European Review for Medical and Pharmacological Sciences 2016; 20: 4048-4054

Fibroblast growth factor 23 serum level in type 2 diabetic italian subjects with peripheral arterial disease and critical limb ischemia

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Abstract. – OBJECTIVE: Fibroblast growth factor 23 (FGF23) was demonstrated to be involved in the occurrence and development of cardiovascular disease (CVD). The aim of this study was to investigate the potential role of FGF23 on presence and severity of peripheral arterial disease (PAD) in type 2 diabetic patients.

PATIENTS AND METHODS: In this study, we analyzed FGF23 serum levels in 413 type 2 diabetic patients with PAD and in 598 diabetic controls without lower limbs atherosclerosis.

RESULTS: We found that FGF23 median serum levels were significantly higher in patients than in diabetic controls (69.3 (58.8-75.1) pg/mL in PAD and 42.98 (37.1-49.8) pg/mL in subjects without PAD ($p < 0.001$) and were significantly and independently associated with critical limb ischemia (CLI) [OR, 7.69 (2.64-16.31); $p = 0.001$].

CONCLUSIONS: We have found, for the first time, that FGF23 could be associated with presence and severity of PAD in Italian patients with type 2 diabetes.

Key Words:
Type 2 diabetes, Fibroblast growth factor 23, FGF-23, Osteoprotegerin, OPG, Peripheral artery disease, PAD, Critical limb ischemia, CLI.

Introduction

Peripheral arterial disease (PAD) is a local manifestation of a systemic atherosclerotic disorder, increases 2-6-fold in both cerebrovascular and cardiovascular diseases and is associated with an annual mortality rate of 4%-6%[1]. Atherosclerosis is a chronic inflammatory disorder in the walls of large and medium arteries and is characterized by the interaction between molecular and environmental factors, as well as between chronic inflammation and altered immune function[2,3].

Fibroblast growth factor 23 (FGF23), is a hormone secreted by osteocytes and by osteoblasts, is involved in the regulation of phosphorus homeostasis, vitamin D metabolism, and bone mineralization; it inhibits activation of calcitriol [1,25(OH)2D], induces urinary phosphorous excretion and suppresses parathyroid hormone (PTH) synthesis[4,5].

FGF23 is involved in endothelial dysfunction and vascular calcification[6,7]: high serum FGF23 levels were an independent risk of coronary artery disease (CAD) and mortality in patients with chronic kidney disease[8] and were associated with vascular and endothelial dysfunction[9,10]. On the other hand, increased FGF23 levels have been associated with left ventricular hypertrophy[11] and prevalent cardiovascular disease in elderly individuals with normal renal function[12]. Type 2 diabetes (T2D) has been associated with higher rates of cardiovascular disease, thus showing the evaluation of FGF23 and its relationship with cardiovascular disease in T2D is of interest. We identified FGF23 serum levels as an independent risk factor for unstable carotid plaque in diabetic patients with internal carotid stenosis[13].

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The present work evaluated whether serum FGF23 levels are associated with presence and severity of PAD in a Italian diabetic population, and correlates with other inflammatory cytokines [High-Sensitivity C-reactive protein (Hs-CRP), interleukin-6 (IL-6)] and osteoprotegerin (OPG).

Patients and Methods

Patients

Patients and controls were recruited among subjects consecutively admitted to the Department of Internal Medicine of the "A. Gemelli" Catholic University Hospital of Rome, Italy and to the Department of Medicine of the "St. M. Goretti" Hospital, Latina, Italy, from November 1, 2013, to June 30, 2016. In the group of patients were enrolled, in a retrospective study, subjects who had a history of PAD. Inclusion criteria for the PAD group were Caucasian race and presence of PAD at Fontaine's stage II, III, or IV. Diagnosis of PAD was performed according to criteria established by the Ad Hoc Committee on Reporting Standards of the Society for Vascular Surgery and the International Society for Cardiovascular Surgery[14]. All patients had an ankle-brachial index (ABI) lower than 0.8 and underwent bilateral high-resolution B-mode ultrasonography evaluation (Eco-color-Doppler Acuson 128XP/10, Acuson, Mountain View, CA, USA, with an 4 MHz transducer). The severity of PAD was defined according to the Fontaine's staging system: patients were considered affected by stage II when they presented *claudicatio intermittens*, by stage III when they presented rest pain, and by stage IV when ischemic trophic lesions of the lower limbs were present. Following the recommendations of the Inter-Society Consensus for the Management of Peripheral Arterial Disease (TASC II), patients with ischemic rest pain, ulcers, or gangrene, attributable to objectively proven PAD, were considered affected by critical limb ischemia (CLI)[15].

Five hundred sixty-three subjects matched for age and gender were enrolled as controls, with an ABI ≥ 1 and normal findings at bilateral high-resolution B-mode ultrasonography evaluation. Subjects without peripheral arterial disease (WPAD) had no family history of PAD. The body mass index (BMI) was calculated as weight/height^2 (kg/m^2). The presence of T2D was indicated by a previous diagnosis and confirmed by fasting blood glucose > 126 mg/dL, glycated hemoglobin > 5.8% or by use of anti-diabetic drugs or insulin. A systolic blood pressure > 130 mmHg and a diastolic blood pressure > 85 mmHg or taking antihypertensive medications were used to define the presence of hypertension. If a patient had a serum cholesterol value of > 220 mg/dL or had been taking cholesterol-lowering medications, he was defined as ipercholestelolemic patient. If patients had never smoked or if they had stopped smoking ≥ 1 year before the study, they were indicated as nonsmokers. All remaining patients were classified as smokers. We determined the estimated glomerular filtration rate (eGFR) using the Chronic Kidney Disease Epidemiology Collaboration equation (eGFR = 175 x standardized $S_{creatinine}^{-1.154}$ × age$^{-0.203}$ × 1.212 [if black] × 0.742 [if female]), in which the GFR is expressed as mL/min per 1.73 m^2 of body surface area and $S_{creatinine}$ is expressed in mg/dL[6]. The eGFR was categorized according to established clinical cutoff points: ≥ 90, 60 to 89, and 15 to 59 mL/min per 1.73 m^2. No one of individuals in our sample had an eGFR < 60 ml/min. Patients with malignant neoplasm, severe renal (eGFR < 60 ml/min) or liver disease, serous membrane chamber fluid, severe edema, hypothyroidism and osteoporosis were also excluded from the study. No one of the patients was taking estrogen supplements, thyroxin, glucocorticoids, immunosuppressive drugs, bisphosphonates and anticoagulants. Approval for this study was provided by the Ethics Committees of the "A. Gemelli" Catholic University Hospital of Rome, Italy, and "St. M. Goretti" Hospital, Latina, Italy. Informed consent was obtained from enrolled patients.

Biochemical Investigation

For every patient were determined serum creatinine, fasting cholesterol, triglycerides and low-density lipoprotein, white blood cell count. From all the individuals involved, after an overnight fast, blood samples were collected. By centrifugation of blood samples, stored at −80°C until assayed, serum was separated and treated. Using a second-generation C-terminal human enzyme-linked immunosorbent assay (Immutopics, San Clemente, CA, USA), we determined plasma FGF23 levels. The coefficient of variation was 9.8%. By using a high-sensitivity ELISA kit (Biocheck Laboratories, Toledo, OH, USA) we determined HsCRP levels. We used as a capture antibody a monoclonal mouse anti-human OPG antibody and a biotinylated polyclonal goat anti-human OPG antibody, for detection. The intra- and inter-assay coefficients of variation were

3.6% and 10.6%, respectively. The sensitivity, defined as the mean ± 3 SD of the 0 standard, was calculated to be 0.15 pmol/ml. By using the Quantikine ELISA kit (R&D Systems, Minneapolis, MN, USA) we assessed IL-6 levels. In each patient, the serum levels were measured twice and the results were averaged.

Statistical Analysis

Demographic and clinical data between the groups were compared by a Chi-squared test and by a *t*-test. FGF-23, HsCRP, OPG and IL-6 serum levels were compared by the Mann-Whitney test. Using a multivariate stepwise logistic regression analysis, two models were tested. The first one adjusted for conventional risk factors (parameters in Table I), while in the second model, FGF-23 and OPG were included for testing. All analyses were performed using the STATA version 11.0 for Windows (Statistics/Data Analysis, Stata Corporation, College Station, TX, USA). Statistical significance was established at $p < 0.05$.

Results

Table I shows the demographic and clinical data of patients with and without PAD. There were no significant differences between the groups in terms of sex ($p = 0.223$), age ($p = 0.148$), diabetes duration ($p = 0.174$), eGFR ($p = 0.253$), phosphate and calcium levels ($p = 0.301$ and 0.675 respectively). In contrast, the BMI, current and former smoking, hypertension, coronary artery disease (CAD), history of ischemic stroke (HIS) and hypercholesterolemia were significantly more frequent in patients than control subjects (Table I).

Interestingly, FGF-23, HsCRP, OPG and IL-6 median serum levels, were significantly higher in PAD patients than in control WPAD (Table II). In particular the FGF-23 serum levels were 69.3 (58.8-75.1) pg/mL in PAD and 42.98 (37.1-49.8) pg/mL in WPAD subjects ($p < 0.001$), the HsCRP median serum levels were 8.23 (6.74-9.89) mg/L in PAD and 3.77 (2.34-5.31) mg/L in WPAD individuals ($p < 0.001$), the OPG levels were 5.04 (3.78-6.12) pmol/L in PAD and 2.21 (1.43-3.21) pmol/L in controls ($p < 0.001$) and the IL-6 median serum levels were 53.8 (51.2-56.1) pg/mL in diabetic patients with PAD and 31.5 (28.3-35.9) pg/mL in diabetic controls ($p < 0.001$) (Table II).

Subsequently, we divided the 413 diabetic patients with PAD in stable PAD (Fontaine's stage II, without rest pain) (n = 221) and CLI (unsta-

Table I. Demographic and clinical data of study participants.

	PAD (n = 413)	WPAD (n = 563)	*p*-value
Men/female, n	207:206	282:381	0.223†
Age (years ± SD)	70.9 ± 3.1	71.2 ± 3.6	0.148*
Diabetes mellitus duration, (years ± SD)	12.7 ± 3.7	12.2 ± 3.1	0.174*
BMI (Kg/m^2 ± SD)	30.1 ± 6.3	27.5 ± 4.2	< 0.01*
Smoking (current), n (%)	164 (39.7)	171 (30.4)	0.031†
Smoking (former), n (%)	181 (43.8)	178 (31.6)	0.019†
Hypertension, n (%)	254 (61.5)	239 (42.4)	< 0.01†
CAD, n (%)	201 (48.7)	177 (31.4)	< 0.01†
HIS, n (%)	154 (37.2)	109 (19.3)	< 0.01†
Hypercholesterolemia, n (%)	272 (65.8)	272 (48.7)	0.023†
LDL-C (mg/dL ± SD)	201 ± 18.6	137 ± 14.5	< 0.01†
Triglycerides (mg/dL± SD)	203 ± 22.7	134 ± 14.2	< 0.01†
Statins, n (%)	258 (62.5)	249 (44.2)	< 0.01†
Antihypertensive drugs, n (%)	244 (59.1)	223 (39.6)	< 0.01†
Antidiabetic treatment			
Diet only, n (%)	43 (10.4)	60 (10.7)	0.437†
Oral Agents, n (%)	203 (49.1)	275 (48.8)	0.374†
Insulin therapy, n (%)	165 (39.9)	222 (39.4)	0.381†
eGFR (mL/min/1.73 m^2 ± SD)	67.5 ± 13.4	71.3 ± 14.1	0.253
Phosphate (mg/dL ± SD)	3.32 ± 1.63	2.97 ± 1.11	0.301
Calcium, mg/dL	9.7 ± 0.6	9.6 ± 0.5	0.675

BMI, body mass index, CAD, coronary artery disease; HIS, history of ischemic stroke; LDL-C, low-density lipoprotein cholesterol; eGFR = estimated glomerular filtration rate. *Statistical test performed with Student's *t*-test. †Chi-square test for categorical values.

Table II. Serum levels in diabetic patients with and without PAD.

Variables	PAD (n = 413)	W/PAD (n = 563)	*p*-value
FGF-23, pg/mL (IQR)	69.3 (58.8-75.1)	42.98 (37.1-49.8)	< 0.001*
HsCRP, mg/L (IQR)	8.23 (6.74-9.89)	3.77 (2.01-4.87)	< 0.001*
OPG, pmol/L (IQR)	5.04 (3.78-6.12)‡	2.21 (1.43-3.21)‡	< 0.001*
IL-6, pg/mL (IQR)	53.8 (51.2-56.1)	31.5 (28.3-35.9)	< 0.001*

Table III. Serum levels in diabetic patients with and without CLI.

	Fontaine's II (n = 221)	CLI (n = 192)	
FGF-23, pg/mL (IQR)	37.3 (34.2-41.2)	73.29 (68.1-77.7)	< 0.001*
HsCRP, mg/L (IQR)	3.64 (2.01-4.75)	8.62 (7.12-9.71)	< 0.001*
OPG, pmol/L (IQR)	3.87 (2.66-4.54)‡	6.95 (5.36-8.32)‡	< 0.001*
IL-6, pg/mL (IQR)	31.4 (28.7-33.1)	55.2 (52.4-58.1)	< 0.001*

FGF-23, fibroblast growth factor-23; HsCRP, high-sensitivity C-reactive protein; OPG, Osteoprotegerin; IL-6, Interleukin-6. *χ^2 test for categorical values. †OR adjusted for age, sex, hypertension, hypercholesterolemia, CAD, PAOD; smoking. ‡Median (interquartile range). §Mann-Whitney U test.

ble PAD with rest pain, ulcers and gangrene) (n = 192) groups. Table IV analyzed FGF23, HsCRP, OPG and IL-6 median serum levels in patients with Fontaine's stage II and CLI. We found that the median levels of the these cytokines were significantly higher in subjects with CLI than in individuals with Fontaine's stage II [FGF23, 73.29 (68.1-77.7) pg/mL *vs.* 37.3 (34.2-41.2) pg/mL, $p < 0.001$; HsCRP, 8.62 (7.12-9.71) mg/L *vs.* 3.64 (2.01-4.75) mg/L, $p < 0.001$; OPG, 6.95 (5.36-8.32) pmol/L *vs.* 3.87 (2.66-4.54) pmol/L, $p < 0.001$; IL-6, 55.2 (52.4-58.1) pg/mL *vs.* 31.4 (28.7-33.1) pg/mL, $p < 0.001$] (Table III).

Table IV. Multivariable stepwise logistic regression model for presence of CLI.

		Variable OR (95% CI)	*p*-value
Model 1	Sex	1.12 (0.89-1.23)	0.253
	Age	1.04 (0.77-1.13)	0.387
	Smoking (current)	22.68 (3.23-67.76)	< 0.01
	Smoking (former)	18.03 (2.16-32.45)	0.001
	Hypertension	21.01 (3.65-75.29)	< 0.001
	Hypercholesterolemia	11.43 (2.33-24.67)	0.001
	Triglycerides	3.78 (1.43-2.12)	0.001
	LDL-C	9.39 (1.37-67.25)	0.021
	HsCRP	32.54 (7.01-96.23)	< 0.001
	IL-6	6.05 (2.14-14.44)	0.001
Model 2	Sex	1.04 (0.76-1.12)	0.163
	Age	1.25 (1.02-1.33)	0.249
	Smoking (current)	48.4 (2.30-156.1)	< 0.001
	Smoking (former)	7.76 (2.05-17.54)	0.175
	Hypertension	29.14 (4.12-125.01)	< 0.001
	Hypercholesterolemia	13.23 (2.37-36.81)	0.002
	Triglycerides	5.51 (1.87-8.39)	0.002
	LDL-C	14.69 (3.73-112.76)	0.002
	HsCRP	59.46 (2.99-321.39)	< 0.001
	IL-6	6.22 (1.89-14.65)	< 0.001
	OPG	5.45 (1.98-12.06)	0.021
	FGF23	7.69 (2.64-16.31)	0.001

Finally, a multivariable stepwise logistic regression analysis revealed that, in model 1, making adjustments for traditional cardiovascular risk factors and established inflammatory cytokines, smoking (current and former), hypertension, hypercholesterolemia, triglycerides, LDL-C, HsCRP and IL-6 levels were independent determinants of CLI in T2D patients with PAD. When FGF-23 and OPG were included in the multivariable analysis (model 2), FGF-23 and OPG remained independently associated with CLI in PAD patients, and the majority of the conventional risk factors in model 1 persisted in being determinants of internal carotid artery stenosis (ICAS) in model 2 (Table IV).

Discussion

To our knowledge, this study shows for the first time that high serum levels FGF23 are independently associated with presence and severity of PAD in Italian patients whit T2D. Our findings are consistent with previous reports that have found increased levels of FGF23 are an independent risk factor for unstable carotid atherosclerosis[13] and adverse cardiovascular events[9]. We observed that the FGF23 median serum levels were significantly higher in T2D patients with PAD than in diabetic controls [69.3 (58.8-75.1) pg/mL and 42.98 (37.1-48.8) pg/mL ($p < 0.001$) respectively]. Interestingly, after all 413 diabetic patients with PAD had been divided into groups, Fontaine's stage II and CLI, we found that the FGF23 median levels were significantly higher in patients with CLI than in those in Fontaine's stage II [73.29 (68.1-77.7) pg/mL *vs.* 37.3 (34.2-41.2) pg/mL, $p < 0.001$]. On the other hand, we found that OPG, HsCRP and IL-6 serum levels were associated with PAD and their levels increased gradually with increasing its severity.

Finally, a multivariable stepwise logistic regression analysis revealed FGF23 and OPG remained independently associated with CLI in diabetic patients (model 2) and the majority of the conventional risk factors in model 1 persisted in being determinants of CLI in model 2.

The potential role of FGF23 in the pathogenesis of atherosclerosis may be explained by its involvement in the complex process of vascular calcification. Vascular calcifications play a critical role in the atherosclerotic process and in particular in the acute cardiovascular or cerebrovascular events and in the rupture of unstable plaques, which leads to critical atherosclerotic stenosis[16]. FGF23 levels were associated with the development of artery calcification in the presence of chronic kidney disease[17,18] even if, recently, high FGF23 serum levels were associated, in patients with CAD, with coronary calcification independent of classical cardiovascular risk factors and with preserved renal function[9]. Since vascular calcifications are associated with atherosclerosis, this could explain the association between elevated FGF23 and the severity of atherosclerotic stenosis. FGF23 serum levels in our diabetic population were significantly increased and associated with presence and severity of PAD. The relationship was not dependent on the serum phosphates level, which still remained within the normal range in the majority of the patients. In addition, a recent report[19] demonstrated an independent association between circulating FGF23 concentrations and the severity and extent of CAD. A positive association with high FGF23 levels was found with the recurrent cardiovascular disease in The Heart Soul Study[20], with the risk of incident heart failure (HF) and total cardiovascular events in the Cardiovascular Health Study[21] and with cardiovascular mortality in the Uppsala Longitudinal Study of Adult Men[22]. FGF23 levels had the greatest effect on soluble Klotho levels, markers of bone mineral metabolism, and arterial stiffness in a diabetic nephropathy population[10].

In line with previous studies, our findings also revealed that OPG had a significant association with presence and severity of PAD, in patients with T2D[23,24]. High concentrations of OPG were associated with heart failure[25], symptomatic carotid stenosis[26], unstable angina[27] and vulnerable carotid plaques[28]; OPG modulates, within bone, the release of cathepsins and could have an important role on plaque stability[29]. Inflammation plays an important role in the pathogenesis of atherosclerosis[30] and our paper confirms that pro-inflammatory cytokines are strongly associated with the development of atherosclerotic diseases[31].

There are several limitations in the present report. First, we performed a case-control study, and an enrollment and survival bias should be considered. Second, we collected data from an European cohort, with other cardiovascular diseases. Also, the comorbidities might represent confounding factors and the generalization of our findings to other age groups or ethnicities is not clear. Moreover, the population size is relatively small and

could lead to false positive results. These data need to be confirmed in larger populations and they should also be tested in groups of different ethnic origins. Furthermore, it is not allowed to rule out the possibility of additional confounding by other factors such as bone status, 1,25-dihydroxy-vitamin D or PTH levels. Finally, we have not considered in our analysis calcium, vitamin D supplements and dietary phosphate intake.

Conclusions

Our work identifies FGF23 serum levels as an independent risk factor for presence and severity of PAD in an Italian population with T2D. The association was independent of traditional cardiovascular risk factors and kidney function. This finding suggested that serum FGF23 levels are an important risk factor for lower limbs atherosclerosis in patients with T2D. Moreover, it suggests that serum FGF23 is a potential biomarker for PAD and CLI. Further studies are needed to evaluate the functional role of FGF23 in the development of atherosclerosis disease.

Conflict of Interest

The authors confirm that there are no known conflicts of in¬terest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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