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Relationship of low plasma klotho with poor grip strength in older community-dwelling adults: the InCHIANTI study

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Abstract

Handgrip strength is a strong indicator of total body muscle strength and is a predictor of poor outcomes in older adults. The aging suppressor gene *klotho* encodes a single-pass transmembrane protein that is secreted as a circulating hormone. In mice, disruption of *klotho* expression results in a syndrome that includes sarcopenia, atherosclerosis, osteoporosis, and shortened lifespan, and conversely, overexpression of *klotho* leads to a greater longevity. The objective was to determine whether plasma klotho levels are related to skeletal muscle strength in humans. We measured plasma klotho in 804 adults, 65 years, in the InCHIANTI study, a longitudinal population-based study of aging in Tuscany, Italy. Grip strength was positively correlated with plasma klotho at threshold <681 pg/mL. After adjusting for age, sex, education, smoking, physical activity, cognition, and chronic diseases, plasma klotho (per 1 standard deviation increase) was associated with grip strength (beta = 1.20, standard error = 0.35, $P = 0.0009$) in adults with plasma klotho <681 pg/mL. These results suggest that older adults with lower plasma klotho have poor skeletal muscle strength.

Keywords

aging; klotho; muscle strength; sarcopenia

Aging is accompanied by sarcopenia, defined as the loss of skeletal muscle mass and muscle strength (25). Humans lose ~20-40% of both skeletal muscle mass and strength from 20 to 80 years of age (2, 5). Poor skeletal muscle strength is predictive of disability (10, 27) and mortality (28). Handgrip strength is strongly correlated with other measures of muscle strength and is often considered representative of total body muscle strength (28). Among the many underlying causes of sarcopenia are increased oxidative stress and inflammation, hormonal changes, anorexia of aging, and lack of physical activity (5).

Klotho, a recently discovered hormone, may play a potential role in the pathogenesis of sarcopenia. The aging suppressor gene *klotho* encodes a single-pass transmembrane protein that is predominantly expressed in the distal tubule cells of the kidney, parathyroid glands, and choroid plexus of the brain. The *klotho* gene was named after one of the three Fates in

Greek mythology, the goddess who spins the thread of life. *Klotho* was originally identified in a mutant mouse strain that could not express klotho, developed multiple disorders resembling human aging, and had a shortened life span (17). The aging phenotypes included atherosclerosis, endothelial dysfunction, decreased bone mineral density, sarcopenia, skin atrophy, and impaired cognition (13, 18, 30).

In vivo gene delivery of *klotho* has been shown to protect against endothelial dysfunction in an atherosclerotic mouse model (29) and hypertension and renal damage in a hypertensive rat model (33). Overexpression of *klotho* in transgenic mice resulted in a significant extension of life span compared with wild-type mice (19). These findings suggest that some phenotypes of aging may be ameliorated and lifespan increased by increased expression of *klotho*.

There are two forms of klotho, membrane and secreted, and each has different functions. Membrane klotho acts as an obligate co-receptor for fibroblast growth factor (FGF)-23, a bone-derived hormone that induces phosphate excretion into urine (32). Secreted klotho is involved in regulation of nitric oxide production in the endothelium (29, 30), calcium homeostasis in the kidney (3, 14), inhibition of intracellular insulin and insulin-like growth factor-1 signaling (19), and inhibition of transforming growth factor-1 signaling (6). *Klotho* gene transcripts for a putative secreted form of klotho protein were described in 1998 (22). In 2004, Imura and colleagues demonstrated that klotho protein was present in both human sera and cerebrospinal fluid (14). The relationship of circulating klotho with clinical phenotypes in human aging has not been studied because of the lack of a sensitive and reliable assay for measurement of secreted klotho protein in the blood. For example, it is not known whether low plasma klotho levels are associated with poor muscle strength in humans. Recently, a sensitive and specific assay was developed for the measurement of soluble klotho in humans (34).

We hypothesized that low plasma klotho concentrations were independently associated with skeletal muscle strength. To address this hypothesis, we measured plasma klotho levels in a large, population-based study of aging.

MATERIALS AND METHODS

The study participants consisted of men and women, aged 65 and older, who participated in the Invecchiare in Chianti, “Aging in the Chianti Area” (InCHIANTI) study, conducted in two small towns in Tuscany, Italy. The rationale, design, and data collection have been described elsewhere, and the main outcome of this longitudinal study is mobility disability (7). Briefly, in August 1998, 1270 people aged 65 years and older were randomly selected from the population registry of Greve in Chianti (pop. 11,709) and Bagno a Ripoli (pop. 4,704), and of 1,256 eligible subjects, 1,155 (90.1%) agreed to participate. Participants received an extensive description of the study and participated after written, informed consent. The study protocol complied with the Declaration of Helsinki and was approved by the Italian National Institute of Research and Care on Aging Ethical Committee and by the Institutional Review Board of the Johns Hopkins University School of Medicine.

Participants were evaluated again for a three-year follow-up visit from 2001-2003 ($n = 926$) and six-year follow-up visit from 2004-2006 ($n = 844$), and nine-year follow-up visit from 2007-2009 ($n = 768$). Of the 926 participants seen at the three-year follow-up visit, 804 (86.8%) had blood drawn and plasma available for analysis. There were no significant differences in age, sex, other demographic factors, or subsequent mortality between those who did or did not participate in the blood drawing. Plasma klotho was measured at the three-year follow-up visit and not the enrollment visit because of the greater availability of

archived plasma samples from the three-year visit. The three year visit will be referred to as the baseline visit for the present study of klotho and grip strength. There were 775 participants who had measurements of both plasma klotho and grip strength at baseline, and of these, grip strength measurements were available on 608 participants at the six-year visit and 472 participants at the nine-year visit.

Demographic information and information on smoking and medication use were collected using standardized questionnaires. Smoking history was determined from self-report. Daily alcohol intake, expressed in gm/day, was determined based upon the European Prospective Investigation into Cancer and Nutrition food frequency questionnaire that had been validated in the Italian population. Education was recorded as years of school.

All participants were examined by a trained geriatrician. Diseases were ascertained according to standard, pre-established criteria and algorithms based upon those used in the Women's Health and Aging Study for diabetes mellitus, coronary heart disease, chronic heart failure, stroke, and cancer (12). Those who did not have diabetes by the algorithm but had a fasting plasma glucose >125 mg/dL (1) were also considered to have diabetes.

Systolic and diastolic blood pressures were calculated from the mean of three measures taken with a standard mercury sphygmomanometer during the physical examination. Weight was measured using a high-precision mechanical scale. Standing height was measured to the nearest 0.1 cm. Body mass index (BMI) was calculated as weight/height² (kg/m²). Mini-Mental State Examination (MMSE) was administered at enrollment, and an MMSE score <24 was considered consistent with cognitive impairment (8). Chronic kidney disease was defined as estimated glomerular filtration rate of <60 mL/min/1.73 m² using the four-variable Modification of Diet in Renal Disease Study equation of Levey and colleagues (20).

Grip strength was measured with a Jamar dynamometer (Irvington, NY). Before testing, the examiner asked the subject if he/she had pain in one hand or in both (for example, a new acute phase of an arthrosis process or a recent attack of gout), or if he had undergone any surgery to the hand. If the response was affirmative, the affected limb was not tested; if both hands were affected, the test was deferred. The subject was seated in front of a bench with the arm of the test hand supported on the bench with the elbow flexed to 45 degrees. The examiner adjusted the handle of the dynamometer such that the proximal interphalangeal joints of the hand were bent to 90 degrees. The examiner asked the subject to perform a practice test and said: 'Try once to use the device to understand how it works. Squeeze the handle until your hand is closed. It does not matter whether you use your maximum strength. Instead, tell me whether you feel you are in a comfortable position to carry out the task.' The examiner then adjusted the device, if necessary, and started the test saying: 'Now begin the test. When I say go, begin to squeeze with all your might. You are ready. Squeeze. Fine, just relax.' The test lasted about 5 seconds and two sessions were performed. Both hands were tested, unless contraindicated as above.

Grip strength is a strong predictor of mortality (27), thus, the competing risk of mortality was also taken into account in this analysis. At the end of the field data collection, mortality data of the original InCHIANTI cohort were collected using data from the Mortality General Registry maintained by the Tuscany Region. Analyses include those who refused to participate in the follow-up after baseline or those who moved away but were known to be alive at the time of censoring of this analysis.

Blood samples were collected in the morning after a 12-h fast. Aliquots of serum and plasma were immediately obtained and stored at -80° C. Soluble α -klotho was measured in EDTA-plasma using a solid phase sandwich enzyme-linked immunosorbent assay (ELISA) (Immuno-Biological Laboratories, Takasaki, Japan) (34). The minimum level of

detectability of the assay is 6.15 pg/mL. The minimum level is below the plasma concentrations that were found in our study. The intra-assay and inter-assay coefficients of variation were 4.1% and 8.9% for klotho measurements in the investigator's (R.D.S.) laboratory. The designation α -klotho is used to describe the original *klotho* gene and its product (15) and to distinguish it from a homolog which was named β -klotho (16). Throughout this paper, the term klotho refers to α -klotho. Commercial enzymatic tests (Roche Diagnostics) were used for measuring serum total cholesterol, triglycerides, and high-density lipoprotein (HDL) cholesterol concentrations. Low-density lipoprotein (LDL) cholesterol was calculated by the Friedewald formula (9).

Variables are reported as medians (25th, 75th percentiles) or as percentages. An exploratory analysis using scatterplots and lowess smoothing curves suggested a threshold between plasma klotho and grip strength. Nonparametric deviance reduction (26) was used to identify the threshold of 681 pg/mL of plasma klotho in relation to grip strength. Given the threshold of plasma klotho, we used piecewise regression (23) to examine the relationship between plasma klotho and grip strength at the 3-year (baseline), 6-year, and 9-year follow-up visits. In multivariable linear mixed models, no significant plasma klotho and time interactions were found in the individual models. All analyses were performed using SAS (v. 9.1.3, SAS Institute, Inc., Cary, NC) with a type I error of 0.05.

RESULTS

The baseline characteristics of the participants are shown in Table 1. The relationship between plasma klotho and grip strength is shown in Figure 1. As noted in the Methods, nonparametric deviance reduction was used to identify a threshold relationship between plasma klotho and grip strength at baseline of 681 pg/mL. The proportion of participants who were above and below the threshold was 46.8% and 53.2%, respectively. Univariate linear regression models were used to examine the relationship between participant characteristics and grip strength at baseline (Table 2). Piecewise regression was used to examine the relationship between plasma klotho, above and below 681 pg/mL, with grip strength at baseline. Older age, physical inactivity, cognitive impairment, congestive heart failure, stroke, chronic obstructive pulmonary disease, chronic kidney disease, depression, cancer, and plasma klotho, per 1 SD increase, for participants $\geq$ 681 pg/mL had significant negative association with grip strength. Current smoking and plasma klotho, per 1 SD increase, for participants $<$ 681 pg/mL, were significantly and positively associated with grip strength.

Multivariable linear mixed models using piecewise regression were used to examine the relationship between plasma klotho and other covariates with grip strength at baseline and at 3 and 6 years following the baseline visit (Table 3). In models adjusting for age and sex (model 1), additionally for education, smoking, and physical activity (model 2), and finally adding MMSE score, stroke, heart failure, chronic kidney disease, cancer, and depression, in participants with plasma klotho $<$ 681 pg/mL, an increase in plasma klotho was significantly associated with increased grip strength (per 1 SD increase, $\beta = 1.20$, standard error = 0.35, $P = 0.0009$). Plasma klotho $\geq$ 681 pg/mL had no significant relationship with grip strength once adjusted for other covariates in the three models above.

We examined the relationship between tertiles of plasma klotho ($<$ 575, 575-763, $>$ 763 pg/mL) and decline in grip strength over six years of follow-up. Mean (standard deviation) decline in grip strength from the lowest to the highest tertile of plasma klotho was 8.27 (4.83), 7.54 (5.04), and 7.25 (4.70) kg, respectively. In multivariable linear mixed models adjusting for age, sex, baseline grip strength, and other covariates above, tertile of plasma klotho at baseline was not significantly associated with decline in grip strength (data not

shown). The proportion of participants who died during six years of follow-up from lowest to the highest tertile of plasma klotho was 31.1%, 24.2%, and 17.1% ($P<0.0001$). The mortality results from this cohort are being reported in detail in a separate paper.

DISCUSSION

The present study shows that older community-dwelling adults with low plasma klotho concentrations have poor grip strength. To our knowledge, this is the first study to describe the relationship between circulating klotho hormone and skeletal muscle strength in humans. The findings are consistent with sarcopenia that has been described in the *klotho* mouse model of aging (13, 18). Plasma klotho concentrations at baseline were associated with grip strength measurements measured during a six year study period. Although plasma klotho was not an independent predictor of decline in grip strength over six years of follow-up, there was a strong competing risk of mortality. Participants with low plasma klotho concentrations were at a higher risk of death, as described in greater detail in a separate paper (31).

Sarcopenia is characterized by loss of skeletal muscle strength and muscle mass due to enhanced catabolic activity in muscle. Two major pathways are involved in the catabolism of protein in muscle cells. In the autophagic-lysosomal pathway, macromolecules and organelles are absorbed into autophagosomes and degraded after fusion with lysosomes and subsequent lysosomal degradation (24). In the ubiquitin-proteasomal pathway, proteins are conjugated to multiple ubiquitins and then degraded within the proteasome complex (4). Insulin/insulin-like growth factor-1 (IGF-1) signaling regulates both pathways through Akt and FoxO3 in skeletal muscle (11, 21). Circulating klotho is believed to regulate insulin/IGF-1 signaling, but the biological mechanism has yet to be identified (18). A recent study suggests that the autophagic-lysosomal pathway is activated in mutant *klotho* mice, but there was no evidence for activation of the ubiquitin-proteasomal pathway or insulin/IGF-1 signaling in *klotho* mice (13).

The strengths of the present study are that the participants were drawn from a population-based sample of older adults living in the community. Grip strength was carefully assessed using standardized methodology. Findings were based upon baseline and six years of follow-up data. Although the analyses were adjusted for potential confounders such as age and chronic diseases, as with any epidemiological study, there may be unmeasured confounding factors that could influence the relationship between plasma klotho and muscle strength. The study populations consisted of white adults from the rural Chianti region of Tuscany, Italy, and whether these findings can be extrapolated to populations in other parts of the world and to minorities is not known.

Future studies are needed to examine the relationship between circulating klotho and skeletal muscle mass and changes in skeletal muscle mass over time.

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Figure 1.
Scatterplot of plasma klotho and grip strength at baseline, with lowess smoothing curve.

Table 1Baseline characteristics of adults, 65 years, in the InCHIANTI study¹

Characteristic		Median (25 th , 75 th Percentile) or %
Age, y		75.0 (71.0, 80.0)
Sex (%)	Male	44.2
	Female	55.8
Education, y		5.0 (4.0, 6.0)
Current smoker (%)		11.0
Body mass index (kg/m ²)		26.4 (23.7, 28.7)
Physical activity (%)	Inactive	26.1
	Low	48.6
	Moderate-High	25.3
Grip strength		26.5 (20.3, 36.5)
Plasma klotho (pg/mL)		664 (521, 811)
Mini-Mental State Exam Score <24 (%)		29.0
Hypertension (%)		72.3
Coronary artery disease (%)		6.1
Heart failure (%)		7.7
Peripheral artery disease (%)		13.7
Stroke (%)		7.6
Diabetes mellitus (%)		14.3
Cancer (%)		8.7
Chronic kidney disease (%)		28.6
Depression (%)		28.5

¹Baseline for this study is the three-year follow-up visit of this prospective study cohort.

Table 2

Univariate linear regression models of plasma klotho and other variables with grip strength at baseline

Characteristic		Beta	SE	P
Age, y		-0.78	0.05	<0.0001
Male sex		15.2	0.60	<0.0001
Education, y		0.88	0.11	<0.0001
Current smoking		4.23	1.26	0.0008
Body mass index, kg/m ²		-0.01	0.10	0.91
Physical activity	Inactive	-14.95	1.00	<0.0001
	Low	-7.80	0.86	<0.0001
MMSE score <24		-8.65	0.85	<0.0001
Hypertension		-1.01	0.90	0.26
Coronary heart disease		0.13	1.69	0.94
Congestive heart failure		-7.13	1.52	<0.0001
Peripheral artery disease		-1.80	1.17	0.12
Stroke		-6.90	1.58	<0.0001
Diabetes mellitus		-1.99	1.15	0.08
Chronic obstructive pulmonary disease		-3.68	0.88	<0.0001
Chronic kidney disease		-3.68	0.89	<0.0001
Depression		-7.28	0.85	<0.0001
Cancer		-3.36	1.42	0.02
Plasma klotho (per 1 SD increase)	<681 pg/mL	2.69	0.58	<0.0001
	681 pg/mL	-1.14	0.39	0.004

Table 3

Multivariable linear mixed models for plasma klotho and grip strength

	Model 1 Adjusted for age, sex			Model 2 Adjusted for age, sex, education, smoking, physical activity			Model 3 Adjusted for age, sex, education, smoking, physical activity, MMSE score, stroke, heart failure, chronic kidney disease, cancer, and depression		
	Beta	SE	P	Beta	SE	P	Beta	SE	P
Plasma klotho (per 1 SD increase)	<681 pg/mL	0.37	0.0007	1.24	0.36	0.0008	1.20	0.35	0.0009
	681 pg/mL	0.25	0.65	-0.14	0.25	0.58	-0.19	0.24	0.44