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# Circulating Klotho levels can predict long-term macrovascular outcomes in type 2 diabetic patients

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ABSTRACT

*Background and aims:* Type 2 diabetes is a global health problem that is associated with a wide variety of vascular complications and associated morbidity and mortality. Klotho is an enzyme and transmembrane protein, and increasing evidence suggests that Klotho may contribute to reduced oxidative stress, improved endothelial function, and vasoprotection. To date, the physiological role of Klotho in vascular complications associated with diabetes is unclear.

*Methods:* We prospectively recruited 252 patients with type 2 diabetes, who visited an outpatient clinic at our hospital between 2009 and 2011. Patients in the top and bottom tertiles of circulating Klotho levels were enrolled for analysis.

*Results:* Of the 168 patients enrolled, 45.8% were male, the mean age was 57.2 years, and the average duration of diabetes was 7.58 years. In multiple regression analysis, a high Klotho level was associated with a reduced risk of developing coronary artery disease and cerebrovascular accidents. Klotho level was also an independent predictor for the development of macroangiopathies within the 7-year study period.

*Conclusions:* Our results suggest that circulating Klotho level is a predictor of long-term macrovascular outcomes in patients with type 2 diabetes.



## 1. Introduction

Patients with type 2 diabetes often have multiple comorbidities, especially those with poorly controlled blood glucose, blood pressure, and serum lipid profile [1,2]. Vascular endothelial cell dysfunction, a complication of diabetes characterized by macro- and microangiopathies, affects multiple organs and causes considerable morbidity and mortality in patients with diabetes mellitus [3,4]. Accelerated atherosclerosis of coronary, carotid, and peripheral arteries plays a key role in macroangiopathies and can lead to coronary artery disease (CAD), cerebrovascular accidents (CVAs), and peripheral artery occlusive disease (PAOD). Retinal, renal, and vasa nervorum microangiopathies play important roles in the pathogenesis of retinopathy, nephropathy, and neuropathy, respectively. Such macro- and microvascular complications can result in a poor prognosis [1]. Early interventions to treat such abnormalities are therefore important, and an early biomarker to allow for such interventions is needed.

Klotho is an enzyme and a transmembrane protein encoded by the *KL* gene in humans. In mice, it has been shown to be involved in the development of a syndrome resembling human aging [5]. The *KL* gene is mainly expressed in the kidneys and brain [6]. It provides some control over an organism's sensitivity to insulin, and appears to play a role in nitric oxide production and hyperphosphatemia [7,8]. Klotho deficiency in mice has been shown to lead to multiple disorders including atherosclerosis, vascular calcification, stroke, osteoporosis, ectopic calcification, skin atrophy, and chronic vascular disease, in addition to a short life-span and infertility [9,10]. Membrane-bound and secreted Klotho may have distinct

*Abbreviations:* ABI, ankle-brachial index; BMI, body mass index; CAD, coronary artery disease; CI, confidence of interval; CVA, cerebrovascular accident; FGF23, fibroblast growth factor-23; LVH, Left ventricular hypertrophy; OR, odds ratio; PAOD, peripheral artery occlusive disease; RAAS, renin-angiotensin-aldosterone system; SD, standard deviations.

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functions. Membrane-bound Klotho has been shown to be a necessary coreceptor for fibroblast growth factor-23 (FGF23) and to regulate calcium and phosphate metabolism in the kidneys [11,12]. Secreted Klotho has been shown to be involved in growth factor signaling pathways, including Wnt transforming growth factor β1 and insulin-like growth factor-1, in ion transport, it has been implicated in regulation of the renin-angiotensin system and has antioxidative effects [11,13]. Recent human studies have shown that a higher circulating level of Klotho is associated with lower risks of metabolic syndrome, renal disease, and cardiovascular disease [14–17]. The protective effects of Klotho on renal insults and renal fibrosis in chronic kidney disease have been well described [18]. Many experimental models have shown positive correlations between the expression of Klotho and statin therapy. In addition, previous studies have suggested that the potential role of Klotho in the reduction of oxidized low-density lipoprotein (ox-LDL)-induced oxidative stress may be correlated to the benefits of statin therapy in ameliorating endothelial dysfunction and atherosclerosis [19,20]. However, clinical studies focusing on Klotho and vascular complications in diabetes have reported inconsistent results [14,21–23], and the clinical impact of Klotho on macro- and microangiopathies is still unclear.

We hypothesized that circulating Klotho level could be used as an early biomarker of vascular complications in patients with type 2 diabetes. Therefore, in this study we investigated the associations between circulating levels of Klotho and the development/progression of macro- and microangiopathies based on a longitudinal study design.

## 2. Materials and methods

### 2.1. Ethics statement

This study was conducted in full compliance with the ethical principles of the Declaration of Helsinki, the Good Clinical Practice guidelines, and the applicable local regulatory requirements. Subjects were invited to participate in this study when they attended a health screening program. A trained endocrinologist examined all of the patients during screening and informed them about the consent procedure. Written informed consent was obtained from all subjects prior to their participation. This study was approved by the Institutional Review Board of Chang Gung Memorial Hospital (approval no. 100–1116B).

### 2.2. Study design and population

This study was performed between October 2009 and January 2011 at Chang Gung Memorial Hospital, Keelung, Taiwan. Patients with type 2 diabetes mellitus between the ages of 18 and 70 years who attended an outpatient clinic in the hospital were recruited for the study. The exclusion criteria were patients with a urinary tract infection over the past 3 months, poorly controlled hypertension, pregnancy, liver cirrhosis, polycystic kidney disease, and systemic lupus erythematosus. The patients completed a baseline survey including demographic and clinical laboratory data. The patients were then invited to attend follow-up visits approximately every year thereafter. The primary study outcomes were the development of macro- and microangiopathies. Follow-up examinations were conducted for 7 years, and physical parameters, assessments of macro- and microangiopathies, as well as blood and urine laboratory test results were recorded yearly.

### 2.3. Follow-up

During the recruitment period, 259 patients completed the baseline survey and were invited to attend yearly follow-up visits. Of those invited, 252 attended and seven declined. During the 7-year study period, 22 patients developed CAD, 29 had CVAs (including 27 with ischemic stroke and two with hemorrhagic stroke), 12 developed left ventricle hypertrophy (LVH), 8 developed or had progression of PAOD, 12 developed or had progression of retinopathy, and 67 developed or had progression of neuropathy. At the end of the study, 218 patients had successfully completed the 7-year annual follow-up, 22 had died, and 12 were lost to follow-up.

### 2.4. Definition of outcomes

The patients were defined as having CAD if they had angina pectoris with a positive exercise test result, myocardial infarction, angiographic evidence of (>75%) coronary artery stenosis after the administration of intra-coronary nitroglycerine 50–200 μg, percutaneous coronary revascularization, coronary artery bypass grafting or a positive myocardial perfusion scan, as previously described [24]. CVAs were diagnosed based on brain computed tomography findings or magnetic resonance imaging [25], and the patients with a CVA were defined as those with an ischemic or hemorrhagic stroke episode during the study period. LVH was diagnosed based on the results from echocardiography. Left ventricular (LV) mass was calculated using the formula recommend by the American Society of Echocardiography, and LV mass index was calculated as LV mass divided by body surface area (in $m^2$). LVH was defined as a LV mass index >115 g/$m^2$ for men and >95 g/$m^2$ for women [26]. PAOD was diagnosed according to the ankle-brachial index (ABI), and was defined as the lowest values of right and left ABIs as measured by Doppler ultrasound. The right and left ABIs were determined as the right and left ankle systolic pressures divided by the highest brachial systolic pressure, respectively. PAOD was defined as an ABI ≤0.9 or >1.3 [27]. Retinopathy was diagnosed based on the findings of a fundoscopic examination [28]. Neuropathy was diagnosed based on the findings of a nerve test, including pinprick, temperature sensation, vibration sensation, and 10-g monofilament tests [29]. Doppler ultrasound, fundoscopic examinations, and nerve tests were routinely performed at least yearly to assess PAOD, retinopathy, and neuropathy. The associated tests to diagnose CAD, CVA and LVH were performed when clinical evidence was noted. Macrovascular complications included CAD, CVAs and PAOD; microvascular complications included neuropathy and retinopathy. The treatment targets for blood pressure, LDL, HbA1C and body mass index (BMI) were defined according to current guidelines [29].

### 2.5. Measurement of circulating Klotho level

Blood samples were centrifuged for 15 min at 3000 rpm within 30 min of collection. Plasma was removed, and the samples were stored at −70 °C until analysis. Serum Klotho level was measured using an enzyme-linked immunosorbent assay (Cusabio Human Klotho ELISA kit, CSB-E13235h, China) at baseline and at the end of the study. The inter- and intra-assay coefficients of variation were <8% and <10%, respectively. The detection limit of this assay was 39 pg/mL [30]. The reference interval for the Klotho assay was 4700–437600 pg/mL in a healthy population [31]. In our laboratory, each Klotho assay was performed in duplicate according to the manufacturer's instructions, and the mean value was used for further statistical analysis.

### 2.6. Statistical analysis

Values were expressed as means and standard deviations (SDs) for continuous data, and categorical data were expressed as