 ± 5.4	—	16.7 ± 12.6
Q1/median/Q3	8.1/13.1/16.1	4.1/15.3/21.2	5.4/8.7/11.2	9.4/11.3/15.9		7.9/14.2/19.2
TmP/GFR, mmol/L/GFR	0.85 ± 0.16	0.89 ± 0.23	0.86 ± 0.25	0.72 ± 0.16	—	0.87 ± 0.21
Q1/median/Q3	0.74/0.81/0.97	0.69/0.85/1.13	0.42/0.91/1.03	0.60/0.78/0.85		0.70/0.88/1.02

Q1, 0.25 quartile; Q3, 0.75 quartile; 1,25D, 1,25-dihydroxy-vitamin D_3; FGF23, carboxy-terminal fibroblast growth factor 23; PTH, intact parathyroid hormone; TmP/GFR, tubular maximum phosphate reabsorption per millilitre of glomerular filtrate.

study is underpowered for this specific type of analysis (which was not the primary focus of the analysis).

Phosphate, calcium and 25-hydroxy vitamin D

Mean serum phosphate levels increased only modestly in patients at higher CKD stages: 0.96 mmol/L ± 0.17 mmol/L in CKD 1 to 1.69 mmol/L ± 0.48 mmol/L in CKD5 (Table 3). Hyperphosphataemia (>1.1 mmol/L) was present in 46% of the participating subjects, mostly in patients with CKD Stages 4 and 5, as expected. The LOWESS function of serum phosphate versus eGFR indicated a baseline at early CKD stages and an exponential increase at advanced CKD stages (Figure 2A). The departure point of the curve from the baseline was at an eGFR of 35 mL/min (95% CI 22–49 mL/min) estimated by fitting a segmented non-linear regression model. TmP/GFR remained normal and unchanged for patients at CKD Stage 1–3 and then declined at Stage 4.

The ionized calcium levels remained unchanged across CKD Stages 1–5 and the LOWESS function remained within the normal range (1.10–1.30 mmol/L) (Figure 2B). The serum levels of 25D also remained unchanged across CKD Stages 1–5 (Figure 2C).

Differences among the CKD stages and healthy volunteers

Klotho levels were lower among CKD Stage 5 patients compared with age-matched healthy volunteers when applying a *post hoc* test adjusted for multiple testing and using volunteers as a reference group. The Klotho levels decreased approximately by half from CKD 1 to 5 (mean difference −504.1 pg/mL (95% CI −747.4 to −260.8;

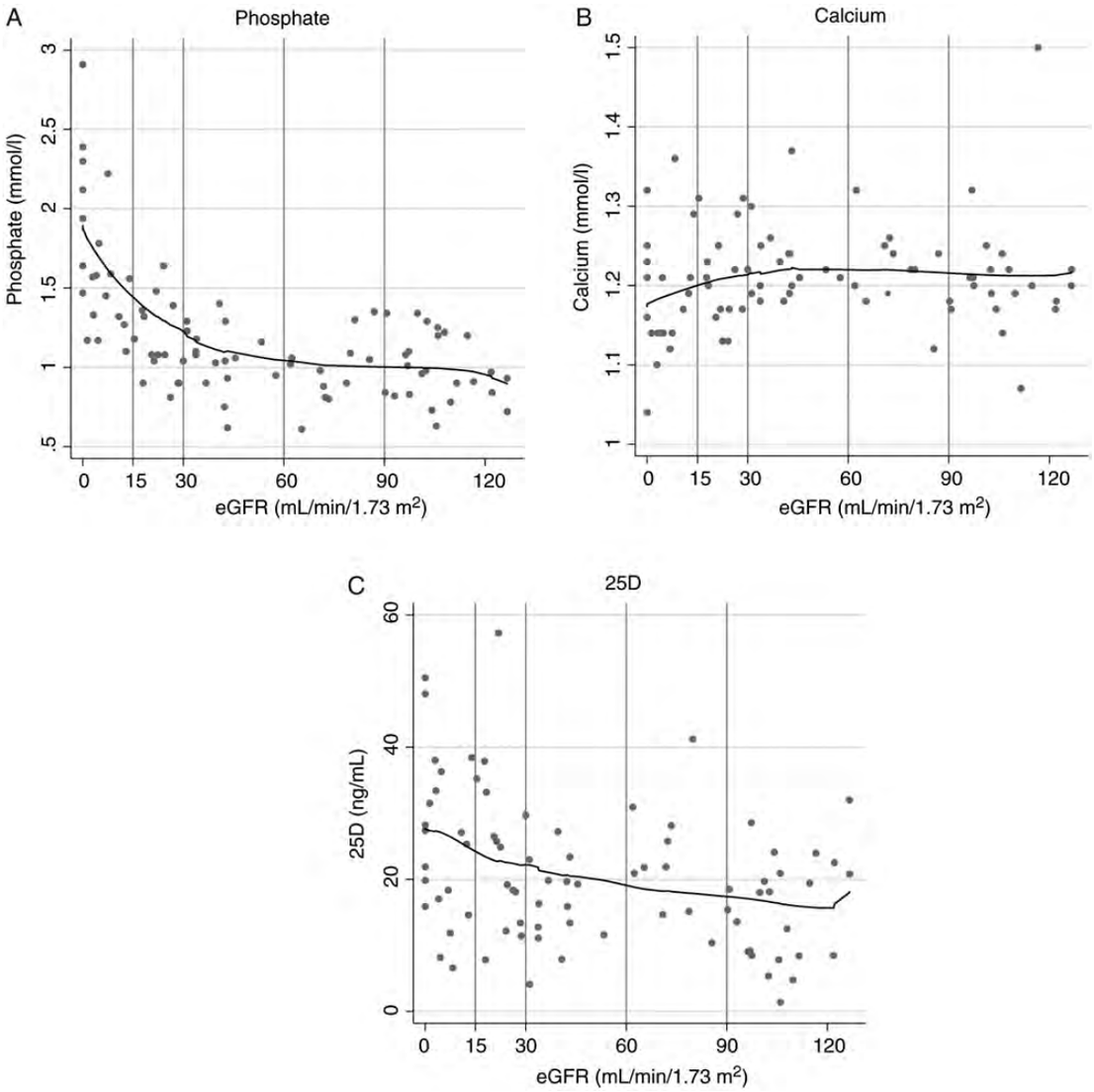

Fig. 2. Scatter plot graphs with LOWESS lines of (A) serum phosphate, (B) ionized calcium and (C) 25-hydroxy-vitamin D (25D), versus eGFR in CKD patients. Each symbol represents one patient.

$P < 0.05$). Serum levels of FGF23 in patients at CKD Stages 4 and 5 were different from those obtained in healthy volunteers ($P < 0.05$), whereas for PTH the serum levels were only different from healthy volunteers at CKD Stage 5 ($P < 0.05$). Only patients at CKD Stage 5 had serum phosphate levels different from those observed in healthy volunteers, similar to what was seen for PTH. TmP/GFR values were similar among CKD patients and volunteers (Table 3).

Discussion

In our present study, we illustrate the concurrent respective patterns of serum levels of the six key actors which govern mineral metabolism in renal insufficiency, i.e. Klotho, 1,25D, FGF23, PTH, phosphate and calcium: serum levels of soluble alpha-Klotho and 1,25D decrease in parallel with the progressive decline in glomerular filtration rate, whereas serum levels of FGF23 rise (Figure 3). All do so before a rise in serum levels of PTH occurs. Ultimately, e.g. at CKD Stage 4 and onwards, serum levels of phosphate start rising, whereas serum levels of ionized calcium remain unchanged across all CKD stages included in this cross-sectional study. The study also confirms the low levels of serum Klotho in haemodialysis patients with secondary hyperparathyroidism [34].

It is of importance to mention that the cross-sectional study design precludes definitive conclusions on the temporal sequence. Furthermore, the relatively low number of study subjects and the substantial variation of PTH and

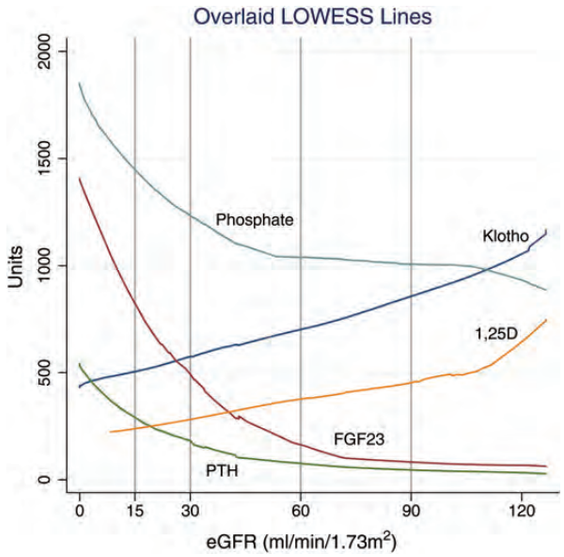

Fig. 3. Overlaid LOWESS lines for Klotho (pg/mL), 1,25D (ng/L × 10^{-3}), carboxy-terminal fibroblast growth factor 23 (FGF23, RU/mL), intact parathyroid hormone (PTH, ng/mL) and serum phosphate (mol/L × 10^{-3}) versus eGFR (mL/min/1.73 m^2) in CKD patients.

FGF23 levels preclude a precise estimation of the departure from the baseline. However, it is noteworthy that the sequence suggested by the present study on 87 patients confirms that recently published on 3879 patients by Isakova *et al*. [18] who pointed to the fact that the rise in serum FGF23 might precede the rise in serum PTH in the course of development of renal insufficiency. As to the rise in FGF23 itself in a state of Klotho deficiency, it has been envisioned as the consequence of phosphorous accumulation as recently demonstrated by preclinical studies which showed the absence of said rise in Klotho −/− mice fed a low phosphate diet [21]. However, several investigators have observed a modest reduction in serum phosphate during early CKD [31,34] and our own data also point in this direction.

The present study illustrates the fact that serum levels of both 1,25D and Klotho decline 'hand-in-hand' with progression of renal insufficiency. Given the current limitations of the respective techniques, it remains speculative to declare which of both levels declines first, i.e. what is the cause and what is the consequence: on the one hand, Tsujikama *et al.* suggested that Klotho might have an enzymatic ability to modify a receptor or ligand autonomously influencing the activity of 1-alpha hydroxylase; on the other hand, the same authors demonstrated that 1,25D itself modulates the expression of Klotho [31].

Earlier studies reported Klotho levels in CKD patients and volunteers: on the one hand, Sugiura *et al.* [35] reported serum Klotho levels of 1413 pg/mL among 30 elderly CKD patients (serum creatinine values of 1.63 ± 1.35 mg/dL) and 404 pg/mL among 10 healthy adults, aged 20–44 years, suggesting an elevation of Klotho in CKD patients. The Klotho levels of these healthy volunteers are substantially lower than values previously reported in the literature and thus may indicate selection bias or technical errors. Although the CKD stages of the patients were not reported, the negative lower boundary of the 95% CI of the serum creatinine values suggests that the CKD stages were not evenly distributed and thus the rather high mean Klotho level in CKD is possibly due to an enrichment of patients at early CKD stages. The fact that FGF23 levels were similar in healthy volunteers and CKD patients further supports the hypothesis that patients at CKD Stages 1 and 2 were selected preferentially in that study. On the other hand, Akimoto *et al.* [36] reported that the amount of urinary Klotho levels correlated positively with residual glomerular function in 36 peritoneal dialysis patients, whereas an association between serum Klotho levels and residual function was not found. Similarly, we did not find an association between residual function and serum Klotho levels among 13 non-anuric haemodialysis patients (Spearman $r = 0.2$, $P = 0.4$); however, this is a subgroup analysis that is most likely underpowered.

It has been postulated by some that an initial renal tubular damage leads to down-regulation of the expression of both Klotho and 1-alpha-hydroxylase, and that the ensuing cascade comprising the rise in FGF23 and in PTH is the consequence: indeed, insufficient production of Klotho, known to occur at the level of both the parathyroid gland and the nephron in condition of renal insufficiency [37, 38], leads to peripheral resistance to FGF23 at both anatomical sites, and FGF23 thus can no longer suppress PTH secretion nor maintain phosphate homeostasis, as already suggested by Kuro-o [17].

Alternatively, it has been suggested [18] that the initial FGF23 increase in early CKD actually causes the down-regulation of Klotho via reduced 1,25D. Therefore, further studies, in particular prospective longitudinal studies, remain to be carried out to clarify this point and to determine the temporal sequence of the observed hormonal changes in as much as the vitamin D administration itself might play a role.

In conclusion, our data elevate the newly assayable serum level of soluble alpha-Klotho to the rank of potentially important marker for early detection of kidney damage and it triggers new hopes for effective monitoring and future therapeutic interventions.

Supplementary data

Supplementary data are available online at http://ndt.oxfordjournals.org.

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