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Original Article

Serum osteocalcin levels are inversely associated with the presence of nonalcoholic fatty liver disease in patients with coronary artery disease

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Abstract: Osteocalcin plays roles in energy, glucose, and lipid metabolism. Consequently, the relationship between osteocalcin level and nonalcoholic fatty liver disease (NAFLD) is of interest. The present study explored the possible correlation between serum osteocalcin levels and NAFLD in patients with CAD. The study enrolled 174 inpatients diagnosed with CAD by coronary angiography (CAG). The presence of fatty liver disease was confirmed by abdominal ultrasonography. NAFLD was diagnosed using the working definition of the revised guidelines for the management of NAFLD published by the Chinese Liver Disease Association. Serum osteocalcin levels were determined using electrochemiluminescent immunoassays. Patients with NAFLD had lower serum osteocalcin levels than those without NAFLD [16.2 (14.2-23.8) vs. 20.7 (15.6-26.2) ng/mL, P<0.05]. After adjustment for gender and age, serum osteocalcin levels correlated with the presence of NAFLD (r=-0.260, P=0.010), fasting plasma glucose level (r=-0.230, P=0.023) and glycated hemoglobin A1c level (r=-0.229, P=0.023). Osteocalcin was an independent factor for the presence of NAFLD (β=-0.097, P=0.025). These data suggested that serum osteocalcin levels were negatively associated with the presence of NAFLD in patients with CAD.

Keywords: Osteocalcin, nonalcoholic fatty liver disease, coronary artery disease

Introduction

Traditionally, bone was considered a relatively inert tissue, providing structural support for the body and participating in mineral metabolism. In the past decade, however, it has become clear that bone is also an active endocrine organ regulating several metabolic processes via the release of bone-derived hormones, including osteocalcin [1], which is a small protein (49 amino acids in humans) synthesized exclusively by osteoblasts. Osteocalcin is initially synthesized as a pre-pro-peptide; several post-translational modifications follow, including excision of the pre-pro-peptide and vitamin K-dependent γ-carboxylation of specific glutamic acid residues. Fully carboxylated osteocalcin has a high affinity for hydroxyapatite and is stored principally in the bone mineral matrix. However, osteocalcin is also present in blood, in both fully and partially carboxylated, and even completely uncarboxylated forms [2]. Osteocalcin was long regarded as a marker of bone formation [3, 4], but mounting evidence now suggests that it plays important roles in obesity, metabolic syndrome, and type 2 diabetes. Thus osteocalcin is involved in the crosstalk between bone and energy metabolism [5].

High incidences of cardiovascular disease (CVD) and nonalcoholic fatty liver disease (NAFLD) pose major threats to public health worldwide. An intimate link is evident between CVD and NAFLD, which share a pathophysiological foundation (insulin-resistance, usually accompanied by obesity and disorders of glucose and lipid metabolism) [6, 7]. Osteocalcin is involved in glycolipid and energy metabolism, and several human studies have found that serum osteocalcin levels are inversely associ-

ated with both the presence and severity of coronary artery disease (CAD) [8-11]. However, any link between serum osteocalcin levels and the presence of NAFLD remains unclear, although several researchers have suggested that such a link exists. This has not been tested in patients with CVD. Therefore, this study was designed to investigate the relevance between serum osteocalcin levels and NAFLD in patients diagnosed with CAD using coronary angiography (CAG).

Materials and methods

Study participants

During July 2008 and January 2010, inpatients of the Cardiology Department of the Sixth People's Hospital affiliated with Shanghai Jiao Tong University who underwent coronary angiography and were diagnosed with CAD were recruited for this study. Each participant completed a standardized questionnaire exploring previous and present illnesses, medication history, and smoking status. Those with the following conditions were excluded: (1) acute or chronic viral hepatitis, drug-induced or autoimmune liver disease; (2) alcoholism (≥140 g alcohol per week [men] or ≥70 g per week [women]) [12]; (3) a recent myocardial infarction (<3 months prior); (4) acute coronary syndrome; (5) congestive heart failure (New York Heart Association Class III-IV); (6) chronic kidney disease; (7) hyper- or hypo-thyroidism; (8) a need for total parenteral nutrition; (9) cancer; (10) a fracture within 1 year prior; and (11) current therapeutic use of drugs known to affect bone or calcium metabolism, such as corticosteroids.

The study was approved by the Ethics Committee of Sixth People's Hospital and complied with the Declaration of Helsinki. All subjects provided written informed consent.

Anthropometric evaluations and laboratory measurements

Weight (in kg) and height (in m) were recorded and used to calculate the Body Mass Index (BMI; kg/m^2). Waist circumference (W) was measured at the midpoint between the inferior border of the lowest rib and the upper margin of the iliac crest, in the mid-axillary line. Blood pressure (BP) was measured with a sphygmomanometer. Patients were instructed to fast overnight (10 h), then fasting and 2-h-postprandial venous blood samples were collected. Fasting plasma glucose (FPG) and 2-h-postprandial glucose (2 h PG) levels were measured using the glucose oxidase method. A Bio-Rad Variant II high-pressure liquid chromatographic platform (Hercules, CA, USA) was used to detect glycated hemoglobin A1c (HbA1c). Fasting serum insulin (FINS) levels were determined by radioimmunoassay (Linco Research, St. Charles, MO, USA) and used to calculate Homeostasis Model of Assessment-Insulin Resistance (HOMA-IR), as follows: [FPG (mmol/L) × FINS (mU/L)]/22.5. A Hitachi 7600-020 auto-analyzer (Tokyo, Japan) was used to measure the levels of the liver function markers aspartate aminotransferase (AST), alanine aminotransferase (ALT), and gamma-glutamyl transpeptidase (GGT) (by enzymatic methods); and lipid levels including those of triglycerides (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C) (again by enzymatic methods). C-reactive protein (CRP) levels were determined via particle-enhanced immunonephelometry using the Cardiophase hs-CRP Reagent (Siemens Healthcare Diagnostics, Newark, NJ, USA). Serum adiponectin levels were measured with a quantitative sandwich enzyme-linked immunosorbent assay (ELISA) (Antibody and Immunoassay Services, The University of Hong Kong); the intra- and inter-assay coefficients of variation were 7.34% and 8.64%, respectively. Total serum osteocalcin levels were quantified via electrochemiluminescent immunoassay (Roche Diagnostics GmbH, Mannheim, Germany); the intra- and inter-assay coefficients of variation were 1.2-4.0% and 1.7-6.5%, respectively.

Coronary arteriography and CAD diagnosis

Coronary arteriography was performed using the standard Judkins technique [13]. All major coronary arteries were examined on at least two orthogonal views. Angiograms were evaluated by two experienced cardiologists blinded to study design and clinical information. CAD was diagnosed when stenosis was evident in ≥50% of the luminal diameter of a major coronary artery, *i.e.*, the left main coronary artery, left anterior descending artery or its first diagonal branch, left circumflex artery or its first

Table 1. Subject characteristics (Data were expressed as mean ± SD or median (interquartile range))

Variables	Non-NAFLD 130	NAFLD 44	P
Men/women	89/41	34/10	0.267
Age (years)	67.7±9.8	64.5±11.0	0.070
BMI (kg/m^2)	23.4±2.7	26.5±2.9	<0.001
Waist circumference (cm)	87.8±8.9	95.5±7.7	<0.001
SBP (mmHg)	130 (120-144)	140 (130-153)	0.003
DBP (mmHg)	75 (70-80)	80 (70-90)	0.006
FPG (mmol/L)	5.3 (4.9-6.1)	6.4 (5.4-7.7)	<0.001
2 h PG (mmol/L)	7.8 (6.4-11.4)	11.5 (8.6-15.3)	<0.001
HbA1c (%)	6.1 (5.7-6.7)	6.9 (6.2-8.0)	<0.001
HOMA-IR	3.8 (2.5-5.0)	5.7 (4.2-9.2)	<0.001
TC (mmol/L)	4.1±1.0	4.7±1.2	0.002
TG (mmol/L)	1.4 (1.0-1.7)	2.3 (1.6-3.7)	<0.001
HDL-c (mmol/L)	1.1 (0.9-1.3)	0.9 (0.8-1.1)	<0.001
LDL-c (mmol/L)	2.8±0.9	3.1±1.0	0.103
CRP (mg/L)	0.9 (0.5-2.7)	2.1 (1.1-4.5)	0.001
ALT (UI/L)	16.0 (11.0-25.3)	26.0 (18.0-36.5)	<0.001
AST (UI/L)	20.0 (16.0-25.0)	22.0 (19.0-25.8)	0.080
GGT (UI/L)	21.0 (14.0-35.0)	35.0 (23.3-49.5)	<0.001
Adiponectin (mg/L)	8.2 (5.1-13.5)	5.6 (4.2-9.6)	0.001
Anti-diabetic drugs, N (%)	31 (23.8)	15 (34.1)	0.183
Anti-hypertensives, N (%)	81 (62.3)	36 (81.8)	0.017
Statins therapy, N (%)	39 (30.0)	17 (38.6)	0.289

Abbreviations: NAFLD, nonalcoholic fatty liver disease; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; FPG, fasting plasma glucose; 2 h PG, 2 h postprandial plasma glucose; HbA1c, glycosylated haemoglobin A1c; HOMA-IR, homeostasis model assessment of insulin resistance; TC, total cholesterol; TG, triglyceride; HDL-C, high-density lipoprotein-cholesterol; LDL-C, low-density lipoprotein-cholesterol; CRP, C-reactive protein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, gamma-glutamyl transferase.

obtuse marginal branch, or right coronary artery.

Diagnostic criteria for NAFLD

As it was impractical to perform liver biopsies on all of the study participants, fatty liver disease was diagnosed by B ultrasonography of the liver. The ultrasonographic criteria included a diffusely increased near-field ultrasound echo (a 'bright liver'); a liver echo greater than that of the kidney; vascular blurring; and gradual attenuation of the far-field ultrasound echo [12]. NAFLD was diagnosed using the working definition of the revised guidelines for the management of NAFLD published by the Chinese Liver Disease Association [12].

Statistical analysis

All statistical analyses were performed with SPSS ver. 19.0 (SPSS, Chicago, USA). The one-sample Kolmogorov-Smirnov test was used to explore the normality of data distribution. Normally distributed data were expressed as means ± standard deviations, and skewed data as medians (with inter-quartile ranges). Clinical data were compared between the two groups using the unpaired Student's *t*-test (for normally distributed data) or Mann-Whitney *U*-test (for skewed data), and the chi-square test was used to compare categorical variables. Spearman correlation coefficients between serum osteocalcin levels and various clinical parameters were calculated in simple and partial correlation analyses. Multivariate logistic regression analysis was used to identify factors that were independently associated with the presence of NAFLD. All *P*-values were two-tailed and the threshold for statistical significance was set at 0.05.

Results

Clinical characteristics of study participants

This study enrolled 174 inpatients (mean age 66.9±10.1 (range 38-86) years; 123 men, 51 postmenopausal women), who were confirmed by CAG to have CAD. The subjects were subdivided into two groups by NAFLD status. As shown in **Table 1**, compared with non-NAFLD subjects, those with NAFLD had significantly higher BMI, W, blood pressure, FPG, 2 h PG, HbA1c, HOMA-IR, TC, TG, CRP, ALT, and GGT levels. These patients also used more anti-hypertensive drugs, but had significantly lower HDL-C levels (P<0.05). The subjects with NAFLD also had significantly lower serum osteocalcin levels

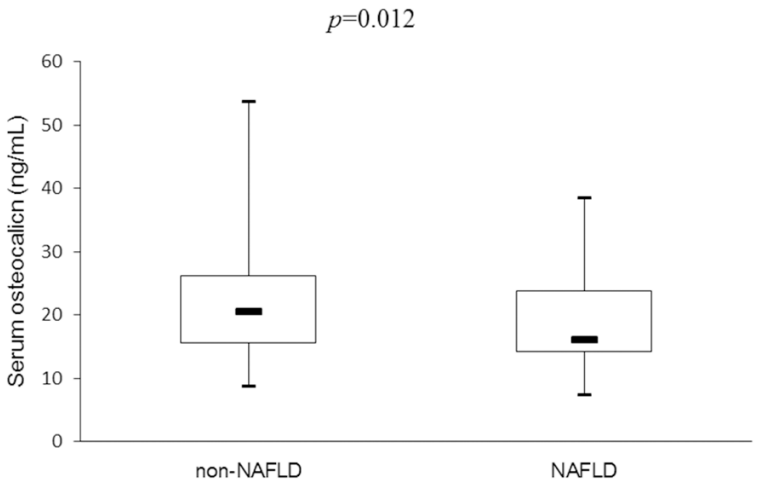

Figure 1. Comparison of serum osteocalcin levels between non-NAFLD and NAFLD groups.

Table 2. Association between serum osteocalcin levels and anthropometric parameters and biochemical indices

Variable	Osteocalcin		Adjusted for sex and age	
	r	P	*r*	P
Male gender	-0.239	0.002		
Age	-0.028	0.715		
NAFLD	-0.192	0.011	-0.260	0.010
BMI	-0.048	0.530	-0.063	0.539
Waist circumference	-0.144	0.058	-0.037	0.716
SBP	0.109	0.153	0.081	0.425
DBP	-0.009	0.906	-0.088	0.391
FPG	-0.215	0.004	-0.230	0.023
2 h PG	-0.076	0.320	-0.141	0.166
HbA1c	-0.122	0.111	-0.229	0.023
HOMA-IR	-0.083	0.293	-0.099	0.334
CRP	0.041	0.602	-0.182	0.073
ALT	-0.012	0.874	-0.018	0.862
AST	-0.038	0.618	0.006	0.955
GGT	0.016	0.836	0.156	0.126
TC	0.030	0.751	-0.010	0.919
TG	0.097	0.294	-0.050	0.623
HDL-C	-0.037	0.687	-0.063	0.535
LDL-C	0.110	0.235	0.047	0.647
Adiponectin	0.017	0.828	-0.030	0.772

Abbreviations: NAFLD, nonalcoholic fatty liver disease; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; FPG, fasting plasma glucose; 2 h PG, 2 h postprandial plasma glucose; HbA1c, glycosylated haemoglobin A1c; HOMA-IR, homeostasis model assessment of insulin resistance; CRP, C-reactive protein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, gamma-glutamyl transferase; TC, total cholesterol; TG, triglyceride; HDL-C, high-density lipoprotein-cholesterol; LDL-C, low-density lipoprotein-cholesterol.

than those without NAFLD [16.2 (14.2-23.8) vs. 20.7 (15.6-26.2) ng/mL, P<0.05; **Figure 1**].

Association between serum osteocalcin levels and NAFLD

We first sought correlations between serum osteocalcin levels and anthropomorphic and biochemical variables. We found that serum osteocalcin levels were negatively associated with male gender (r=-0.239, P=0.002), the presence of NAFLD (r=-0.192, P=0.011), and FPG (r=-0.215, P=0.004). After adjustment for gender and age, the latter two associations remained (NAFLD: r=-0.260, P=0.010 and FPG: r=-0.230, P=0.023), and serum osteocalcin levels correlated with HbA1c also (r=-0.229, P=0.023) (**Table 2**).

We next performed multivariate logistic regression analysis using the independent variables osteocalcin, gender, age, BMI, W, blood pressure, HbA1c, HOMA-IR, lipid, CRP, liver enzyme, adiponectin, use of therapeutic statins, use of anti-hypertensives, and use of anti-diabetic drugs. We found that BMI (β=0.345, P<0.001), HbA1c (β=0.641, P=0.004), and TG (β=1.002, P<0.001) were independent positive factors for the presence of NAFLD, whereas the only independent negative factor was serum osteocalcin levels (β=-0.097, P=0.025) (**Table 3**).

Discussion

Since it was realized that osteocalcin regulates glycolipid and energy metabolism, many studies have focused on the relationship between osteocalcin and NAFLD, in efforts to find new ways to prevent and treat the condition. A case-control study found that serum osteocalcin levels were significantly lower in

Table 3. Independent factors of NAFLD identified by multivariate logistic regression analysis

Independent variable	*β*	*S.E.*	*OR*	P	*95% C.I*
Osteocalcin	-0.097	0.044	0.907	0.025	0.833-0.988
BMI	0.345	0.098	1.412	<0.001	1.166-1.710
HbA1c	0.641	0.220	1.898	0.004	1.233-2.923
TG	1.002	0.277	2.723	<0.001	1.584-4.682

Variables included in the original model were osteocalcin, gender, age, BMI, W, SBP, DBP, HbA1c, HOMA-IR, TC, TG, HDL-C, LDL-C, CRP, ALT, AST, GGT, adiponectin; use of therapeutic statins, use of anti-hypertensives, and use of anti-diabetic drugs. Abbreviations: BMI, body mass index; W, waist circumference; SBP, systolic blood pressure; DBP, diastolic blood pressure; HbA1c, glycosylated haemoglobin A1c; HOMA-IR, homeostasis model assessment of insulin resistance; TC, total cholesterol; TG, triglyceride; HDL-C, high-density lipoprotein-cholesterol; LDL-C, low-density lipoprotein-cholesterol; CRP, C-reactive protein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, gamma-glutamyl transferase.

patients with biopsy-proven NAFLD than in healthy controls; serum osteocalcin levels were inversely associated with both ALT and AST concentrations and were the only independent predictor of the extent of hepatocyte ballooning in NALFD patients [14]. Large-scale population-based studies found that, in men, serum osteocalcin levels were lower in those with NAFLD than without NAFLD; a lower level was associated with the presence of NAFLD [15]. In women, serum osteocalcin levels were independently and inversely associated with the presence of NAFLD in both pre- and post-menopausal women without osteopenia or osteoporosis [16]. Animal studies showed that subcutaneous infusion or intermittent intraperitoneal injection of osteocalcin into wild-type mice fed high-fat diets improved hepatic steatosis, degeneration caused by ballooning, and fibrosis [17-19]. Osteocalcin attenuated endoplasmic reticulum stress via the NF-κB signaling pathway, and robustly reduced the liver expression levels of pro-inflammatory and pro-fibrotic genes, alleviating NAFLD [18, 19]. Such findings suggested that osteocalcin influences the development of NAFLD not only phenomenologically, but also mechanistically. Similarly, in this study, we found that serum osteocalcin levels were significantly lower in CAD patients with NAFLD than in those without NAFLD; furthermore, serum osteocalcin levels were independently associated with the presence of NAFLD in CAD patients. Therefore, osteocalcin may influence the development of NAFLD in patients with CAD. Although several studies described associations between serum osteocalcin levels and NAFLD, other studies yielded contradictory results. Previously, we found that although serum osteocalcin levels were inversely correlated with the presence of NAFLD (P<0.01), the association did not remain significant upon logistic regression analysis [20]. Separately, Rubén *et al*. [21] and Sinn *et al*. [16] reported that serum osteocalcin levels were not associated with NAFLD in severely obese patients or in pre- and post-menopausal women with osteopenia or osteoporosis. These findings suggest that the relationship between serum osteocalcin levels and NAFLD may be complex, influenced by race, bone mineral density, weight, and concomitant diseases.

Recent work in the mouse has shown that osteocalcin are closely associated with glucose and lipid metabolism [17, 22], and it is widely accepted that perturbations in these aspects of metabolism are common causes of NAFLD. Compared to wild-type mice, osteocalcin-deficient animals exhibited reduced insulin secretion, glucose tolerance, and insulin sensitivity; but increased body weight, fat mass, and adipocyte numbers. Mice with osteocalcin gain-of-function had a near-opposite phenotype [22]. Human epidemiological studies have yielded similar findings; serum osteocalcin levels were inversely associated with body mass, blood glucose level, and insulin-resistance [23-25]. Likewise, in the present study, serum osteocalcin levels were linked to the FPG levels and HbA1c levels. In addition, body mass, TG and HbA1c levels were independently associated with the presence of NAFLD in CAD patients, suggesting that osteocalcin influences disease development by regulating glucose metabolism.

Several limitations of this study should be noted. The study was cross-sectional in nature, the sample size relatively small, and some inherent bias may have been masked. In addition, we did not perform any liver biopsies, which remain the “gold standard” for NAFLD diagnosis. Rather, we used ultrasonography to this end; this technique is of low sensitivity when used to detect hepatic steatosis.

In conclusion, we found that serum osteocalcin levels were independently and negatively associated with the presence of NAFLD in CAD patients. As the study was cross-sectional in nature, we do not know whether osteocalcin influences the incidence of NAFLD in CAD patients, or whether NAFLD developing in such patients changes serum osteocalcin levels. Further large-scale prospective clinical studies are required.

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Disclosure of conflict of interest

None.

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