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Research Article

Serum osteoprotegerin levels in patients with type 2 diabetes and non-alcoholic fatty liver: A biomarker for the risk of cardiovascular disease

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Abstract. Background: Non-alcoholic steatohepatitis (NASH) is a more advanced, non-benign stage of the non-alcoholic fatty liver disease (NAFLD). Diabetes mellitus type 2 (DMT2) is a major cardiovascular risk factor through atherosclerotic macroangiopathy injury. The combination of the two entities with multiple common pathophysiological components gives way to a phenotype posing high cardiometabolic risk through genetic, molecular, metabolic, inflammatory, and organ changes. Osteoprotegerin (OPG) has proven to be a promising and powerful independent predictor for cardiovascular disease. To date, little to no research has looked at the serum OPG levels in patients with DMT2 and NAFLD - in its spectrum of conditions. Objective: The purpose of this study was to investigate the OPG serum levels and their meaning in a group of diabetic patients with NAFLD in various degrees of severity, compared to a control group; and the links between OPG serum levels and the degree of liver injury, besides the correlations between OPG serum levels and the cardiovascular risk factors. Materials and methods: This cross-sectional study included 150 subjects among which 110 diabetic patients with DMT2, and 40 healthy controls. The patient population (N=110) was divided into three groups: group 1 – patients with DMT2 and NASH (n=35); group 2 – patients with DMT2 and NAFLD (n=38), and group 3 – patients with DMT2, without liver disease (i.e., normal liver) (n=37). The diagnosis for liver injury was performed by means of liver function tests, ultrasonography, and a panel of serological biomarkers to predict NASH phenotype. Anthropometric indicators, clinical and biochemical parameters were determined; the measurement of basal serum OPG was performed through the sandwich ELISA protocol. Results: The mean value for serum OPG level in the diabetic patients (n=110) was 1894.33 pg/ml, significantly higher compared to control population (n=40), where the level of serum OPG was 1355 pg/ml ($p < 0.001$). The highest value of serum OPG levels was found in the group of DMT2 patients with NASH (2237 pg/ml±544, $p < 0.001$), statistically significant when compared with every other group of diabetic patients. The serum OPG level for the DMT2 patients with NASH was positively correlated with: age (r=0.38; p=0.013); hypertension (r=0.452, p=0.03); fasting plasma glucose (r=0.341, p=0.012); hsCRP (r=0.47, p=0.02); diabetic nephropathy – mircroalbuminuria (r=0.219, p=0.012) and macroalbuminuria (r=0.451, p=0.021); with NT-proBNP (r=0.301; p=0.027); and with the Framingham risk score (r=0.42, p=0.003). Negative correlations were found between serum OPG and eGFR (r=-0.29, p=0.019). No correlations were found between OPG and the following parameters: gender (r=0.012, p=0.55), BMI (r=0,345, p=0.067), liver transaminases (r=0.245, p=0.06), and HbA1c (r=0.06, p=0.58), respectively. Conclusions: The OPG serum concentration is significantly higher in the diabetic patients compared to controls, as the presence of NAFLD and NASH tends to increase the OPG values. The positive correlation of serum OPG levels with multiple cardiovascular risk factors in patients with DMT2 and NAFLD/NASH, indicate that OPG may be useful as additional biomarker to assess the cardiovascular risk in this particular group of patients.

Key Words: non-alcoholic fatty liver disease, non-alcoholic steatohepatitis, osteoprotegerin, diabetes mellitus type 2, cardiovascular disease risk factors, biomarkers.

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Introduction

Non-alcoholic fatty liver disease (NAFLD) comprises a broad spectrum of liver conditions, which can range from simple steatosis to non-alcoholic steatohepatitis (NASH), and even cirrhosis (Matteoni et al 1999), considered at present a component of the metabolic syndrome (MetS) (Marchesini et al 2011).

NAFLD is one of the most common causes of chronic liver disease, worldwide. Amid the epidemic of obesity and type 2 diabetes mellitus (DMT2) – diabesity – the prevalence of NAFLD is expected to grow (Farag et al 2011).

Most commonly, fatty liver is diagnosed by liver function tests as well as by ultrasound examination. Fatty liver is associated with several risk factors for atherosclerosis, the latter being themselves components of MetS: hypertension, diabetes, or dyslipidemia.

In its most serious form (i.e., steatohepatitis), the fatty liver is associated with cardiovascular risk factors, and cardiovascular

events generating morbidity and mortality, both in people with diabetes and those without diabetes. Although multiple common mechanisms between NAFLD and risk factors for atherosclerosis were previously described, primarily related to insulin resistance (IR), a possible direct relationship between fatty liver and atherosclerosis continues to be investigated (Farag et al 2011).
Osteoprotegerin (OPG) is a soluble protein, belonging to a superfamily of receptors of the tumor necrosis factor (TNF), acting as soluble pseudoreceptor for the receptor activator of ligand, nuclear factor kβ (RANKL, receptor-activator of nuclear factor kβ-ligand) to prevent activation of osteoclasts and thus bone resorption (Simonet et al 1997).
OPG is seen also in osteoblasts, as well as in other tissues and organs: heart, kidney, liver, spleen, bone marrow, adipose tissue, and endothelial cells. Later, various studies noted that OPG acts as a trap-receptor, whereby the activation of nuclear factor kβ-ligand (NF-kβ), one of the main signalling pro-inflammatory ways, is prevented, mainly due to induction of expression of pro-inflammatory genes with the production of cytokines, chemokines, and adhesion molecules (Boyle et al 2003).
The inhibitory action of NF-kβ over the pro-inflammatory pathway is an important component in regulating bone metabolism, and important metabolic effects. On the one hand, OPG exerts an anti-inflammatory action and, on the other hand, an anti-apoptotic role that prevents programmed cell death.
Since OPG mRNA is present also in the arterial wall, it was suggested that increased serum levels of OPG, reflect high content of OPG in the arterial wall, in patients with diabetes (Olesen et al 2005). In addition, others have identified an increased content of OPG in the calcified coronary plaques, and this presence has been associated with the severity of coronary disease, and with the incidence of cardiovascular events, independent of conventional risk factors (Dhore et al 2003; Jono et al 2002; Kiechl et al 2004).
This data indicate that increased levels of serum OPG are a biomarker of atherosclerotic lesions. Moreover, increased levels of OPG have been reported both in patients with unstable angina (Schoppet et al 2003), and in patients with acute myocardial infarction (Crisafulli et al 2005).
On the other hand, recent studies have shown that low levels of serum OPG from patients with NAFLD were associated with the progression toward NASH (Yilmaz et al 2010).
Past research findings indicate that high levels of OPG are associated with diabetes (Browner et al 2001), while others demonstrated that elevated levels of serum OPG is a predictor of higher mortality in patients with type 1 diabetes (DMT1) and diabetic nephropathy (Jorsal et al 2008). Moreover, in a prospective study conducted over an 18-month time lapse, it was shown that elevated levels of serum OPG in patients with DMT2 was linked with increased incidence of cardiovascular events (Anand et al 2006).
Given that patients with DMT2 have a high prevalence of fatty liver, sometimes with severe forms, and that DMT2 is a major risk factor for cardiovascular disease – macroangiopathy – the purpose of this study was to examine the serum levels of OPG in a group of diabetic patients with and without fatty liver impairment, compared to a control group of healthy subjects with similar characteristics, but without diabetes.

Materials and methods

Subjects

The population of this study included a total of 150 participants, of which 110 consecutive patients were diagnosed with DMT2. All participants were recruited from clinical settings of the Medical Clinic IV and Polyclinic CFR Cluj-Napoca.
Three groups of patients with DMT2 were enrolled in the present study:
Group 1: 35 patients with DMT2 and NASH;
Group 2: 38 patients with DMT2 and NAFLD;
Group 3: 37 patients with DMT2 without liver disease (with normal results on liver function test and without signs of liver steatosis as determined by ultrasound);
Group 4: control group, consisting of 40 healthy subjects, matched with the study patients, based on age and gender.
Exclusion criteria from this study were alcohol consumption (> 20 g/day), the presence of viral hepatitis B, C, autoimmune hepatitis, hemochromatosis, chronic medication (amiodarone, tamoxifen), and other rare liver diseases, malignancies. Prior to enrolment in the study, each participant has signed an informed consent form. The study was approved by the Ethics Committee of UMF "Iuliu Haţieganu" Cluj-Napoca, and was conducted in accordance with the Declaration of Helsinki.

Methodology

Diabetes was defined according to the guidelines of ADA (American Diabetes Association 2010).
Liver ultrasound was performed after 12 hours of fasting, and each subject was examined in supine and left lateral decubitus position, during deep apnoea. An experienced examiner, blinded to clinical data, indicated the presence or absence of fatty infiltration of the liver, as well as the degree of severity of fatty liver disease. Hepatic steatosis was defined by means of ultrasonography, with the following characteristics: echogenicity of the right renal cortex compared with the liver (absent = 1; present = 0), brightness of liver parenchyma (ranging from normal to severe), degree of reflectivity from the diaphragm (diaphragm was well visualized = 0; markedly decreased reflectivity of the diaphragm = 1); beam attenuation with standard settings; and intrahepatic vessels with bright walls on ultrasound (present = 0, absent = 1). The diagnosis of hepatic steatosis was established based on these measurements.
The fatty liver index was based on four markers: BMI, waist circumference, triglycerides, and gamma-GT, recognized in the literature as markers used to accurately identify NAFLD (Jiang et al 2013, Bedogni et al 2006).
Based on recent studies, serological biomarkers such as adiponectin and IL-6 were used to diagnose NASH, as they provide a positive, reliable, and accurate predictive value to detect NASH (Festi et al 2013; Grigorescu et al 2012).
Data on participants' lifestyle, diet, medical history, smoking status, drug therapy followed – antidiabetic agents (oral insulin), antihypertensives, lipid-lowering medication, calcium and vitamin supplements used, was collected by means of a structured survey that was administered to the patients during the clinical assessment. Weight, waist circumference (WC) height, were assessed at the time of physical examination of each patient.

Table 1. The clinical and demographic characteristics of the study patients and controls

Variable	DM type 2 + NASH	DM type 2 + NAFLD	DM type 2 only	Controls
N (subjects)	35 (31.82%)	38 (34.54%)	37 (33.64%)	40
Male	23	27	21	28
Female	12	11	16	12
Age (years)	57.9±7.5	56.7±6.6	55.7±4.8	54.4±2.9
Weight (kg)	87.9±12.3	82.7±10.3	81.4±8.9	78.4±11.4
BMI (kg/m^2)	33.5±7.3	31.6±6.7	30.4±3.8	27.1±4.9
WC (cm)	114.8±13.2	107.1±8.9	98.5±9.1	93.7±8.6
Duration of diabetes (years)	8.90±4.5	7.89±5.4	6.22±2.4	–
SBP (mmHg)	128.5±22.3	126.9±15.1	124.7±14.6	122.8±16.2
DBP (mmHg)	83.7±8.4	82.5±6.1	80.3±4.2	71.6±5.1
HbA1C (%)	7.8±3.4	7.1±2.9	6.9±3.3	–

Blood pressure was assessed and determined as the average of at least 2 measurements, in accordance with the required protocols (Manchia et al 2007).

Blood samples were drawn in the fasting state, and all the routine blood chemistry analysis were assayed by laboratory techniques. Urine was collected for 24 hours to determine the albumin excretion, which allowed for the determination of the ACR (albumin/creatinine ratio); normal values for 24 hours urine albumin excretion is < 30 mg/24 hours; microalbuminuria 30-300 mg/24 hours; macroalbuminuria > 300 mg/24 hours. Estimated Glomerular Filtration Rate (eGFR) was determined using the MDRD method (Levey et al 1999), normal values > 60 ml/min /1.73m^2.

All the biological data and biomarkers measured were determined in the MedLife Laboratory, and in the Department of Biochemistry, UMF Cluj-Napoca.

The serum NTpro-BNP was measured using the ELISA technique. Serum NT pro-BNP and serum OPG levels were determined with ELISA sandwich method, using the kit from R & D system (Human osteoprotegerin / TNFRSF11B DuoSet ELISA, R & D system). NT pro-BNP normal values are below 300 ng/ml, this being a well-validated independent marker for cardiovascular disease, and independent of other prognostic markers.

The serum adiponectin and IL-6 were determined with the ELISA kits from R&D sys-tem – Human IL-6 DuoSet ELISA, and Human Total Adiponectin/Acrp30 Quantikine ELISA Kit, following the manufacturer recommendations. For this assay, the detection limit for OPG was in the range of [62.50 - 4000 pg/mL], for IL-6 it was [9.36-600pg/mL], and [3.90 - 250 ng/mL] for adiponectin, respectively.

The measurements were carried out using sera dilution, while all samples were meas-ured in duplicate. All samples were processed blindly to the clinical status of participants.

Statistical analysis

Data are expressed as the mean ± standard deviation. To compare the quantitative var-iables for two groups, we used the t test; and the one-way Anova test to compare the quantita-tive variables for more than three groups. In addition, the Pearson test was done to assess the correlations between two continuous variables; the Spearman test was also used to analyze the monotonic relationship between two continuous or ordinal variables. The chi-square and Fischer's exact tests were used for categorical variables to test for the homogeneity of proportions in the groups under study.

All statistical analyses were performed using the following softwares: GraphPad Prism ver-sion 6.0 b, Prism 6 for Windows GraphPad Prism, and Excel (Microsoft Office 2010).

Results

The group of DMT2 patients included 110 persons who were divided into 3 groups, based on the diagnostic criteria for NAFLD, as follows:

• group 1: 35 (31.82%) patients with DMT2 and NASH (23 men and 12 women);

• group 2: 38 (35.54%) patients with DMT2 and NAFLD (27 men and 11 women);

• group 3: 37 (33.64%) patients with DMT2 without NAFLD criteria of diagnostic (21 men and 16 women);

• group 4 (control): 40 subjects (28 men an 12 women).

The clinical and demographic parameters for all four groups are presented in Table 1.

From among the diabetic patients, 35.45% were females, and 64.55% males. The average age of the patients was 56.17 years old. The average duration of diabetes was 7.67 years. The average of HbA1c was 7.26%.

Regarding the smoking status of our subjects included in the study, no woman included in the study was a current smoker nor had any history of smoking; as for the diabetic male patients (n = 71 of 110 patients), 15 were current smokers (21.12%), 33 were former smokers (46.48%), and 23 (32.39%) never smoked. In the control group, 8 subjects were current smokers (20%), 16 were formers smokers (40%), and 16 never smoked (40%).

Osteoprotegerin mean serum levels were higher in the patient group (n = 110; 1894.33 ± 334.66 pg/mL) as compared to the control group, where the levels of OPG were 1355 ±212 pg/mL. This difference is statistically significant ($p < 0.001$) (Figure 1).

When we compared the OPG serum levels in each one of the three DMT2 patient groups, we found the highest level of OPG in the patients with DMT2 and NASH (group 1), followed by NAFLD (group 2), and the patients without NAFLD (group 3) ($p = 0.001$ [group 1 vs. group 3, and group 1 vs. group 2]; and $p = 0.0262$ [group 2 vs. group 3]).

Table 2. Biochemical characteristics of the four study groups

Variable	DM type 2 + NASH	DM type 2 + NAFLD	DM type 2 only	Controls	p
OPG (pg/ml)	2237±455	1784±236	1662±313	1355±212	< 0.001
Fasting plasma glucose (mg/dl)	153±17.2	146±18.5	134±12.6	98±11.0	0.002
AST (UI/L)	48±5.9	29.1±2.7	26.0±3.6	25±34	0.012
ALT (UI/L)	54.2±7.4	35.4±3.8	32.1±3.4	30.2±2.9	0.023
GGT (UI/L)	68.8±6.9	50.4±7.2	48.3±4.1	38.4±2.9	0.04
Total Cholesterol (mmol/L)	230.5±18.32	224.7±16.9	218.8±22.6	198.4±12.9	0.05
HDL Cholesterol (mmol/L)	37.3±3.9	38.5±3.6	40.2±5.8	48.3±13.01	0.031
LDL Cholesterol (mmol/L)	128.9±8.82	126.5±7.4	121.1±16.5	110.4±28.2	0.045
Triglycerides (mg/dl)	225±32.0	188.4±23.7	162.3±15.8	152.8±75.7	0.04
hsC-reactive protein (mg/dl)	5.8±2.7	2.489±1.5	1.98±1.1	1.89±1.0	0.021
Fibrinogen (mg/dl)	408.9±62.4	332.2±38.9	309.7±25.4	292.6±15.14	0.03
Serum Creatinine (mg/dl)	1.32±0.25	1.28±0.46	1.24±0.29	0.89±0.34	< 0.001
eGFR (ml/min/1,73 m^2), MDRD	68 (32-125)	74 (43-149)	85 (57-151)	102.4 (88 – 145)	0.02
NT-proBNP (ng/ml)	235±75.21	148± 53.9	88±61.3	45.3±28.0	0.011
IL-6 (pg/ml)	3.98±2.75	3.33±2.41	3.04±1.72	1.25±1.96	0.034
Adiponectin (ng/ml)	3814.5±1888.1	5890.1± 2314	6899.1±2218.3	9245.6±4312.8	0.039
Albuminuria(mg/24h)	173 (42-512)	102 (45-197)	87 (33-99)	27 (15-43)	<0.001
Normoalbuminuria*	6 (17.14%)	8 (21.05%)	18 (48.64%)	33 (82.5%)	0.013
Microalbuminuria*	25 (71.42%)	29 (76.31%)	19 (51.35%)	7 (17.5%)	0.011
Macroalbuminuria*	4 (11.42%)	1 (2.63%)	0 (0%)	0 (0%)	0.02
Framingham risk score	17±8.9	16±9.2	15.4±8.6	14.5±8.4	0.04

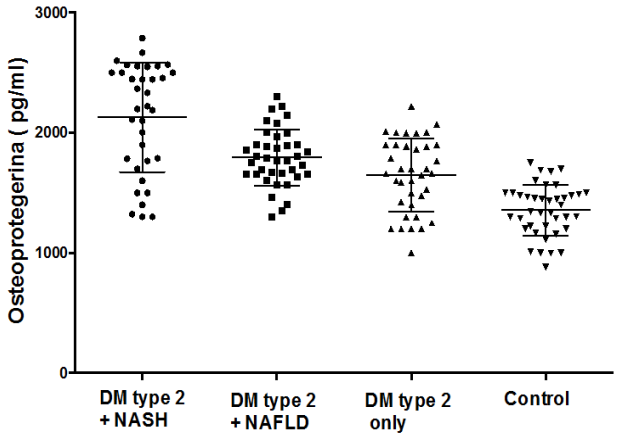

Figure 1. The levels of osteoprotegerin (OPG) serum in patients with DM type 2 and various levels of hepatic steatosis vs. control group

Pearson and Spearman tests were performed to assess the relationships between the serum OPG levels and clinical variables and biological parameters under study, in our groups (Table 3). Serum OPG levels were positively correlated – for all 4 groups – with the following variables: age, hypertension, fasting plasma glucose, and Framingham risk score.

In all three diabetic groups, the levels of seric osteoprotegerin were positively correlated with multiple inflammatory markers: hsCRP, fibrinogen, NT-proBNP, IL-6, adiponectin; and were inversely correlated with eGFR.

For groups 1 and 2 – patients with DMT2 + NASH, and DMT2 + NAFLD – seric OPG was correlated with: seric creatinine, 24 hours albuminuria, micro- and macroalbuminuria, and with WC. In all four groups, the serum levels of OPG did not correlate with the following variables: gender, BMI, liver transaminase, and dyslipidemia.

Discussion

In this cross-sectional study conducted on 150 subjects, we analyzed serum OPG levels in four distinct groups: patients with DMT2 and NASH (group 1), DMT2 and NAFLD (group 2), DMT2 with normal liver (group 3), and the control healthy subjects (group 4), respectively. Our results and analyses indicate elevated levels of serum OPG in all diabetic patients in the 3 groups, with the highest values found in the group with non-alcoholic steatohepatitis (group 1).

High OPG levels were present also in patients with simple steatosis (group 2), and even in those with normal liver (group 3), with significant p-values in all study groups. These results indicate that serum OPG level increases with the degree of fatty liver infiltration; furthermore, an incremental progression of OPG level was noted with the increase of severity degree of liver damage, marked by the appearance of hepatocyte necroinflammation.

OPG serum levels in diabetic patients with non-alcoholic steatohepatitis (group 1) was significantly increased, compared to the patients with DMT2 and simple steatosis (NAFLD; group 2). Our results and the positive correlations between OPG serum levels, waist circumference, and the biological parameters, confirm

Table 3. Correlations between serum osteoprotegerin and clinical and biochemical variables

Variable	DM type 2 + NASH	DM type 2 + NAFLD	DM type 2 only	Controls
Age (years)	r=0.38; p=0.013	r=0.36; p=0.034	r=0.31; p=0.024	r=0.26; p=0.02
Gender	r=0.012; p=0.55	r=0.09; p=0.70	r=0.10; p=0.80	r=0.11; p=0.85
BMI (kg/m^2)	r=0.345; p=0.067	r=0.312; p=0.091	r=0.202; p=0.131	r=0.143;p=0.378
WC (cm)	r=0.391; p=0.031	r=0.372; p=0.046	r=0.190; p=0.052	r=0.082;p=0.073
LFT's elevation	r=0.245; p=0.06	r=0.05; p=0.55	r=0.03; p=0.65	r=0.04;p=0.70
Fasting plasma glucose (mg/dl)	r=0.341; p=0.012	r=0.231; p=0.024	r=0.173; p=0.04	r=0.021;p=0.048
Hypertension	r=0.451; p=0.03	r=0.378; p=0.027	r=0.241; p=0.021	r=0.11;p=0.01
Dyslipidemia	r=0.084; p=0.59	r=0.06; p=0.68	r=0.075; p=0.74	r=0.02; p=0.93
hs CRP(mg/l)	r=0.47; p=0.02	r=0.38; p=0.038	r=0.23; p=0.04	r=0.11;p=0.81
Fibrinogen (g/l)	r=0.25; p=0.03	r=0.14; p=0.01	r=0.12; p=0.04	r=0.11; p=0.06
IL-6 (pg/ml)	r=0.776; p=0.02	r=0.671; p=0.03	r=0.431; p =0.035	r=0.241; p=0.06
Adiponectin (ng/ml)	r=-0.478; p=0.022	r=-0.405; p=0.034	r=-0.311; p=0.037	r=-0.080; p=0.05
NT-proBNP (ng/ml)	r=0.301; p=0.027	r=0.215; p=0.037	r=0.122; p=0.02	r=0.008; p=0.07
HbA1c	r=0.06; p=0.58	r=0.04; p=0.55	r=0.09; p=0.75	r=0.13; p=0.80
Serum Creatinine (mg/dl)	r=0.075; p=0.025	r=0.067; p=0.031	r=0.001; p=0.34	r=0.004; p=0.25
eGFR (ml/min/1.73 m^2)	r=-0.29; p=0.019	r=-0.27; p=0.021	r=-0.19; p=0.05	r=0.067; p=0.12
Albuminuria (mg/24h)	r=0.174; p=0.035	r=0.123; p=0.038	r=0.107; p=0.029	r=0.089; p=0.039
Normoalbuminuria	r=0.094; p=0.15	r=0.066; p=0.23	r=0.056; p=0.34	r=0.044;p=0.44
Microalbuminuria	r=0.219; p=0.012	r=0.116; p=0.023	r=0.089; p=0.036	r=0.077;p=0.04
Macroalbuminuria	r=0.451; p=0.021	r=0.348; p=0.03	–	–
Framingham risk score	r=0.42; p=0.003	r=0.36; p=0.001	r=0.26; p=0.002	r=0.16; p=0.05

and extend the evidence on the role of OPG as a cardiovascular risk biomarker, in a population with high cardiovascular risk – DMT2 patients, with increased risk of hepatic impairment due to the presence of steatosis. Our study revealed a strong link between the levels of OPG and predictors of cardiovascular risk such as increased waist circumference, elevated albuminuria, and NT-proBNP, in patients with DMT2 and steatohepatitis, as reported also by Ninomiya et al. (2009) and Tarnow et al. (2006); however, these authors did not include in their analyses the presence of non-alcoholic fatty liver.

Our study was designed to find whether the serum OPG level could be a biomarker for the risk of cardiovascular disease, in the diabetic patients with NAFLD; and different forms of severity associated with high morbidity and mortality from cardiovascular cause.

OPG is present in the arterial wall in diabetic patients, and it was suggested previously that serum OPG reflects the arterial content in osteoprotegerin, thus representing an indication of arterial atherosclerotic lesions (Olesen et al 2005).

Past research documented elevated OPG levels in coronary calcified plaques (Dhore et al 2003), and from an angiographic perspective, elevated OPG was correlated with the severity of carotid stenosis, and cardiovascular events (Kiechl et al 2004; Ueland et al 2004). Other works have also determined the prognostic value of OPG in patients with heart failure that occurred immediately after an acute myocardial infarction (Knudsen et al 2003). These authors showed that increased plasma levels of OPG are associated with increased mortality post-myocardial infarction, 27 months after the event.

Noteworthy are the correlations between OPG and multiple variables such as the degree of coronary calcification , duration of diabetes, the manifestations of microvascular injury, type 1 and 2 diabetes – retinopathy and peripheral neuropathy (Anand et al 2006; Jorsal et al 2008; Reinhard et al 2010). To this literature, we add the results of our study, reporting correlations between elevated serum OPG levels in diabetic patients and NAFLD (especially in its inflammatory form – NASH), diabetic nephropathy, and kidney function impairment.

In our patients with DMT2 and steatohepatitis, the highest levels of serum OPG were positively correlated with the presence of micro- and macroalbuminuria, both of them recognized as risk factors for cardiovascular and kidney disease.

In a recent study, 193 patients with DMT2 were followed for 16.8 years on average, and it was found that the level of OPG is indeed an important predictor of mortality from various causes, in patients with DMT2; and the effect of OPG level on mortality is independent of other traditional risk factors in that specific population of patients (Moschen et al 2005).

With regard to the direct links between OPG and liver disease, few studies have examined the OPG in patients with liver disease. Among them, Moschen et al. (2005) have found an increase in OPG levels in a sample of 87 cirrhotic patients, as compared with the control group. In the same vein, another study reported a high level of OPG in 20 patients with alcoholic cirrhosis, compared with the control group population (Fábrega et al 2005). In 2010, a study published by Yilmaz and his collaborators indicated low levels of serum OPG in a sample of Turkish patients with NASH, as compared with those suffering from NAFLD

and simple hepatic steatosis, but this study was conducted on a non-diabetic population (Yilmaz et al 2010). This is the only study that documents low levels of OPG in non-diabetic patients with NASH, and also factors of high cardiovascular risk. Our study sought to measure, for the first time, the levels of serum OPG in diabetic NASH/NAFLD patients.

To date, the mechanism explaining the increase of serum OPG levels reported in numerous studies cited above, is still unclear. OPG is a soluble glycoprotein from the superfamily of TNFSF11 (tumor necrosis factor); it plays the role of a soluble receptor which interacts with RANKL / RANK system; by this influence, OPG modulates cell survival (by inhibiting the process of cell apoptosis) and contributes to the mineralization and inflammatory processes. Therefore, OPG is an important regulator of both vascular biology and inflammatory process. High levels of OPG can be interpreted either as a protective response, or as a partial explanation for increased atherosclerosis. Although the exact mechanism at the cellular level is still not fully understood, laboratory data and numerous clinical studies tend to suggest that in fact, OPG is beneficial in that this glycoprotein underlies a compensatory or protection mechanism in the inflammatory process associated to cardiovascular, diabetes or liver diseases. It is also likely that OPG represents a mere marker of the degree of vascular disease, secondary to an inflammatory process that occurs in all the conditions mentioned above.

Consistent with previous studies, we found that the level of OPG is increased in the population of patients with diabetes mellitus, in contrast to subjects in the control group. Our study is among the first ones showing that serum OPG level is increased in patients with DMT2 and NAFLD/NASH, reflecting that hepatic impairment may lead to an increase of OPG and, potentially, an increase in the cardiovascular risk; thus reinforcing the role of OPG as biomarker for the risk of developing cardiovascular disease.

Unlike the results reporting decreased OPG levels in the study by Yilmaz et al. (2010) conducted on a population of Turkish patients, the OPG levels in diabetic patients with NAFLD and NASH from our study were increased. Also, our study reports increased levels of OPG, comparable to those described by numerous other studies.

Nevertheless, the present study has some limitations which should be mentioned. The cross-sectional design of this study does not allow for longitudinal observation of the biomarker's (OPG) evolution over time, and the predictive potential of OPG in relation to morbidity and mortality of cardiovascular cause, for this high-risk population.

Another limitation is the small sample size of the study, and the use of non-invasive diagnostic methods for liver disease, instead of liver biopsy.

Future longitudinal studies with larger groups of patients, using the gold standard to determine liver disease severity, will offer further clarification, thus deepening more our understanding of the meaning and the underlying mechanisms of serum OPG levels, in patients with diabetic NASH with high risk for cardiovascular disease.

Conclusions

In conclusion, the level of serum osteoprotegerin is significantly increased in patients with DMT2 compared to controls, and the presence of NAFLD and NASH fosters an incremental progression in this biomarker for the risk of cardiovascular disease. The positive correlation of OPG serum levels with waist circumference, hypertension, fasting plasma glucose, hsCRP, NT-proBNP, micro- and macroalbuminuria, as well as the Framingham risk score, in DMT2 patients with NASH, support the role of OPG as a potential biomarker for the risk of cardiovascular disease.

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