The Klotho-FGF23-CPP axis and cardiovascular outcomes in diabetic patients with chronic limb threatening ischemia: a prospective study

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Research Article

Keywords: Diabetes mellitus, peripheral artery disease (PAD), Klotho, Fibroblast growth factor 23 (FGF23), Calciprotein particles (CPP).

Posted Date: September 6th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1917942/v2

Abstract

Background: Cardiovascular complications after lower extremity revascularization (LER) are common in diabetic patients with peripheral arterial disease (PAD) and chronic limb threatening ischemia (CLTI). The Klotho-FGF23-CPP axis is associated with endothelial injury and cardiovascular risk.

The aim of this study was to analyze the relationship between Klotho-FGF23-CPP serum levels and the incidence of major adverse cardiovascular events (MACE) and major adverse limb events (MALE) after LER in diabetic patients with PAD and CLTI.

Methods: Baseline levels of Klotho, FGF23, and CPP, and their association with subsequent incidence of MACE and MALE were analyzed in a prospective, non-randomized study in a population of diabetic patients with PAD and CLTI requiring LER.

Results: A total of 220 patients were followed for 12 months after LER. Sixty-three MACE and 122 MALE were recorded during follow-up period. Baseline lower Klotho serum levels (295.3 ± 151.3 pg/mL *vs* 446.4 ± 171.7 pg/mL, $p<0.01$), whereas increased serum levels FGF23 (75.0 ± 11.8 pg/mL *vs* 53.2 ± 15.4 pg/mL, $p<0.01$) and CPP (461.8 ± 168.6 pg/mL *vs* 269.3 ± 184.3 pg/mL, $p<0.01$) were significantly associated with the development of MACE. ROC analysis confirmed the predictive power of the Klotho-FGF23-CPP axis. Furthermore, decreased Klotho serum levels were associated with the occurrence of MALE after LER (329.1 ± 136.8 pg/mL *vs* 495.4 ± 183.9 pg/mL, $p<0.01$).

Conclusions: Klotho-FGF23-CPP axis imbalance is a potential biomarker for increased cardiovascular risk after LER in diabetic patients with PAD and CLTI.

Background

Peripheral arterial disease (PAD) is a common complication of type 2 diabetes mellitus (T2DM) and an independent cardiovascular risk factor[1]. Diabetic patients with PAD have poorer quality of life and life expectancy than patients without PAD. In general, PAD is also more aggressive in people with T2DM than in people without T2DM leading to a higher incidence of tissue loss and amputations[2–4]. One of the most common complications of PAD is chronic limb threatening ischemia (CLTI), which necessitates endovascular revascularization and often results in lower extremity gangrene requiring amputation[5]. Patients with CLTI may also experience major adverse cardiovascular events (MACE) and major adverse limb events (MALE) in the period following revascularization procedure[6]. This phenomenon is primarily due to the fact that atherosclerotic disease in patients with PAD is essentially a polyvascular disease that affects multiple arterial beds in the lower extremities, coronary arteries, and brain[7, 8]. There is also extensive evidence that other pathophysiological mechanisms involving structural protein of cardiac contractility, atherosclerosis associated inflammation response, and platelet activation pathways may play an important role in MACE and MALE after non-cardiac surgical interventions[9, 10]. Despite implementation of optimal medical therapy aimed at managing modifiable risk factors including lifestyle modification, use of lowering lipid therapy, use of antiplatelet therapy, novel anticoagulants, and medications to control diabetes and high blood pressure[11–13], still a large number of patients with PAD undergoing revascularization experience MACE and MALE[1]. In this context, identification of novel pathophysiological pathways as adjuvant risk stratification and/or therapeutic targets is important in further reducing the incidence of MACE and MALE on PAD patients CLTI undergoing revascularization for limb salvage.

Klotho, a membrane-bound protein co-receptor for fibroblast growth factor 23 (FGF23), has been shown to be involved in atherosclerosis and cardiovascular disease[14]. Moreover, Klotho regulates blood glucose and cholesterol levels[15, 16]. Furthermore, the lack of Klotho has been found to promote calcification and accumulation of cholesterol in the arteries, which can lead to coronary heart disease[17]. Interestingly, reduced Klotho serum levels are associated with endothelial dysfunction, oxidative stress, accelerated atherosclerosis, plaque instability with increase in atherosclerosis mediated inflammatory response playing a plausible role in acute cardiovascular[14, 18–21].

Klotho protein functions as the obligate co-receptor FGF23[22], a relatively new member of the fibroblast growth factor family, and has been shown to play a role in the development of atherosclerosis and cardiovascular disease[23, 24]. In fact, in individuals with chronic kidney disease (CKD), FGF23 is associated with endothelial dysfunction, arterial wall calcification, left ventricular hypertrophy, coronary artery disease and cardiovascular mortality[23, 25, 26]. More recently, elevated FGF23 levels have been shown to be associated with the presence and severity of PAD in a diabetic patient population without CKD[24].

Calciprotein particles (CPP), protein complexes containing calcium, phosphate, and the serum protein fetuin A, act as regulator of FGF23 secretion and of Klotho-FGF23 axis[27]. Several data indicate that individuals with elevated levels of CPP have an increased risk for developing atherosclerosis, endothelial dysfunction and cardiovascular disease[28–31].

Given the available data, we hypothesized that Klotho, FGF23, and CPP levels might influence the incidence of cardiovascular complications after endovascular revascularization of the lower limb (LER).

Methods

Study design

The aim of this study was to evaluate the relationship between Klotho, FGF23 and CPP serum levels at moment of LER and the incidence of MACE and MALE in a cohort of T2DM patients with PAD and CLTI. We conducted a prospective, non-randomized study approved by the Ethics Committee of the Fondazione Policlinico Universitario A. Gemelli IRCCS. All included patients gave informed consent to participate in the study, in accordance with the principles of the Declaration of Helsinki.

Study population and clinical assessment

The study population included 220 T2DM patients with PAD and CLTI requiring revascularization from the Fondazione Policlinico Universitario A. Gemelli IRCCS in Rome, Italy. Patients were consecutively enrolled between December 20, 2019, and June 30, 2021.

Inclusion criteria included: age at least 18 years, diagnosed with T2DM for at least 1 year, ankle/brachial index (ABI) less than 0.9, at least one lower extremity artery stenosis greater than 50% detected by ultrasound (US) color Doppler, stage 4 or 5 PAD diagnosis according to Rutherford classification, presence of CLTI and LER indication for target artery stenosis, as previously described[6, 32–35]. Exclusion criteria were: LER within the last 3 months, diabetic foot ulcer with active infection or osteomyelitis, diabetic peripheral neuropathy, homozygous familial hypercholesterolemia, absolute contraindications to antiplatelet therapy, thrombophilia, active cancer, active autoimmune disease, B or C stage liver disease according to Child-Pugh classification, life expectancy less than 12 months, pregnancy.

To classify and stratify patients with diabetic foot ulcers, the Wound, Ischemia, foot Infection (WIfI) classification system was used. If necessary, radiographic studies to rule out osteomyelitis were performed. Diabetic peripheral neuropathy was excluded as previously described[6]. PAD was defined according to the criteria of the Society for Vascular Surgery and the International Society for Cardiovascular Surgery[33]. All patients underwent lower extremity US. Ultrasound evaluation has also been used to confirm significant stenosis in the setting of arterial calcification in patients with an ABI of 1.40 or higher.

For all patients the following clinical and laboratory data were collected. Complete clinical history, including but not limited to coronary heart disease (CAD), cerebrovascular disease (CVD) history, hypertension, smoking status, body mass index (BMI), blood tests, as described below.

All patients were taking lipid-lowering therapy to achieve a low-density lipoprotein cholesterol (LDL-C) target of less than 55 mg/dL.

At time of revascularization, all patients received single antiplatelet therapy and dual antiplatelet therapy (DAPT) for 1 month after revascularization.

Endovascular revascularization procedure and follow-up

LER was performed as previously described[6, 32, 33]. Angioplasty and, if necessary, arterial stenting were defined as successful if the remaining arterial stenosis was less than 30% of the lumen[6]. We excluded from follow-up 19 (7.94%) of 239 patients due to primary treatment failure after revascularization. No major perioperative complications were recorded, as defined by the Society of Interventional Radiology[36]. During the 12-month follow-up period, patients were evaluated at 1, 3, 6, and 12 months after LER to assess the incidence of MACE and MALE outcomes. MACE was defined as a combination of myocardial infarction, stroke and cardiovascular death. MALE refer to the composite of acute limb ischemia, major vascular amputations, limb-threatening ischemia leading to urgent revascularization[6].

Blood test and biochemical analysis

On the day of LER, blood samples were collected from all patients after an overnight fast. Glucose, creatinine, calcium, phosphorus, vitamin D, total cholesterol, LDL-C, triglycerides, and glycated hemoglobin were assessed. Renal function was calculated by the modification of diet in renal disease (MDRD) formula to determine estimated glomerular filtration rate (eGFR). Serum was separated from blood samples by centrifugation and stored at -80°C prior to each assessment. Three commercially available ELISA kits (EH3058 and EH4278 from Wuhan Fine Biotech Co., Ltd.; CSB-E12130h from Cusabio Biotech Co.) were used to determine Klotho, FGF23 and CPP levels, according to the manufacturers' protocol. The intra- and inter-assay coefficients of variation were 3.5% and 10.5%, respectively. Sensitivity, defined as the mean ± 3 SD of the 0 standard, was calculated as 0.15 pmol/mL. Serum levels were measured twice for each patient and the results were averaged.

Statistical analysis

Demographic and clinical data were summarized as means (standard deviations) for continuous variables and counts (percentages) for categorical variables. Where appropriate, chi-square and t-tests were used to compare groups. A logarithmic transformation was applied to not normally distributed variables before performing other analyses. Where appropriate, Klotho, FGF23 and CPP levels were compared using Mann-Whitney, Kruskal-Wallis, and Dunn's multiple comparisons. Multivariate stepwise logistic regression analyses adjusted for traditional atherosclerotic risk factors and Klotho, FGF23 and CPP levels were performed. We calculated the area under the receiver operating characteristic (ROC) curve to test the predictive discrimination of MACE or MALE. We elaborated two additional ROC curves for a model including only traditional risk factors (age, sex, BMI, high blood pressure, diabetes duration, smoking status, Rutherford staging, previous cardiovascular and cerebrovascular events, total cholesterol, LDL cholesterol, triglycerides, FBG, HbA1c) (Model 1) and for a model including all the risk factors plus all three proteins as continuous variables (Model 2). We then compared the areas under the ROC curves using the roccomp function in Stata. All analyses were performed using STATA version 14.0 for MacOS (Statistics/Data Analysis, Stata Corporation) and GraphPad Prism version 9.4.0 for MacOS (GraphPad Software, Inc.). Statistical significance was established at $p < 0.05$.

Results

Characteristics of the study population

A total of 220 patients were followed until the end of the study. Of these, 150 were male (68.2%). The average age was 71.3 ± 9.2 years. The median duration of diabetes was 12.4 ± 4.6 years. The mean BMI was 29.5 ± 3.0 kg/m^2. In total, we enrolled 61 (27.7%) active smokers, 115 (52.3%) former smokers, and 38 (17.3%) never smokers. Considering other cardiovascular risk factors, 120 (54.5%) patients had arterial hypertension, 100 (45.4%) patients had hyperlipidemia, 102 (46.4%) patients had a history of CAD, and 45 (20.4%) patients had a history of CVD. Looking specifically at the characteristics of PAD, the mean ABI score was 0.39 ± 0.1, and the Rutherford staging included 121 (55.0%) category 4 patients and 99 (45%) category 5 patients. Mean glycated hemoglobin was 8.8 ± 1.5%, LDL-C was 100.7 ± 19.4 mg/dL, and mean eGFR was 64.4 ± 14.6 mL/min/1.73m^2. The mean levels of FGF23, CPP, and Klotho were 59.4 ± 17.5 pg/mL, 324.4 ± 199.7 pg/mL, and 403.1 ± 179.3 pg/mL, respectively. The full characteristics of the population are shown in Table 1.

Table 1
Demographic characteristics and clinical data of the study cohort at baseline

Number of patients	**220**
Men/female, n	150:70
Age, years ± SD	71.3 ± 9.2
Diabetes duration, years ± SD	12.4 ± 4.6
BMI, Kg/m^2 ± SD	29.5 ± 3.0
Smoking (current), n (%)	61 (27.7)
Smoking (former), n (%)	115 (52.3)
Never smoked, n (%)	38 (17.3)
Hypertension, n (%)	120 (54.5)
Hypercholesterolemia, n (%)	100 (45.4)
CAD, n (%)	102 (46.4)
CVD, n (%)	45 (20.4)
ABI, ± SD	0.39 ± 0.1
Rutherford II-4, n (%)	121 (55.0)
Rutherford III-5, n (%)	99 (45.0)
WIfI010, n (%)	99 (45.0)
WIfI020, n (%)	35 (15.9)
WIfI110, n (%)	62 (28.2)
WIfI120, n (%)	107 (48.6)
HbA1c, % ± SD	8.8 ± 1.5
FBG, mg/dL ± SD	114.1 ± 19.8
Total cholesterol, mg/dL ± SD	204.1 ± 29.3
LDL cholesterol, mg/dL ± SD	100.7 ± 19.4
Triglycerides, mg/dL ± SD	212.2 ± 36.9
Creatinine, mg/dL ± SD	1.4 ± 0.4

Data are reported as means (standard deviation) for continuous variables and numbers (percentages) for categorical variables. BMI, Body Mass Index; CAD, Coronary Artery Disease; CVD, Cerebrovascular Disease; ABI, Ankle Brachial Index; WIfI, Wound, Ischemia, foot Infection; FBG, Fasting Blood Glucose; eGFR, estimated Glomerular Filtration Rate; Ca, Calcium; Ph, Phosphorus; FGF23, Fibroblast Growth Factor 23; CPP, Calciprotein Particles.

Number of patients	220
eGFR, mL/min/1.73m^2 ± SD	64.4 ± 14.6
Ca, mg/dL ± SD	9.5 ± 0.8
Ph, mg/dL ± SD	3.7 ± 0.8
Vitamin D, ng/mL ± SD	48.5 ± 15.9
FGF23 pg/mL ± SD	59.4 ± 17.5
CPP, pg/mL ± SD	324.4 ± 199.7
Klotho, pg/mL ± SD	403.1 ± 179.3
Data are reported as means (standard deviation) for continuous variables and numbers (percentages) for categorical variables. BMI, Body Mass Index; CAD, Coronary Artery Disease; CVD, Cerebrovascular Disease; ABI, Ankle Brachial Index; WIfI, Wound, Ischemia, foot Infection; FBG, Fasting Blood Glucose; eGFR, estimated Glomerular Filtration Rate; Ca, Calcium; Ph, Phosphorus; FGF23, Fibroblast Growth Factor 23; CPP, Calciprotein Particles.	

Serum levels of Klotho, FGF23 and CPP and incidence of MACE at 12 months

All patients were followed up for 12 months. During follow-up, we observed 63 MACEs.

MACE patients were exclusively male (63, p < 0.01), were predominantly suffering from hypertension (46, p < 0.01), and were active (30, p < 0.01) or ex-smokers (56, p < 0.01). There were no significant differences in history of hypercholesterolemia (p = 0.62), previous CAD (p = 0.06), previous CVD (p = 0.49) or LDL levels (p = 0.50) between people with and without MACE. Furthermore, we did not find any significant differences in diabetes duration (p = 0.62), FBG (p = 0.27) or HbA1c levels (p = 0.08) between the two populations. We documented that serum calcium levels in patients with MACE were slightly higher, but within the normal range (9.7 ± 0.7 mg/dL *vs* 9.4 ± 0.8 mg/dL, p = 0.03).

We found higher levels of FGF23 (75.0 ± 11.8 pg/mL *vs* 53.2 ± 15.4 pg/mL, p < 0.01) and CPP (461.8 ± 168.6 pg/mL *vs* 269.3 ± 184.3 pg/mL, p < 0.01), and lower levels of Klotho (295.3 ± 151.3 pg/mL *vs* 446.4 ± 171.7 pg/mL, p < 0.01) in MACE patients. **Supplemental Table 1** and Fig. 1 provide a comprehensive overview of the characteristics of patients with and without MACE.

We then considered the three components of the MACE composite outcome (**Supplemental Fig. 1**). Considering cardiovascular death, we did not notice any differences in terms of FGF23 (p = 0.12) and Klotho (p = 0.48) levels, while we recorded that the deceased patients had higher CPP (p = 0.03) levels. We found higher levels of FGF23 (p < 0.01) and CPP (p < 0.01), and lower levels of Klotho (p < 0.05) in CAD patients. We also recorded higher levels of FGF23 (p < 0.01) and CPP (p < 0.01), and lower levels of Klotho (p < 0.01) in CVD patients.

Three ROC curves were constructed to predict the incidence of MACE based on Klotho, FGF23, and CPP baseline levels, and the areas under the curve (AUC) were 0.24 (95% CI 0.17, 0.32), 0.87 (95% CI 0.81, 0.92), and 0.78 (95% CI 0.72, 0.84), respectively (**Supplemental Fig. 2**). To test the efficacy of knowing protein levels at baseline, we compared the predictive power of traditional risk factors and risk factors plus Klotho, FGF23, and CPP levels. Including serum protein levels significantly improved the prediction of incident MACE after LER (Fig. 2).

Multivariate analysis showed that Klotho ($p < 0.01$), FGF23 ($p < 0.01$) and CPP ($p < 0.01$) baseline levels were independent determinant of MACE in CLTI patients receiving LER (Table 2). An additional multivariate analysis was performed including all the variables, and we found that male sex ($p < 0.01$), history of CAD ($p < 0.01$), smoking status ($p < 0.01$), total cholesterol ($p < 0.01$), and LDL cholesterol ($p < 0.01$) were independent determinant of MACE. Notably, after adjustment for all traditional risk factors, Klotho ($p < 0.01$), FGF23 ($p < 0.01$) and CPP ($p < 0.01$) levels were still independent determinant of MACE (**Table 3**).

Table 2
Multivariable logistic regression for MACE

	Coef.	St.Err.	t-value	p-value	[95% Conf	Interval]	Sig
FGF23	0.011	0.001	8.41	0.000	0.009	0.014	**
CPP	0.001	0.000	5.86	0.000	0.000	0.001	**
Klotho	-0.001	0.000	-4.22	0.000	-0.001	0.000	**
Constant	-0.391	0.112	-3.50	0.001	-0.611	-0.171	**
Mean dependent var	0.286		SD dependent var	0.453			
R-squared	0.464		Number of obs	220.000			
F-test	62.335		Prob > F	0.000			
Akaike crit. (AIC)	145.792		Bayesian crit. (BIC)	159.367			
*** p < 0.01*							

Table 33
Multivariable logistic regression for MACE

	Coef.	St.Err.	t-value	p-value	[95% Conf	Interval]	Sig
Age	0.002	0.002	0.99	0.325	-0.002	0.006	
Male sex	0.161	0.042	3.82	0.000	0.078	0.244	**
BMI	-0.010	0.007	-1.36	0.175	-0.023	0.004	
Diabetes duration	0.005	0.004	1.34	0.182	-0.003	0.013	
Hypertension	0.063	0.044	1.42	0.158	-0.025	0.151	
Hypercholesterolemia	0.035	0.040	0.88	0.382	-0.044	0.115	
CAD	-0.095	0.038	-2.48	0.014	-0.170	-0.019	*
CVD	-0.016	0.055	-0.29	0.775	-0.124	0.093	
Smoking (current)	0.257	0.049	5.29	0.000	0.161	0.353	**
Smoking (former)	0.381	0.045	8.53	0.000	0.293	0.470	**
Never smoked	0.271	0.058	4.66	0.000	0.156	0.385	**
ABI	0.155	0.947	0.16	0.870	-1.712	2.023	
Rutherford II-4	-0.112	0.084	-1.34	0.183	-0.277	0.053	
Rutherford III-5	0.000	.	.	.	.	.	
WIfI010	0.000	.	.	.	.	.	
WIfI020	-0.009	0.080	-0.12	0.908	-0.168	0.149	
WIfI110	-0.034	0.116	-0.29	0.769	-0.264	0.195	
WIfI120	-0.076	0.175	-0.44	0.663	-0.422	0.269	
Total cholesterol	-0.002	0.001	-2.08	0.039	-0.004	0.000	*
LDL cholesterol	0.003	0.001	2.03	0.044	0.000	0.005	*
Triglycerides	-0.001	0.001	-1.18	0.241	-0.002	0.000	
FPG	-0.001	0.001	-1.59	0.114	-0.003	0.000	
HbA1c	0.013	0.013	0.99	0.323	-0.013	0.038	
Creatinine	-0.048	0.044	-1.09	0.276	-0.135	0.039	
eGFR	-0.001	0.001	-0.88	0.379	-0.004	0.001	
Ca	-0.001	0.024	-0.05	0.963	-0.048	0.045	

	Coef.	St.Err.	t-value	p-value	[95% Conf	Interval]	Sig
Ph	-0.008	0.023	-0.37	0.715	-0.054	0.037	
Vitamin D	-0.001	0.001	-1.17	0.244	-0.004	0.001	
FGF23	0.007	0.001	5.99	0.000	0.005	0.009	**
CPP	0.001	0.000	5.29	0.000	0.000	0.001	**
Klotho	0.000	0.000	-4.25	0.000	-0.001	0.000	**
Constant	0.099	0.544	0.18	0.855	-0.974	1.173	
Mean dependent var	0.286		SD dependent var		0.453		
R-squared	0.716		Number of obs		220.000		
F-test	16.483		Prob > F		0.000		
Akaike crit. (AIC)	58.400		Bayesian crit. (BIC)		160.209		
*** p < 0.01, * p < 0.05*							

Serum levels of Klotho, FGF23 and CPP and incidence of MALE at 12 months

During the 12 months after LER intervention, we registered 122 MALE.

In **Supplemental Table 2** and Fig. 1 are fully reported the characteristics of patients with and without MALE. However, MALE patients were mostly male (90, $p < 0.05$), and suffering from hypertension (74, $p = 0.04$). There were no differences concerning other traditional cardiovascular risk factors. Interestingly, we found higher levels of FGF23 (62.2 ± 17.3 pg/mL *vs* 56.1 ± 17.3 pg/mL, $p < 0.01$), and lower concentrations of Klotho (329.1 ± 136.8 pg/mL *vs* 495.4 ± 183.9 pg/mL, $p < 0.01$) in MALE patients. Conversely, there were no significant differences between the two populations, regarding CPP levels (332.6 ± 202.7 pg/mL *vs* 314.3 ± 196.5 pg/mL, $p = 0.50$).

We then constructed ROC curves on Klotho, FGF23, and CPP baseline levels to predict the incidence of MALE after LER intervention. The three AUC were 0.24 (95% CI 0.17, 0.31), 0.60 (95% CI 0.52, 0.67), and 0.53 (95% CI 0.45, 0.60), respectively (**Supplemental Fig. 1**). As for the MACE outcome, we compared ROCs with traditional risk factors alone and with risk factors plus Klotho, FGF23, and CPP in predicting MALE. Likewise, addition of baseline protein levels significantly improved the predictive power of MALE after LER (Fig. 2).

Multivariate analysis showed that only baseline Klotho levels ($p < 0.01$) were independent determinants of MALE in CLTI patients during the 12 months after LER (Table 4). A further multivariate analysis, including the other variables, showed that hypertension ($p < 0.01$), and Rutherford stage 4 ($p < 0.05$) persisted to be independent determinants of MALE. Remarkably, after adjusting for all factors, Klotho levels ($p < 0.01$) remained independent determinants of MALE during the follow-up (Table 5).

Table 4
Multivariable logistic regression for MALE

	Coef.	St.Err.	t-value	p-value	[95% Conf	Interval]	Sig
FGF23	0.002	0.002	1.10	0.274	-0.002	0.006	
CPP	0.000	0.000	-0.74	0.460	0.000	0.000	
Klotho	-0.001	0.000	-7.22	0.000	-0.002	-0.001	**
Constant	0.980	0.148	6.61	0.000	0.688	1.273	**
Mean dependent var	0.555		SD dependent var		0.498		
R-squared	0.219		Number of obs		220.000		
F-test	20.161		Prob > F		0.000		
Akaike crit. (AIC)	270.403		Bayesian crit. (BIC)		283.978		
*** p < 0.01*							

Table 5
Multivariable logistic regression for MALE

	Coef.	St.Err.	t-value	p-value	[95% Conf	Interval]	Sig
Age	0.002	0.003	0.67	0.503	-0.005	0.009	
Male sex	0.010	0.072	0.14	0.885	-0.131	0.151	
BMI	-0.008	0.012	-0.66	0.509	-0.032	0.016	
Diabetes duration	0.008	0.007	1.17	0.244	-0.005	0.021	
Hypertension	0.206	0.076	2.72	0.007	0.056	0.355	**
Hypercholesterolemia	0.044	0.069	0.64	0.525	-0.092	0.180	
CAD	-0.045	0.065	-0.69	0.492	-0.173	0.083	
CVD	-0.117	0.094	-1.25	0.214	-0.301	0.068	
Smoking (current)	-0.016	0.083	-0.20	0.843	-0.180	0.147	
Smoking (former)	0.010	0.076	0.14	0.891	-0.140	0.161	
Never smoked	0.125	0.099	1.26	0.209	-0.070	0.320	
ABI	2.016	1.612	1.25	0.213	-1.165	5.196	
Rutherford II-4	0.290	0.143	2.04	0.043	0.009	0.572	*
Rutherford III-5	0.000	.	.	.	.	.	
WIfI010	0.000	.	.	.	.	.	
WIfI020	-0.126	0.137	-0.93	0.356	-0.396	0.143	
WIfI110	-0.067	0.198	-0.34	0.735	-0.458	0.324	
WIfI120	-0.315	0.298	-1.06	0.291	-0.903	0.272	
Total cholesterol	-0.001	0.001	-0.74	0.459	-0.004	0.002	
LDL cholesterol	0.002	0.002	1.02	0.309	-0.002	0.006	
Triglycerides	-0.002	0.001	-1.74	0.083	-0.003	0.000	
FPG	-0.002	0.002	-1.33	0.186	-0.005	0.001	
HbA1c	0.006	0.022	0.28	0.781	-0.037	0.049	
Creatinine	-0.131	0.075	-1.74	0.083	-0.278	0.017	
eGFR	-0.001	0.002	-0.50	0.617	-0.005	0.003	

	Coef.	St.Err.	t-value	p-value	[95% Conf	Interval]	Sig
Ca	0.044	0.040	1.10	0.274	-0.035	0.124	
Ph	0.005	0.039	0.14	0.891	-0.072	0.082	
Vitamin D	-0.001	0.002	-0.37	0.714	-0.005	0.003	
FGF23	0.001	0.002	0.60	0.548	-0.003	0.005	
CPP	0.000	0.000	-0.97	0.331	0.000	0.000	
Klotho	-0.001	0.000	-6.91	0.000	-0.002	-0.001	**
Constant	0.417	0.927	0.45	0.653	-1.412	2.246	
Mean dependent var	0.555		SD dependent var	0.498			
R-squared	0.318		Number of obs	220.000			
F-test	3.048		Prob > F	0.000			
Akaike crit. (AIC)	292.670		Bayesian crit. (BIC)	394.479			
*** p < 0.01, * p < 0.05*							

Discussion

In this prospective study, we demonstrate that an imbalance the Klotho-FGF23-CPP axis is associated with higher incidence of adverse cardiovascular outcomes after revascularization in diabetic patients with PAD and CLTI. This is a novel finding, it is consistent with the established role of Klotho-FGF23-CPP axis in atherosclerosis, inflammation and endothelial dysfunction found in other humans and animal models. In particular, decreased Klotho basal serum levels and increased FGF23 and CPP levels were associated with the incidence of MACE after revascularization intervention. In addition, decreased Klotho levels were also associated with the development of MALE.

The Klotho gene (also known as α-Klotho) is located on chromosome 13q12 and it was first identified as an anti-aging factor[37]. It is expressed in the kidney, and even less in the pancreatic islets, lung, liver, skeletal muscle, aorta, brain, and prostate. Klotho acts primarily as a coreceptor of FGF23, but also interacts with other receptor systems, including transforming growth factor-β and insulin receptors, and signaling pathways, including wingless-related integration site (Wnt)[37]. Klotho is associated to CKD but exerts multiple cardiovascular protective effects due to different receptors and regulatory systems. In fact, it induces Akt expression, through but not only the insulin receptor system, thereby reducing oxidative stress and increasing nitric oxide (NO) production in the endothelium and preventing endothelial dysfunction[38, 39]. Notably, CPP exert cytotoxic effects against vascular endothelial cells[31].

Furthermore, Klotho deficiency is associated with the development of hypertension, particularly by increasing the stiffness of the arterial walls[40]. Moreover, Klotho and FGF23 are implicated in calcium balance and arterial calcification, as well as other pro-inflammatory mediators, such as osteoprotegerin[41, 42] and CPP[31]. The Klotho-FGF23 axis is also involved in the development of left ventricular hypertrophy[43]. In fact, Klotho inhibits FGF23- and angiotensin II-induced myocardial hypertrophy[43]. Since these data were primarily obtained in animal models, often genetically modified, it is unclear whether this impairment is due to increased FGF23 activity and/or decreased Klotho function. Nevertheless, both proteins have been shown to play a role in causing the disease. However, soluble Klotho was found to reduce cardiac hypertrophy, independently of FGF23 and phosphate levels, by inhibiting abnormal activity of calcium-dependent signaling in the heart[44]. In addition, Klotho inhibits cardiac remodeling through the Wnt pathway[37]. Interestingly, increased levels of Klotho reduce oxidative damage after myocardial infarction in animal models[45]. Furthermore, reduced Klotho levels are also associated with central obesity, elevated triglycerides, and metabolic syndrome[46]. Not surprisingly, reduced Klotho levels and elevated FGF23 concentrations have been associated with increased hospitalizations for heart failure and the incidence of cardiovascular death in a population of 3555 patients with stable ischemic heart disease[47]. The main findings of our study are consistent with previous data, and show that reduced Klotho serum levels and elevated FGF23 and CPP concentration are associated with the development of MACE in a T2DM population. Diabetic patients with PAD are frequently affected by polyvascular disease and are at high cardiovascular risk[11].

The prospective nature of the present study, one of the first in humans, provides further insight on potential novel risk factors of MACE after revascularization in patients with CLTI. Traditional risk factors including Male gender, smoking, and arterial hypertension were associated to the development of MACE as expected. Remarkably, also calcium levels were slightly, but significantly, elevated in the MACE population. Conceivably, changes in the Klotho-FGF23-CPP axis are responsible, at least in part, for altered calcium balance and increased cardiovascular risk. However, multivariate analysis did not confirm a significant role for calcium levels, while it did corroborate that male sex, history of CAD, and total and LDL cholesterol levels are crucial determinant for MACE risk. This is consistent with previous data regarding classical cardiovascular risk factors and lipid profile as biomarkers for the MACE incidence[6, 7]. Smoking status was also relevant. In fact, present and past smoking, was significant, as well as having never smoked. However, when active smoking and previous smoking habits were coherent with previous evidence, it was surprising that never smoking was also critical for the development of MACE. This unexpected finding could be explained by the relatively small sample size, a selection bias intrinsic to the vascular specialty center in which the study was conduction, and a reporting bias of risk factor exposure. Relatedly, even when these factors were included in the analysis model, the results indicated that baseline levels of Klotho, FGF23, and CPP were independent determinants of MACE incidence after LER. Interestingly, this hypothesis was also supported by the ROC analysis, with the area under the curve showing the predictive power of the three factors. To our knowledge, this is the first demonstration in prospective human study of a role for the Klotho-FGF23-CPP axis in the development of cardiovascular complications after LER in diabetic patients with PAD and CLTI.

Considering all the possible mechanisms involved, we can assume that the reasons for our finding are multiple and interdependent. Endothelial injury and dysfunction associated with reduced Klotho levels and elevated FGF23 concentrations, as well as direct damage caused by CPP, have been shown to justify the worsening of atherosclerosis in diabetic patients with PAD. Imbalance in calcium metabolism may also be associated with aggravation of arterial wall injury, consistent with data from other proinflammatory cytokines involved in calcium and phosphorus metabolism[6]. In addition, oxidation of LDL may play a role, which may also have therapeutic implications. The study population had no target LDL levels, according to the latest guidelines, and statins have, among other pleiotropic effects, the ability to increase circulating Klotho levels. Conceivably, at least a part of patients, those with reduced Klotho concentrations, may benefit more from statin therapy when reaching the same LDL levels. Furthermore, drugs that inhibit the renin-angiotensin system promote Klotho expression and it is plausible this class of drugs could have better effects in patients who have reduced Klotho serum levels, regardless of the effect on blood pressure. Unfortunately, the relatively small sample size did not allow to stratify the outcomes for the different lipid-lowering or antihypertensive treatments, so we are currently unable to confirm this hypothesis. The small number of individual studied could also explain the lack of differences observed when analyzing the baseline levels of Klotho and FGF23 in the first component of the composite outcome MACE, specifically in cardiovascular death. However, the significant difference was maintained for CAD and CVD.

Another finding of the present study was the relationship between baseline Klotho levels and the incidence of MALE after revascularization intervention. Considering the characteristics of the population with and without MALE during follow-up, we observed differences in male sex, hypertension, and Klotho and FGF23 levels. Interestingly, as with the MACE outcome, calcium levels were slightly elevated in the MALE population. However, multivariate analysis confirmed that only hypertension and Rutherford stage 4 had a significant role in the development of MALE. Importantly, reduced Klotho baseline levels were identified as a predisposing factor for MALE incidence even after adjusting for all factors considered in the study. ROC analysis also confirmed the good predictive power of MALE after LER in our patient population. The mechanisms leading to increased MALE in patients with low basal Klotho levels at the time of LER may be diverse, but maybe related to direct endothelial injury, which has been already demonstrated in other models[38, 48, 49]. Surprisingly, FGF23 levels did not prove to be a significant predictor after inclusion in multivariate analysis. This may be related to the main limitation of the study, namely the relatively small sample size. However, Klotho has been shown to exert a range of endothelial protective effects independent of FGF23 activity, which may justify the effects observed in our study population.

In addition to the relatively small population, limitations of the study include that it was conducted at a single center in a Caucasian population. However, significant differences in Klotho-FGF23-CPP axis levels between races were not described, except that FGF23 levels were associated with food insecurity in a black population[50]. Moreover, the small sample size did not even allow us to stratify the protein levels of the different components of the composite endpoint MALE. Another limitation of our study is that we did not assess the association between original arterial lesions and following incident MALE. Also, we excluded patients with primary LER failure from the study to reduce bias, and therefore we did not analyze the serum levels of Klotho, FGF23 and CPP. Furthermore, we did not measure protein levels during follow-up, and we have no way of knowing whether these changed over time. Nevertheless, the aim of this study was to identify possible biomarkers of risk after LER, and further determination over time is not necessary. Finally, we did not analyze the effects of various ongoing pharmacotherapies, especially those that may affect Klotho levels and activity, such as statins, renin-angiotensin system inhibitors, and pioglitazone.

Conclusion

Diabetic patients with PAD and CLTI are at increased risk of cardiovascular complications after LER. Despite the best treatment and follow-up, many individuals, with apparently similar risk factors and undergoing same intervention, experience very different and unpredictable complications. The availability of new biomarkers during revascularization of the limb may change the approach to treatment and follow-up, leading to more aggressive treatment targets or stricter follow-up. Given previous data on the role of the Klohto-FGF23-CPP axis in the cardiovascular environment, we hypothesized that this might be a reliable biomarker of cardiovascular risk even in a CLTI diabetic population requiring LER. We have demonstrated for the first time that Klohto-FGF23-CPP is associated with the development of MACE after LER and that reduced Klotho levels predict MALE after revascularization. These data were obtained in a relatively small but particularly selected population, and need to be confirmed in a larger scenario. Future research will also be able to better elucidate the interactions between the three components of the axis and to highlight whether these effects are independent of each other. It will also be important to know whether current and future therapies can bias the risk from axis imbalance towards, for example, a decrease in Klotho or an increase in FGF23.

Taken together, these data could improve cardiovascular risk stratification in diabetic patients with PAD and help physicians identify personalized treatments.

Abbreviations

ABI ankle/brachial index

AUC area under the curve

BMI body mass index

CAD coronary artery disease

CLTI chronic limb-threatening ischemia

CVD cerebrovascular disease

DAPT double antiplatelet therapy

eGFR estimated glomerular filtration rate

LDL-C low-density lipoprotein cholesterol

LER lower limb revascularization

MACE major adverse cardiovascular events

MALE major adverse limb events

NO nitric oxide

PAD peripheral artery disease

ROC receiver operating characteristics

T2DM type 2 diabetes mellitus

US ultrasound

WIfI wound, ischemia, foot infection

Wnt wingless-related integration site

Declarations

Ethics approval and consent to participate

The study was approved by the Ethics Committee of the Fondazione Policlinico Universitario A. Gemelli IRCCS and adhered to the principles of the Declaration of Helsinki. All the individuals agreed to participate in the study and gave informed consent.

Consent for publication

All authors have read the paper and agree that it can be published.

Guarantor's statement

Dr. Andrea Flex is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Availability of data and materials

The datasets generated during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

Funding

Not applicable.

Authors' contributions

FB participated in the design of the study, performed data analysis and reviewed the manuscript. FA, ALC and MAN carried out the immunoassays. MMR and DP participated in the design of the study and performed statistical analyses. RI performed the endovascular procedures. LE and PD reviewed the manuscript. AG, MM and AF conceived the study, participated in its design and coordination and helped draft the manuscript. All authors read and approved the final manuscript.

Acknowledgements

The authors thank the Italian Ministry of Health, Ricerca Corrente 2021.

References

1. Biscetti F, Nardella E, Rando MM, Cecchini AL, Gasbarrini A, Massetti M, **et al**. **Outcomes of Lower Extremity Endovascular Revascularization: Potential Predictors and Prevention Strategies**. Int J Mol Sci. 2021;**22**:2002.
2. Takahara M. **Diabetes Mellitus and Lower Extremity Peripheral Artery Disease**. JMA J. 2021;**4**:225–31.
3. Biscetti F, Pitocco D, Straface G, Zaccardi F, **de** Cristofaro R, Rizzo P, **et al**. **Glycaemic variability affects ischaemia-induced angiogenesis in diabetic mice**. Clin Sci (Lond). 2011;**121**:555–64.
4. Barnes JA, Eid MA, Creager MA, Goodney PP. **Epidemiology and Risk of Amputation in Patients With Diabetes Mellitus and Peripheral Artery Disease**. Arterioscler Thromb Vasc Biol. 2020;**40**:1808–17.
5. Beckman JA, Schneider PA, Conte MS. **Advances in Revascularization for Peripheral Artery Disease: Revascularization in PAD**. Circ Res. United States; 2021;**128**:1885–912.
6. Biscetti F, Ferraro PM, Hiatt WR, Angelini F, Nardella E, Cecchini AL, **et al**. **Inflammatory Cytokines Associated With Failure of Lower-Extremity Endovascular Revascularization (LER): A Prospective Study of a Population With Diabetes**. Diabetes Care. 2019;**42**:1939–45.
7. Aday AW, Matsushita K. **Epidemiology of Peripheral Artery Disease and Polyvascular Disease**. Circ Res. 2021;**128**:1818–32.
8. Gutierrez JA, Mulder H, Jones WS, Rockhold FW, Baumgartner **I**, Berger JS, **et al**. **Polyvascular Disease and Risk of Major Adverse Cardiovascular Events in Peripheral Artery Disease: A Secondary Analysis of the EUCLID Trial**. JAMA Network Open. 2018;**1**:e185239–e185239.
9. Zhang L-J, Li N, Li Y, Zeng X-T, Liu M-Y. **Cardiac Biomarkers Predicting MACE in Patients Undergoing Noncardiac Surgery: A Meta-Analysis**. Front Physiol. 2019;**9**:1923.

10. Schneider GS, Rockman CB, Berger JS. **Platelet Activation Increases in Patients Undergoing Vascular Surgery**. Thromb Res. 2014;**134**:952–6.
11. Aboyans V, Ricco J-B, Bartelink M-LEL, Björck M, Brodmann M, Cohnert T, **et al**. **2017 ESC Guidelines on the Diagnosis and Treatment of Peripheral Arterial Diseases, in collaboration with the European Society for Vascular Surgery (ESVS): Document covering atherosclerotic disease of extracranial carotid and vertebral, mesenteric, renal, upper and lower extremity arteriesEndorsed by: the European Stroke Organization (ESO)The Task Force for the Diagnosis and Treatment of Peripheral Arterial Diseases of the European Society of Cardiology (ESC) and of the European Society for Vascular Surgery (ESVS)**. Eur Heart J. England; 2018;**39**:763–816.
12. Kithcart AP, Beckman JA. ACC/AHA Versus ESC **Guidelines for Diagnosis and Management of Peripheral Artery Disease: JACC Guideline Comparison**. J Am Coll Cardiol. United States; 2018;**72**:2789–801.
13. Golledge J. **Update on the pathophysiology and medical treatment of peripheral artery disease**. Nat Rev Cardiol. Nature Publishing Group; 2022;**19**:456–74.
14. Brandenburg VM, Kleber ME, Vervloet MG, Larsson TE, Tomaschitz A, Pilz S, **et al**. **Soluble klotho and mortality: the Ludwigshafen Risk and Cardiovascular Health Study**. Atherosclerosis. Ireland; 2015;**242**:483–9.
15. Yao Y, Wang Y, Zhang Y, Liu C. **Klotho ameliorates oxidized low density lipoprotein (ox-LDL)-induced oxidative stress via regulating LOX-1 and PI3K/Akt/eNOS pathways**. Lipids Health Dis. 2017;**16**:77.
16. Ciardullo S, Perseghin G. **Soluble α-Klotho levels, glycemic control and renal function in US adults with type 2 diabetes**. Acta Diabetol. 2022;**59**:803–9.
17. Koga S, Ikeda S, Akashi R, Yonekura T, Kawano H, Maemura K. **Serum soluble Klotho is inversely related to coronary artery calcification assessed by intravascular ultrasound in patients with stable coronary artery disease**. J Cardiol. Netherlands; 2021;**77**:583–9.
18. Donate-Correa J, Martín-Núñez E, Mora-Fernández C, Muros-de-Fuentes M, Pérez-Delgado N, Navarro-González JF. **Klotho in cardiovascular disease: Current and future perspectives**. World J Biol Chem. 2015;**6**:351–7.
19. Moe SM. **Klotho: a master regulator of cardiovascular disease? Circulation**. United States; 2012;**125**:2181–3.
20. Martín-Núñez E, Donate-Correa J, Ferri C, López-Castillo Á, Delgado-Molinos A, Hernández-Carballo C, **et al**. **Association between serum levels of Klotho and inflammatory cytokines in cardiovascular disease: a case-control study**. Aging (Albany NY). 2020;**12**:1952–64.
21. Xia W, Zhang A, Jia Z, Gu J, Chen H. **Klotho Contributes to Pravastatin Effect on Suppressing IL-6 Production in Endothelial Cells**. Mediators Inflamm. 2016;**2016**:2193210.
22. Quarles LD. **FGF-23 and α-Klotho Co-Dependent and Independent Functions**. Curr Opin Nephrol Hypertens. 2019;**28**:16–25.
23. Krupp K, Madhivanan P. **FGF23 and risk of all-cause mortality and cardiovascular events: a meta-analysis of prospective cohort studies**. Int J Cardiol. Netherlands; 2014;**176**:1341–2.

24. Biscetti F, Straface G, Porreca CF, Bertoletti G, Vincenzoni C, Snider F, **et al**. **Increased FGF23 serum level is associated with unstable carotid plaque in type 2 diabetic subjects with internal carotid stenosis**. Cardiovasc Diabetol. 2015;**14**:139.
25. Faul C, Amaral AP, Oskouei B, Hu M-C, Sloan A, Isakova T, **et al**. **FGF23 induces left ventricular hypertrophy**. J Clin Invest. 2011;**121**:4393–408.
26. Jimbo R, Shimosawa T. **Cardiovascular Risk Factors and Chronic Kidney Disease-FGF23: A Key Molecule in the Cardiovascular Disease**. Int J Hypertens. 2014;**2014**:381082.
27. Akiyama K, Miura Y, Hayashi H, Sakata A, Matsumura Y, Kojima M, **et al**. **Calciprotein particles regulate fibroblast growth factor-23 expression in osteoblasts**. Kidney International. 2020;**97**:702–12.
28. Aghagolzadeh P, Bachtler M, Bijarnia R, Jackson C, Smith ER, Odermatt A, **et al**. **Calcification of vascular smooth muscle cells is induced by secondary calciprotein particles and enhanced by tumor necrosis factor-α**. Atherosclerosis. Ireland; 2016;**251**:404–14.
29. Anzai F, Karasawa T, Komada T, Yamada N, Miura Y, Sampilvanjil A, **et al**. **Calciprotein Particles Induce IL-1β/α-Mediated Inflammation through NLRP3 Inflammasome-Dependent and -Independent Mechanisms**. Immunohorizons. United States; 2021;**5**:602–14.
30. Chen W, Fitzpatrick J, Monroy-Trujillo JM, Sozio SM, Jaar BG, Estrella MM, **et al**. **Associations of Serum Calciprotein Particle Size and Transformation Time With Arterial Calcification, Arterial Stiffness, and Mortality in Incident Hemodialysis Patients**. Am J Kidney Dis. 2021;**77**:346–54.
31. Kutikhin AG, Feenstra L, Kostyunin AE, Yuzhalin AE, Hillebrands J-L, Krenning G. **Calciprotein Particles: Balancing Mineral Homeostasis and Vascular Pathology**. Arterioscler Thromb Vasc Biol. 2021;**41**:1607–24.
32. Biscetti F, Nardella E, Rando MM, Cecchini AL, Angelini F, Cina A, **et al**. **Association between omentin-1 and major cardiovascular events after lower extremity endovascular revascularization in diabetic patients: a prospective cohort study**. Cardiovasc Diabetol. 2020;**19**:170.
33. Biscetti F, Nardella E, Rando MM, Cecchini AL, Bonadia N, Bruno P, **et al**. **Sortilin levels correlate with major cardiovascular events of diabetic patients with peripheral artery disease following revascularization: a prospective study**. Cardiovasc Diabetol. 2020;**19**:147.
34. Biscetti F, Bonadia N, Santini F, Angelini F, Nardella E, Pitocco D, **et al**. **Sortilin levels are associated with peripheral arterial disease in type 2 diabetic subjects**. Cardiovasc Diabetol. 2019;**18**:5.
35. Biscetti F, Nardella E, Bonadia N, Angelini F, Pitocco D, Santoliquido A, **et al**. **Association between plasma omentin-1 levels in type 2 diabetic patients and peripheral artery disease**. Cardiovasc Diabetol. 2019;**18**:74.
36. Sacks D, Marinelli DL, Martin LG, Spies JB. **Reporting standards for clinical evaluation of new peripheral arterial revascularization devices**. J Vasc Interv Radiol. United States; 2003;**14**:S395-404.
37. Tyurenkov IN, Perfilova VN, Nesterova AA, Glinka Y. **Klotho Protein and Cardio-Vascular System. Biochemistry (Mosc)**. United States; 2021;**86**:132–45.
38. Chung C-P, Chang Y-C, Ding Y, Lim K, Liu Q, Zhu L, **et al**. **α-Klotho expression determines nitric oxide synthesis in response to FGF-23 in human aortic endothelial cells**. PLoS One. 2017;**12**:e0176817.

39. Flex A, Biscetti F, Iachininoto MG, Nuzzolo ER, Orlando N, Capodimonti S, **et al**. **Human cord blood endothelial progenitors promote post-ischemic angiogenesis in immunocompetent mouse model**. Thromb Res. 2016;**141**:106–11.
40. Kanbay M, Demiray A, Afsar B, Covic A, Tapoi L, Ureche C, **et al**. **Role of Klotho in the Development of Essential Hypertension**. Hypertension. United States; 2021;**77**:740–50.
41. Giovannini S, Tinelli G, Biscetti F, Straface G, Angelini F, Pitocco D, **et al**. **Serum high mobility group box-1 and osteoprotegerin levels are associated with peripheral arterial disease and critical limb ischemia in type 2 diabetic subjects**. Cardiovasc Diabetol. 2017;**16**:99.
42. Biscetti F, Giovannini S, Straface G, Bertucci F, Angelini F, Porreca C, **et al**. **RANK/RANKL/OPG pathway: genetic association with history of ischemic stroke in Italian population**. Eur Rev Med Pharmacol Sci. 2016;**20**:4574–80.
43. Han X, Cai C, Xiao Z, Quarles LD. **FGF23 induced left ventricular hypertrophy mediated by FGFR4 signaling in the myocardium is attenuated by soluble Klotho in mice**. J Mol Cell Cardiol. 2020;**138**:66–74.
44. Xie J, Yoon J, An S-W, Kuro-o M, Huang C-L. **Soluble Klotho Protects against Uremic Cardiomyopathy Independently of Fibroblast Growth Factor 23 and Phosphate**. J Am Soc Nephrol. 2015;**26**:1150–60.
45. Xu Z, Zheng S, Feng X, Cai C, Ye X, Liu P. **Klotho gene improves oxidative stress injury after myocardial infarction**. Exp Ther Med. 2021;**21**:52.
46. Cheng Y-W, Hung C-C, Fang W-H, Chen W-L. **Association between Soluble α-Klotho Protein and Metabolic Syndrome in the Adult Population**. Biomolecules. 2022;**12**.
47. Bergmark BA, Udell JA, Morrow DA, Jarolim P, Kuder JF, Solomon SD, **et al**. **Klotho, fibroblast growth factor-23, and the renin-angiotensin system - an analysis from the PEACE trial**. Eur J Heart Fail. 2019;**21**:462–70.
48. Buendía P, Carracedo J, Soriano S, Madueño JA, Ortiz A, Martín-Malo A, **et al**. **Klotho Prevents NFκB Translocation and Protects Endothelial Cell From Senescence Induced by Uremia**. J Gerontol A Biol Sci Med Sci. United States; 2015;**70**:1198–209.
49. Saito Y, Yamagishi T, Nakamura T, Ohyama Y, Aizawa H, Suga T, **et al**. **Klotho protein protects against endothelial dysfunction**. Biochem Biophys Res Commun. United States; 1998;**248**:324–9.
50. Pool LR, Kershaw KN, Gordon-Larsen P, Gutiérrez OM, Reis JP, Isakova T, **et al**. **Racial Differences in the Associations Between Food Insecurity and Fibroblast Growth Factor 23 in the Coronary Artery Risk Development in Young Adults Study**. J Ren Nutr. United States; 2020;**30**:509–17.

Figures

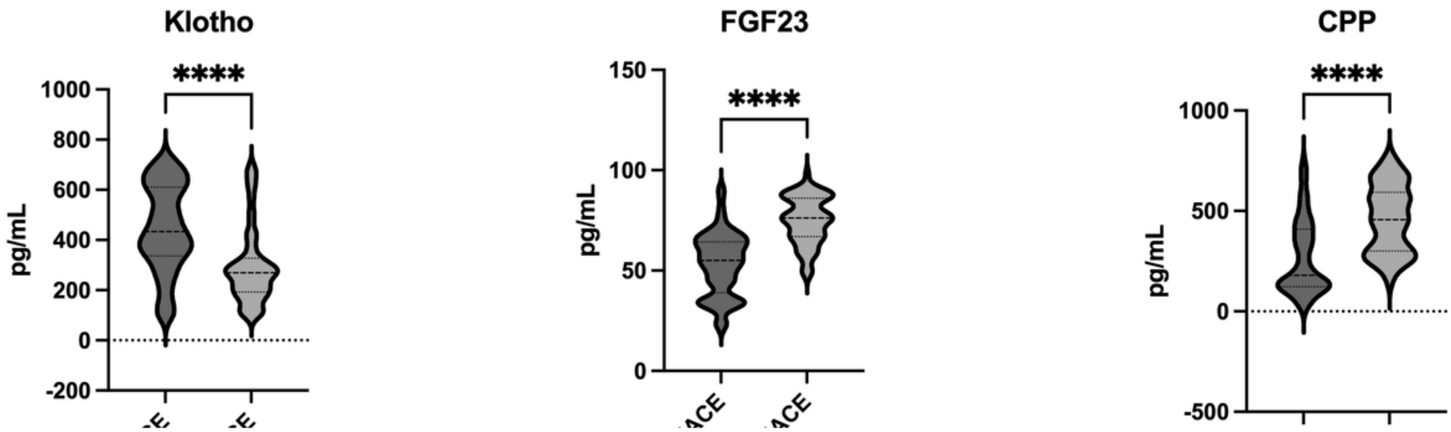

Figure 1

Klotho, FGF23 and CPP levels according to MACE and MALE outcomes. On the violin plots, shape shows the distribution, central line represents the median, upper line represents the upper interquartile range (IQR) and the lower line represents the lower IQR. **** = p<0.0001, ** = p<0.01.

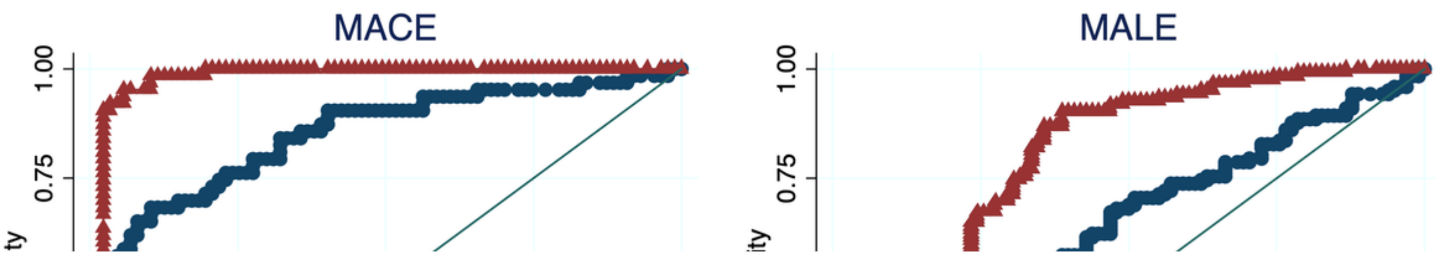

Figure 2

On the left, receiver operating characteristic (ROC) curves comparing the performance of a model without (Model 1) and with proteins (Model 2) in predicting MACE. The true-positive rate (sensitivity) is plotted as a function of the false-positive rate (1 - Specificity). $p<0.001$.

On the right, receiver operating characteristic (ROC) curves comparing the performance of a model without (Model 1) and with proteins (Model 2) in predicting MALE. The true-positive rate (sensitivity) is plotted as a function of the false-positive rate (1 - Specificity). $p<0.001$.

Supplementary Files

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