Fibroblast Growth factor 23 and α -Klotho protein are associated with adverse clinical outcomes in non dialysis chronic kidney disease stage 1-5 patients

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Abstract

Background: Fibroblast Growth Factor 23 (FGF-23) and α -Klotho contribute to the patho-genesis of chronic kidney disease - mineral and bone disorders (CKD-MBD). The aim of our study was to evaluate the association of FGF-23 and α -Klotho levels with CKD-MBD parameters, as well as with renal prognosis and mortality, in CKD patients stage 1-5, not in renal replacement therapy (RRT). **Methods:** 128 patients were included. At enrollment GFR was measured (mGFR) and plasma levels of carboxyl terminal FGF-23 (cFGF-23) and soluble α -Klotho (sKlotho) were determined by ELISA. Abdominal aorta calcification (AAC) score was assessed in lateral abdominal X ray. The composite end point (event) was initiation of RRT or death. Follow-up was five years (median 36, range 2-60 months). **Results:** mGFR significantly correlated with cFGF-23 and sKlotho negatively and positively respectively ($p < 0.0001$ for both). Multiple regression analysis showed an independent correlation of cFGF-23 with mGFR, 25-OH vitamin D, presence of diabetes mellitus and AAC score, of sKlotho with mGFR and phosphate and of AAC score with sKlotho and cFGF-23. Multivariate regression tree analysis, led to the formation of three regression groups (A, B, C) based on two "cut off" values: mGFR levels of 60.85 ml/min/1.73m² and serum phosphate levels of 3.7 mg/dl. These groups significantly correlated with the five CKD stages and additionally cFGF-23 and sKlotho plasma levels ($p < 0.0001$ for both). During follow-up, 40 out of the 128 patients, (31.2%) either initiated RRT or died (31 and 9 respectively). Kaplan Meier survival analysis showed that groups of patients with cFGF-23 levels less than median and those with sKlotho more than median value showing a more favorable course, regarding outcome ($p = 0.0003$ for cFGF-23, $p = 0.004$ for sKlotho). In Cox regression analysis the association of cFGF-23 ($p = 0.04$), sKlotho ($p = 0.008$) and AAC score ($p = 0.01$) with the presence of "event" remained significant after adjustment for traditional and CKD- related covariates. **Conclusions:** In CKD patients stage 1-5, cFGF-23 and sKlotho levels are associated with adverse clinical outcomes. mGFR and serum phosphate, in association with sKlotho levels, may provide a "new classification" of CKD patients, which appears to be predictive of outcome.

Background

Mineral and bone disorders (MBD) are a well known complication of chronic kidney disease (CKD) that appears in parallel with the deterioration of renal function and recent evidence suggests that alterations of Fibroblast Growth Factor 23 (FGF-23) and α -Klotho protein are the earliest detectable biochemical abnormalities of CKD-MBD [1-3].

FGF-23, produced by osteocytes and osteoblasts, consists the most well studied phosphatonin, promoting renal phosphate excretion, by down-regulation of luminal sodium-phosphate co-transporter in the proximal renal tubular cells. FGF-23 also suppresses calcitriol (1,25-dihydroxy-vitamin D) synthesis, by inhibiting the enzyme renal 1 α -hydroxylase, which catalyzes calcitriol production, and also by stimulating the enzyme 24-hydroxylase, involving in calcitriol degradation [4,5]. As a result, FGF-23 contributes to the development of secondary hyperparathyroidism (SHPT), while on the other hand, FGF-23 inhibits parathyroid hormone (PTH) production and secretion, forming a negative regulatory mechanism [4,6]. Beginning in early CKD, FGF-23 progressively increases, reaching in patients with end

stage renal disease (ESRD) values that can be up to 1000-fold higher than in subjects with normal renal function [5]. FGF-23 induces a negative phosphate balance by functioning either as a phosphaturic peptide or as a counter-regulatory hormone for calcitriol, which in turn results in reduced intestinal absorption of phosphate. Thus, FGF-23 contributes to the characteristic profile of patients with CKD stage 2-4: normal serum phosphate levels, increased fractional excretion of phosphate and calcitriol deficiency [5]. Finally, FGF-23 was found to be associated with the rate of CKD progression and the risk for the development of incident CKD and progression to ESRD [7-9]. Apart from its classic actions, FGF-23 has also been shown to induce hypertrophy of cardiomyocytes and to impair cardiac contractility. Accordingly, a number of studies in CKD patients, as well as in other patient populations have demonstrated association of its levels with left ventricular hypertrophy, atrial fibrillation and all-cause and cardiovascular mortality [7,9,10-15]. However, it appears that FGF-23 has no effect on vasculature and several studies failed to prove a correlation between FGF-23 levels and markers of atherosclerosis such as vascular calcifications and arterial stiffness [16,17].

α -Klotho consists a single pass transmembrane protein, mainly produced in the kidney, which functions as an obligatory co-receptor for FGF-23, in its classic target organs, kidneys and parathyroid glands [18,19]. There are two basic forms of α -Klotho protein. The first is the "transmembrane" one, that is expressed primarily in the plasma membrane of distal renal tubular cells, although there is strong evidence that is also expressed in proximal tubule [19]. The second is generated from extracellular domain cleavage by two membrane anchored proteases (secretases), which belong to a disintegrin and metalloprotease family (ADAM 10 and 17). The above process leads to the release of this part into the extracellular space, which is thereafter detectable as a single peptide in blood, urine and cerebrospinal fluid [19]. This form of protein is usually referred as "soluble" α -Klotho, which has other functions than the transmembrane one, through glucosidase or sialidase activity including suppression of growth factor signaling, protection from oxidative stress and regulation of ion channels and transporters [18,20]. It should be mentioned that α -Klotho has a key role in phosphate metabolism, not only as an obligatory co-receptor of FGF-23, but also by acting as a phosphaturic factor independently of FGF-23 [21]. During the last decade, α -Klotho protein is gaining more and more interest and attention of nephrology community, as an early biomarker in the course of kidney damage, either in acute kidney injury or in CKD [20]. α -Klotho plasma levels in CKD, in contrast to FGF-23, show a progressive decrease in parallel with CKD progression to later stages [19]. However, it is not fully determined at which level of glomerular filtration rate (GFR) this decrease becomes obvious and also which other parameters of CKD-MBD coexist with α -Klotho plasma levels fall. Today, it is well accepted that these two important biomarkers, FGF-23 and α -Klotho, along with renal function, consist a significant bone-kidney axis, with a notable role in CKD, which has a direct relationship with deranged phosphate metabolism [22]. The number of studies investigating the probable association of α -Klotho levels with CKD progression and mortality is limited and the results are sometimes contradictory. Thus, a study showed that low α -Klotho levels are related with deterioration of renal function and increased mortality [23] whereas other studies failed to confirm these findings [24]. In addition, experimental studies have shown that α -Klotho exerts cardioprotective effects in uremia and attenuates vascular calcification [25,26]. However, in CKD patients the effects of α -Klotho on vasculature

are not clear [27,28,29]. The aim of our study was the longitudinal assessment of FGF-23 and α -Klotho levels in CKD patients stage 1-5 and to investigate their associations with parameters of CKD-MBD, as well with the presence and the extend of vascular calcifications. Moreover, the association of FGF-23 and α -Klotho with clinical outcome and mortality was investigated.

Methods

Patients

Between February 2011 and November 2013, 128 adult patients (85 male, median age 67 years, range 18-86) with CKD stages 1-5, not on RRT, who were attended as outpatients of the Department of Nephrology at Papageorgiou General Hospital, consecutively entered the study. Inclusion criteria were: age >18 years, absence of active inflammatory, hepatic or malignant disease and no use of corticosteroids or other immunosuppressive medication at the time of the study entry. Patients' primary diseases were: diabetic kidney disease in 53 cases (41.4%), hypertensive kidney disease in 40 (31.3%), chronic glomerulonephritis in 8 (6.2%), tubulointerstitial nephropathy in 8 (6.2%), polycystic kidney disease in 6 (4.8%), and other or undetermined disease in 13 (10.1%) cases. All patients were classified into 5 subgroups according to criteria of the National Kidney Foundation-Kidney Disease Outcomes Quality Initiative 2002 for CKD staging (NKF-K/DOQI) [30]. At the time of the study, patients were receiving the following medications: One hundred nineteen (93%) one or more antihypertensive agents (diuretics, calcium channel blockers, angiotensin converting enzyme inhibitors, angiotensin II receptor antagonists, beta blockers, alpha-adrenergic blocking agents and centrally acting anti-hypertensive agents) either for the treatment of pre-existing hypertension (HTN) or as an anti-proteinuric or heart failure therapy, 71 (55.4%) HMG-CoA reductase inhibitors, 57 (44.5%) an antiplatelet agent, 28 (21.9%) short or/and long acting insulin and 26 (20.3%) one or more hypoglycemic agent. None of the patients was receiving recombinant human erythropoietin therapy, phosphate binders or other treatment for SHPT. HTN was defined as a systolic blood pressure (SBP) of 140 mm Hg or more or/and a diastolic blood pressure (DBP) of 90 mm Hg or more, or the use of antihypertensive drugs. Fifty nine patients (46%) had a previous history of diabetes mellitus (DM), independently of their primary disease, and also 59 (46%) had a previous history or clinical signs of cardiovascular disease (CVD). The last was defined as the history of coronary artery disease or angina pectoris, ischemic/hemorrhagic stroke or transient cerebrovascular attack and peripheral artery disease. Thirty three patients suffered from coronary artery disease, with myocardial infarction in 17 and coronary artery bypass or percutaneous transluminal coronary angioplasty in 20 of them, 15 patients suffered from ischemic/hemorrhagic stroke or transient cerebrovascular ischemia and 30 patients from peripheral arterial disease.

Thirty seven healthy subjects without CKD, recruited among apparently healthy blood donors of the Blood Transfusion Center at Hippokration General Hospital, served as control group to obtain reference values for carboxyl terminal FGF-23 (cFGF-23) and soluble α -Klotho (sKlotho). Our study was approved by institutional ethic committees and all participants gave a written informed consent. All procedures were conducted in accordance with the Declaration of Helsinki (2008 Amendment).

Somatometric and hemodynamic characteristics

Body mass index (BMI), body surface area (BSA) and waist to hip ratio were calculated in all patients. BMI was estimated from patients' body weight in kilograms (kg) and height in meters (m), according to the formula: $\text{BMI (kg/m}^2\text{)} = \text{body weight/height}^2$. BSA was estimated from patients' height in centimeters (cm) and body weight in kg, using the Haycock formula: $\text{BSA (m}^2\text{)} = 0.024265 \times \text{height}^{0.3964} \times \text{body weight}^{0.5378}$. Waist to hip ratio was calculated from the quotient of waist to hip perimeter. SBP and DBP, were recorded at the first visit of the patients, while mean blood pressure (MBP) was calculated according to the formula: $[(\text{DBP} \times 2) + \text{SBP}] : 3$.

Laboratory methods

Blood and urine samples were taken at baseline in the morning, under fasting conditions. Blood samples for the measurement of cFGF-23, sKlotho, 25-hydroxy- (25-OH) and 1,25-dihydroxy- (1,25-OH₂) vitamin D were separated by immediate centrifugation (1500 g for 10 min), aliquoted and stored at -70 °C until assay. Hematological analysis was performed in an automated hematological analyzer (ADVIA 2120i, Siemens) and biochemical tests in an automated biochemical analyzer (Architect-c 16000, Abbott Diagnostics). Albuminuria was determined from albumin to creatinine ratio (ACR) in an untimed "spot" urine specimen and was expressed in mg/gr. Fractional excretion of phosphate (FEP) was calculated according to the formula: $\text{FEP} = [\text{spot urine phosphate (mg/dl)} \times \text{serum creatinine (mg/dl)}] / [\text{serum phosphate (mg/dl)} \times \text{spot urine creatinine (mg/dl)}]$. The ratio of tubular maximum reabsorption rate of phosphate to GFR (TmP/GFR) was also calculated in mmol/L, using serum phosphate and FEP, according to the nomogram of Walton and Bijvoet [31]. Serum intact molecule of PTH (iPTH) was assayed by the method of enhanced chemiluminescence (Immulite 2000, Siemens). Serum C reactive protein (CRP) level was measured by nephelometry (Beckmann Coulter). Serum beta-2 microglobulin was determined by a microparticle immunoenzymatic assay (Axysm, Abbott Diagnostics). Serum levels of 25-OH and 1,25-OH₂ vitamin D were determined with a radioimmunoassay method (RIA) in a γ camera counter. Plasma levels of cFGF-23 and sKlotho were measured by an enzyme-linked immunosorbent assay (ELISA), using commercially available standard kits (human cFGF-23, Immutopics Inc, San Clemente CA and human sKlotho, Immuno-Biological Laboratories Co Ltd, Fujioka-Shi, Japan). Plasma from patients were diluted 1:10 and 1:2, respectively, for the quantitation of cFGF-23 and sKlotho. Plasma from controls was tested undiluted for the quantitation of cFGF-23. The concentrations of these proteins were calculated by reference to standard curves, performed with the corresponding recombinant molecule. All plasma samples were tested in duplicate. The sensitivity of the ELISA system was 1.5 RU/ml and 6.15 pg/ml for cFGF-23 and sKlotho respectively. Routine laboratory measurements were determined in Central Laboratory, while 25-OH and 1,25-OH₂ vitamin D serum levels measurements in 3rd Department of Nuclear Medicine of the Aristotle University of Thessaloniki at Papageorgiou General Hospital. cFGF-23 and sKlotho plasma levels measurements were determined in the Research Laboratory of the Department of Nephrology, Aristotle University of Thessaloniki at Hippokration General Hospital.

Renal function

The level of renal function was determined by measuring GFR (measured GFR, mGFR) at baseline visit, using a radioisotopic method. This procedure was made by means of plasma clearance of a labeled, exogenous filterable tracer, namely chromium-51-ethylene diamino tetraacetic acid ($^{51}\text{Cr-EDTA}$) using the single injection-two sample technique according to a standardized protocol. [32] All measurements were performed in the morning between 09.00-13.00. Patients' preparation included abstinence from xanthin containing beverages and heavy protein intake before the test. Avoidance of strenuous exercise and moderate hydration were instructed during the procedure. Regularly taken medications were recorded but not interrupted. A dose of 80 μCi $^{51}\text{Cr-EDTA}$ was given intravenously with extreme concern for extravasation and venous blood samples were obtained from the contralateral arm at 2 and 4 hours post-injection. The activity of 1 ml of plasma from each sample and of 1 ml of a standard solution was adequately counted in a well type γ -counter. GFR, in ml/min, was calculated according to the one-compartment "slope-intercept" method with Brochner-Mortensen correction for the neglected early, fast, non-renal, component of tracer plasma disappearance. mGFR values were normalized for 1.73 m^2 of BSA and expressed as ml/min/ 1.73 m^2 .

Aortic calcifications

All the patients were examined for the presence of abdominal aorta wall calcifications, at the level of the four lumbar vertebrae (L1-L4), as these were depicted in a lateral X-ray of the abdomen, performed with radiation data 81kV and 50-65 mAs in an X-ray machine of Multix type (Siemens). Each patient was graded with a score, depending on the severity of the calcified lesions in front of each lumbar vertebra, which was characterized as abdominal aorta calcification (AAC) score [33]. According to this score, the quantification of each calcified lesion was assessed separately for the posterior and anterior wall of the aorta, using as boundaries a theoretical line in the intervertebral space above and below each vertebra. Subsequently, the posterior and anterior score were summed together for each vertebra. AAC score was graded as follows: 0 point: no calcified deposits, 1 point: small scattered calcified deposits filling less than 1/3 of the longitudinal wall of aorta, 2 points: calcified deposits extended in 1/3 or more, but less than 2/3 of the longitudinal wall of aorta, and finally 3 points: calcified deposits extended in more than 2/3 of the longitudinal wall of aorta. Score ranged from 0 for no calcified lesions to 24 for all the vertebrae severely affected.

Follow up study

The composite end point of the study was either initiation of RRT or death, whichever came first. These two parameters were both characterized as "event". After initial assessment, patients were followed up for five years (median time 36, range 2-60 months). During follow-up, patients were censored in case of RRT initiation. All deaths were recorded accurately. Each death was reviewed, all available medical information was collected including hospitalization records and an underlying cause was assigned. In the case of an out-of-hospital death, family members were interviewed by telephone to better ascertain the circumstances surrounding death. Sudden death was defined as unexpected natural death within one hour from symptom onset and without a prior condition that would appear fatal. At the end of follow,

apart from mGFR in the beginning of the study, an estimated value of GFR (eGFR) was also recorded, characterized as final GFR (fGFR), using the estimated formula of Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI). The CKD-EPI equation, expressed as a single equation, was: $eGFR = 141 \times \min(Scr/\kappa, 1)^\alpha \times \max(Scr/\kappa, 1)^{-1.209} \times 0.993^{age} \times 1.018$ [if female] or $\times 1.159$ [if black], where Scr is serum creatinine, κ is 0.7 for females and 0.9 for males, α is -0.329 for females and -0.411 for males, min indicates the minimum of Scr/ κ or 1, and max indicates the maximum of Scr/ κ or 1 [34]. For those who finally presented with “event”, their fGFR came of from their last creatinine measurement just before the occurrence of event. The value of fGFR was recorded for each patient at the end of his/her follow up period.

Statistical analysis

Statistical analysis was performed with the open source software “R”, version 3.4.0 (R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0). Data were reported as mean value $\pm$ SD (normally distributed data), median with range (non-normally distributed data) or percentage frequency, as appropriate. The normality was examined by Shapiro-Wilk test. Non-normally distributed variables were log-transformed before entering analyses. A Pearson's correlation and a Spearman's rank correlation test were used to assess the correlation between normally and not normally distributed variables respectively. The significance of differences in means between two binary categorical groups was assessed by Student's t-test or Wilcoxon-Mann-Whitney test, as appropriate. Among three or more groups, mean differences were assessed by analysis of variance (ANOVA) test, or Kruskal-Wallis test as appropriate. Multiple regression analysis with a forward elimination procedure was used to assess the influence of some of the examined variables on cFGF-23, sKlotho and AAC score. Classic variables (sex, age, BMI, presence of HTN, DM and history of CVD), as well those showing significant univariate correlation with cFGF-23, sKlotho or AAC score ($p < 0.05$) such as waist to hip ratio, mGFR, hemoglobin, transferrin saturation% (TSAT%), phosphate, albumin, total alkaline phosphatase (ALP), high density lipoprotein (HDL), ACR, CRP, FEP, TmP/GFR, calcium excretion in 24 hour urine collection, iPTH, 25-OH and 1,25-OH₂ vitamin, were used. When cFGF-23, sKlotho or AAC score were not used as the independent variable, then they were used in the multiple regression analysis as a dependent one along with the rest. Also, multiple regression was used to assess the influence of variables on fGFR using again the classic ones and those with significant univariate correlation with fGFR ($p < 0.05$). These were: sex, age, BMI, presence of HTN, DM, history of CVD, waist to hip ratio, hemoglobin, calcium, phosphate, total ALP, HDL, ACR, CRP, FEP, TmP/GFR, iPTH, 25-OH and 1,25-OH₂ vitamin, cFGF-23, sKlotho and AAC score.

Multivariate regression tree consists a simple, yet powerful analytic tool that helps to determine the most “important” variables in a particular dataset [35]. In our study, this type of analysis, using the most important variables (mGFR, hemoglobin, phosphate, ACR, FEP, calcium excretion in 24 hour urine collection, iPTH, 1,25-OH₂ vitamin and cFGF-23) that were significantly correlated with s-Klotho ($p < 0.05$), revealed the formation of three distinct groups, clearly distinguished from each other, which were based on mGFR and serum phosphate levels. In details, the three groups, named regression group A, B and C, were obtained in two steps: the 1st division was made between $mGFR \geq 60.85$ (regression group A) and

mGFR<60.85 ml/min/1.73 m². Subsequently, the group with the lowest mGFR was further divided into two other subgroups, according this time to the value of serum phosphate, which was chosen by the procedure of tree analysis to be the value of 3.7 mg/dl. The patients with mGFR<60.85 ml/min/1.73 m² and serum phosphate <3.7 mg/dl consisted regression group B, while those with serum phosphate ≥3.7 mg/dl the regression group C. Contingency tables were used in order to summarize the relationships between [categorical variables](#) that were compared to each other. Pearson's chi-square test was used to examine the significance of the association in contingency tables. Event free survival was analyzed by Kaplan Meier curves according to cFGF-23 and sKlotho plasma levels more or less than the median value, to five CKD stages, to three regression groups and to tertiles of AAC scores (1st: 0-4, 2nd: 5-15, 3rd: 16-24). Probability of survival was also analyzed by the Cox proportional hazard model to estimate the relationship of cFGF-23, sKlotho plasma levels and AAC score with outcome. An univariate analysis in a non-adjusted model was also conducted for all the examined variables, in relation to outcome, while for cFGF-23, sKlotho and AAC score an adjusted model, using the following variables as co-variables, was also used. These co-variables were: sex, age, BMI, waist to hip ratio, presence of DM or history of CVD, hemoglobin, albumin, CRP, ACR, and TmP/GFR. p values <0.05 were considered statistically significant in all comparisons.

Results

Initial epidemiological, somatometric and hemodynamic parameters, as well, comorbidity factors and mGFR, at baseline are shown in **Table 1**. Apart from age, HTN and mGFR, the rest of variables did not show significant difference among the five CKD stages. Initial hematological and biochemical parameters in serum and urine are shown in **Table 2**. Hemoglobin, creatinine, phosphate, total ALP, HDL, ACR and beta-2 microglobulin differed significantly among the five CKD stages. Initial CKD-MBD parameters are shown in **Table 3**. FEP, TmP/GFR, calcium and phosphate excretion in 24 hour urine collection, 1,25-OH₂ vitamin D, cFGF-23, sKlotho and AAC score differed significantly among the five CKD stages. Mean values of cFGF-23 and sKlotho in the control group were 47.4 ± 53.3 RU/ml and 855.6 ± 230.1 pg/ml respectively, which differed significantly with those in the CKD group (p<0.0001 for both). Differences were significant from stage 3 to 5 (data are not shown).

Correlations of cFGF-23 and sKlotho with studied parameters

Unadjusted univariate correlation analysis revealed that cFGF-23 significantly correlated, either positively or negatively, with the following variables: age (r=0.30, p=0.0005), waist to hip ratio (r=0.24, p=0.005), mGFR (r=-0.59, p<0.0001), hemoglobin (r=-0.38, p<0.0001), TSAT% (r=-0.20, p=0.02), creatinine (r=0.58, p<0.0001), phosphate (r=0.23, p=0.006), albumin (r=-0.19, p=0.02), total ALP (r=0.23, p=0.008), HDL (r=-0.28, p=0.001), ACR (r=0.26, p=0.002), CRP (r=0.26, p=0.002), beta-2 microglobulin (r=0.58, p<0.0001), FEP (r=0.49, p<0.0001), TmP/GFR (r=-0.21, p=0.01), calcium excretion in 24 hour urine collection (r=-0.27, p=0.001), iPTH (r=0.42, p<0.0001), 25-OH vitamin D (r=-0.29, p=0.0008), 1,25-OH₂ vitamin D (r=-0.36, p<0.0001) and AAC score (r=0.35, p<0.0001). Also, cFGF-23 correlated negatively with sKlotho (r=-0.24, p=0.004). Values of mGFR lower than median (41.5 ml/min/1.73m²) and history of CVD correlated with

higher cFGF-23 plasma levels ($p<0.0001$ and $p=0.0001$ respectively). cFGF-23 plasma levels differed significantly among CKD stages and also among the tertiles of AAC score ($p<0.0001$ and $p=0.002$ respectively). Multiple regression analysis showed that cFGF-23 was independently associated with mGFR ($p=0.001$), 25-OH vitamin D ($p=0.002$), sex ($p=0.01$), presence of DM ($p=0.02$), AAC score ($p=0.04$) and finally with hemoglobin ($p=0.05$) (cFGF-23 multiple $r=0.64$, $p<0.0001$). Because of the intercorrelation between mGFR and phosphate ($r=-0.35$, $p<0.0001$), mGFR and CRP ($r=-0.28$, $p=0.001$) and mGFR and TmP/GFR ($r=0.21$, $p=0.01$), when the parameter of mGFR was excluded from the above model, phosphate, CRP and TmP/GFR turned to be independently associated with cFGF-23 ($p=0.004$, $p=0.008$ and $p<0.0001$ respectively). Furthermore, in the context of strong correlation between mGFR and FEP ($r=-0.66$, $p<0.0001$), as well between FEP and TmP/GFR ($r=-0.62$, $p<0.0001$), when both mGFR and TmP/GFR were removed from the model, FEP became independently associated with cFGF-23 ($p<0.0001$).

Unadjusted univariate correlation analysis revealed that sKlotho significantly correlated, either positively or negatively, with the following variables: mGFR ($r=0.52$, $p<0.0001$), hemoglobin ($r=0.24$, $p=0.005$), creatinine ($r=-0.53$, $p<0.0001$), phosphate ($r=-0.42$, $p<0.0001$), ACR ($r=-0.30$, $p=0.0004$), beta-2 microglobulin ($r=-0.42$, $p<0.0001$), FEP ($r=-0.32$, $p=0.0002$), calcium excretion in 24 hour urine collection ($r=0.26$, $p=0.002$), iPTH ($r=-0.21$, $p=0.01$) and 1,25-OH₂ vitamin D ($r=0.38$, $p<0.0001$). Values of serum phosphate levels lower than median (3.53 mg/dl) and mGFR higher than median correlated with higher sKlotho plasma levels ($p=0.001$ and $p<0.0001$ respectively). Presence of DM correlated with lower sKlotho plasma levels ($p=0.02$). Multiple regression analysis showed that sKlotho was independently associated with mGFR ($p<0.0001$) and phosphate ($p=0.001$), (sKlotho multiple $r=0.53$, $p<0.0001$). Similarly with cFGF-23 analysis, because of the strong intercorrelation between mGFR and FEP, as well mGFR and AAC score ($r=-0.32$, $p=0.0001$), when mGFR was excluded from this model, FEP and AAC score became independently associated with sKlotho ($p=0.002$ and $p=0.03$ respectively). In addition, age also turned to be independently associated ($p=0.03$), while phosphate became much more significantly associated with sKlotho ($p<0.0001$).

Correlations of AAC score with studied parameters

Unadjusted univariate analysis revealed that AAC score significantly correlated, either positively or negatively with the following variables: age ($r=0.54$, $p<0.0001$), waist to hip ratio ($r=0.31$, $p=0.0002$), BMI ($r=0.17$, $p=0.04$), mGFR ($r=-0.32$, $p=0.0001$), hemoglobin ($r=-0.25$, $p=0.004$), TSAT% ($r=-0.27$, $p=0.001$), creatinine ($r=0.27$, $p=0.002$), phosphate ($r=0.26$, $p=0.002$), HDL ($r=-0.18$, $p=0.03$), beta-2 microglobulin ($r=0.36$, $p<0.0001$), FEP ($r=0.34$, $p<0.0001$), calcium excretion in 24 hour urine collection ($r=-0.23$, $p=0.007$), iPTH ($r=0.28$, $p=0.001$), 25-OH vitamin D ($r=-0.21$, $p=0.01$) and cFGF-23 ($r=0.35$, $p<0.0001$). Presence of DM and history of CVD correlated with higher AAC score ($p<0.0001$ for both). Multiple regression analysis showed that AAC score was independently associated with age ($p<0.0001$), phosphate ($p=0.007$), history of CVD and presence of DM ($p=0.004$ and $p=0.007$ respectively), sKlotho ($p=0.03$) and cFGF-23 ($p=0.05$) (AAC score multiple $r=0.65$, $p<0.0001$).

Classification of patients to 3 groups according to mGFR values and serum phosphate levels

A multivariate regression tree analysis, using sKlotho as response variable, led to the formation of three distinct groups (regression group A, B, C). mGFR levels of 60.85 ml/min/1.73m² and serum phosphate levels of 3.7 mg/dl were the two “cut off” values on which the classification of the three groups was based. It was remarkable that these regression groups significantly correlated with the five CKD stages, according to the test of a 3x5 contingency table (Pearson chi-square test $p<0.0001$). As it is shown in **Figure 1**, regression group A corresponded exclusively to CKD stage 1 and 2, while the regression groups B and C shared the other three stages, with regression group B frequency to decrease, while regression group C frequency to increase progressively from CKD stage 3 to 5. cFGF-23 and sKlotho plasma levels differed significantly among the three regression groups ($p<0.0001$ for both) in a positive and in a negative direction respectively (**Figure 2**).

Correlation of the five CKD stages with the three regression groups A, B and C in percentage (left panel) and in number of patients (right panel) with a test of 3x5 contingency table (Pearson's chi-square test $p<0.0001$)

cFGF-23 (left panel) and sKlotho (right panel) plasma levels differed significantly among the three regression groups A, B, C in a positive and in a negative direction respectively (Kruskal-Wallis test, $p<0.0001$ for both)

Regarding the two parameters of renal phosphate excretion, significant difference among the three groups was also shown for FEP and for TmP/GFR values ($p<0.0001$ for both). Higher FEP values characterized regression group B and C, compared with group A, while TmP/GFR differed in a “biphasic way”, with higher values in regression group A, lower in group B and again higher values in group C (**Figure 3**). AAC score also differed significantly among the regression groups in a positive direction ($p=0.0003$).

The above tree analysis did not reveal similar results, using cFGF-23 as response variable.

Fractional excretion of phosphate (FEP) (left panel) and ratio of tubular reabsorption of phosphate to glomerular filtration rate (TmP/GFR) (right panel) values differed significantly among the three regression groups in a positive direction and in a biphasic way respectively ($p<0.0001$ for both)

Associations of cFGF-23 and sKlotho plasma levels with clinical outcomes

Univariate linear regression analysis showed that cFGF-23 and sKlotho were significantly associated with fGFR, negatively and positively respectively, at the end of the follow up period ($p<0.0001$ for both) (**Figure 4**). Moreover, multiple regression analysis, using fGFR as the independent variable, showed that fGFR was independently associated with sKlotho ($p<0.0001$), cFGF-23 ($p=0.02$), presence of HTN ($p=0.002$), iPTH ($p=0.002$), FEP ($p=0.003$), 1,25-OH₂ vitamin D ($p=0.01$), HDL ($p=0.04$) and phosphate ($p=0.05$) (fGFR multiple $r=0.75$, $p<0.0001$).

Univariate linear regression analysis showed that cFGF-23 (left panel) and sKlotho (right panel) were significantly correlated with fGFR value, at the end of follow up period ($p<0.0001$ for both)

During the follow up period (median 36, range 2-60 months), 40 out of the 128 patients, (31.2%) either initiated RRT or died (31 and 9 respectively). These 9 deaths were due to cardiovascular causes. Patients who reached the composite end point or "event" compared with those who did not, showed significantly higher serum creatinine ($p<0.0001$), phosphate ($p=0.01$), total ALP ($p=0.03$), CRP ($p=0.05$), beta-2 microglobulin ($p<0.0001$) and iPTH ($p<0.0001$) levels, waist to hip ratio ($p=0.01$), ACR ($p=0.04$), FEP ($p<0.0001$) and AAC score ($p=0.01$) values and finally cFGF-23 plasma levels ($p<0.0001$). On the contrary, the same patients showed significantly lower mGFR ($p<0.0001$), TmP/GFR ($p=0.002$) and calcium excretion in 24 hour urine collection ($p=0.05$) values, serum hemoglobin ($p=0.001$), calcium ($p=0.008$), 25-OH ($p=0.002$) and 1,25-OH₂ vitamin D levels ($p=0.001$) and finally sKlotho plasma levels ($p=0.0001$).

All the patients were also classified into four groups, according to the median value of cFGF-23 and sKlotho plasma levels (218.8 RU/ml and 479.8 pg/ml respectively). Finally, four different combinations were formed, using less or more than median value of each variable. These groups were named: 1. less FGF-23/more Klotho, 2. less FGF-23/less Klotho, 3. more FGF-23/more Klotho, 4. more FGF-23/less Klotho. A 2x4 contingency table showed that there was a significant difference, regarding the presence of "event", between the group of less FGF-23/more Klotho and the group of more FGF-23/less Klotho. In the first one, out of 38 patients, only 4 (10.5%) reached the composite end point, while in the last, out of 39 patients, 23 (58.9%) reached it. In the other two intermediate groups, less FGF-23/less Klotho and more FGF-23/more Klotho, the number of patients who reached the composite end point was similar (23% and 28% respectively) (Total chi-square test, $p<0.0001$) (**Table 4**). In addition, according to the median value of cFGF-23 plasma levels and the three regression groups (A,B,C), six more combinations were made: less or more than median value of cFGF-23 with regression group A, B and C respectively. A 2x6 contingency table showed that there was a significant difference, regarding the presence of "event", between the group of less FGF-23/regression group A and the group of more FGF-23/regression group C. The first had the best outcome, as only 1 (4.3%) out of the 23 patients reached the composite end point, while the worst outcome was associated with the second one, where out of 32 patients, 17 (53.1%) reached the composite end point (Total chi-square test, $p<0.0001$) (**Table 4**).

Kaplan Meier survival analysis showed significant difference, regarding patients' outcome, according to "event". Patients with cFGF-23 plasma levels more than median value had worse outcome, compared with those with less than median, while those with sKlotho plasma levels more than median value had better outcome compared with those with less than median ($p=0.0003$ and $p=0.004$ respectively) (**Figure 5**). Kaplan Meier survival curves also differed significantly, as to the presence of "event", among the five CKD stages, the three regression groups and the tertiles of AAC score ($p<0.0001$, 0.006 and 0.01 respectively) (**Figure 6**).

Kaplan Meier survival analysis showed significant difference, as to patients' outcome, according to "event", for values more and less than median of cFGF-23 (left panel) and sKlotho (right panel) plasma levels ($p=0.0003$ and $p=0.004$ respectively). Patients with cFGF-23 higher and sKlotho plasma levels lower than median value, showed worse outcome compared with those with the opposite combination.

Kaplan Meier survival analysis showed significant difference, as to patients' outcome, according to "event", for the five CKD stages, the three regression groups A, B and C and the three tertiles of AAC score ($p<0.0001$, $p=0.006$ and $p=0.01$ respectively)

Univariate Cox regression analysis confirmed the difference in outcome, according to the presence of "event" for cFGF-23, sKlotho and AAC score ($p=0.0001$, $p=0.001$ and $p=0.003$ respectively). Presence of DM, history of CVD, mGFR, hemoglobin, calcium, phosphate, total ALP, TmP/GFR, iPTH, 25-OH and 1,25-OH₂ vitamin D, also differed, as to the outcome of patients, in the univariate model (**Table 5**). In a multivariate Cox regression analysis, after adjusting for covariates including sex, age, BMI, waist to hip ratio, presence of DM, history of CVD, hemoglobin, albumin, CRP, ACR, and TmP/GFR, the association between cFGF-23, sKlotho and AAC score with "event" remained significant ($p=0.04$, $p=0.008$ and $p=0.01$ respectively) (**Table 6**).

Discussion

It is well known that FGF-23 and sKlotho protein, have a major role in regulation of phosphate homeostasis [36] and alterations of their concentrations are the earliest observed abnormalities in CKD-MBD [3,5]. In accordance with the above, the present study demonstrated that cFGF-23 levels increase and sKlotho protein fall significantly from CKD stage 3 and thereafter.

cFGF-23 and sKlotho associations with cardiovascular risk factors

Several important associations were noted between cFGF-23 or sKlotho and both traditional and CKD-related cardiovascular risk factors, which deserve some comments. Firstly, an independent correlation of cFGF-23 with the presence of DM was observed, which is consistent with the results of a large previous study, based on data from the Chronic Renal Insufficiency Cohort (CRIC) study, that demonstrated significantly higher levels in the diabetic group of patients in each stratum of eGFR [37]. In addition, cFGF-23 showed a positive correlation with waist to hip ratio, but not with BMI, which consists a novel finding, since it has been demonstrated before, in patients with normal renal function and in other community-based groups, but not in those with different CKD stages [38,39]. Of note, the above finding appears in contrast with recent studies in hemodialysis patients that showed an inverse relationship between FGF-23 and adiposity. Given the well documented association of FGF-23 with mortality, a probable explanation is that this discrepancy reflects the reverse epidemiology of traditional cardiovascular risk factors, including obesity, previously reported in hemodialysis patients which however is not observed in non dialysis patients [40,41]. A negative correlation was found between cFGF-23 and TSAT%, which underlines an interaction between iron and FGF-23 production [42]. This finding, which has not been previously reported

in CKD stage 1-5 patients, is in accordance with studies in hemodialysis and transplanted patients, in whom the presence of iron deficiency promoted a rise in FGF-23 levels and on the contrary, iron intravenous administration reduced its levels [43,44]. Of note, a recent paper, again in non dialysis CKD patients, found that high serum FGF-23 levels were associated with an increased risk for anemia [45]. Finally, serum levels of 25-OH vitamin D were significantly higher in the group of patients with lower than median cFGF-23 value and also lower than median value of serum phosphate. Moreover, an independent association was noted between cFGF-23 and 25-OH vitamin D, which has not been previously reported in CKD population. The combined effect of these two parameters on renal outcome has been studied previously [46]. Although, FGF-23 is negatively associated predominately with 1,25-OH₂ vitamin D, it is not clear if the same inverse relationship exists between FGF-23 and the precursor form of vitamin D. It is well known that FGF-23 suppresses calcitriol synthesis through decrease of 1 α -hydroxylase and increase of 24-hydroxylase production and accordingly several studies demonstrated a negative correlation between FGF-23 and 1,25-OH₂ vitamin D. The association between cFGF-23 and 25-OH vitamin D can be explained by the common catabolism of both 25-OH and 1,25-OH₂ vitamin D, by the enzyme 24-hydroxylase [47]. On the other hand, the absence a significant independent correlation between 1,25-OH₂ vitamin D and cFGF-23 in the present study, may be due to the presence of high intercorrelation between the two forms of vitamin D and also may indicate a major role of 25-OH vitamin D in CKD-MBD.

In univariate analysis, sKlotho levels correlated with mGFR, hemoglobin, creatinine, phosphate, ACR, beta-2 microglobulin, FEP, calcium excretion in 24 hour urine collection, iPTH and 1,25-OH₂ vitamin D, as in other previous studies. However, in contrast to sKlotho presumed phosphaturic action and previous reports [21], the present study demonstrated a negative correlation of sKlotho with FEP. This could be attributed to the fact that phosphaturia characterizes more advanced CKD stages which are also characterized by lower sKlotho plasma levels. The significant difference of sKlotho plasma levels among CKD stages, as well the independent correlation of sKlotho with mGFR and phosphate in multiple regression analysis, confirmed the already recognized relationship of sKlotho with renal function and phosphate [48]. In contrast with other studies, patients with DM had lower sKlotho plasma levels [49]. This was probably related to the presence of diabetic kidney disease in the majority of these patients. Moreover, it remains unclear, up to now, whether sKlotho plasma levels have an upward or a downward course in diabetic patients, as previous reports have shown inconsistent results [50].

Associations of cFGF-23 and sKlotho with AAC score

Several studies, mainly in dialysis patients, have investigated the association of FGF-23 with the presence and severity of arterial calcifications with conflicting results [16,17,51]. This is, at least partially, explained by differences in patient populations, in vascular beds examined and in methods used to access calcifications and cardiovascular risk factors. The present study results showed a significant difference of cFGF-23 plasma levels among the tertiles of AAC score and an independent correlation with AAC score, which concurs with some, although not all, previous reports [52]. It should be noted that FGF-23 does not

appear to have an association with medial calcification, or their surrogate marker such as arterial stiffness [17]. However, FGF-23 is associated with inflammation through several mechanisms and AAC score detects both intimal and medial calcifications, indiscriminately. Accordingly, the observed association between cFGF-23 and AAC score in our study may reflect FGF-23 effect mainly on the process of intimal calcification. In addition, multiple regression analysis, using this time AAC score as the independent variable, showed that cFGF-23 and sKlotho were both associated with AAC score. Recent experimental studies have shown that sKlotho attenuates calcification process, through up-regulation of its gene in the presence of intermedin 1-53 and also through its endogenous expression in human arteries, where in turn sKlotho functions as an inhibitor of calcification process in the context of vitamin D receptors (VDRs) activation [26,29]. The above correlations may provide evidence that these two biomarkers may contribute through different pathways to the excessive calcification burden of CKD patients.

s-Klotho plasma levels association with regression groups, according to mGFR values and serum phosphate levels

Multivariate regression tree analysis, using the variable of sKlotho as response variable, revealed the formation of three distinct groups (regression group A, B, C) according to the values of patients' mGFR and serum phosphate levels (cut off values 60.85 ml/min/1.73m² and 3.7 mg/dl respectively). It is worth emphasizing that these three groups significantly correlated with the five CKD stages. A significant difference of cFGF-23 and sKlotho plasma levels was observed among the three regression groups, although the difference for sKlotho was much more evident than that for cFGF-23. The above relationship among mGFR, serum phosphate and sKlotho plasma levels suggests that sKlotho and phosphate, collaborate tightly in CKD progression. As it was shown, the transition from regression group A to groups B and C was based only on the level of mGFR, while the difference between group B and group C was further based on serum phosphate level. This observation may demonstrate that even "normal" serum phosphate levels, such as the value of 3.7 mg/dl, could be combined on the one hand with more advanced CKD stages and on the other with lower sKlotho and as expected with higher cFGF-23 plasma levels. This interplay between sKlotho and phosphate in CKD has gained interest in the field of CKD-MBD and may be indicative of the need for early intervention in the regulation of serum phosphate levels, even when these are within normal limits [53]. Also, it was shown that the two parameters of renal phosphate handling, FEP and TmP/GFR, differed significantly among the three regression groups. In particular, the patients in the regression groups B and C presented with higher FEP values, compared with those in group A. The same finding was also observed among the five CKD stages, with FEP values increasing as CKD progresses to later stages. This is compatible with the expected response of remaining or healthy renal tubules to excrete more phosphate, in order to avoid its accumulation with the deterioration of renal function. On the other hand, the variable of TmP/GFR, which consists a "theoretical" renal phosphate threshold, corresponding to the "theoretical" lower limit of serum phosphate levels, below which all filtered phosphate would be reabsorbed, also reflects in a way patients' phosphaturia, with lower values to be translated into increased renal phosphate excretion. In our data, TmP/GFR showed higher values in

patients of regression groups A and C and lower in those of group B. This finding may indicate that during the early CKD stages (included in regression group A), as well during the advanced ones (included in regression group C), the variable of TmP/GFR, as an index of the degree of phosphaturia, showed increased values, compatible either of no need of increased renal phosphate excretion in case of early CKD or of a possible difficulty in achieving phosphaturia in case of advanced CKD. Furthermore, we observed that when the values of serum phosphate were equal or more than 3.7 mg/dl in conjunction with a reduced mGFR value of less than 60.85 ml/min/1.73m², as in the case of regression group C, the value of plasma sKlotho was in its lowest level. Of note, the above tree analysis did not reveal similar results using cFGF-23 as response variable. Accordingly, plasma cFGF-23 levels in regression group C did not show a “clear” rise compared with those in the previous regression group B, but only with those in the first group A, which included patients with preserved mGFR. **(Figure 2)** These new interesting findings may denote that the role of sKlotho, compared with that of FGF-23, may be more decisive to phosphate handling in CKD patients. Moreover, the above sorting of patients, according to mGFR and serum phosphate levels may suggest that these two parameters together could be useful in the development of a “different CKD classification” which furthermore presents an impressive correlation with the classic CKD staging and with sKlotho.

Association of cFGF-23/sKlotho plasma levels with clinical outcomes

Univariate analysis showed an association between cFGF-23 and sKlotho with eGFR at the end of the follow-up period (fGFR). It is interesting that univariate linear regression analysis showed that cFGF-23 and sKlotho were significantly associated with fGFR, which remained significant in multiple regression analysis, after adjustment for several covariates, known to be associated with CKD progression. The association of FGF-23 with CKD progression has been previously reported; however the number of studies investigating the prognostic value of sKlotho levels, is very limited and their results are contradictory [23,24]. During the follow up period, 31.2% of patients either initiated RRT or died from cardiovascular causes. Our results showed that, apart from well recognized traditional and CKD-related risk factors [54,55] including mGFR, hemoglobin, FEP, TmP/GFR, 25-OH and 1,25-OH₂ vitamin D, cFGF-23 and sKlotho also differed significantly between the group of patients who reached the composite end point or the “event”, and the group of “no event”. In addition, contingency table analysis reconfirmed that cFGF-23 and sKlotho plasma levels together, affected patients’ outcome. In particular, the four groups that were formed according the combinations of the median values of both biomarkers, differed significantly as to the presence of “event”, with the group of less FGF-23/more Klotho and the one of more FGF-23/less Klotho to show the greater difference between them. Almost the same results were shown when the combinations groups were made between the median value of cFGF-23 and each one of the three regression groups (A,B,C). Again, a significant difference, related to the presence of “event”, between the group of less FGF-23/regression group A and the group of more FGF-23/regression group C was obvious. Kaplan Meier survival analysis results were also consistent with the above findings, regarding patients’ outcome. The difference was obvious from the curves of patients with cFGF-23 plasma levels more than median and those with sKlotho plasma levels less than median value, which showed a worse outcome in

comparison with the opposite ones. Survival analysis also showed that the presence of "event" differed significantly among the five CKD stages, the three regression groups and the tertiles of AAC score. It is worth mentioning that either the observed significant difference between the group of less FGF-23/regression group A and the group of more FGF-23/regression group C by contingency tables, or the survival difference among regression group A, B and C by Kaplan Meier, may denote that the tree analysis classification of our patients consists an interesting finding, since not only mGFR, serum phosphate and sKlotho plasma levels are related with this classification, but also the outcome of the patients. Additionally, all the above results underline the important role of FGF-23/sKlotho not only in CKD progression but in cardiovascular mortality too. Multivariate Cox regression analysis also showed that cFGF-23, sKlotho and AAC score were independently significantly associated with adverse outcome. Of note, several studies have shown an independent correlation of FGF-23 with all-cause mortality but those assessing cardiovascular mortality are limited [7,9,12,13]. Additionally, there are only two previous studies in hemodialysis patients which showed a correlation of sKlotho levels with all-cause and cardiovascular mortality [56,57] and only one in non dialysis CKD stages 2-4 patients in which an association between sKlotho and cardiovascular outcomes was not found [58].

The main limitation of our study is the relatively small patient number and accordingly the small number of cardiovascular deaths, during the follow up period. In addition, cFGF-23 and sKlotho were measured only at baseline, with no further assessment. However, our results are strengthened by the large number of measured clinical and laboratory parameters, known to affect outcome in CKD patients, the extensive statistical adjustment and the adequate follow-up period.

Conclusions

In CKD patients stage 1-5, cFGF-23 and sKlotho levels closely correlate with many CKD-MBD parameters and are independently associated with progression to ESRD and cardiovascular mortality. In addition, mGFR levels, serum phosphate and sKlotho values may divide patients into "new" CKD stages, which have a possible prognostic value in the outcome of these patients. Finally, it would be reasonable to support that in CKD patients, the determination of both FGF-23 and sKlotho plasma levels provides significant additional prognostic information, apart from a variety of traditional and novel risk factors. Studies are required to determine whereas modification of their levels towards "normal", through different therapeutic approaches, would improve clinical outcomes in this high-risk patient population.

Abbreviations

AAC score, abdominal aortic calcification score
ACR, albumin to creatinine ratio
ALP, alkaline phosphatase
BMI, body mass index
BSA, body surface area
cFGF-23, carboxyl terminal fibroblast growth 23
CKD, chronic kidney disease
CKD-MD, chronic kidney disease - mineral and bone disorders
CRP, C-reactive protein
CVD, cardiovascular disease
DBP, diastolic blood pressure
DM, diabetes mellitus
eGFR, estimated glomerular filtration rate
ELISA, enzyme-linked immunosorbent assay
ESRD, end stage renal disease
FEP, fractional excretion of phosphate
fGFR, final glomerular filtration rate
GFR, glomerular filtration rate
Hb, hemoglobin
HDL, high density lipoprotein
HTN, hypertension
LDL, low density lipoprotein
MBP, mean blood pressure
mGFR, measured glomerular filtration rate
PTH, intact parathyroid hormone
RIA, radioimmunoassay
SBP, systolic blood pressure
SHPT, secondary hyperparathyroidism
sKlotho, soluble Klotho

TmP/GFR, ratio of tubular maximum reabsorption of phosphate to glomerular filtration rate

TSAT%, transferrin saturation%

Declarations

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Availability of data and materials The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions EM, ET, PP, SP and AP conceived and designed the experiments; EM, GA, AP performed the experiments; EM and AP wrote the paper. All authors have read and approved the manuscript.

Ethics approval and consent to participate Our study was approved by institutional ethic committees (Papageorgiou General Hospital, Democritus University of Thrace, and Aristotle University of Thessaloniki). All participants gave a written informed consent. All procedures were conducted in accordance with the Declaration of Helsinki (2008 Amendment).

Consent for publication Not applicable.

Competing interests The authors declare that they have no competing interests.

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Tables

Table 1. Initial epidemiological, somatometric and hemodynamic parameters, comorbidity factors and mGFR values of the 128 patients in the five CKD stages

	All	CKD 1 (n=6)	CKD 2 (n=18)	CKD 3 (n=66)	CKD 4 (n=32)	CKD 5 (n=6)	p
Age, years	67 (18-86)	41 (18-61)	66 (23-78)	68 (35-86)	69.5 (31-81)	66.5 (33-78)	0.0004
Male, gender	85 (66.4%)	4 (66.6%)	15 (83.3%)	43 (65.1%)	21 (65.6)	3 (50%)	NS
BMI kg/m ²	29.1 (18.9-55.2)	28.2 (22.0-33.9)	28.3 (18.9-38.3)	29.5 (21.6-55.2)	28.4 (20.8-41.4)	31.3 (24.7-44.5)	NS
Waist to hip ratio	0.99 ± 0.09	0.94 ± 0.07	0.96 ± 0.09	1.00 ± 0.08	1.02 ± 0.12	1.04 ± 0.08	NS
SBP, mmHg	140 (90-200)	115 (100-180)	120.00 (90-160)	140 (90-200)	140 (90-170)	140 (90-160)	NS
DBP, mmHg	80 (60-120)	75 (60-100)	80 (70-90)	80 (60-120)	80 (60-90)	75 (69-90)	NS
MBP, mmHg	99.1 (70.0-143.3)	88.3 (76.7-120.0)	93.3 (76.7-110.0)	100.0 (76.7-143.3)	99.2 (70.0-113.3)	100.0 (76.7-110.0)	NS
HTN	107 (83.5%)	3 (50.0%)	12 (66.6%)	57 (86.3%)	30 (93.7%)	5(83.3%)	0.02
DM	59 (46.0%)	1 (16.6%)	7 (38.8%)	29 (43.9%)	20 (62.5%)	2 (33.3%)	NS
CVD	59 (46.0%)	0 (0%)	6 (33.3%)	31 (46.9%)	19 (59.3%)	3 (50.0%)	NS
mGFR ml/min/1.73m ²	41.50 (8.50-119.50)	105.35 (90.40-119.50)	68.50 (60.60-87.70)	46.25 (30.20-59.60)	23.65 (15.70-29.60)	12.50 (8.50-14.10)	<0.0001

Values are expressed as number of patients with percentage (%), mean ± SD, or median with range, as appropriate.

(Abbreviations: BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; MBP, mean blood pressure; HTN, hypertension; DM, diabetes mellitus; CVD, cardiovascular disease; mGFR, measured glomerular filtration rate)

	All	CKD 1 (n=6)	CKD 2 (n=18)	CKD 3 (n=66)	CKD 4 (n=32)	CKD 5 (n=6)	p
Hb, gr/dl	12.91 ± 1.72	14.03 ± 1.50	13.69 ± 1.53	13.15 ± 1.74	12.01 ± 1.45	11.57 ± 0.99	0.0003
TSAT%	25 (5- 84)	36.5 (22- 65)	32.5 (8- 55)	25 (12- 84)	22.5 (5- 53)	26.5 (15- 42)	NS
Creatinine, mg/dl	1.54 (0.52- 6.17)	0.59 (0.52- 1.25)	1.01 (0.66- 1.75)	1.42 (0.76- 2.88)	2.33 (1.49- 3.69)	3.57 (2.50- 6.17)	<0.0001
Ca, mg/dl	9.27 ± 0.56	9.11 ± 0.61	9.30 ± 0.49	9.36 ± 0.51	9.22 ± 0.69	8.73 ± 0.35	NS
P, mg/dl	3.64 ± 0.73	3.79 ± 0.46	3.14 ± 0.64	3.50 ± 0.63	4.04 ± 0.69	4.43 ± 1.01	<0.0001
Albumin, gr/dl	4.09 ± 0.39	4.06 ± 0.73	4.20 ± 0.45	4.11 ± 0.35	4.04 ± 0.38	3.82 ± 0.31	NS
Total ALP, IU/L	72.5 (26-187)	51 (40- 117)	66 (48- 105)	72 (26- 187)	80 (26- 133)	97.5 (48- 133)	0.03
Total cholesterol, mg/dl	175.5 (109- 272)	198 (137- 231)	166.5 (111- 270)	185.5 (109- 272)	166.5 (110- 223)	170 (143- 258)	NS
Triglycerides, mg/dl	135 (39- 442)	152 (111- 347)	115.5 (39-268)	139 (57- 442)	135 (64- 353)	147.5 (62- 320)	NS
HDL cholesterol, mg/dl	40 (20- 74)	43 (34- 55)	44.5 (28-74)	40 (20- 73)	38.5 (26- 58)	42 (36-68)	0.05
LDL cholesterol, mg/dl	98.5 (42-194)	102.5 (52-165)	90 (54- 186)	111 (46- 194)	96.5 (42- 146)	95 (45- 177)	NS
ACR, mg/gr	98.5 (1.9- 6891.0)	267.4 (7.0- 2832.4)	49.2 (9.4- 6891.0)	49.6 (1.9- 2340.0)	290.3 (7.1- 2947.8)	965.5 (134.7- 1395.4)	0.001
CRP, mg/dl	0.39 (0.12- 9.41)	0.25 (0.18- 5.50)	0.28 (0.12- 2.23)	0.40 (0.14- 4.97)	0.47 (0.15- 9.41)	0.45 (0.25- 2.06)	NS
Beta-2 microglobulin, mg/dl	3.51 (0.92- 11.73)	1.27 (0.92- 1.80)	1.94 (0.95- 3.92)	3.17 (1.35- 6.55)	5.68 (2.57- 10.43)	7.46 (7.07- 11.73)	<0.0001

Values are expressed as mean ± SD or median with range, as appropriate.

(Abbreviations: Hb, hemoglobin; TSAT%, transferrin saturation%; Ca, calcium; P, phosphate; ALP, alkaline phosphatase; HDL, high density lipoprotein; LDL, low density lipoprotein; ACR, albumin to creatinine ratio; CRP, C-reactive protein)

Table 3. Initial CKD-MBD parameters of the 128 patients in the five CKD stages

	All	CKD 1 (n=6)	CKD 2 (n=18)	CKD 3 (n=66)	CKD 4 (n=32)	CKD 5 (n=6)	p
FEP	0.19 (0.02-0.54)	0.07 (0.03-0.19)	0.11 (0.02-0.35)	0.17 (0.05-0.48)	0.26 (0.03-0.51)	0.36 (0.15-0.54)	<0.0001
TmP/GFR, mmol/L	1.35 (0.62-2.00)	1.83 (0.90-1.93)	1.25 (0.84-1.60)	1.12 (0.62-2.00)	1.10 (0.65-2.00)	0.95 (0.86-1.58)	0.008
Ca24h, mg	52.1 (5.1-473.0)	128.0 (25.6-473.0)	132.5 (17.5-210.0)	52.6 (5.1-186.2)	40.2 (16.5-105.0)	47.5 (22.0-63.0)	<0.0001
P24h, mg	580.0 (177.1-1882.0)	889.7 (509.6-1881.9)	690.2 (338.5-1750.4)	592.4 (183.0-1300.0)	495.8 (177.1-1134.0)	671.0 (423.0-1668.5)	0.01
iPTH, pg/ml	108.0 (11.9-646.3)	33.3 (14.5-65.0)	65.1 (27.4-118.0)	108.0 (11.9-646.3)	154.5 (44.3-573.1)	224.3 (52.8-316.0)	<0.0001
25-OH vitamin D, ng/ml	18.9 (2.9-62.0)	15.5 (2.9-39.0)	21.4 (5.8-38.5)	19.5 (3.0-62.0)	15.0 (3.9-38.0)	15.0 (4.0-35.0)	NS
1,25-OH ₂ vitamin D, pg/ml	15.5 (1.0-46.0)	20.3 (11.2-46.0)	25.6 (10.0-40.0)	17.8 (1.5-46.0)	6.8 (1.0-34.0)	8.6 (1.0-29.0)	<0.0001
cFGF-23, RU/ml	218.8 (41.0-1819.0)	107.0 (41.0-275.0)	114.9 (67.5-217.4)	218.8 (49.0-1273.0)	351.5 (120.0-1819.2)	542.5 (200.0-743.5)	<0.0001
sKlotho, pg/ml	479.8 (144.2-1345.0)	910.5 (481.8-1345.0)	757.4 (363.0-1236.4)	492.6 (144.3-836.4)	356.8 (213.0-594.3)	431.8 (281.8-575.5)	<0.0001
AAC score (0-24)	6 (0-24)	0.0 (0.0-13.0)	0.0 (0.0-20.0)	7.00 (0.0-24.0)	8.5 (0.0-24.0)	9.5 (0.0-24.0)	0.01

Values are expressed as median with range, as appropriate.

(Abbreviations: FEP, fractional excretion of phosphate; TmP/GFR, ratio of tubular maximum reabsorption of phosphate to glomerular filtration rate; Ca24h, calcium excretion in 24 hour urine collection; P24h, phosphate excretion in 24 hour urine collection; iPTH, intact parathyroid hormone; 25-OH, 25 hydroxy- and 1,25-OH₂, 1,25 dihydroxy- vitamin D; cFGF-23, carboxyl terminal fibroblast growth 23; sKlotho, soluble Klotho; AAC score, abdominal aortic calcification score)

Table 4. Presence of “event” among different combinations of cFGF-23, sKlotho and regression groups

	Total	“No event” (0)	“Event” (1)
c-term FGF-23 less than median/ s-Klotho more than median	38	34	4 (10.5%)
c-term FGF-23 less than median/ s-Klotho less than median	26	20	6 (23%)
c-term FGF-23 more than median/ s-Klotho more than median	25	18	7 (28%)
c-term FGF-23 more than median/ s-Klotho less than median	39	16	23 (58.9%)
Total	128	88	40 (31.2%)

	Total	“No event” (0)	“Event” (1)
c-term FGF-23 less than median/ regression group A	23	22	1 (4.3%)
c-term FGF-23 less than median/ regression group B	26	21	5 (19.2%)
c-term FGF-23 less than median/ regression group C	15	11	4 (26.6%)
c-term FGF-23 more than median/ regression group A	1	1	0 (0%)
c-term FGF-23 more than median/ regression group B	31	18	13 (41.9%)
c-term FGF-23 more than median/ regression group C	32	15	17 (53.1%)
Total	128	88	40 (31.2%)

A 2x4 contingency table test showing the relationship between combinations of less or more than cFGF-23/sKlotho median values, with the presence of "event" (upper panel). A 2x6 contingency table test showing the relationship between combinations of less or more than cFGF-23 median value and regression group A,B,C with the presence of "event" (lower panel). (Pearson's chi-square test, $p < 0.0001$ for both).

(Abbreviations: cFGF-23, carboxyl terminal fibroblast growth 23; sKlotho, soluble Klotho)

Table 5. Univariate Cox regression analysis for cFGF-23, sKlotho, AAC score and for other variables according to the presence of "event" in a non adjusted model

Variable	Hazard Ratio	95% CI	p
cFGF-23	1.002	1.001-1.002	0.0001
sKlotho	0.997	0.995-0.999	0.001
AAC	1.066	1.022-1.111	0.003
DM	2.302	1.211-4.375	0.009
CVD	2.940	1.514-5.712	0.0009
mGFR	0.926	0.901-0.952	<0.0001
Hb	0.727	0.601-0.880	0.0008
Ca	0.488	0.279-0.853	0.01
P	1.827	1.251-2.669	0.002
Total ALP	1.016	1.006-1.016	0.004
TmP/GFR	0.228	0.070-0.736	0.009
iPTH	1.005	1.003-1.007	<0.0001
25-OH vitamin D	0.950	0.918-0.983	0.001
1,25-OH ₂ vitamin D	0.944	0.912-0.978	0.0005

(Abbreviations: cFGF-23, carboxyl terminal fibroblast growth 23; sKlotho, soluble Klotho; AAC score, abdominal aortic calcification score; DM, diabetes mellitus; CVD, cardiovascular disease; mGFR, measured glomerular filtration rate, Hb, hemoglobin; Ca, calcium; P, phosphate; ALP, alkaline phosphatase; TmP/GFR, ratio of tubular maximum reabsorption of phosphate to glomerular filtration rate; iPTH, intact parathyroid hormone; 25-OH, 25 hydroxy- and 1,25-OH₂, 1,25 dihydroxy- vitamin D)

Table 6. Cox regression analysis with non adjusted and adjusted model for cFGF-23, sKlotho and AAC score according to the presence of "event"

Variable	Non adjusted		Adjusted	
Composite end point or "event"				
	HR (95% CI)	p	HR (95% CI)	p
cFGF-23	1.002 (1.001-1.002)	0.0001	1.001 (1.000-1.001)	0.04
sKlotho	0.997 (0.995-0.999)	0.001	0.996 (0.994-0.999)	0.008
AAC score	1.066 (1.022-1.111)	0.003	1.084 (1.019-1.154)	0.01

The model was adjusted for sex, age, BMI, waist to hip ratio, presence of DM, history of CVD, hemoglobin, albumin, CRP, ACR, and TmP/GFR. (Abbreviations: BMI, body mass index;; DM, diabetes mellitus; CVD, cardiovascular disease; CRP, C-reactive protein; ACR, albumin to creatinine ratio; TmP/GFR, ratio of tubular maximum reabsorption of phosphate to glomerular filtration rate; cFGF-23, carboxyl terminal fibroblast growth 23; sKlotho, soluble Klotho; AAC score, abdominal aortic calcification score)

Figures

Figure 1

Correlation of the five CKD stages with the three regression groups A, B, C.

Figure 2

Difference of cFGF-23 and sKlotho among the three regression groups A, B, C.

Figure 3

Difference of FEP and TmP/GFR among the three regression groups A, B, C.

Figure 4

Correlation of cFGF-23 and sKlotho with fGFR

Figure 5

Kaplan Meier curves of composite end point or “event” according to median values of cFGF-23 and sKlotho plasma levels.

Figure 6

Kaplan Meier curves of composite end point or “event” according to CKD stages, regression groups and AAC score tertiles