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ORIGINAL

Serum osteocalcin is inversely associated with lower extremity atherosclerotic disease in Chinese patients with type 2 diabetes mellitus

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Abstract. Serum osteocalcin (OCN) is closely related to metabolic risk factors, and the relationship between OCN and atherosclerosis has been investigated. However, it is still controversial. Herein, we explored the potential correlation between serum total OCN and lower extremity atherosclerotic disease (LEAD) in 326 hospitalized Chinese patients with type 2 diabetes mellitus (T2DM). Femoral intima-media thickness (F-IMT) and lower limb atherosclerotic plaque were assessed through color Doppler ultrasound. Subjects with LEAD had significantly lower serum OCN levels compared with those without LEAD (14.54 [14.10–14.89] ng/mL *versus* 16.79 [15.86–18.04] ng/mL, $p < 0.001$). Spearman's correlation analysis revealed that serum OCN levels were positively associated with high density lipoprotein cholesterol (HDL-C) and negatively associated with fasting plasma glucose (FPG), 2-hour postprandial plasma glucose (2hPG), hemoglobin A1c (HbA1c), waist-to-hip ratio (WHR) and F-IMT. Multiple logistic analysis revealed that OCN (OR 0.938, 95% confidence interval (CI 0.933–0.950, $p = 0.003$) and glomerular filtration rate (GFR) (OR 0.990, 95% CI 0.985–0.996, $p = 0.003$) were independently and inversely associated with LEAD, while age (OR 1.140, 95% CI 1.127–1.148, $p < 0.001$), diabetes duration (OR 1.068, 95% CI 1.039–1.080, $p < 0.005$) and uric acid (UA) (OR 1.005, 95% CI 1.002–1.007, $p = 0.032$) were independently and positively associated with LEAD. Additionally, multiple linear regression analysis revealed that serum OCN levels were negatively associated with F-IMT (standardized $\beta = -0.180$, $p = 0.002$). In Chinese patients with T2DM, serum OCN levels were independently and inversely correlated with LEAD.

Key words: Type 2 diabetes mellitus, Lower extremity atherosclerotic disease, Osteocalcin, Femoral intima-media thickness

TYPE 2 DIABETES MELLITUS (T2DM) incidence is increasing in China at an alarming rate, imposing a considerable burden on public health. The latest epidemiological survey showed that the diabetes prevalence in individuals above 20 years old was 9.7%, with a prediabetes prevalence reaching 15.5% in the Chinese population [1]. As a frequently encountered macrovascular complication of diabetes, lower extremity atherosclerotic disease (LEAD) counts among the main factors causing foot ulceration and amputation [2]. Moreover, LEAD constitutes an important indicator of atherosclerotic disease burden, and is associated with high mortality from cardiovascular and cerebrovascular causes [3]. Early detection and treatment of LEAD are critical to prevent amputation and mortality in the diabetic population. Even though LEAD is an independent predictor of cardiovascular and cerebrovascular ischemic events, this particular manifestation of systemic atherosclerosis is largely under-diagnosed and undertreated [4]. Therefore, it is vital for diabetic patients to recognize LEAD and control its risk factors as early as possible.

Osteocalcin (OCN), a bone formation marker produced by osteoblastic cells and derived from procollagen metabolism, is a specific and sensitive parameter of bone remodeling involved in bone mineralization and calcium homeostasis [5]. OCN is reported to participate in the regulation of adipose-related gene expression and influence glucose tolerance, fat consumption, and insulin resistance [6]. In recent years, many studies noted the association between serum OCN and atherosclerosis. Ogawa-Furuya N *et al.* found that the serum OCN level is inversely associated with abdominal aortic calcification in men with T2DM [7]. Kim KM *et al.*

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showed that lower uncarboxylated OCN level is significantly associated with coronary artery disease [8]. A study by Deng H *et al.* indicated that OCN is inversely related to carotid atherosclerosis in middle-aged male individuals [9].

Except for the above findings that OCN was associated with metabolic dysfunction and the well-established link between metabolic disorders and atherosclerosis disease, no clinical studies have reported the potential connection between OCN and LEAD. Thus, the aim of this study was to clarify the possible link between serum OCN levels and LEAD in T2DM patients to provide some information on the diagnosis and treatment of LEAD.

Research Design and Methods

Study population

For this study, 326 hospitalized T2DM patients in the international medical ward of the Department of Geriatrics and the Department of Endocrinology and Metabolism of the first Affiliated Hospital of University of Science and Technology of China during January 2018 to May 2019 were enrolled. They were admitted for uncontrolled hyperglycemia or diabetic complications. The diagnostic criteria of diabetes were based on the American Diabetes Association standards [10]. Patients with type 1 diabetes mellitus, gestational diabetes, or other specific types of diabetes were excluded. Additionally, individuals with hepatic dysfunction (acute vital hepatitis, alanine aminotransferase, or aspartate aminotransferase >1.5-fold the upper limit of normal), renal dysfunction (serum creatinine ≥115 μmol/L, or glomerular filtration rate (GFR) <60 mL/min/1.73 m^2), hyper- or hypothyroidism, acute infection, malignant tumor, psychiatric disease, or a history of fracture in the past year were also excluded. In addition, none had been treated with calcium supplements, vitamin D preparations, hormone therapy, antiresorptive therapy, thiazides, steroids or other medications that might affect bone mass. All participants were asked to report their clinical information regarding diabetes duration, alcohol consumption, smoking status, and medications. This study was approved by the Ethics Committee of The First Affiliated Hospital of University of Science and Technology of China (Anhui Provincial Hospital). All subjects provided written informed consent before participation in the study.

Clinical and biochemical measurements

All subjects completed a questionnaire that collected general background information including present and previous illness, medication, alcohol consumption and smoking status. Hypertension was defined as systolic blood pressure (SBP) ≥140 mmHg or diastolic blood pressure (DBP) ≥90 mmHg or history of antihypertensive medicine administration. Height, weight, waist circumference, hip circumference and blood pressure were assessed on a standardized form by the same physician during the health check-up. Body mass index (BMI) was calculated as body weight (in kg) divided by the square of the height (in m). Waist-to-hip ratio (WHR) was calculated as waist circumference divided by hip circumference.

Fasting blood samples were collected from each subject after 10 h of overnight fasting for measurement. Approximately 2 h after eating breakfast, 2-hour plasma glucose (2hPG) was assessed. Blood samples were transported to the central laboratory of The First Affiliated Hospital of University of Science and Technology of China as needed after collection. All serum samples were stored at 4°C and analyzed within a day of sampling. Fasting plasma glucose levels (FPG) and 2hPG were determined by the glucose oxidase method (Automatic Biochemistry Analyzer; Beckman Coulter). Uric acid (UA), creatinine and serum lipid components including total cholesterol (TC), triglyceride (TG), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), total calcium (Ca) and phosphorus (P) were assessed by routine procedures on an Auto Analyzer (Hitachi 7600-020; Hitachi, Tokyo, Japan). Hemoglobin A1c (HbA1c) was estimated by high-performance liquid chromatography on an HLC-723G7 analyzer (Tosoh Corporation, Japan). Serum C-reactive protein (CRP) was measured by particle-enhanced immunonephelometric assay (Dade Behring Inc, Newark, NJ, USA). Serum total OCN and serum insulin were measured using electrochemiluminescence immunoassay (coefficient of variation <4.0% and <5.0% for OCN and insulin, respectively). Homeostasis model assessment index for insulin resistance (HOMA-IR) = [Insulin × FPG]/22.5 [11]. GFR was estimated using the Modification of Diet in Renal Disease method [12].

Ultrasonography measurements

All participants underwent color Doppler ultrasound examinations of lower limb arteries using an Acuson Sequoia 512 scanner (Siemens Medical Solutions, Mountain View, CA, USA) equipped with a 5–13 MHz linear array transducer. Ultrasound examination included measurement of atherosclerotic plaque and femoral intima-media thickness (F-IMT). Seven arteries in each lower limb, including the femoral artery, deep femoral artery, superficial femoral artery, popliteal artery, anterior tibial artery, posterior tibial artery, and peroneal artery, were checked for atherosclerotic plaque. Intima-media thickness (IMT) was defined as the distance between the

leading edge of the lumen-intima echo and the leading edge of the media-adventitia echo [13]. F-IMT was defined as the mean value of IMTs of the bilateral femoral arteries [14].

Determination of LEAD

According to the Mannheim consensus [13], atherosclerotic plaque was defined as the presence of a focal structure encroaching into the arterial lumen at least 0.5 mm, or at least 50% greater than the thickness of the surrounding vessel wall, or an IMT ≥1.5 mm. LEAD was defined when atherosclerotic plaques were present in any of the lower limb artery segments listed above [15].

Statistical analysis

Statistical analyses were performed using SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). All variables underwent a normality test. Normally distributed data and F-IMT data are presented as mean ± standard deviation (SD), and skewed data are expressed as the median with interquartile range. Categorical variables are expressed as a percentage (%). Inter-group comparisons of normally distributed data and skewed data were carried out by the unpaired Student's *t* test and Mann–Whitney *U* test, respectively. The Chi square test was used for inter-group comparisons of categorical variables. The association of OCN and anthropometric and biochemical parameters was analyzed by Spearman tests. Multiple linear regression analysis was conducted to identify factors independently correlated with the serum OCN levels. Multivariable logistic regression analysis was performed to identify multiple factors associated with LEAD. The threshold of statistical significance was set at 0.05 for two-tailed *p*-values.

Results

A total of 326 T2DM participants were enrolled in the present study [median age: 59 (48–63) years], including 157 subjects with LEAD and 169 subjects without LEAD. As shown in Table 1, compared with those without LEAD, SBP, UA and F-IMT in the LEAD group were significantly higher, whereas the levels of HDL-C and GFR were significantly lower (all $p < 0.05$). In addition, subjects with LEAD were older and also exhibited a longer diabetes duration than those without LEAD ($p < 0.05$). Other variables did not differ significantly between the two groups (all $p > 0.05$; Table 1).

Among the entire study population, the median (interquartile range) serum OCN levels were 15.78 (15.23–16.34) ng/mL. There was no significant gender difference in serum OCN levels (16.02 [15.34–16.57] ng/mL in men *versus* 15.39 [14.89–15.64] ng/mL in women, $p > 0.05$). Subjects with LEAD had significantly lower serum OCN levels compared with those without LEAD (14.54 [14.10–14.89] ng/mL *versus* 16.79 [15.86–18.04] ng/mL, $p < 0.001$). Subgroup analysis confirmed the significant difference in serum OCN levels between subjects with and without LEAD separately in men and women (14.68 [14.2–15.07] ng/mL *versus* 18.59 [16.87–19.02] ng/mL in men, $p = 0.002$; 13.95 [13.78–14.86] ng/mL *versus* 16.94 [15.82–17.54] ng/mL in women, $p = 0.008$; Fig. 1).

The associations between OCN and anthropometric and biochemical parameters were analyzed. In the LEAD group, Spearman's correlation analysis demonstrated a negative correlation between OCN and FPG ($r = –0.385$, $p < 0.001$), OCN and 2hPG ($r = –0.323$, $p < 0.001$), OCN and HbA1c ($r = –0.314$, $p < 0.001$), and OCN and F-IMT ($r = –0.310$, $p < 0.001$). In the Non-LEAD group, the analysis demonstrated a negative correlation between OCN and FPG ($r = –0.178$, $p = 0.005$), OCN and 2hPG ($r = –0.135$, $p = 0.034$), OCN and HbA1c ($r = –0.185$, $p = 0.034$), OCN and Insulin ($r = –0.128$, $p = 0.033$) and OCN and F-IMT ($r = –0.186$, $p = 0.027$). Moreover, the associations between OCN and other atherosclerosis risk factors were also investigated. In the LEAD group, a significant correlation between OCN and SBP ($r = –0.135$, $p = 0.070$) was found. However, in the Non-LEAD group, there was no significant correlation between OCN and other risk factors. In contrast, as shown in Table 2, in the whole study population, OCN was found to have significant positive correlation with HDL-C and negative correlation with FPG, 2hPG, HbA1c, WHR and F-IMT.

In multiple logistic analysis, the presence of LEAD was defined as the dependent variable and age, BMI, WHR, SBP, HbA1c, Insulin, TG, HDL-C, LDL-C, UA, Ca, P, GFR, serum OCN, diabetes duration and current smoking were defined as independent variables. The analysis identified age (OR 1.140, 95% CI 1.127–1.148, $p < 0.001$), diabetes duration (OR 1.068, 95% CI 1.039–1.080, $p < 0.005$), UA (OR 1.005, 95% CI 1.002–1.007, $p = 0.032$), GFR (OR 0.990, 95% CI 0.985–0.996, $p = 0.003$) and serum OCN levels (OR 0.938, 95% confidence interval [CI] 0.933–0.950, $p = 0.003$) as independently associated with LEAD in patients with T2DM (Table 3).

To further identify factors independently affecting serum OCN levels, a multiple linear regression model was used. The dependent variable was serum OCN levels, and the independent variables were HDL-C, FPG, 2hPG, HbA1c, WHR and F-IMT. The analysis revealed that HbA1c (standardized $\beta = –0.210$, $p = 0.001$) and F-IMT (standardized $\beta = –0.180$, $p = 0.002$) were independently and negatively correlated with serum OCN levels. Furthermore, these relationships remained significant after

Table 1 Characteristics of the study participants

Variable	Total	without LEAD	with LEAD
N (men/women)	326 (166/160)	169 (86/83)	157 (80/77)
Age (years)	59 (48–63)	50 (42–57)	63 (55–69)*
BMI (kg/m^2)	24.91 ± 2.92	25.25 ± 3.01	24.75 ± 2.84
WHR	0.92 ± 0.23	0.91 ± 0.20	0.93 ± 0.25
SBP (mmHg)	132 (123–140)	125 (117–133)	133 (125–142)*
DBP (mmHg)	81 (73–84)	78 (72–85)	80 (73–83)
FPG (mmol/L)	7.81 ± 2.32	7.74 ± 2.02	7.85 ± 2.45
2hPG (mmol/L)	13.75 ± 4.74	13.58 ± 4.36	13.92 ± 4.83
HbA1c (%)	8.85 ± 2.07	8.82 ± 1.37	8.91 ± 1.89
Insulin (mU/L)	5.41 (4.78–9.53)	5.58 (4.82–9.60)	5.36 (4.62–9.48)
HOMA-IR	1.48 (1.26–2.10)	1.50 (1.31–2.09)	1.45 (1.35–1.98)
TG (mmol/L)	1.54 (1.19–1.91)	1.52 (1.22–1.89)	1.60 (1.06–1.94)
TC (mmol/L)	1.98 ± 0.95	2.05 ± 0.98	1.87 ± 0.82
HDL-C (mmol/L)	1.02 (0.89–1.25)	1.08 (0.91–1.38)	1.01 (0.85–1.20) *
LDL-C (mmol/L)	2.64 ± 0.77	2.58 ± 0.65	2.71 ± 0.80
UA (μmol/L)	315.24 ± 78.25	308.85 ± 75.71	351 ± 82.75*
Ca (mmol/L)	2.32 ± 0.12	2.31 ± 0.12	2.32 ± 0.12
P (mmol/L)	1.24 ± 0.13	1.22 ± 0.12	1.20 ± 0.11
GFR (mL/min/1.73 m^2)	95.78 ± 22.86	102.23 ± 24.24	91 ± 23.75*
CRP (mg/L)	0.84 (0.43–1.52)	0.85 (0.48–1.57)	0.82 (0.46–1.55)
F-IMT	0.78 ± 0.12	0.74 ± 0.11	0.88 ± 0.10*
Current smoker, *n* (%)	84 (25.76)	44 (26.03)	40 (25.47)
Current drinker, *n* (%)	53 (16.26)	27 (15.97)	26 (16.56)
Dyslipidemia, *n* (%)	111 (32.08)	53 (31.36)	54 (33.04)
Hypertension, *n* (%)	235 (72.70)	122 (72.19)	115 (73.24)
Anti-hypertensive therapy, *n* (%)	123 (37.73)	63 (37.28)	60 (38.21)
Anti-diabetic therapy, *n* (%)	244 (74.85)	126 (74.56)	118 (75.16)
Lipid-lowing therapy, *n* (%)	55 (16.87)	29 (17.16)	26 (16.56)
Diabetes duration (years)	9 (0.85–12)	5 (0.80–10)	11 (1.20–18)*

* $p < 0.05$ *versus* without LEAD

additional adjustment for gender and factors potentially affecting serum OCN levels (Ca, P, and GFR; both $p < 0.05$; Table 4).

Discussion

This study demonstrated that serum OCN levels were significantly lower in Chinese T2DM patients with LEAD. Our analyses showed that decreased serum OCN level was an independent risk factor for LEAD, and serum OCN level was negatively associated with F-IMT. Additionally, a negative correlation between serum OCN levels and HbA1c was observed.

Evidence indicates that the skeleton works not only as a structural scaffold but also as an endocrine organ. As an osteoblast-secreted small peptide, OCN was able to bind to hydroxyapatite in bone constitution and was also reported to be active in glucose and fat metabolism. For example, a lower level of OCN was found to decrease glucose tolerance [16], which was consistent with our findings that OCN had negative significant Spearman's correlation with FPG and 2hPG. The mechanism by which OCN could regulate glucose metabolism has been studied: OCN, especially undercarboxylated OCN, can

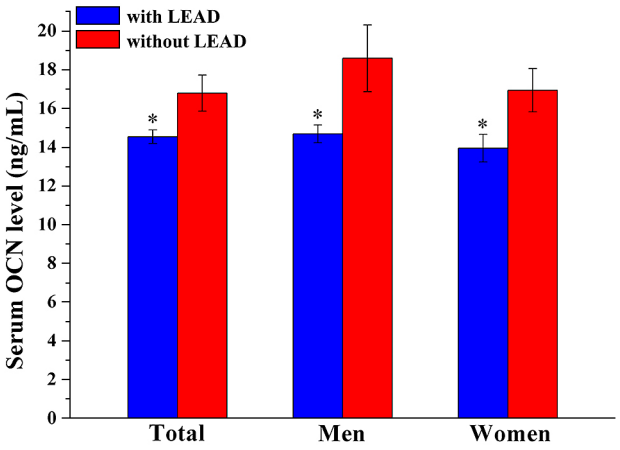

Fig. 1 Comparison of serum OCN levels between diabetes patients with and without LEAD.
Data are shown as median with 25th and 75th percentiles.
* $p < 0.05$ *versus* without LEAD.

Table 2 Correlations of OCN with various clinical and biochemical markers in the whole study population

Markers	*r*	*p*
Age	0.021	0.234
BMI	–0.079	0.136
WHR	–0.045	0.015
SBP	–0.048	0.238
DBP	–0.079	0.108
FPG	–0.324	<0.005
2hPG	–0.258	<0.005
Insulin	–0.089	0.078
HbA1c	–0.245	<0.005
TG	–0.058	0.283
TC	0.024	0.571
HDL-C	0.072	0.012
LDL-C	–0.145	0.158
UA	–0.217	0.125
Ca	0.154	0.356
P	0.142	0.368
GFR	0.123	0.389
CRP	–0.114	0.077
F-IMT	–0.245	0.012

significantly induce insulin genes (*Ins 1* and *Ins 2*) and insulin proliferating genes (*CyclinD2*, *Cdk4*) expression [17] while also increasing adiponectin expression, thus increasing insulin secretion and reducing insulin resistance, and consequently reducing blood sugar and fat content [18]. Studies have previously reported that bone formation is known to be decreased in the setting of high glucose levels. In healthy individuals, ingestion of 75 g of glucose leads to a decrease in markers of both bone formation and resorption [19], and *in vitro* data show that exposure to high glucose levels impairs osteoblast function [20]. In this study, multiple stepwise regression analysis revealed that HbA1c was independently affecting serum OCN levels, and the relationship remained significant after additional adjustment for gender and Ca, P and GFR.

Multiple studies have investigated the association between OCN and atherosclerosis. Some studies suggest that OCN may actually be protective against early atherosclerosis. In a Japanese study, serum OCN was negatively associated with carotid intima-media thickness (C-IMT) in T2DM men [21]. In another study, serum levels of OCN were inversely associated with the severity of coronary artery disease in Chinese men [22]. Whereas in a cross-sectional study with 461 Chinese subjects, the group diagnosed with atherosclerotic plaques detected through angiography had increased OCN concentration compared to the group without atherosclerotic plaques [23]. Chi *et al.* found that peritoneal dialysis patients with higher serum OCN levels had higher central arterial stiffness as measured by carotid–femoral pulse wave velocity (PWV), and that the serum OCN level was an independent marker of central arterial stiffness in peritoneal dialysis patients [24]. The conflicting results of the above-mentioned studies may be caused by the differences in different genders, populations and the methods of examining the presence of atherosclerosis, including coronary artery calcification score, PWV, and C-IMT. In this study, ultrasound was used to measure the atherosclerotic plaque and F-IMT, and it was observed that OCN (OR 0.938, 95% confidence interval (CI) 0.933–0.950, $p = 0.003$) was independently and inversely correlated with the presence of LEAD. However, the OR of serum OCN with LEAD seemed still very small, its contribution on LEAD may be slight. In addition, multiple linear regression analysis revealed that the serum OCN level was negatively associated with F-IMT (standardized $\beta = –0.180$, $p = 0.002$). This is the first study that investigates the correlation between serum OCN levels and LEAD. Consistent with previous studies [25], we also found that these traditional risk factors, including age, diabetes duration, and UA were positively and independently associated with the presence of LEAD in T2DM patients.

The biological effects of OCN on the development of atherosclerotic are not fully known. A small number of studies have examined the effect of total OCN administration *in vivo* on the vasculature in animal models of

Table 3 Independent factors for LEAD identified by multivariate logistic regression analysis

Variables	*β*	*SE*	OR (95% CI)	*p*
Age	0.157	0.026	1.140 (1.127–1.148)	<0.001
Diabetes duration	0.080	0.015	1.068 (1.039–1.080)	<0.005
UA	0.044	0.016	1.005 (1.002–1.007)	0.032
GFR	–0.032	0.008	0.990 (0.985–0.996)	0.003
OCN	–0.023	0.007	0.938 (0.933–0.950)	0.003

Notation: Independent variables included in the model were age, BMI, WHR, SBP, HbA1c, Insulin, TG, HDL-C, LDL-C, UA, GFR, serum OCN, diabetes duration and current smoking.

Table 4 Multivariate regression analyses of factors associated with serum OCN levels

Variable	Standardized *β*	*t*	*p*
Model 1			
HbA1c	–0.210	–3.98	0.001
F-IMT	–0.180	–3.25	0.002
Model 2 (adjusted for gender)			
HbA1c	–0.189	–3.76	0.013
F-IMT	–0.167	–2.86	0.007
Model 2 (adjusted for Ca, P, and GFR)			
HbA1c	–0.245	–4.02	0.003
F-IMT	–0.178	–3.31	0.002

Notation: Independent variables included in the model were HDL-C, FPG, 2hPG, HbA1c, WHR and F-IMT.

disease [26, 27]. Total OCN treatment had a protective effect on high-fat-diet-induced hypertension by reducing mean and DBP by ~5 and 7 mmHg, and there was also a concomitant improvement in body weight, FPG levels, glucose tolerance, circulating lipids, and markers of inflammation in animals receiving OCN treatment [28]. In another study, the administration of total OCN repaired the alteration to PWV, concurrently with improvements in FPG and circulating lipids [27].

It is unclear whether OCN has a direct role in the vasculature, independent of metabolic outcomes, or whether the association is mediated indirectly, *via* the metabolic effects of OCN. *In vitro*, it has been demonstrated that OCN may upregulate nitric oxide synthesis *via* activation of the phosphoinositide 3-kinase (PI3K)—protein kinase B (Akt)—endothelial nitric oxide synthase (eNOS) signaling pathway in human endothelial cells, which may have a protective effect against endothelial dysfunction. Thus, these findings indicate that OCN may have a protective function in the vasculature, independent from its influence on metabolic outcomes. Nevertheless, OCN's receptor in the vasculature is yet to be identified. Previous studies have identified the G protein-coupled receptor family C, group 6, subtype A (GPRC6A) as the OCN receptor in the tissues of mice [28]. In humans, GPRC6A has been identified as the OCN receptor in the testes [29]. There is minimal evidence regarding the role of GPRC6A as the receptor for OCN in the vasculature of animals [30], but whether OCN interacts with this receptor is presently unknown. Overall, future studies should aim to elucidate whether GPRC6A is the receptor for OCN in the vasculature of humans.

There were some limitations of our study. Firstly, this study was limited by the cross-sectional design and relatively small sample size, which made it difficult to clarify the causal relationship between decreased serum OCN levels and the presence of LEAD. Secondly, the study population was restricted to patients with T2DM. Hence, further prospective studies are warranted to confirm and generalize the present findings in a larger population, including those without diabetes. Thirdly, in the blood, OCN is found in two forms: carboxylated OCN, which is mainly involved in bone mineralization, and undercarboxylated OCN, which is mainly involved in energy metabolism and controlled by vitamin K concentration. In this study, only the total OCN concentration

was measured, and the different form of OCN and the concentration of vitamin K were not considered [31].

In conclusion, serum OCN levels independently and negatively correlate with LEAD in Chinese patients with T2DM. Further studies and investigations are needed to understand the effects of OCN on atherosclerotic diseases.

Author Contributions

QHL: Study design, sample identification for OCN analysis, data collection, database management, statistical analysis, data interpretation, writing of manuscript. QHL had full access to the data in the study and final responsibility for the decision to submit for publication. JZ: Study conception and design, sample collection and OCN analysis, data collection, statistical analytic plan input, data interpretation. JJL: Study design, sample collection, data collection, statistical analytic plan input, data interpretation, writing of manuscript. DMK: Study conception and design, sample collection and OCN analysis, data collection, statistical analytic plan input, data interpretation. HYZ: Study design, statistical analysis, data interpretation, revision of manuscript. Authors have no relevant conflicts of interest.

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