RESEARCH ARTICLE

Associations between Thyroid Hormones, Calcification Inhibitor Levels and Vascular Calcification in End-Stage Renal Disease

Christiaan Lucas Meuwese[1], Hannes Olauson[1], Abdul Rashid Qureshi[1], Jonaz Ripsweden[2], Peter Barany[1], Cees Vermeer[3], Nadja Drummen[3], Peter Stenvinkel[1] *

1 Department of Renal Medicine, CLINTEC, Karolinska Institutet, Stockholm, Sweden, 2 Medical Imaging and Technology, CLINTEC, Karolinska Institutet, Stockholm, Sweden, 3 VitaK, Maastricht University, Maastricht, The Netherlands

* peter.stenvinkel@ki.se

Abstract

Introduction

Vascular calcification is a common, serious and elusive complication of end-stage renal disease (ESRD). As a pro-calcifying risk factor, non-thyroidal illness may promote vascular calcification through a systemic lowering of vascular calcification inhibitors such as matrix-gla protein (MGP) and Klotho.

Methods and Material

In 97 ESRD patients eligible for living donor kidney transplantation, blood levels of thyroid hormones (fT3, fT4 and TSH), total uncarboxylated MGP (t-ucMGP), desphospho-uncarboxylated MGP (dp-ucMGP), descarboxyprothrombin (PIVKA-II), and soluble Klotho (sKlotho) were measured. The degree of coronary calcification and arterial stiffness were assessed by means of cardiac CT-scans and applanation tonometry, respectively.

Results

fT3 levels were inversely associated with coronary artery calcification (CAC) scores and measures of arterial stiffness, and positively with dp-ucMGP and sKlotho concentrations. Subfractions of MGP, PIVKA-II and sKlotho did not associate with CAC scores and arterial stiffness. fT4 and TSH levels were both inversely associated with CAC scores, but not with arterial stiffness.

Discussion

The positive associations between fT3 and dp-ucMGP and sKlotho suggest that synthesis of MGP and Klotho is influenced by thyroid hormones, and supports a link between non-thyroidal illness and alterations in calcification inhibitor levels. However, the absence of an association between serum calcification inhibitor levels and coronary calcification/arterial stiffness and the fact that MGP and Klotho undergo post-translational modifications

underscore the complexity of this association. Further studies, measuring total levels of MGP and membrane bound Klotho, should examine this proposed pathway in further detail.

Introduction

Patients with chronic kidney disease (CKD) are exposed to a greatly increased risk of cardio-vascular morbidity and mortality compared to the general population[1] Underlying mechanisms linking CKD to CVD are incompletely understood but encompass both traditional and novel risk factors.[1, 2] In contrast to the situation in the general population, the predominant vascular pathology in CKD is arterial media calcification.[3]

In the genesis of uremic vascular calcification, osteochondrocytic differentiation of vascular smooth muscle cells (VSMC) has appeared as a cornerstone process.[4] The osteoblast like VSCMCs produce bone proteins and release pre-calcified membrane matrix vesicles,[4] which normally contain calcification inhibitors, such as Matrix Gla protein (MGP), preventing them to exert their calcifying actions.[5] In order to act as a calcification inhibitor, MGP must first be activated by posttranslational gammaglutamate carboxylation. As this process is vitamin K dependent and patients with ESRD typically have a poor vitamin K status, plasma levels of desphospho-uncarboxylated MGP (dp-ucMGP) are generally elevated.[6] In more severe states of vitamin K insufficiency, also blood-clotting factors are affected and uncarboxylated clotting factors, or PIVKAs (Proteins Induced by Vitamin K Absence), are detectable in the circulation. The most commonly detected PIVKA is descarboxyprothrombin, also known as PIVKA-II.

Another recently discovered factor of interest is Klotho, a membrane-bound protein expressed at the highest levels in renal tubules, parathyroid glands and choroid plexus. Membrane-bound Klotho functions as co-receptor for Fibroblast growth factor-23 (FGF23), allowing for high-affinity binding to FGF-receptors.[7] FGF23-Klotho signalling is essential for phosphate and vitamin D homeostasis, and is severely dysregulated in CKD. Klotho can also be shedded from the cell surface into circulation by α-secretases to form soluble Klotho (sKlotho). In vitro, sKlotho was demonstrated to inhibit sodium-dependent phosphate uptake in VSMC and thereby prevent phosphate-induced vascular calcification.[8]

Finally, hormonal derangements in ESRD include a systemic lowering of serum free triiodothyronine (fT3) and thyroxine (fT4) concentrations, making up part of the non-thyroidal illness spectrum.[9] Presence of non-thyroidal illness in ESRD has been strongly associated with cardiovascular mortality,[10] and also with vascular calcification,[11–13] whereby posing it as a candidate cardiovascular risk factor that can be manipulated in ESRD. These observations are reinforced by studies in the general population showing associations between subclinical thyroid hormone alterations and an increased coronary calcification.[14] The increased cardiovascular risk due to non-thyroidal illness could be explained by the promotion of endothelial dysfunction, vasoconstriction and lipid alterations by a systemic low thyroid hormone state. [15] These pathways seem, however, not fully able to explain the specific presence of media calcification.

Recent in vitro studies have suggested functional links between thyroid hormones, MGP and Klotho. First, Sato et al.[16] observed that physiological concentrations of T3 facilitate MGP gene expression in smooth muscle cells, an effect that is likely mediated by thyroid hormone response element in the promotor region of the MGP gene.[16] Similarly, Klotho synthesis was reported to be under control of thyroid hormone stimulation.[17] These findings lead us to speculate that nonthyroidal illness could initiate an increased vascular calcification
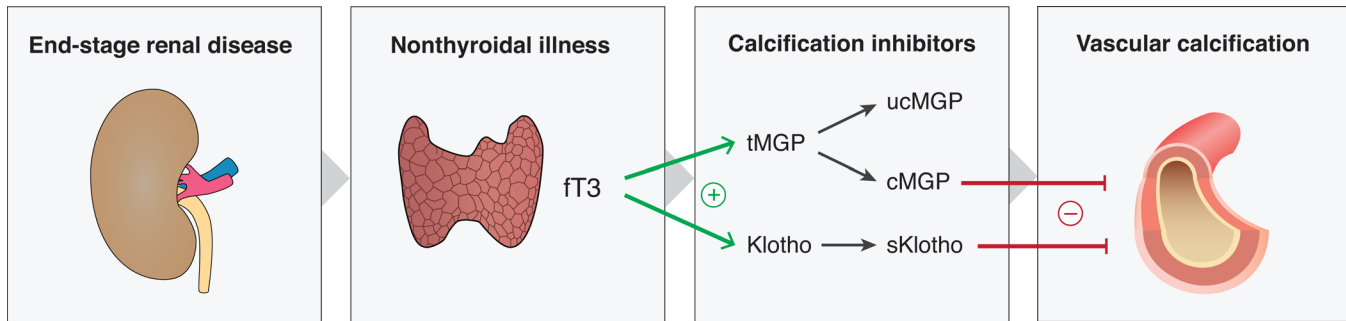
Fig 1. A hypothesis on the impact of non-thyroidal illness on vascular calcification.

doi:10.1371/journal.pone.0132353.g001

through down-regulation of the protective effects by MGP and Klotho (Fig 1). Therefore, we studied; 1) the association between thyroid hormone concentrations and (i) vascular calcification/arterial stiffness, (ii) sKlotho and (iii), MGP as well as; 2) the association of plasma MGP and sKlotho concentrations with surrogate markers of vascular calcification/arterial stiffness in a carefully phenotyped cohort of younger ESRD patients.

Methods and Materials

Patients

Between 2009 and August 2014 adult ESRD patients selected to undergo living donor kidney transplantation (LD-RTx) at Karolinska University Hospital in Stockholm, Sweden, were invited to participate in the study. Ethical approval for the study protocol was obtained from the regional ethical committee in Stockholm County, Sweden who approved the consent procedure. Patients provided written informed consent. Out of 101 patients who met the inclusion criteria and gave informed consent, two were excluded because of warfarin usage and another two due to incomplete thyroid hormone data, leaving 97 patients for the present study. Of these 97 patients, the most common causes of CKD were polycystic kidney disease (n = 18) glomerulonephritis (n = 29), diabetic nephropathy (n = 6), interstitial nephritis (n = 7), other causes (n = 23), and unknown (n = 14). The most commonly used forms of medication were erythropoietin (78.4%), beta-blockers (55.7%), non-calcium-based phosphate binders (53.6%) and loop-diuretics (60.9%). CVD was defined as having coronary artery, cerebrovascular, and/or peripheral artery disease. Subjective global assessment (SGA) was determined as previously exemplified.[18]

Laboratory measurements

Venous blood was collected after an overnight fast; all blood samples were centrifuged immediately and stored at -70°C until required for analysis. Serum high-sensitivity C-reactive protein (CRP), calcium, phosphate, creatinine, and albumin levels were determined using routine methods at the Department of Laboratory Medicine, Karolinska University Hospital. Serum levels of IL-6 were quantified using an Immulite system (Siemens Healthcare Diagnostics, Los Angeles, CA, USA). Plasma analyses of thyroid hormones were performed using a Roche Modular E/Cobas E analyser and commercially available electrochemiluminescence immunoassays for fT3 [analytical sensitivity (AS), 0.4 pmol/L; total coefficients of variation (CVs), 6.27% and 3.36% at 3.16 and 10.03 pmol/L, respectively], fT4 (AS, 0.3 pmol/L; total CVs, 2.05% and 3.03% at 12.01 and 34.70 pmol/L, respectively) and TSH (AS, 0.014 mU/L; total CVs, 2.16%

and 2.42% at 0.995 and 5.530 mU/L, respectively). Results are expressed as the average of two measurements.

Vitamin K status was assessed by measuring dp-ucMGP levels in citrate plasma using the InaKtif MGP iSYS kit (IDS, Boldon), a pre-commercial automated assay based on the conformation-specific dual antibody ELISA. [6] Total uncarboxylated MGP (t-ucMGP) was measured using a competitive (single antibody) ELISA, in which the capture antibody is directed against ucMGP, and plasma ucMGP is quantified from its competition with a labelled tracer peptide.[7] Both assays were performed at VitaK, Maastricht University, The Netherlands. Plasma concentrations of PIVKA-II were determined by an enzyme immunoassay (Eisai Co., Tokyo, Japan) according to the manufacturer's instructions. Soluble Klotho levels were determined using a commercially available ELISA kit (Immuno-Biological Laboratories Co., Ltd, Japan) according to the manufacturer's instructions.

Cardiac CT-scans

Cardiac CT scans were performed using a 64-channel detector scanner [LightSpeed VCT; General Electric (GE) Healthcare, Milwaukee, WI, USA] in cine mode. Scans were ECG-gated and a standard non-contrast protocol was used with a tube voltage of 100 kV, tube current of 200 mA, 350 ms rotation time, 2.5 mm slice thickness and displayed field of 25 cm. Data were processed and analysed using an Advantage Workstation 4.4 (GE Healthcare). Smartscore 4.0 (GE Healthcare) was used to assess CAC scores. Calcified plaques were considered to be present if values crossed the standard threshold of 130 Hounsfield units. CAC scores were expressed in Agatston units (AU) as previously described in detail.[19] Total CAC score was calculated as the sum of the CAC scores in the left main artery, the left anterior descending artery, the left circumflex artery and the right coronary artery.

Arterial Stiffness

Arterial elasticity was assessed by means of applanation tonometry (SphygmoCor device, AtCor Medical, Sydney, Australia). Measurements were conducted in a climate-controlled room and after patients had rested for five minutes in a supine position. On basis of 20 consecutive measurements at the radial site, the device constructs a peripheral arterial waveform, which is transcalculated into an aortic waveform using a validated transfer formula [20]. Aortic systolic and diastolic pressures and pulse pressure were calculated on basis of these derived aortic wave shapes. The difference between the first and second systolic peaks of the aortic waveform defined the aortic augmentation pressure. The ratio of diastolic and systolic pressure time index defined Buckberg's subendocardial viability ratio (SEVR).

Statistical analyses

Patient characteristics were presented across groupings based on tertiles of fT3 (cut-off values: 2.73 and 3.71 pmol/L) using means and standard deviations (SD), medians and interquartile ranges (IQR) and numbers and percentages, accordingly. Linear trends across groups were tested by means of the appropriate tests.

Associations between thyroid hormone levels and arterial stiffness and calcification inhibitor parameters were examined by means of scatter plots and linear regression analyses. Because of non-normality, t-ucMGP, dp-ucMGP, PIVKA, and sKlotho levels were logarithmically transformed. Models were adjusted for age, sex, diabetes mellitus, dialysis vintage, SGA, and IL-6 levels. The association between fT3 levels and CAC scores was studied using logistic regression analyses. For this purpose, CAC scores were dichotomized across 400 AU. In these analyses, age sex, and diabetes mellitus were considered as potential confounders. The cut-off

values of 100 and 400 AU were chosen on basis of the distribution of our population and previous recommendations.[21] For illustrational purposes, histograms were created showing the percentage of patients having CAC scores over 100 and 400 AU across tertiles of fT3 distribution. The coherence between the different systemic calcification inhibitors and thyroid hormones was studied using Spearman correlation coefficients.

Statistical analyses were performed using STATA version 12.1 (StataCorp LP, Texas, USA). Betas with 95% confidence intervals (CIs) not containing 0, and odds ratios with 95% CIs not including 1 were considered statistically significant. For all other tests, a p-value lower than 0.05 was considered to indicate statistical significance. Figures were created using PRISM 5.02 (GraphPad Software Inc, La Jolla, CA, USA 1992).

Results

Baseline and outcome characteristics

The cohort comprised 97 individuals with an average (SD) age of 45.1 (14.0) years. Sixty three (64.9%) of patients received dialysis treatment for a median (IQR) duration of 1.0 (0.5–2.0) years before LD-Rtx. The others underwent preemptive Rtx. Patients in the lowest fT3 tertile had more frequently pre-existing CVD and on average lower albumin and phosphate levels as compared with the higher tertiles (Table 1). Calcium levels were not significantly different between groups.

Thyroid hormones and arterial stiffness/vascular calcification

In Table 2, the distribution of arterial stiffness parameters across fT3 tertiles is presented. With decreasing fT3 levels, patients had significantly higher pulse pressure (PP) and tended to have lower aortic augmentation pressure (AP). Regression analyses in Table 3 show a significant inverse association between fT3 levels and PP (β [95%CI]: -4.2 [-7.3 to -1.1] p = 0.008), which sustained after adjustment for confounding factors (-3.5 [-6.7 to -0.3] p = 0.031). For the association between fT3 and diastolic BP and Aortic AP, a trend was seen (-1.4 [-2.9 to 0.0] p = 0.052) while no such link became apparent for the associations between fT3 and systolic BP, Aortic augmentation index (Aix) and SEVR.

Cardiac CT-scans (n = 65) showed that 26.2, 15.4 and 13.9 percent of patients had CAC scores over 100, 400 and 800 AU, respectively. A dose response association was observed between fT3 tertiles and CAC scores; a significantly larger part of patients in the lower fT3 tertiles vs. those in the highest tertile had CAC scores >400 AU (Fig 2). Logistic regression analyses confirmed this association and showed that, per pmol/L increase in fT3, the adjusted odds [95% CI] for having CAC scores >400 AU was 0.19 [0.05–0.74, p = 0.017] lower. Linear regression analyses showed an average (95% CI) decrease in CAC scores (β: -179 [: -356 to -1] p = 0.048) with an increment of one pmol/L in fT3 concentration, persisting after adjustment (β: -164 [-329 to 1] p = 0.051). TSH levels showed an inverse association with CAC scores; (per logTSH increase, adjusted odds for having CAC levels >100 AU and >400 AU decreased with 0.3 [0.1–0.8, p = 0.019] and 0.4 [0.1–1.0, p = 0.049], respectively (S1 Appendix)). Higher fT4 levels associated with an increased adjusted odds for having CAC scores > 100 AU by 13 [1.0–1.6, p = 0.021]. Neither TSH nor fT4 levels were associated with parameters of vascular stiffness (S1 Appendix).

Thyroid hormones and plasma calcification inhibitors

With an increase in fT3 tertile, and although not statistically significant, a gradual increase in plasma levels of dp-uc-MGP and sKlotho was noted (Table 2). Plasma levels of t-ucMGP, and

Table 1. Baseline characteristics according to fT3 tertiles.

	Lowest tertile fT3 < 2.73 pmol/L n = 32	Middle tertile 2.73 > fT3 < 3.71 pmol/L n = 32	Highest tertile fT3 > 3.71 pmol/L n = 33	p-for trend
General characteristics				
Men, n %[¶]	17 (53.1)	19 (59.4)	26 (78.8)	0.095
Age, years[#]	46.2 (13.9)	44.5 (14.7)	44.5 (13.8)	0.414
CVD, n %[¶]	9 (28.1)	4 (12.5)	5 (15.2)	0.128
Diabetes Mellitus, n %[¶]	10 (31.3)	4 (12.5)	5 (15.2)	0.067
SGA, n %[1, ¶]	9 (30)	9 (29)	3 (9.7)	0.121
Previously on dialysis, %	19 (59.4)	22 (67.8)	22 (66.7)	0.789
Dialysis vintage, years[2,*]	1.0 (0.4–2.0)	0.7 (0.5–1.3)	1.0 (0.5–2.7)	0.709
Medication				
Beta-blockers, n %[¶]	17 (53.1)	20 (62.5)	17 (51.5)	0.987
Phosphate binders, n %[¶]	18 (56.3)	17 (53.0)	17 (51.5)	0.810
Laboratory measurements				
Albumin, g/L[#]	34.7 (3.0)	36.0 (3.6)	37.3 (3.5)	0.005
Interleukin-6, pg/L	1.1 (0.5–1.8)	0.9 (0.4–2.2)	1.3 (0.8–2.1)	0.429
PTH, pg/mL	296 (218–439)	222 (96–360)	196 (156–449)	0.118
Calcium, mmol/L[#]	2.3 (0.2)	2.3 (0.2)	2.3 (0.3)	0.541
Phosphate, mmol/L[#]	1.5 (0.3)	1.8 (0.7)	1.7 (0.5)	0.031
Thyroid hormone profile				
TSH, mIU/L[*]	0.75 (0.30–1.05)	0.76 (0.42–0.96)	1.20 (0.63–1.67)	0.106
fT3, pmol/L[#]	2.24 (0.37)	3.22 (0.27)	4.30 (0.53)	-
fT4, pmol/L[#]	15.27 (4.68)	15.39 (2.33)	14.92 (2.13)	0.801

1. SGA: subjective global assessment > 1.

2. Vintage was calculated in those who were previously on dialysis.

* Medians plus interquartile ranges (IQR). Differences were tested by means of a non-parametric trend test.

Means plus standard deviations (SD). Differences were tested by means of univariate regression analyses

¶ Numbers and percentages. Differences were tested by means of Chi squire tests.

doi:10.1371/journal.pone.0132353.t001

PIVKA did not differ between the different fT3 tertiles. Fig 3 illustrates scatterplots of the associations between fT3 and t-ucMGP (A), dp-ucMGP (B), PIVKA-II (C) and sKlotho (D). As shown in Table 3, results from linear regression analyses confirmed a statistically significant association between fT3 and dp-uc-MGP (β: 0.15 [0.05 to 0.25] p = 0.004) and between fT3 and sKlotho (β: 0.12 [0.01 to 0.22] p = 0.031). Only the association between fT3 and Log(dp-ucMGP) persisted after adjustment for age, sex, diabetes mellitus, cardiovascular disease, vintage, IL-6 levels, SGA, PIVKA-II and albumin levels. FT3 levels were not significantly associated with t-uc-MGP and PIVKA-II. fT4 and TSH concentrations were not associated with calcification inhibitor levels (S1 Appendix). As expected, PIVKA-II associated with dp-ucMGP (Rho = 0.32, p = 0.01).

Plasma calcification inhibitors and arterial stiffness/vascular calcification

Fig 4 illustrates percentages of individuals with CAC scores over 100 and 400 AU across tertiles of t-ucMGP (A), dp-ucMGP (B), PIVKA-II (C), and sKlotho (D). For sKlotho, a larger part of

Table 2. Vascular calcification characteristics of patients according to fT3 status.

	Lowest tertile	Middle tertile	Highest tertile	p-for trend
	fT3 < 2.67 pmol/L	fT3 2.67–3.79 pmol/L	fT3 > 3.79 pmol/L	
	n = 32	n = 32	n = 33	
Arterial stiffness				
Systolic BP, mmHg [#]	142 (24)	138(18)	140(20)	0.531
Diastolic BP, mmHg[#]	80 (12)	82(11)	86(11)	0.058
Pulse pressure, mmHg [#]	63(16)	57(13)	54(14)	0.014
Aortic AP, mmHg [*,1]	7.0(4.0–12.0)	7.0(3.0–10.0)	6.0(0.0–11.0)	0.079
Aortic Aix, mmHg[*,1]	19.0(12.0–23.0)	20.0 (9.0–25.0)	16.0(1.0–25.0)	0.388
SEVR, % [*,1]	149.2(29.7)	137.0(28.6)	150.8(33.8)	0.830
Serum calcification inhibitors				
t-ucMGP, nmol.L [*,1]	2932(2017–3737)	2690(2160–3507)	2703(1800–3553)	0.675
dp-ucMGP, pmol/L [*,1]	1543(1194–2082)	1723(1274–2419)	2301(2011–3428)	0.001
PIVKA-II, mAU/mL [*,2]	39.5(26.0–54.0)	32.0(21.0–44.0)	43.0(28.5–56.0)	0.675
sKlotho, pg/ml [*]	315(239–513)	324(225–414)	421(363–561)	0.129

BP; Blood pressure, AP: aortic augmentation pressure, Aix: aortic augmentation index, SEVR: Subendocardial viability ratio.

1. Measurements were available in 28, 24 and 27 patients respectively.

2. Measurements were available in 18, 19 and 24 patients respectively.

* Medians plus interquartile ranges (IQR). Differences were tested by means of a non-parametric trend test.

Means plus standard deviations (SD). Differences were tested by means of univariate regression analyses

doi:10.1371/journal.pone.0132353.t002

patients in the lower tertiles had CAC scores >400 AU, although this association did not reach statistical significance (p-for trend = 0.226). With regards to measures of arterial stiffness we found that higher dp-ucMGP levels were associated with a higher aortic AP in crude (β: 3.7 [0.2 to 6.8] p = 0.021) but not in adjusted models (β: 2.2 [-1.0 to 5.4] p = 0.179). No other associations between plasma calcification inhibitors and vascular calcification/arterial stiffness were found.

Discussion

In a selected group of younger ESRD patients, fT3 levels were inversely associated with CAC scores and measures of arterial stiffness. Moreover, fT3 levels were positively associated with circulating levels of dp-uc-MGP and Klotho. However, neither sKlotho, dp-ucMGP nor PIVKA-II were associated with CAC scores and arterial stiffness.

We also report markedly elevated median PIVKA-II and dp-ucMGP levels and that these markers for vitamin K status (hepatic and vascular vitamin K status, respectively) were positively associated.[22] Thus, our data support previous literature that ESRD patients have severe vitamin K deficiency and are at high risk of vascular calcification.[23] Average circulating levels of dp-ucMGP (measured in the same laboratory) were reported to be markedly lower (about 400 pmol/L) in healthy volunteers of the same age group. [24] Impaired carboxylation of MGP (i.e. high levels of dp-ucMGP) has previously been reported to be associated with intimal and medial vascular calcification also in other patient populations prone to calcification, such as patients with aortic stenosis [25]. Although circulating levels of t-ucMGP were markedly lower in ESRD patients investigated in the present study than in healthy controls [6], we report no difference in CAC scores and grades of arterial stiffness across the range of t-ucMGP levels.

Table 3. Regression analyses on the associations between fT3 and measures of arterial stiffness and calcification inhibitors.

|	Crude model		Adjusted[1]	
	Beta (95%CI)	p-value	Beta (95%CI)	p-value
Linear regression analyses				
Arterial stiffness				
Systolic BP [#]	-1.8(-6.2 to 2.6)	0.423	-1.6(-6.2 to 3.0)	0.485
Diastolic BP	2.4(-0.2 to 5.0)	0.066	1.9(-0.3 to 9.5)	0.067
Pulse pressure	-4.2(-7.3 to -1.1)	0.008	-3.5(-6.7 to -0.3)	0.031
Aortic AP	-1.4(-2.9 to 0.0)	0.052	-1.3(-2.8 to 0.2)	0.081
Aortic Aix	-1.8(-4.2 to 0.7)	0.153	-1.8(-4.3 to 0.7)	0.159
SEVR	1.8(-5.4 to 9.0)	0.624	0.8(-7.6 to 9.1)	0.858
Laboratory parameters				
Log(t-uc-MGP)	-0.04(-0.16 to 0.07)	0.443	0.00(-0.13 to 0.13)	0.971
Log(dp-uc-MGP)	0.15(0.05 to 0.25)	0.004	0.13(0.02 to 0.24)	0.019
Log(PIVKA)	0.06(-0.06 to 0.19)	0.312	0.05(-0.10 to 0.21)	0.482
Log(sKlotho)	0.12(0.01 to 0.22)	0.031	0.09(-0.04 to 0.21)	0.187
Logistic regression analyses[2]				
CAC > 100	0.58(0.31 to 1.09)	0.090	0.34(0.13 to 0.92)	0.033
CAC > 400	0.35(0.14 to 0.88)	0.026	0.19(0.05 to 0.74)	0.017

BP; Blood pressure, AP: aortic augmentation pressure, Aix: aortic augmentation index, SEVR: subendocardial viability ratio.

1 Adjusted for sex, age, diabetes mellitus, IL-6, vintage, and SGA.

2 The logistic regression analyses for the association between fT3 and CAC scores were adjusted for age and sex.
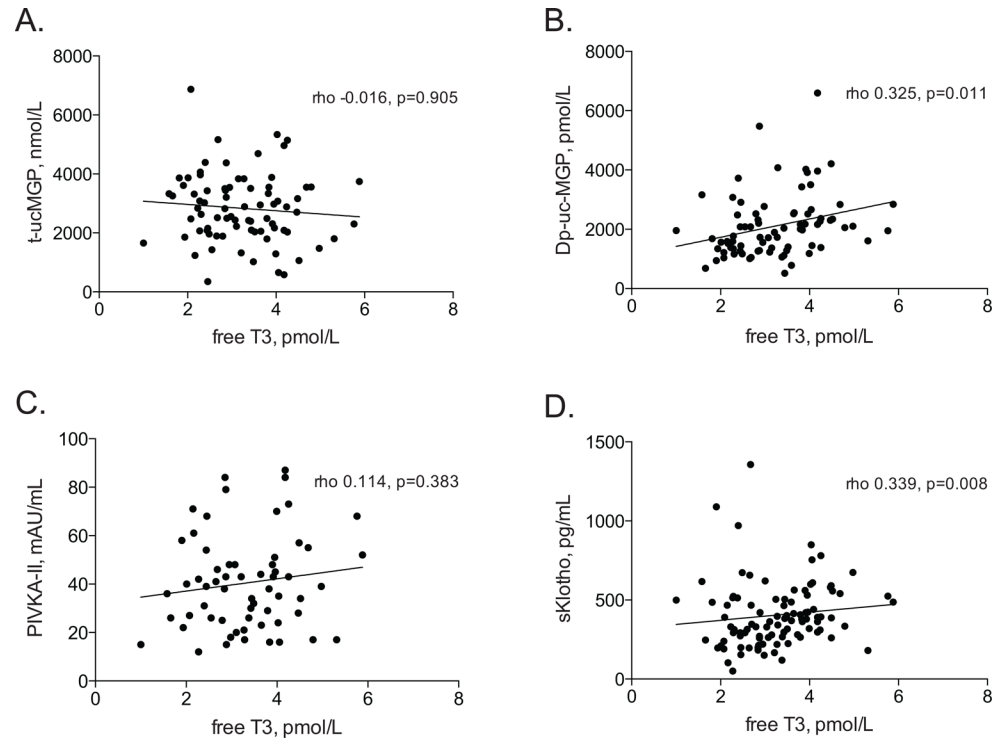
doi:10.1371/journal.pone.0132353.t003

The proportion of patients with elevated CAC scores was lower in the current cohort than in previous studies in patients on peritoneal dialysis (PD) [13, 26, 27] and hemodialysis (HD) [28]. This divergence could be explained by the selective nature of our cohort as LD-RTx recipients are selected by virtue of their younger age, relatively good physical condition and low
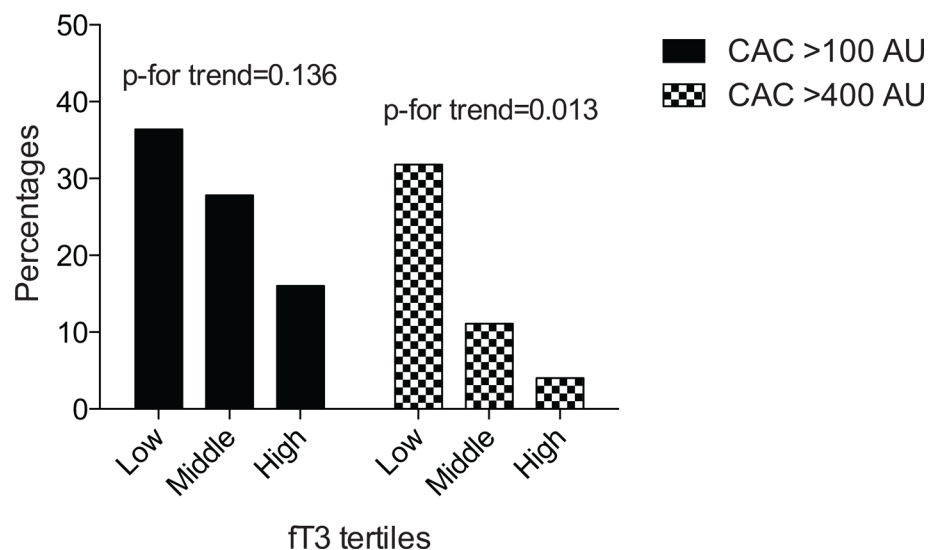
Fig 2. Coronary artery calcification scores across tertiles of fT3.
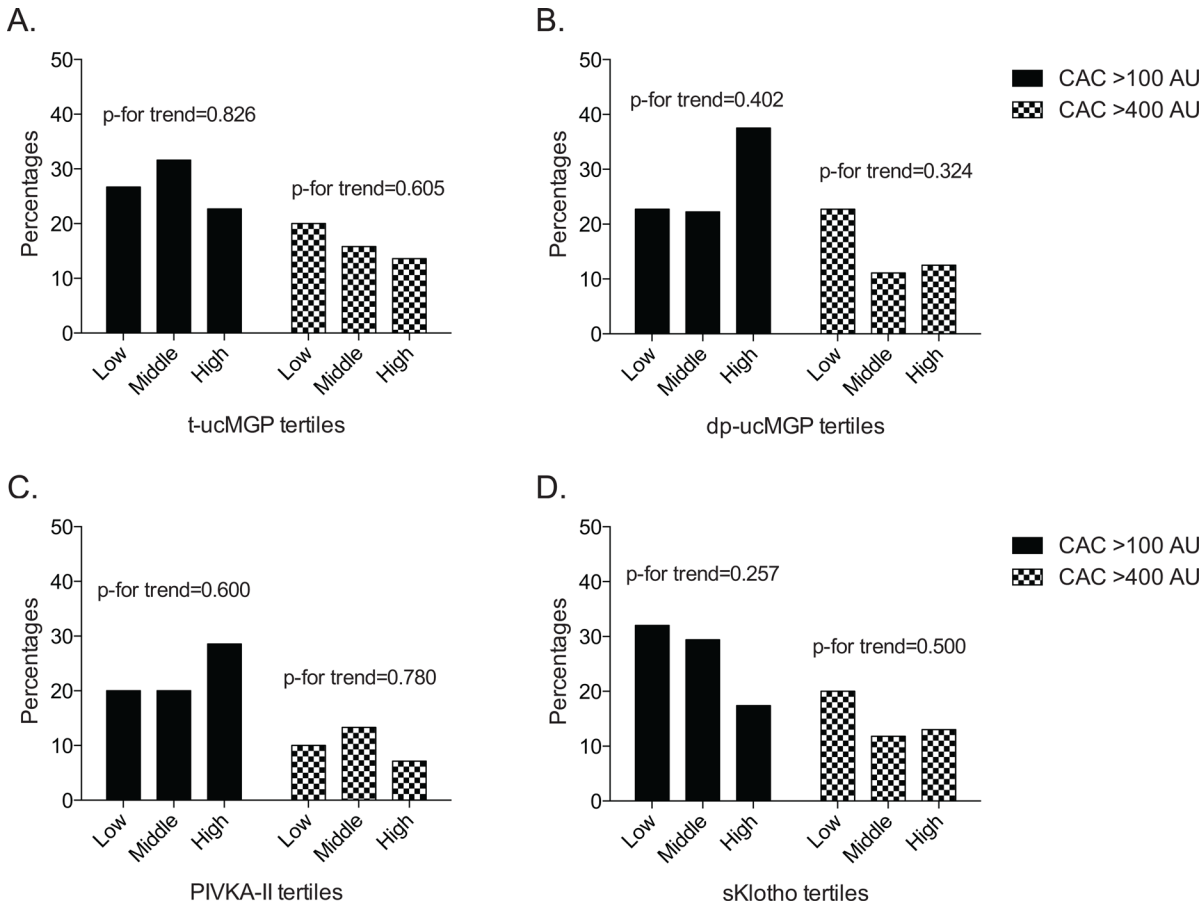
doi:10.1371/journal.pone.0132353.g002

Fig 3. Associations between fT3 and t-ucMGP (A), dp-ucMGP (B), PIVKA (C), and sKlotho (D).

comorbidity burden. Also, they have often spent little or no time on maintenance dialysis. A comparison of baseline characteristics between the different cohorts indeed supports this reasoning. Our data show that fT3 levels were inversely associated with CAC scores and arterial stiffness, a finding consistent with our previous report in prevalent dialysis patients.[13] In adjusted analyses, we also observed a statistically significant association in which lower TSH levels were accompanied by higher CAC scores. This observation fits the concept of non-thyroidal illness in which a general down-regulation of the hypothalamic-pituitaric-thyroidal (HPT)-axis occurs in more severe forms and a fT3 levels corresponds to lower TSH levels.[29]

Given prior observations of an up-regulation of MGP [16] and Klotho [17] by stimulation of fT3 in vitro and their established function as calcification inhibitors, we hypothesized on a possible intermediating role of MGP and sKlotho in the causal path between non-thyroidal illness and vascular calcification (Fig 1). The current study is the first to address this hypothesis in the setting of uremia. We observed that fT3 levels were positively associated with dp-ucMGP, supporting the hypothesis of an increased expression of MGP upon fT3 stimulation as suggested by Sato et al. [16]. Combined with limited availability of vitamin K in the uremic milieu the extra synthesis of MGP, driven by fT3, could lead to higher levels of dp-ucMGP. Intriguing in the interpretation of this association is that the uremic milieu is typified by elevated dp-ucMGP rather than low levels [6], which in turn have been associated with higher mortality hazards.[30] This association could therefore not intermediate the link between non-thyroidal illness and an increased vascular calcification in ESRD and implies much more complex interactions. Intervention studies with thyroid hormones in vitamin K-replete controls and vitamin K deficient uremic subjects to study changes in total and dp-ucMGP may be one way to resolve this.

Fig 4. Percentage of patients with elevated CAC scores > 100 and > 400 across t-ucMGP (A), dp-ucMGP (B), PIVKA (C), and sKlotho (D) tertiles.

In this selected cohort, t-ucMGP and dp-ucMGP levels were not associated with surrogate markers of vascular calcification, such as CAC score, or arterial stiffening, which contrasts to previous findings in older and sicker ESRD patients.[31, 32] When directly scoring degree of medial calcification in arterial biopsies however, significantly higher levels of uc-dpMGP were found in younger ESRD patients with moderate and severe vascular calcification assessed by histology of abdominal arterial biopsies (unpublished observation Stenvinkel et al. 2015). It could be speculated that CAC score reflects intimal coronary calcification whereas dp-ucMGP concentrations are more associated with the risk of medial vascular calcification. Published studies demonstrate a high sensitivity of CAC for the presence of coronary artery disease but a lower specificity for obstructive CAC. [33] Moreover, previous autopsy studies have shown that the correlation between femoral and coronary atherosclerosis was weak, especially in younger individuals. [34, 35] Finally, although higher dp-ucMGP predicted higher total, non-cancer and cardiovascular mortality in a recent study of 2318 participants from a Flemish population study, it predicted a lower risk for coronary events [36]. Although this discrepant finding may reflect bias due to competing events, it may also imply a differential impact on different outcomes.

In the present study fT3 levels were significantly associated with sKlotho concentrations, a finding that partly accords with a previous in vitro study on murine adipocytes showing an increased synthesis of the membrane-bound form of Klotho upon T3 stimulation.[17] The

same study however failed to observe a concomitant increase in sKlotho levels.[17] We have previously demonstrated that the kidney is the principal source of sKlotho, and that renal expression of membrane-bound Klotho correlate closely to sKlotho levels.[37] However, in the uremic milieu the correlation between the membrane-bound isoform and sKlotho is weak or absent and most studies have failed to find a consistent correlation between sKlotho and renal function.[38] This could speculatively be explained by altered shedding of membrane-bound Klotho in the uremic milieu, or by methodological problems with the current assays. In our analyses, a weak inverse trend was observed between sKlotho tertiles and CAC scores, which accords with an animal study describing higher vascular calcium content in Klotho-haploinsufficient mice with CKD versus wild type equivalents.[8] In contrast, sKlotho levels were not independently associated with the presence of cardiovascular disease in a Dutch sample of 127 dialysis patients.[39] Nevertheless, the current association between thyroid hormones and the calcification process implicates a role for fT3 in regulation of sKlotho. In line with this, a recent study in rats with calcified aortas due to the effects of nicotine and vitamin D3 found that T3 supplementation resulted in an upregulation of Klotho levels and fast reduction in aortic tissue calcium content with improvement of hemodynamic function.[40]

Although our study was conducted in a carefully phenotyped cohort of ESRD patients, several limitations must be discussed. Firstly, analyses were cross-sectional whereby limiting the possibility of causal inference. Secondly, whereas LD-Rtx recipients are younger, less sedentary and with lower comorbidity, CAC scores and arterial stiffness were on average less severe than in older and sicker prevalent dialysis patients. This reduces the power of analysis by a lower number of cases. Nevertheless, associations between fT3 and CAC scores were of convincing strength. Thirdly, in our analyses we assumed that blood levels of calcification inhibitors and thyroid hormones reflect the situation at a tissue level. This may however be more complex as several modulating steps take place on a tissue level.

In summary, we found fT3, as an indicator of nonthyroidal illness, to be inversely associated with CAC scores and measures of with arterial stiffness in this selected cohort of younger ESRD patients. Whereas fT3 associated positively with serum sKlotho and dp-ucMGP levels, sKlotho, t-ucMGP, dp-ucMGP and PIVKA-II levels did not associate with CAC scores and arterial stiffness. Given the complexity of this association, studies measuring vascular calcification inhibitory status at a tissue level as well as longitudinal studies are needed to provide further clarification on this possible pathway.

Supporting Information

S1 Appendix. Regression analyses on the associations between fT4 and TSH and measures of arterial stiffness/coronary calcification and calcification inhibitors.
(DOCX)

S2 Appendix. Kärl-Tx data file.
(XLSX)

Author Contributions

Conceived and designed the experiments: CLM HO ARQ JR PB CV ND PS. Performed the experiments: HO JR CV ND PS. Analyzed the data: CLM HO ARQ CV ND PS. Contributed reagents/materials/analysis tools: CLM HO PB CV PS. Wrote the paper: CLM HO ARQ JR PB CV ND PS. Extensive discussions about the content and interpretation of the data: CLM HO ARQ JR PB CV ND PS.

References

1. Stenvinkel P. Chronic kidney disease: a public health priority and harbinger of premature cardiovascular disease. J Intern Med. 2010; 268(5): 456–67. doi: 10.1111/j.1365-2796.2010.02269.x PMID: 20809922

2. Stenvinkel P, Carrero JJ, Axelsson J, Lindholm B, Heimburger O, Massy Z. Emerging biomarkers for evaluating cardiovascular risk in the chronic kidney disease patient: how do new pieces fit into the uremic puzzle? Clin J Am Soc Nephrol. 2008; 3(2): 505–21. doi: 10.2215/CJN.03670807 PMID: 18184879

3. Lanzer P, Boehm M, Sorribas V, Thiriet M, Janzen J, Zeller T, et al. Medial vascular calcification revisited: review and perspectives. Eur Heart J. 2014; 35(23): 1515–25. doi: 10.1093/eurheartj/ehu163 PMID: 24740885

4. Shanahan CM. Mechanisms of vascular calcification in CKD-evidence for premature ageing? Nat Rev Nephrol. 2013; 9(11): 661–70. doi: 10.1038/nrneph.2013.176 PMID: 24018414

5. Wallin R, Schurgers LJ, Loeser RF. Biosynthesis of the vitamin K-dependent matrix Gla protein (MGP) in chondrocytes: a fetuin-MGP protein complex is assembled in vesicles shed from normal but not from osteoarthritic chondrocytes. Osteoarthritis Cartilage. 2010; 18(8): 1096–103. doi: 10.1016/j.joca.2010.05.013 PMID: 20510384

6. Cranenburg EC, Koos R, Schurgers LJ, Magdeleyns EJ, Schoonbrood TH, Landewe RB, et al. Characterisation and potential diagnostic value of circulating matrix Gla protein (MGP) species. Thromb Haemost. 2010; 104(4): 811–22. doi: 10.1160/TH09-11-0786 PMID: 20694284

7. Urakawa I, Yamazaki Y, Shimada T, Iijima K, Hasegawa H, Okawa K, et al. Klotho converts canonical FGF receptor into a specific receptor for FGF23. Nature. 2006; 444(7120):770–4. PMID: 17086194

8. Hu MC, Shi M, Zhang J, Quinones H, Griffith C, Kuro-o M, et al. Klotho deficiency causes vascular calcification in chronic kidney disease. J Am Soc Nephrol. 2011; 22(1): 124–36. doi: 10.1681/ASN.2009121311 PMID: 21115613

9. Kaptein EM. Thyroid hormone metabolism and thyroid diseases in chronic renal failure. Endocr Rev. 1996; 17(1): 45–63. PMID: 8641223

10. Zoccali C, Mallamaci F, Tripepi G, Cutrupi S, Pizzini P. Low triiodothyronine and survival in end-stage renal disease. Kidney Int. 2006; 70(3): 523–8. PMID: 16775599

11. Tatar E, Kircelli F, Asci G, Carrero JJ, Gungor O, Demirci MS, et al. Associations of triiodothyronine levels with carotid atherosclerosis and arterial stiffness in hemodialysis patients. Clin J Am Soc Nephrol. 2011; 6(9): 2240–6. doi: 10.2215/CJN.02540311 PMID: 21836150

12. Tatar E, Sezis DM, Kircelli F, Gungor O, Yaprak M, Asci G, et al. The association between thyroid hormones and arterial stiffness in peritoneal dialysis patients. Int Urol Nephrol. 2011; 44(2): 601–6. doi: 10.1007/s11255-011-0034-7 PMID: 21779917

13. Meuwese CL, Carrero JJ, Cabezas-Rodriguez I, Heimburger O, Barany P, Lindholm B, et al. Non-thyroidal illness: a risk factor for coronary calcification and arterial stiffness in patients undergoing peritoneal dialysis. J Intern Med. 2013; 274(6): 584–93. doi: 10.1111/joim.12107 PMID: 23815158

14. Zhang Y, Kim BK, Chang Y, Ryu S, Cho J, Lee WY, et al. Thyroid hormones and coronary artery calcification in euthyroid men and women. Art, Thromb, and Vasc Biol. 2014; 34(9): 2128–34.

15. Meuwese CL, Dekkers OM, Stenvinkel P, Dekker FW, Carrero JJ. Nonthyroidal illness and the cardiorenal syndrome. Nat Rev Nephrol. 2013; 9(10): 599–609. doi: 10.1038/nrneph.2013.170 PMID: 23999398

16. Sato Y, Nakamura R, Satoh M, Fujishita K, Mori S, Ishida S, et al. Thyroid hormone targets matrix Gla protein gene associated with vascular smooth muscle calcification. Circ Res. 2005; 97(6): 550–7. PMID: 16100044

17. Mizuno I, Takahashi Y, Okimura Y, Kaji H, Chihara K. Upregulation of the klotho gene expression by thyroid hormone and during adipose differentiation in 3T3-L1 adipocytes. Life Sci. 2001; 68(26): 2917–23. PMID: 11411791

18. Meuwese CL, Dekker FW, Lindholm B, Qureshi AR, Heimburger O, Barany P, et al. Baseline levels and trimestral variation of triiodothyronine and thyroxine and their association with mortality in maintenance hemodialysis patients. Clin J Am Soc Nephrol. 2012; 7(1): 131–8. doi: 10.2215/CJN.05250511 PMID: 22246282

19. Agatston AS, Janowitz WR, Hildner FJ, Zusmer NR, Viamonte M Jr., Detrano R. Quantification of coronary artery calcium using ultrafast computed tomography. J Am Coll Cardiol. 1990; 15(4): 827–32. PMID: 2407762

20. Chen CH, Nevo E, Fetics B, Pak PH, Yin FC, Maughan WL, et al. Estimation of central aortic pressure waveform by mathematical transformation of radial tonometry pressure. Validation of generalized transfer function. Circulation. 1997; 95(7): 1827–36. PMID: 9107170

21. Rumberger JA, Brundage BH, Rader DJ, Kondos G. Electron beam computed tomographic coronary calcium scanning: a review and guidelines for use in asymptomatic persons. Mayo Clin Proc. 1999; 74 (3): 243–52. PMID: 10089993

22. Cranenburg EC, Schurgers LJ, Uiterwijk HH, Beulens JW, Dalmeijer GW, Westerhuis R, et al. Vitamin K intake and status are low in hemodialysis patients. Kidney Int. 2012; 82(5): 605–10. doi: 10.1038/ki. 2012.191 PMID: 22648294

23. Krueger T, Westenfeld R, Ketteler M, Schurgers LJ, Floege J. Vitamin K deficiency in CKD patients: a modifiable risk factor for vascular calcification? Kidney Int. 2009; 76(1): 18–22. doi: 10.1038/ki.2009. 126 PMID: 19387474

24. Theuwissen E, Magdeleyns EJ, Braam LA, Teunissen KJ, Knapen MH, Binnekamp IA, et al. Vitamin K status in healthy volunteers. Food & Function. 2014; 5(2): 229–34.

25. Ueland T, Gullestad L, Dahl CP, Aukrust P, Aakhus S, Solberg OG, et al. Undercarboxylated matrix Gla protein is associated with indices of heart failure and mortality in symptomatic aortic stenosis. J Intern Med. 2010; 268(5): 483–92. doi: 10.1111/j.1365-2796.2010.02264.x PMID: 20804515

26. Ammirati AL, Dalboni MA, Cendoroglo M, Draibe SA, Fernandes Canziani ME. Coronary artery calcification, systemic inflammation markers and mineral metabolism in a peritoneal dialysis population. Nephron Clin Pract. 2006; 104(1): c33–c40. PMID: 16685142

27. Stompor T, Rajzer M, Pasowicz M, Krasniak A, Sulowicz W, Kawecka-Jaszcz K, et al. Coronary artery calcification, common carotid artery intima-media thickness and aortic pulse wave velocity in patients on peritoneal dialysis. Int J Artif Organs. 2006; 29(8): 736–44. PMID: 16969750

28. Barreto DV, Barreto FC, Carvalho AB, Cuppari L, Cendoroglo M, Draibe SA, et al. Coronary calcification in hemodialysis patients: the contribution of traditional and uremia-related risk factors. Kidney Int. 2005; 67(4): 1576–82. PMID: 15780114

29. Warner MH, Beckett GJ. Mechanisms behind the non-thyroidal illness syndrome: an update. J Endocrinol. 2010; 205(1): 1–13. doi: 10.1677/JOE-09-0412 PMID: 20016054

30. Schlieper G, Westenfeld R, Kruger T, Cranenburg EC, Magdeleyns EJ, Brandenburg VM, et al. Circulating nonphosphorylated carboxylated matrix gla protein predicts survival in ESRD. J Am Soc Nephrol. 2011; 22(2): 387–95. doi: 10.1681/ASN.2010040339 PMID: 21289218

31. Cranenburg EC, Brandenburg VM, Vermeer C, Stenger M, Muhlenbruch G, Mahnken AH, et al. Uncarboxylated matrix Gla protein (ucMGP) is associated with coronary artery calcification in haemodialysis patients. ThrombHaemost. 2009; 101(2): 359–66.

32. Delanaye P, Krzesinski JM, Warling X, Moonen M, Smelten N, Medart L, et al. Dephosphorylated-uncarboxylated Matrix Gla protein concentration is predictive of vitamin K status and is correlated with vascular calcification in a cohort of hemodialysis patients. BMCNephrol. 2014; 15: 145.

33. Budoff MJ, Gul KM. Expert review on coronary calcium. Vascular Health and Risk Manag. 2008; 4(2): 315–24.

34. Mitchell JR, Schwartz CJ. Relationship between arterial disease in different sites. A study of the aorta and coronary, carotid, and iliac arteries. BMJ. 1962; 1(5288): 1293–301. PMID: 14474632

35. Sollberg LA, McGarry PA, Moossy J, Strong JP, Tejada C, Loken AC. Severity of atherosclerosis in cerebral arteries, coronary arteries, and aortas. Ann New York Acad of Sci. 1968; 149(2): 956–73.

36. Liu YP, Gu YM, Thijs L, Knapen MH, Salvi E, Citterio L, et al. Inactive matrix gla protein is causally related to adverse health outcomes: a mendelian randomization study in a flemish population. Hypertension. 2015; 65(2): 463–70. doi: 10.1161/HYPERTENSIONAHA.114.04494 PMID: 25421980

37. Olauson H, Larsson TE. FGF23 and Klotho in chronic kidney disease. Curr Opin Nephrol Hypertens. 2013; 22(4): 397–404. doi: 10.1097/MNH.0b013e32836213ee PMID: 23666415

38. Seiler S, Wen M, Roth HJ, Fehrenz M, Flugge F, Herath E, et al. Plasma Klotho is not related to kidney function and does not predict adverse outcome in patients with chronic kidney disease. Kidney Int. 2013; 83(1): 121–8. doi: 10.1038/ki.2012.288 PMID: 22895520

39. Buiten MS, de Bie MK, Bouma-de KA, van DB, Dekker FW, Jukema JW, et al. Soluble Klotho is not independently associated with cardiovascular disease in a population of dialysis patients. BMC Nephrol. 2014; 15(1): 197.

40. Zhang J, Chang JR, Duan XH, Yu YR, Zhang BH. Thyroid hormone attenuates vascular calcification induced by vitamin D3 plus nicotine in rats. Calc Tiss Int. 2015; 96(1): 80–7.