Tohoku J. Exp. Med., 2015, **237**, 17-23

Low Serum Level of Klotho Is an Early Predictor of Atherosclerosis

Nursen Keles,[1] Mustafa Caliskan,[1] Burcu Dogan,[2] Necibe Nur Keles,[3] Macit Kalcik,[4] Feyza Aksu,[1] Osman Kostek,[5] Soe Moe Aung,[6] Banu Isbilen[7] and Aytekin Oguz[5]

[1]Department of Cardiology, Goztepe Training and Research Hospital, Istanbul Medeniyet University, Istanbul, Turkey
[2]Department of Family Physicians, Goztepe Training and Research Hospital, Istanbul Medeniyet University, Istanbul, Turkey
[3]Eyup Government Hospital Neurology Clinics, Istanbul, Turkey
[4]Department of Cardiology, Iskilip Atif Hoca State Hospital, Corum, Turkey
[5]Department of Internal Medicine, Goztepe Training and Research Hospital, Istanbul Medeniyet University, Istanbul, Turkey
[6]Department of Cardiology, Fatih University, Istanbul, Turkey
[7]Department of Biochemistry, Goztepe Training and Research Hospital, Istanbul Medeniyet University, Istanbul, Turkey

The Klotho gene, identified as an 'aging suppressor' gene, encodes a single-pass transmembrane protein. The extracellular domain of Klotho is cleaved and released in the blood stream, where it may function as a vasculoprotective hormone. Carotid artery intima-media thickness (CIMT), flow-mediated dilation (FMD) of the brachial artery and epicardial fat thickness (EFT) have been reported as early predictors of atherosclerosis. We aimed to investigate the relationship between serum Klotho levels and early atherosclerotic predictors, including EFT, FMD and CIMT in healthy adults. Fifty healthy volunteers were enrolled in this study, consisting of 21 males and 29 females with median age of 32 years. They were free of known risk factors for cardiovascular diseases. Serum Klotho levels were determined by the ELISA method. The study population was divided into two groups (n = 25 for each) according to the median serum Klotho level (459.4 pg/mL): higher Klotho (HK) group (613.6 pg/mL; ranges of 501.2-772.6 pg/mL) and lower Klotho (LK) group (338.7 pg/mL; ranges of 278.8-430.3 pg/mL). EFT was measured by transthoracic echocardiography, and CIMT and FMD were measured with standard procedures. The LK group showed lower values of FMD ($p = 0.012$) and larger values of EFT ($p = 0.01$) and CIMT ($p < 0.001$), compared to the HK group. Thus, the low serum Klotho levels were associated with increased EFT and CIMT and with the decreased FMD in the study population. We propose that the lower serum Klotho level is a newly identified predictor of atherosclerosis.

Keywords: atherosclerosis; carotid artery intima-media thickness; epicardial fat thickness; flow-mediated dilation; Klotho
Tohoku J. Exp. Med., 2015 September, **237** (1), 17-23.

Introduction

The anti-aging properties of the Klotho gene are well known. The gene is mainly expressed in the distal convoluted tubules of the kidney, parathyroid glands and choroid plexus of the brain (Kuro-o et al. 1997). The protein was originally identified in a mutant mouse strain lacking the Klotho gene, which developed multiple disorders resembling human aging and had a shortened life span (Kuro-o et al. 1997). In an atherosclerotic mouse model, in vivo gene delivery of Klotho protected against endothelial dysfunction (Saito et al. 2000). Overexpression of Klotho in transgenic mice significantly extended their life span when compared to that of wild-type mice (Kurosu et al. 2005). The Klotho gene encodes a single-pass transmembrane protein (Kuro-o 2011). The extracellular portion of Klotho is cleaved and released to the blood stream where it may function as a vasculoprotective hormone possibly by improving endothelial function (Moe and Drueke 2008). A recent study showed that higher Klotho levels were associated

Received April 3, 2015; revised and accepted July 23, 2015. Published online August 20, 2015; doi: 10.1620/tjem.237.17.
Correspondence: Nursen Keles, M.D., Department of Cardiology, Goztepe Training and Research Hospital, Istanbul Medeniyet University, Doktor Erkin Caddesi, Kadiköy, Istanbul, Turkey.
e-mail: drnursenkeles@yahoo.com.tr

with lower prevalence of cardiovascular disease (Semba et al. 2011).

Epicardial adipose tissue is a visceral fat tissue, deposited around the heart on the free wall of the right ventricle, left ventricular apex and atrium. A recent study showed that epicardial adipose tissue plays an important role in the development and progression of coronary artery disease (Schejbal 1989).

The structure of the coronary artery wall is dynamic. By increasing its external diameter, the internal lumen size can be maintained, even during the development of atherosclerotic plaques (Glagov et al. 1987; Davies 1996). An increase in the carotid artery intima-media thickness (CIMT) is regarded as a marker of early atherosclerosis (Persson et al. 1994). Endothelial dysfunction is also thought to be an early event in the atherosclerotic process. Flow-mediated dilation (FMD) has been used as a non-invasive method for detecting endothelial dysfunction (Anderson and Mark 1989; Laurent et al. 1990; Celermajer et al. 1992; Sorensen et al. 1995).

In the present study, we investigated the relationship between serum Klotho levels of adults and early predictors of cardiovascular diseases, such as epicardial fat thickness (EFT), FMD and CIMT. We aimed to determine whether high Klotho serum levels are protective against atherosclerosis.

Methods

Study population

Fifty healthy volunteers were included in this study: 21 males and 29 females with median age of 32 years (ranges of 27-38 years). Each volunteer was questioned about the major cardiovascular risk factors. The exclusion criteria were the presence of coronary artery disease; hypertension; obstructive sleep apnea; stroke; congestive heart failure; systemic diseases, such as hematologic, hepatic and renal diseases; diabetes mellitus, impaired glucose tolerance, dyslipidaemia, excessive alcohol consumption (> 120 g/day) and obesity, body mass index (BMI) of > 30 kg/m². Other exclusion criteria were using any vasoactive drugs, having an ST segment or T-wave changes specific for myocardial ischemia and a pathological Q wave and left bundle branch block on electrocardiography (ECG).

Classification of participants according to their serum Klotho levels

The study population was divided into two subgroups according to their serum Klotho levels. The median serum Klotho level of the study population was defined, and the division of the study population was done according to this median serum Klotho value. Patients who had higher than median Klotho levels were included in the higher Klotho (HK) group and those with lower than median Klotho levels were included in the lower Klotho (LK) group. There were 25 volunteers in each subgroup.

The study was performed according to the recommendations set forth by the Declaration of Helsinki on Biomedical Research Involving Human Subjects. Written informed consent was obtained from each subject, and the institutional ethics committee approved the study protocol.

Biochemical assessment

Venous blood samples were obtained from each patient after overnight fasting, a 24-h period of abstinence from alcohol and vigorous physical exercise for the determination of serum biochemical parameters. Total cholesterol, high-density lipoprotein, low-density lipoprotein and triglyceride were measured using enzymatic methods. Serum fasting glucose, urea, creatinine, protein, albumin, parathyroid hormone, alkaline phosphatase, haemoglobin A1c and 25 (OH) vitamin D values were also measured.

Measurement of serum Klotho level

Blood samples were left to clot and then centrifuged at 1,500 g for 10 min. Serum samples were stored at −80°C until analysis, and serum Klotho levels were measured within 2 months after the serum samples were obtained. Serum Klotho was determined with a Klotho ELISA kit (Cusabio Biotech Co., Ltd., P.R. China), according to the manufacturer's guideline. The minimum detectable level of the test was 0.156 ng/mL. The intra- and inter-assay coefficients of variability of the test were < 8% and < 10%, respectively. Pedersen et al. (2013) found that normal mean values of serum Klotho level were 472 pg/mL (2.5-97.5% reference limits; 204-741 pg/mL) with no difference between genders by using ELISA method.

Echocardiographic examination

Transthoracic echocardiographic examination was performed with a GE Vivid 7 (Horten, Norway) echocardiography machine. Two-dimensional, M-mode and tissue Doppler echocardiographic examinations were performed. The left ventricular (LV) ejection fraction (EF), LV end-diastolic diameter (LVEDD), LV end-systolic diameter (LVESD), end-diastolic interventricular septal (IVS) and posterior wall (PW) thicknesses were measured on the parasternal long-axis view. All measurements were done on M-mode images.

The early diastolic peak flow velocity (E), late diastolic peak flow velocity (A) and E wave deceleration time were measured using transmitral Doppler imaging. The pulsed-wave Doppler mode was used for the Doppler tissue-imaging (DTI). Filters were set to exclude high-frequency signals, and the Nyquist limit was adjusted to a velocity range of −15 to −20 cm/s. Gains were minimized to achieve minimal background noise. All the DTI recordings were obtained during normal respiration. The velocities were recorded by placing a 5-mm sample volume on the lateral side of the mitral annulus, and they were recorded for 5-10 cardiac cycles at a sweep speed of 100 mm/s. Indexes of regional systolic function, namely, the time velocity integral of the myocardial systolic (Sm) wave and diastolic function parameters, such as myocardial early (Em) and atrial peak (Am) velocities (m/s), were measured. The isovolumic relaxation time (IVRT) was measured as the time interval between the end of Sm and the onset of Em. All regional systolic and diastolic function parameters were measured in three consecutive cardiac cycles and averaged (Sohn et al. 1997). The same blinded investigator performed the echocardiography, and two blinded cardiologists analysed the echocardiogram recordings.

Measurement of epicardial fat thickness

The EFT was measured by transthoracic echocardiography as a hypoechoic space on the free wall of the right ventricle from the parasternal long-axis views at end-diastole and averaged over three cardiac cycles (Fig. 1A).

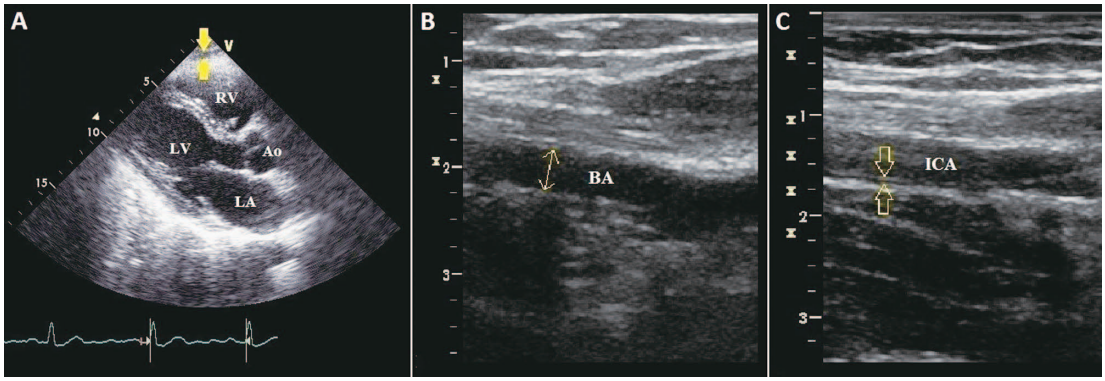

Fig. 1. Measurements of EFT, CIMT and FMD.
Images from a subject in the higher Klotho group summarizing the measurement of epicardial fat thickness by means of transthoracic echocardiography as a hypoechoic space on the free wall of the right ventricle from parasternal long-axis views at end-diastole (A), measurement of brachial artery diameter at end diastole when maximum lumen diameter was visualized longitudinally using a linear array transducer (B) and measurement of carotid intima-media thickness as the distance between the media-adventitia interface and the lumen-intima interface of the carotid artery (C). Ao, aorta; BA, brachial artery; ICA, internal carotid artery; LA, left atrium; LV, left Ventricle; RV, right ventricle.

Vascular assessment

The FMD of the brachial artery in response to transient ischemia was studied using high-resolution ultrasound (Harris et al. 1995; Avena et al. 1998). Each patient was placed in a supine position for 10 min of rest before the measurement. The patient's right arm was relaxed in an extended position, and the FMD of the brachial artery was measured 2-5 cm above the antecubital fossa. The brachial artery was visualized longitudinally using a 17-5 MHz linear array transducer (GE Vivid 7, Horten, Norway), and the B-mode and pulsed Doppler spectral curve were recorded. The baseline diameter of the brachial artery was measured, and a cuff was placed around the forearm distal to the imaged artery segment. The cuff was inflated to about 30 mmHg above the systolic blood pressure for 5 min. The diameter of the maximal brachial artery was determined from six recordings taken every minute after the cuff release. All the measurements were taken at end diastole, incident with the R wave on the ECG. The baseline and peak value diameters acquired during ischemia-induced hyperaemia were used for the evaluation of the percentage FMD (flow-mediated vasodilatation) using the formula: FMD = (the maximum diameter − the baseline diameter)/baseline diameter × 100) (Fig. 1B).

Measurement of CIMT

The CIMT was measured with a Logiq 5 ultrasound scanner equipped with a linear probe (General Electric Medical Systems, Wallingford, CT, USA). A single trained sonographer blinded to the patients' data performed the sonographic evaluations. The patients lay supine in a quiet, dark room. The left common carotid arteries were examined with the patient's head in the midline position, tilted slightly upward. The probe was placed about 1 cm proximal to the bifurcation of the common carotid artery, and the maximum lumen diameter was visualized in the longitudinal plane. The CIMT was defined as the distance between the media-adventitia interface and the lumen-intima. Two parallel echogenic lines separated by an anechoic space can be visualized in the anterior wall of the carotid artery (Urakawa et al. 2006) (Fig. 1C).

Statistical analysis

The statistical analysis was performed with computer software (SPSS version 13.0, SPSS Inc., Chicago, IL, USA). Numeric variables were analysed with a Student's *t*-test and a Mann-Whitney *U*-test. Categorical variables were analysed with a Chi-square test and Fisher's exact test, as appropriate. The data were expressed as medians (25-75 percentiles) for numeric variables and as percentage (%) for categorical variables. Correlation analysis was performed using Spearman's correlation test. A p value < 0.05 was considered statistically significant.

Results

The volunteers was 10 males and 15 females with the median age of 31 years (ranges of 28-37 years) in the HK group and 11 males and 14 females with the median age of 33 years (25-39 years) in the LK group. There was no significant difference between the ages of the two study groups (p = 0.71). There was also no between-group differences in their BMI (26.6 [21.6-31.4] vs. 26.7 [27.8-28.4] kg/m^2; p = 0.99) or systolic and diastolic blood pressure (BP) values (105 [100-130] vs. 110 [110-140] mmHg; p = 0.94; 70 [60-70] vs. 70 [70-80] mmHg; p = 0.15, respectively). The two study groups had similar values of biochemical parameters (Table 1).

The median serum Klotho level of the study population was 459.4 pg/mL. The serum Klotho levels were 613.6 pg/mL (ranges of 501.2-772.6) in the HK group, while the levels were 338.7 pg/mL (ranges of 278.8-430.3) in the LK group (Table 1). All echocardiographic measurements, including the IVS thickness, PW thickness, LVEDD, LVESD and EF, were similar between the two groups (Table 2). When the diastolic functional parameters, including mitral E and A waves and lateral Em and Am, were compared, there was no significant difference between the groups. However, the mitral E deceleration time of the LK group was significantly longer than that of the HK group (164 [118-274] vs. 141 [118-148] msn; p = 0.01) (Table 2).

The EFT values of the LK group were significantly larger than those of the HK group (0.75 [0.58-0.77] vs. 0.62

Table 1. Comparison of baseline characteristics of study population.

	All Subjects (n = 50)	HK Group (n = 25)	LK Group (n = 25)	p value
Age, years	32 (27-38)	31 (28-37)	33 (25-39)	0.71
Gender, M/F	21/29	10/15	11/14	0.77
BMI (kg/m²)	26.6 (22.5-29.3)	26.6 (21.6-31.4)	26.7 (27.8-28.4)	0.99
Current smoker (%)	15 (30)	6 (12)	9 (18)	0.34
SBP (mm Hg)	105 (100-140)	105 (100-130)	110 (110-140)	0.94
DBP (mm Hg)	70 (60-80)	70 (60-70)	70 (70-80)	0.15
FBG (mg/dL)	93 ± 10	92 ± 9	93 ± 10	0.64
HbA1c (%)	5.5 ± 0.2	5.4 ± 0.2	5.5 ± 0.2	0.34
LDL-C (mg/dL)	118 ± 37	117 ± 14	120 ± 33	0.78
HDL-C (mg/dL)	50 ± 10	51 ± 10	50 ± 10	0.61
Triglyceride (mg/dL)	102 ± 53	98 ± 50	106 ± 57	0.58
Urea (mg/dL)	27 ± 8	28 ± 7	26 ± 8	0.78
Creatinine (mg/dL)	0.9 ± 0.22	0.91 ± 0.2	0.9 ± 0.23	0.44
Protein (mg/dL)	7.1 ± 1.3	6.9 ± 1.8	7.3 ± 0.5	0.31
Albumin (mg/dL)	4.4 ± 0.3	4.4 ± 0.2	4.4 ± 0.3	0.61
Calcium (Eq/L)	9.8 ± 0.4	9.8 ± 0.3	9.8 ± 0.4	0.95
Phosphate (mEq/L)	3.4 ± 0.5	3.4 ± 0.4	3.5 ± 0.5	0.61
Parathormone	38.4 ± 15.4	41.6 ± 18.3	35.1 ± 12.1	0.24
TSH	1.9 ± 1.1	1.6 ± 0.9	2.1 ± 1.2	0.12
GFR	101 (89.5-118)	99 (85-110)	111 (95.5-131)	0.27
25 (OH) vitamin D	15.6 (12.5-26.3)	15.6 (13.6-27.5)	15.6 (9.9-22.9)	0.58
Klotho (pg/mL)	459.4 (335.1-617.2)	613.6 (501.2-772.6)	338.7 (278.8-430.3)	< 0.001

BMI, body mass index; DBP, diastolic blood pressure; F, female; FBG, fasting blood glucose; GFR, glomerular filtration rate; HK, higher Koltho; LK, lower Klotho; M, male; SBP, systolic blood pressure; TSH, thyroid stimulating hormone.

Table 2. Comparison of echocardiographic characteristics between two groups.

	HK Group (n = 25)	LK Group (n = 25)	p value
EF (%)	69 (64-76)	61 (55-68)	0.89
IVS thickness (mm)	7.5 (7-8.5)	8 (7-10)	0.27
PW thickness (mm)	8.5 (7.5-10)	8.5 (7-10)	0.42
LVEDD (mm)	45 (37-50)	44 (43-54)	0.63
LVESD (mm)	27 (24-28)	31 (28-35)	0.14
E (m/s)	0.81 (0.77-0.95)	0.57 (0.52-0.72)	0.11
A (m/s)	0.65 (0.53-0.66)	0.77 (0.60-0.78)	0.69
DT (msn)	141 (118-148)	164 (118-274)	**0.01**
IVRT (msn)	111 (104-114)	126 (118-131)	0.09
Sm lateral (cm/s)	0.07 (0.07-0.11)	0.08 (0.06-0.15)	0.97
Em lateral (cm/s)	0.13 (0.12-0.15)	0.12 (0.07-0.13)	0.15
Am lateral (cm/s)	0.08 (0.06-0.09)	0.08 (0.03-0.23)	0.72
EFT (cm)	0.62 (0.43-0.70)	0.75 (0.58-0.77)	**0.01**
FMD (%)	16.7 (9.6-34.3)	8.8 (4.5-18.1)	**0.012**
CIMT (mm)	0.49 (0.42-0.60)	0.70 (0.61-0.83)	**< 0.001**

CIMT, carotid intima-media thickness; DT, deceleration time; EF, ejection fraction; EFT, epicardial fat thickness; FMD, flow mediated dilatation; IVS, interventricular septum; IVRT, isovolumic relaxation time; HK, higher Klotho; LK, lower Klotho; LVEDD, left ventricular end-diastolic diameter; LVESD, left ventricular end-systolic diameter.

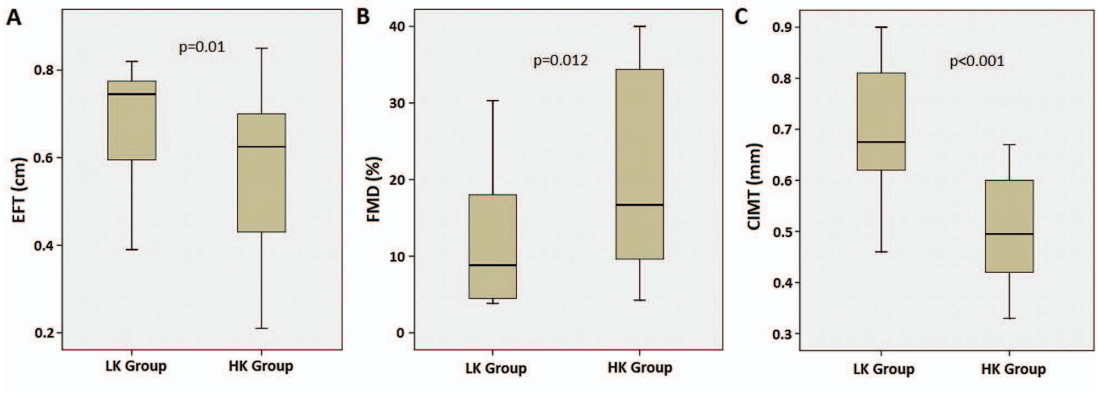

Fig. 2. Comparisons of EFT, CIMT and FMD values between lower and higher Klotho groups.
The box-plot graphs comparising epicardial fat thickness (EFT) (A), flow mediated dilatation (FMD) (B) and carotid intima-media thickness (CIMT) (C) values in higher Klotho and lower Klotho groups each consisting of 25 subjects.

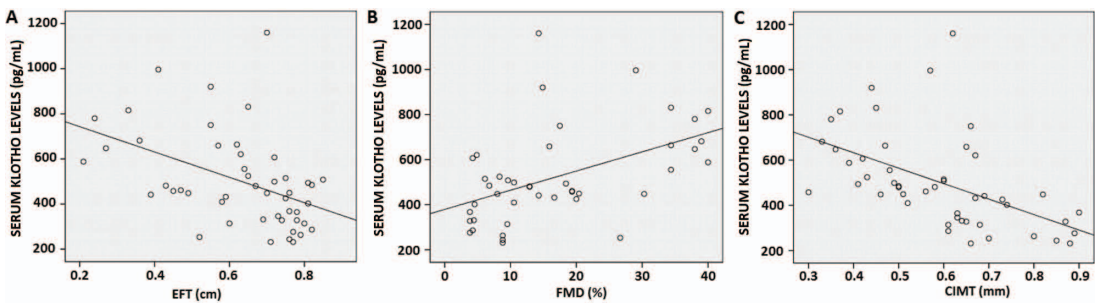

Fig. 3. Correlations of serum Klotho levels with FMD, EFT and CIMT.
The scatter plot graphs summarizing the positive correlation between serum Klotho levels and flow mediated dilatation (FMD) percentage values (B) and negative correlation between serum Klotho levels with epicardial fat thickness (EFT) (A) and carotid intima-media thickness (CIMT) values (C) of the study population (n = 50).

[0.43-0.70] cm; p = 0.01) (Fig. 2A). There was a significant between-group difference in the percentages of FMD, with the HK group having a significantly higher percentage than the LK group (16.7% [9.6-34.3%] vs. 8.8% [4.5-18.1%]; p = 0.012) (Fig. 2B). There was also a significant difference between the CIMT values of the wo groups, with the LK group having significantly higher CIMT values than the HK group (0.70 [0.61-0.83] vs. 0.49 [0.42-0.60] mm; p < 0.001) (Fig. 2C).

Considering the whole study population, there was a moderate positive correlation between serum Klotho levels and FMD percentage values (*r*: 0.483, p = 0.001) and negative correlations with EFT and CIMT values (*r*: −0.456; p = 0.001 and *r*: −0.522; p < 0.001, respectively) (Fig. 3A-C).

Discussion

In this study, we found a significant association between lower serum Klotho levels and early predictors of atherosclerosis, such as EFT, FMD and CIMT, in adult subjects with no known risk factors for cardiovascular diseases.

Studies of mice have demonstrated that a lack of Klotho gene expression results in sarcopenia, atherosclerosis, osteoporosis and a shortened lifespan (Saito et al. 1998) and that overexpression of Klotho leads to greater longevity (Kuro-o et al. 1997). Two forms of Klotho, membrane and secreted, have been described, and each has different functions (Saito et al. 1998). Membrane Klotho acts as an obligate co-receptor for fibroblast growth factor −23, a bone-derived hormone that induces phosphate excretion into urine (Urakawa et al. 2006). Secreted Klotho participates in the regulation of nitric oxide production in the endothelium (Saito et al. 1998, 2000), calcium homeostasis in the kidney (Chang et al. 2005; Imura et al. 2007) and inhibition of intracellular insulin and insulin-like growth factor-1 signalling (Kurosu et al. 2005). Although it is not expressed in blood vessels, the Klotho protein protects against endothelial dysfunction by increasing nitric oxide availability. A previous study demonstrated that Klotho-deficient mice had reduced arteriolar vasodilatation and aortic relaxation responses to acetylcholine and lower urinary excretion of NO_2 and NO_3 (Saito et al. 1998). Imura et al. (2004) found that the Klotho protein was present in both human serum and cerebrospinal fluid.

The endothelium is a large paracrine organ that secretes a variety of factors involved in the regulation of vascular tone, cell growth, platelet and leukocyte interactions and thrombogenicity (Galley and Webster 2004). The endothelium can sense and respond to numerous internal

and external stimuli via cell membrane receptors and signal transduction mechanisms, leading to the synthesis and release of various vasoactive, thromboregulatory and growth factor substances (Drexler 1998). A non-invasive technique has been widely used to evaluate flow-mediated vasodilation, an endothelium-dependent function, in the brachial artery. Endothelial dysfunction plays an important role in the development of atherosclerosis (Anderson and Mark 1989; Laurent et al. 1990; Celermajer et al. 1992; Sorensen et al. 1995). With this technique, nitric oxide release is provoked, and subsequent vasodilation is imaged and quantitated as an index of vasomotor function.

In a previous study, lower Klotho levels were associated with a lower FMD percentage and higher CIMT values in patients with chronic kidney disease (Kitagawa et al. 2013). In the present study, we also found that higher serum Klotho levels were related to a higher FMD percentage and lower CIMT values in a normal population similar to Kitagawa's study on patients with chronic kidney disease.

The association between epicardial fat and cardiovascular diseases had been studied before (Jeong et al. 2007). Due to the anatomic and functional proximity of epicardial fat to the myocardium and its dynamic metabolic activity, interactions between the heart and epicardial fat have been suggested (Iacobellis and Willens 2009). Epicardial fat can be imaged and measured using standard two-dimensional echocardiography.

Echocardiographic EFT is more closely related to visceral adiposity than to general obesity. EFT has been shown to be correlated with metabolic syndrome, insulin resistance, coronary artery disease and subclinical atherosclerosis (Iacobellis et al. 2014). In the present study, we investigated the relationship between serum Klotho levels and EFT, which is a novel and simple tool for cardiometabolic risk prediction. We found that subjects with lower Klotho levels had significantly thicker epicardial fat tissue.

There are limitations in the presents study. This study was cross-sectional, and we did not perform a prospective observation. In addition, we did not analyze the effect of confounders other than serum Klotho levels as potential predictors of atherosclerosis. Therefore, we cannot assume any reason-result relation. On the other hand, the results indicate the correlation between lower serum Klotho levels and atherosclerosis.

In conclusion, we found that lower serum Klotho levels were directly correlated with EFT and CIMT and inversely correlated with FMD percentage values in a healthy population. We conclude that low serum Klotho levels may be a new early predictor of atherosclerosis.

Conflict of Interest

The authors declare no conflict of interest.

References

Anderson, E.A. & Mark, A.L. (1989) Flow-mediated and reflex changes in large peripheral artery tone in humans. *Circulation*, **79**, 93-100.

Avena, R., Mitchell, M.E., Nylen, E.S., Curry, K.M. & Sidawy, A.N. (1998) Insulin action enhancement normalizes brachial artery vasoactivity in patients with peripheral vascular disease and occult diabetes. *J. Vasc. Surg.*, **28**, 1024-1031.

Celermajer, D.S., Sorensen, K.E., Gooch, V.M. Spiegelhalter, D.J., Miller, O.I., Sullivan, I.D., Lloyd, J.K. & Deanfield, J.E. (1992) Non-invasive detection of endothelial dysfunction in children and adults at risk of atherosclerosis. *Lancet*, **340**, 1111-1115.

Chang, Q., Hoefs, S., van der Kemp, A.W., Topala, C.N., Bindels, R.J. & Hoenderop, J.G. (2005) The β-glucuronidase Klotho hydrolyzes and activates the TRPV5 channel. *Science*, **310**, 490-493.

Davies, M.J. (1996) Stability and instability: two faces of coronary atherosclerosis. The Paul Dudley White Lecture 1995. *Circulation*, **94**, 2013-2020.

Drexler, H. (1998) Factors involved in the maintenance of endothelial function. *Am. J. Cardiol.*, **82**, 3S-4S.

Galley, H.F. & Webster, N.R. (2004) Physiology of the endothelium. *Br. J. Anaesth.*, **93**, 105-113.

Glagov, S., Weisenberg, E., Zarins, C.K., Stankunavicius, R. & Kolettis, G.J. (1987) Compensatory enlargement of human atherosclerotic coronary arteries. *N. Engl. J. Med.*, **316**, 1371-1375.

Harris, L.M., Faggioli, G.L., Shah, R., Koerner, N., Lillis, L., Dandona, P., Izzo, J.L., Snyder, B. & Ricotta, J.J. (1995) Vascular reactivity in patients with peripheral vascular disease. *Am. J. Cardiol.*, **76**, 207-212.

Iacobellis, G., Diaz, S., Mendez, A. & Goldberg, R. (2014) Increased epicardial fat and plasma leptin in type 1 diabetes independently of obesity. *Nutr. Metab. Cardiovasc. Dis.*, **24**, 725-729.

Iacobellis, G. & Willens, H.J. (2009) Echocardiographic epicardial fat: a review of research and clinical applications. *J. Am. Soc. Echocardiogr.*, **22**, 1311-1319.

Imura, A., Iwano, A., Tohyama, O., Tsuji, Y., Nozaki, K., Hashimoto, N., Fujimori, T. & Nabeshima, Y. (2004) Secreted Klotho protein in sera and CSF: implication for post-translational cleavage in release of Klotho protein from cell membrane. *FEBS Lett.*, **565**, 143-147.

Imura, A., Tsuji, Y., Murata, M., Maeda, R., Kubota, K., Iwano, A., Obuse, C., Togashi, K., Tominaga, M., Kita, N., Tomiyama, K., Iijima, J., Nabeshima, Y., Fujioka, M., Asato, R., et al. (2007) Alpha-Klotho as a regulator of calcium homeostasis. *Science*, **316**, 1615-1618.

Jeong, J.W., Jeong, M.H., Yun, K.H., Oh, S.K., Park, E.M., Kim, Y.K., Rhee, S.J., Lee, E.M., Lee, J., Yoo, N.J., Kim, N.H. & Park, J.C. (2007) Echocardiographic epicardial fat thickness and coronary artery disease. *Circ. J.*, **71**, 536-539.

Kitagawa, M., Sugiyama, H., Morinaga, H., Inoue, T., Takiue, K., Ogawa, A., Yamanari, T., Kikumoto, Y., Uchida, H.A., Kitamura, S., Maeshima, Y., Nakamura, K., Ito, H. & Makino, H. (2013) A decreased level of serum soluble Klotho is an independent biomarker associated with arterial stiffness in patients with chronic kidney disease. *PLoS One*, **8**, e56695.

Kuro-o, M. (2011) Phosphate and Klotho. *Kidney Int. Suppl.*, **79**, 20-23.

Kuro-o, M., Matsumura, Y., Aizawa, H., Kawaguchi, H., Suga, T., Utsugi, T., Ohyama, Y., Kurabayashi, M., Kaname, T., Kume, E., Iwasaki, H., Iida, A., Shiraki-Iida, T., Nishikawa, S., Nagai, R. & Nabeshima, Y.I. (1997) Mutation of the mouse Klotho gene leads to a syndrome resembling ageing. *Nature*, **390**, 45-51.

Kurosu, H., Yamamoto, M., Clark, J.D., Pastor, J.V., Nandi, A., Gurnani, P., McGuinness, O.P., Chikuda, H., Yamaguchi, M., Kawaguchi, H., Shimomura, I., Takayama, Y., Herz, J., Kahn, C.R., Rosenblatt, K.P., et al. (2005) Suppression of aging in mice by the hormone Klotho. *Science*, **308**, 1829-1833.

Laurent, S., Lacolley, P., Brunel, P., Laloux, B., Pannier, B. & Safar, M. (1990) Flow-dependent vasodilation of brachial artery in essential hypertension. *Am. J. Physiol.*, **258**, H1004-1011.

Moe, S.M. & Drueke, T. (2008) Improving global outcomes in mineral and bone disorders. *Clin. J. Am. Soc. Nephrol*., **3**, S127-S130.

Pedersen, L., Pedersen, S.M., Brasen, C.L. & Rasmussen, L.M. (2013) Soluble serum Klotho levels in healthy subjects. Comparison of two different immunoassays. *Clin. Biochem.*, **46**, 1079-1083.

Persson, J., Formgren, J., Israelsson, B. & Berglund, G. (1994) Ultrasound-determined intima-media thickness and atherosclerosis. Direct and indirect validation. *Arterioscler. Thromb*., **14**, 261-264.

Saito, Y., Nakamura, T., Ohyama, Y., Suzuki, T., Iida, A., Shiraki-Iida, T., Kuro-o, M., Nabeshima, Y., Kurabayashi, M. & Nagai, R. (2000) In vivo Klotho gene delivery protects against endothelial dysfunction in multiple risk factor syndrome. *Biochem. Biophys. Res. Commun*., **276**, 767-772.

Saito, Y., Yamagishi, T., Nakamura, T., Ohyama, Y., Aizawa, H., Suga, T., Matsumura, Y., Masuda, H., Kurabayashi, M., Kuro-o, M., Nabeshima, Y. & Nagai, R. (1998) Klotho protein protects against endothelial dysfunction. *Biochem. Biophys. Res. Commun.*, **248**, 324-329.

Schejbal, V. (1989) Epicardial fatty tissue of the right ventricle: morphology, morphometry and functional significance. *Pneumologie*, **43**, 490-499.

Semba, R.D., Cappola, A.R., Sun, K., Bandinelli, S., Dalal, M., Crasto, C., Guralnik, J.M. & Ferrucci, L. (2011) Plasma klotho and cardiovascular disease in adults. *J. Am. Geriatr. Soc.*, **59**, 1596-1601.

Sohn, D.W., Chai, I.H., Lee, D.J., Kim, H.C., Kim, H.S., Oh, B.H., Lee, M.M., Park, Y.B., Choi, Y.S., Seo, J.D. & Lee, Y.W. (1997) Assessment of mitral annulus velocity by Doppler tissue imaging in the evaluation of left ventricular diastolic function. *J. Am. Coll. Cardiol.*, **30**, 474-480.

Sorensen, K.E, Celermajer, D.S., Spiegelhalter, D.J., Georgakopoulos, D., Robinson, J., Thomas, O. & Deanfield, J.E. (1995) Non-invasive measurement of human endothelium dependent arterial responses: accuracy and reproducibility. *Br. Heart J.*, **74**, 247-253.

Urakawa, I., Yamazaki, Y., Shimada, T., Iijima, K., Hasegawa, H., Okawa, K., Fujita, T., Fukumoto, S. & Yamashita, T. (2006) Klotho converts canonical FGF receptor into a specific receptor for FGF23. *Nature*, **444**, 770-774.