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Research Article

Thoracic Aorta Calcification and Circulating a-Klotho Levels in Patients with Chronic Kidney Disease

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Abstract

Background: Evidence suggests that the anti-aging protein a-Klotho is a central modulator of mineral homeostasis. Circulating a-Klotho exerts endocrine activity and has been implicated in the process of vascular calcification, which is accelerated in patients with chronic kidney disease (CKD) and portends an unfavorable prognosis. However, the role of a-Klotho in this process is still unclear.

Methods: In this study we enrolled a total of 58 adult patients with CKD. Group 1 included 28 participants with CKD stage V and group 2 included 28 participants with CKD stage III.

Results: Participants in group 1 had lower levels of circulating a-Klotho compared to group 2 (376; 279 - 570 pg/mL vs. 722; 477 - 892 pg/mL; P < 0.001), were of younger age (55.5; 45 - 62.5 years vs. 69; 64 - 73.5 years; P < 0.001), had lower body mass index (BMI) (25.6; 23.8 - 27.5 kg/m^2 vs. 28.2; 25.7 - 31.1 kg/m^2; P=0.041), higher serum phosphate (4.85; 4.05 - 5.7 mg/dL vs. 3.45; 2.95 - 3.85 mg/dL; P < 0.001), higher calcium-phosphate product (42; 35.4 - 50 mg^2/dL^2 vs. 32.6; 28.8 - 35.3 mg^2/dL^2; P < 0.001), higher calcitonin levels (55.3; 16.8 - 131 pg/mL vs. 4.3; 1.65-8.95 pg/mL; P < 0.001) and higher parathyroid hormone (PTH) levels (28.9; 14.7 - 45 pmol/L vs. 7.05; 4.45 - 9.35 pmol /L; P < 0.001). In multivariate analysis, age and BMI were found to correlate with severity of total calcification in the thoracic aorta.

Conclusions: No statistically significant difference was documented between the two groups in terms of thoracic aorta calcification. Age and BMI were identified as predictor of thoracic aorta calcification.

Keywords: *a-Klotho; Vascular Calcification; Thoracic Aorta Calcification; Chronic Kidney Disease*

Abbreviations

CKD: Chronic Kidney Disease; CS: Calcium Score; Asc: Ascending; Desc: Descending; LMS: Left Main Stem; LAD: Left Anterior Descending Artery; LCx: Left Circumflex Artery; RCA: Right Coronary Artery; 25 (OH) D: 25 Hydroxyvitamin D; PTH: Parathyroid Hormone; HTN: Hypertension; DM: Diabetes Mellitus

Introduction

It is widely endorsed that aortic calcification, as a manifestation of vascular calcification, results in vascular stiffness, reduction in vascular compliance and as a consequence, left ventricular hypertrophy [1-3]. Patients with chronic kidney disease (CKD) represent a distinct population with high prevalence of vascular calcification which confers a high morbidity and mortality [4,5]. This represents an accelerated process of inappropriate deposition particularly of calcium phosphate salts into the vascular tissues with a pattern of distribution in the tunica intima and/or tunica media aortic wall [6,7]. This process can become evident even at early stages of CKD [8].

Under normal conditions the homeostasis of minerals is orchestrated by a multitude of factors preventing abnormal deposition of minerals in soft tissues. In cases of imbalance between the inhibitors and inducers of calcification such as in cases of CKD, vascular calcification ensues [9-11]. One of the emerging regulating factors is a-Klotho. It was in 1997 when Kuro-o., *et al.* published their study on a previously unknown gene, the mutation of which led to a clinical syndrome that resembled aging and encompassed growth retardation, short lifespan, arteriosclerosis, ectopic calcification, osteoporosis, skin atrophy and emphysema in mice homozygous for the transgene [12]. The main transcript of this gene, a-Klotho, has been regarded as the anti-aging protein, the overexpression of which has been related to extended lifespan in mice and it led to the identification of diverse endocrine pathways linking aging and mineral metabolism [13]. Mineral metabolism dysregulation and vascular calcification, a major finding in aging [14] and independent predictor of cardiovascular mortality and morbidity [15], are all hallmark characteristics in patients with CKD, a well-established state of pre-mature aging [16,17].

It was further documented that levels of circulating a-Klotho diminish with worsening renal function since early in the process [18]. Therefore, a role has been suggested for a-Klotho in the process of vascular calcification in patients with CKD although, data are relatively scarce, equivocal and even conflicting [19,20]. In this study we explored the relationship between circulating a-Klotho levels and severity of calcification in the thoracic aorta, coronary arteries and aortic valve in patients at different stages of CKD, namely between stage V (end-stage renal disease) and stage III CKD with no previous history of CV disease.

Methods

Study design and laboratory investigations

We enrolled 56 patients with CKD at AHEPA University Hospital, Greece, between January 1, 2016 and December 31, 2017. Patients with 1) active cancer; 2) systemic inflammatory or granulomatous disease; and 3) primary hyperparathyroidism were excluded. Group 1 included 28 patients with end-stage renal disease, CKD stage V (eGFR < 15 mL/min/1.72m^2) under renal replacement therapy in the form of regular intermittent hemodialysis. Group 2 included 28 outpatients (OP) with CKD stage III (eGFR ≥ 30 mL/min/1.72m^2, < 60 mL/min/1.72m^2) and stable eGFR for the last at least 3 months prior to enrollment. The 4-variable, abbreviated modification of diet in renal disease (MDRD) study equation (eGFR = 186 × serum creatinine-1.154 × age-0.203 × 0.742 if female) was used for the calculation of eGFR [21,22]. We decided to compare patients with distinctly different stages of CKD, namely stage III and stage V CKD. The decision was made in view of the inferred differences between these two stages as, comparing patients with CKD of stage V and stage V would call a significantly higher number of enrollees to reach statistical significance.

Clinical data were collected through the primary physician, the Medical Records and, patients interview. Baseline demographic and clinical data were recorded as follows: age, gender, primary kidney disease, body mass index, blood pressure, medication history, and comorbid diseases. For laboratory measurements, blood samples were drawn in the morning after an overnight fast of at least 8 hours for the OP group. For all patients in the CKD-V group, dry weight was used as reference weight and, blood samples were drawn at the beginning of the hemodialysis session. For the majority of these patients, the hemodialysis session in the middle of the week was selected (either Wednesday or Thursday) for the blood samples.

After centrifuging whole blood at 3,000 rpm for 10 minutes at 4ºC, supernatant serum was immediately stored in aliquots in deep freeze (-80ºC) for future analysis. No freeze-thaw-freeze cycle of the stored serum samples occurred at any point of the study. Human soluble a-Klotho was measured using a commercially available for research use, solid phase sandwich enzyme-linked immunosorbent assay (ELISA) kit (IBL International GmbH, Hamburg, Germany). It has been previously tested, and the intra-assay and inter-assay coef-

ficient of variation (CV) was < 10% [23]. The serum soluble α-Klotho levels with this assay range between 93.75 and 6,000 pg/mL, cross reactivity with human a-Klotho is 100% (specificity) and sensitivity is as low as 6.15 pg/mL. Serum parathyroid hormone (PTH) was assayed by a double ('sandwich') electro-chemiluminescence immunoassay (Roche Diagnostics GmbH, Mannheim, Germany); intra-assay and inter-assay CV are < 10% and serum PTH levels with this assay range between 5.50 and 2300 pg/mL. Serum 25 hydroxyvitamin D (25(OH)D) was analyzed with the radioimmunoassay method (DIA source ImmunoAssays S.A., Belgium); intra-assay and inter-assay CV are < 10% and detection limit is 1.5 ng/mL. Serum calcitonin was analyzed by a double ('sandwich') immunoradiometric assay (Institute of Isotopes Ltd, Budapest, Hungary); intra-assay and inter-assay CV are < 10%, serum calcitonin levels with this assay range between 0 and 2,000 pg/mL and sensitivity is 1.5 pg/mL.

Imaging investigations

All patients had a non-contrast cardiac multi-slice computed tomography (MSCT) using a 128-slice scanner (CT Optima 660, GE Healthcare) on the day or within a week of the blood samples draw. Agatston score was used for the quantitative evaluation of the degree of calcification of the thoracic aorta, coronary arteries and aortic valve [24]. Prospective electrocardiogram triggering (75% of R-R interval) was used with a slice thickness of 2.5 mm. The scan range extended from the level of clavicles down to the diaphragm, including the thoracic aorta in all its length. The following scanning parameters were applied: collimation width: 32 × 0.625 mm; rotation time: 330 ms; tube voltage: 120 kV; and maximum effective tube current: 64 mA. Image reconstruction was gated retrospectively to 75% of R-R interval. MSCT images were reconstructed using a cardiac standard filter with a slice thickness of 2.5 mm. The slice thickness of the images reconstructed and reviewed was 0.625 mm for optimal isotropic imaging. MSCT data sets were transferred to an offline workstation (AW workstation, GE Healthcare) for image analysis. Thoracic aorta calcium score, was determined 2-dimensionally using the calcium score data sets on the workstation and, defined by Agatston units (AU). The SmartScore 4.0 (General Electric Company, France) software package was used for the quantification of Agatston score. Total thoracic aorta calcium score was determined by summing the individual lesion scores of each of the main portions of the aorta.

Statistical analysis

We described continuous variables as median (25^{th} - 75^{th} percentile) and categorical variables as N (%). We compared variables between groups using the nonparametric Mann-Whitney U test for continuous and Fisher's exact test for categorical variables. We examined bivariate correlations of thoracic aorta calcification scores with clinical variables using the Spearman correlation coefficient. To identify determinants of calcification, we log-transformed the corresponding calcium scores, assuming a value of 0 (log-transformed 1) for absent calcium and, used stepwise linear regression as above. Because of the small sample size, all regression estimates, and confidence intervals were calculated with resampling (jackknife) to ensure robust variable selection and stable estimates. STATA 14.2 (StataCorp LP, College Station, TX) software package was used for all analyses.

The study adhered to the tenets of the declaration of Helsinki, as revised in 2013 and it was approved by the Ethics Committee of AHEPA University Hospital, Aristotle University of Thessaloniki. All patients provided written informed consent prior to enrollment. The study is registered on ClinicalTrials.gov (Identification number NCT03858413).

Results

28 patients with CKD stage V under hemodialysis and 28 patients with stable CKD stage III totaling 56 patients, participated in the study. Baseline clinical and biochemical characteristics are presented in table 1. Renal function as expressed by eGFR averaged 24.2 mL/min/1.73m^2 in the total study population (median: 19.6; 25^{th} - 75^{th} percentile: 5.9 - 42.2) and included patients with CKD stage V (N = 28), median eGFR 5.9 mL/min/1.73m^2 (5.1 - 7.2) and CKD stage III (N = 28), median eGFR 42.2 mL/min/1.73m^2 (36.1-45.4). 38 patients (68%) were males (16 with CKD-III and 22 with CKD-V). Mean age in the CKD-III group was 67.9 years (69; 64 - 73.5 years) and mean age in the CKD-V group was 54.2 years (55.5; 45 - 62.5 years); there was statistically significant difference between the two groups with overall, younger patients in the CKD-V group (P < 0.001).

Characteristic	Both Groups (N = 56)	CKD-V (N = 28)	CKD-III (N = 28)	P-value
Age (years)	63 (52, 71)	55.5 (45, 62.5)	69 (64, 73.5)	< 0.001
Male gender (%)	68	79	57	
Body mass index (kg/m^2)	27.2 (23.9, 29.8)	25.6 (23.8, 27.5)	28.2 (25.7, 31.1)	0.041
Serum a-Klotho (pg/mL)	523 (328, 794)	376 (279, 570)	722 (477, 892)	< 0.001
Thoracic aorta CS	1004 (107, 3810)	1004 (48, 5300)	1232 (214, 3413)	0.935
Asc aorta CS	0 (0, 37)	0 (0, 78)	0 (0, 20.5)	0.643
Aortic arch CS	570 (15.5, 1811)	742 (0.5, 1747)	497 (100, 1811)	0.863
Desc aorta CS	258 (10.5, 1766)	88.5 (10.5, 2047)	426 (12, 1398)	0.844
Aortic valve CS	21 (0, 122)	39.5 (0, 215)	8.5 (0, 89)	0.304
25 (OH) D (ng/mL)	20.2 (13.1, 25.8)	18.3 (13.1, 28.4)	20.8 (12.8, 24.5)	0.909
Calcitonin (pg/mL)	11.3 (4.1, 55.3)	55.3 (16.8, 131)	4.3 (1.65, 8.95)	< 0.001
Estimated Glomerular Filtration Rate (mL/min/1.73m^2)*	19.6 (5.9, 42.2)	5.9 (5.1, 7.2)	42.2 (36.1, 45.4)	< 0.001
Total Cholesterol (mg/dL)	157 (131, 189)	143 (127, 171)	172 (153, 197)	0.006
LDL Cholesterol (mg/dL)	84.5 (63, 103)	69.5 (59, 92)	91.5 (69.5, 111)	0.007
Triglycerides (mg/dl)	139 (88.5, 168)	151 (122, 223)	107 (78, 148)	0.002
Calcium (mg/dL)	9.06 (8.77, 9.51)	8.93 (8.3, 9.36)	9.3 (8.86, 9.64)	0.031
Phosphate (mg/dL)	3.9 (3.3, 4.85)	4.85 (4.05, 5.7)	3.45 (2.95, 3.85)	< 0.001
Calcium X Phosphate product (mg^2/dL^2)	35.4 (30.6, 42)	42 (35.4, 50)	32.6 (28.8, 35.3)	< 0.001
Total protein (gr/dL)	7.02 (6.59, 7.34)	6.81 (6.52, 7.34)	7.08 (6.75, 7.35)	0.376
Albumin (gr/dL)	4.32 (4.06, 4.56)	4.17 (3.98, 4.55)	4.38 (4.19, 4.56)	0.176
PTH (pmol/L)	11.5 (7.05, 28.9)	28.9 (14.7, 45)	7.05 (4.45, 9.35)	< 0.001
TSH (μIU/mL)	2.5 (1.64, 3.51)	2.02 (1.48, 4.04)	2.5 (2.2, 3.17)	0.974
FT3 (pmol/L)	3.95 (3.3, 4.75)	4.15 (3.65, 4.8)	3.8 (2.45, 4.45)	0.058
FT4 (pmol/L)	14.9 (12.6, 17.8)	14.9 (12.9, 17.6)	14.5 (12.3, 18.7)	0.987
Hemoglobin (gr/dL)	11.9 (10.8, 13.3)	10.9 (10.5, 11.9)	12.9 (11.6, 13.8)	< 0.001
HTN (%)	85.7	89.3	82.1	0.449
Hyperlipidemia (%)	53.6	53.6	53.6	1
DM (%)	41.1	17.9	64.3	< 0.001

Table 1: *Patient characteristics of group 1 (CKD-V) in comparison to group 2 (CKD-III) and in the total population studied.*

Values for continuous variables represent median (25th, 75th percentile).

** Calculated with the Modification of Diet in Renal Disease study equation.*

CS: Calcium Score; Asc: Ascending; Desc: Descending; LMS: Left Main Stem; LAD: Left Anterior Descending Artery; LCx: Left Circumflex Artery; RCA: Right Coronary Artery; 25(OH)D: 25 Hydroxyvitamin D; PTH: Parathyroid Hormone; HTN: Hypertension; DM: Diabetes Mellitus.

Serum a-Klotho was significantly lower in the CKD-V group compared to the CKD-III group (376; 279-570 pg/mL vs. 722; 477 - 892 pg/mL, respectively; P < 0.001).

In a bivariate analysis age (ρ = 0.528, P < 0.001) and albumin (ρ = -0.264, P = 0.05) were shown to be significantly associated with calcium score in the thoracic aorta. In-segment analysis showed that FT3 is negatively correlated with degree of calcium in the ascending aorta (ρ = -0.282, P = 0.035) and aortic arch (ρ = -0.283, P = 0.035). No correlation was documented between a-Klotho levels and calcification of the thoracic aorta (Figure 1). In multivariate analysis, age, BMI, hyperlipidemia and HDL were identified as predictors of extent of thoracic aorta calcification (Table 2).

Variable	Coefficient	95% Confidence Intervals	P
Age (years)	0.058	0.04, 0.08	< 0.001
BMI (kg/m^2)	-0.065	-0.12, -0.01	0.018
Hyperlipidemia*	0.829	0.22, 1.44	0.008
HDL (mg/dL)	-0.023	-0.04, -0.01	0.004

Table 2: *Multivariate analysis performed to identify predictors of calcium score in the thoracic aorta.*

**Defined as LDL cholesterol ≥ 190 mg/dL without Diabetes Mellitus or LDL cholesterol 70 - 189 mg/dL with concomitant Diabetes Mellitus or already on lipid-lowering treatment.*

BMI: Body Mass Index; HDL: High Density Lipoprotein.

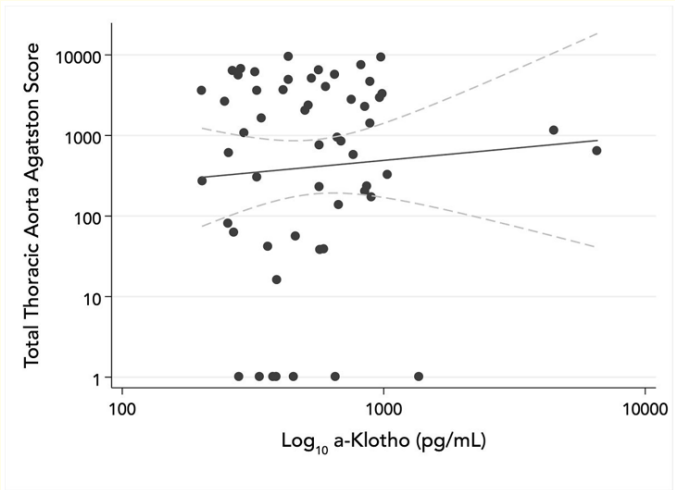

Figure 1: *Correlation of a-Klotho with thoracic aorta calcification.*

Discussion

In this observational study including 56 patients with CKD, we describe the association between circulating a-Klotho levels and calcification in the thoracic aorta. Circulating a-Klotho levels were significantly lower in the CKD-V group compared to the CKD-III group in our cohort. However, no statistically significant difference was noted between the two groups in terms of thoracic aorta, coronary arteries or aortic valve calcification.

Klotho is part of the FGF-23/Klotho, 1,25-dihydroxyvitamin D or calcitriol (1,25$(OH)_2$D) and PTH axis and have a central role in predominantly regulating the mineral homeostasis, namely, regulate the calcium and phosphate concentration within very strict and narrow ranges and in a very delicate balance to commensurate with the body demands. The main type of Klotho is a-Klotho, a type I, single-pass transmembrane protein of 1012 amino acids in humans [12]. Membrane Klotho is an obligatory co-factor for FGF-23, a 251-amino acid hormone predominantly produced from osteocytes and osteoblasts during active bone remodeling [25] and increase urinary phosphate excretion and suppresses synthesis of 1,25$(OH)_2$D. Apart from the membrane form of a-Klotho, there is the circulating form which is a product of shedding from proteases and secretases [26,27]. This circulating form has been shown to have hormonal properties independent from FGF-23.

There is mounting evidence that vascular calcification is an independent predictor of cardiovascular morbidity and mortality in CKD [28-30]. It is suggested that circulating a-Klotho is pivotal in the process of vascular calcification because it (1) regulates phosphate levels

[31] and (2) protects against vascular calcification by inactivating the FGFR1/ERK signaling pathway in bone marrow-derived mesenchymal stem cells [32]. Decreased levels of circulating serum Klotho have been associated with increased arterial stiffness [18].

This study confirms previous knowledge that circulating a-Klotho levels deplete as renal impairment declines [33-36]. No statistically significant difference in the calcification severity between the two groups. This could be postulated to be explained by the severity of CKD in the CKD-III group as, the majority of the participants were in stage IIIb CKD (eGFR ≥ 30 mL/min/1.72m^2, < 45 mL/min/1.72m^2), namely, renal function in those participants was moderately to severely impaired rather than mildly to moderately impaired and closer to CKD IV and V stages.

Our study has several limitations. This was a cross-sectional study with 56 participants in total and hence, no temporal causation could be inferred. As patients were randomly selected, the significant difference in age between the two groups may account to some extent for the non-significant difference in calcification severity in the thoracic aorta. The subgroup of CKD stage V included younger patients and, the calcification severity was not different compared to patients with CKD stage III. It could be postulated that if patients were age-matched there could have been a significant difference in the extent of calcification. We believe that future studies trying to shed more light on the differential severity in calcification should include more patients to enhance power and a more diverse CKD cohort in an effort to include participants with all different stages of CKD severity. In addition, long-term follow-up of these patients would provide evidence of temporal association between circulating a-Klotho levels and calcification in the heart vasculature. Future studies should age-match the population of the sub-groups to exclude age as a confounding factor. Furthermore, all participants were of Caucasian ancestry secondary to the race distribution at the site of the study. Therefore, no conclusions can be drawn for patients with CKD of Black or other race.

Conclusion

In conclusion, this study showed that circulating a-klotho levels deplete with progression of CKD but no significant correlation was found in severity of thoracic aorta calcification between patients with CKD-IIIb and CKD V.

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Conflict of Interest

The authors have no conflicts of interest to disclose.

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