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Original Article

Association of serum 25-hydroxy vitamin D and osteocalcin levels with non-alcoholic fatty liver disease

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Abstract: Considering the strong association of non-alcoholic fatty liver disease (NAFLD) with obesity and the metabolic syndrome, recent researches have witnessed a significant scientific interest into the potential role of vitamin D or osteocalcin in NAFLD, but the relationship between them remains controversial. The present study was designed to investigate the association of serum 25-hydroxy vitamin D (25-OH-VitD) and osteocalcin levels with NAFLD. A total of 398 participants who were referred to our endocrinology outpatient clinics and health examination center were recruited from November 2014 to February 2015. After screening, 368 patients were included in the study finally. Serum 25-OH-VitD was determined by radioimmunoassay and serum osteocalcin was determined by immunoradiometric assay. NAFLD was diagnosed by hepatic ultrasonographic examination. The association of serum 25-OH-VitD and osteocalcin with NAFLD was evaluated. Among the participants, 190 did not have NAFLD (non-NAFLD group), 178 had NAFLD (NAFLD group). The levels of serum 25-OH-VitD and osteocalcin were lower in NAFLD group than those in non-NAFLD group ($P < 0.001$). Serum 25-OH-VitD and osteocalcin levels were associated with the NAFLD ($P < 0.001$). Serum 25-OH-VitD and osteocalcin levels were protective factors for the development of NAFLD ($P < 0.001$). Our findings suggest that lower serum 25-OH-VitD and osteocalcin levels are associated with the presence of NAFLD.

Keywords: 25-hydroxy vitamin D, osteocalcin, non-alcoholic fatty liver disease

Introduction

Non-alcoholic fatty liver disease (NAFLD) is a clinical syndrome characterized by no causes for secondary hepatic fat accumulation such as excessive alcohol consumption, use of steatogenic medication or hereditary disorders. Recently, with the Westernization of lifestyles, NAFLD represents an emerging health problem in the world. The prevalence of NAFLD among adults in the general population in China is approximately 15%-30% [1], and the prevalence in the developed region is close to 40% [2, 3]. Previous studies have shown that NAFLD is strongly associated with obesity, dyslipidemia, insulin resistance and type 2 diabetes mellitus [4, 5], and NAFLD is now considered the hepatic manifestation of the metabolic syndrome [6].

Besides the recognized risk factors, such as hyperglycemia and dyslipidemia, some new circulating biomarkers of bone metabolism have been found to be altered in patients with NAFLD recently, which suggested that there be an interaction between bone metabolism and liver metabolism. The skeleton, classically viewed as a structural scaffold necessary for mobility, a regulator of calcium-phosphorus homeostasis and maintenance of the hematopoietic function has now been identified as a new endocrine organ, it can secrete some hormones which involved in various metabolic diseases such as diabetes, obesity and NAFLD [7, 8]. Recently, a series of clinical epidemiological evidence suggested that NAFLD link with low bone mineral density [9-12], but the underlying mechanisms remained unclear, some researches indicated the presence of a complex interplay between the bone and liver, that is, Bone-liver Axis, and in which play an important role was some bone metabolism-related molecules including vitamin D and osteocalcin [8, 13].

Given the strong association of NAFLD with obesity and the metabolic syndrome, recent researches have witnessed a significant scientific interest into the potential role of vitamin D or osteocalcin in NAFLD, but the relationship between them remains controversial unfortunately. A recently published meta-analysis summarized that NAFLD subjects were 26% more likely to be vitamin D deficient compared to matched controls [14]. Some epidemiologic evidences supported a link between vitamin D deficiency and NAFLD [15-17], on the contrary, other studies got the opposite results [18, 19]. Similar to this situation, the relationship between osteocalcin and NAFLD was also contradictory [20-22], even in our population [23, 24]. Additionally, previous studies only paid attention to the correlation between a single hormonal signal (vitamin D or osteocalcin) and NAFLD, and there is no statistical analysis of these two hormonal signals in the same population, so the aim of this study is to detect the serum levels of 25-hydroxy vitamin D (25-OH-VitD), the most stable form of vitamin D, and osteocalcin, analyze the association of 25-OH-VitD and osteocalcin with NAFLD in the same population, at the same time.

Materials and methods

Subjects

This study was approved by Institutional Review Board and Ethics Committee of The First Affiliated Hospital of Bengbu Medical College, Bengbu, Anhui, China. Informed consent was obtained from each participant. From November 2014 to February 2015, 398 subjects who were referred to our endocrinology outpatient clinics and health examination center were recruited. Participants were excluded from this study based on the following criteria [1]: (1) an average alcohol consumption of ≥ 20 g ethanol/day in men (≥ 140 g/week) and 10 g ethanol/day in women (≥ 70 g/week); (2) viral hepatitis B and viral hepatitis C; (3) cirrhosis and hepatic carcinoma; (4) other chronic liver diseases such as autoimmune hepatitis, Wilson's disease and hemochromatosis; (5) current use of drugs known to influence 25-OH-VitD metabolism, including glucocorticoids and calcium/vitamin D supplements. After screening, 368 patients were included in the study finally.

Anthropometric measurements

Each subject underwent a physical examination, including measurements of height, weight, waist circumference (WC) and blood pressure (BP). Measurements of weight and height were used to calculate the body mass index (BMI) [= (kg/m^2)]. WC was measured on the midaxillary line between the lower border of the rib cage and the upper margin of the iliac crest. BP was obtained from the average of three measurements made with a standard mercury sphygmomanometer at 3-minute intervals.

Laboratory examination

Fasting blood samples were obtained after a 10-hour fast by venipuncture of the large antecubital veins. The samples were then centrifuged immediately, and the plasma was separated and stored at -80°C. Fasting plasma glucose (FPG) was measured by a glucose oxidase method. Glycosylated hemoglobin A1c (HbA1c) was determined using a high-performance liquid chromatographic method (Bio-Rad, Hercules, CA, USA). Serum 25-OH-VitD concentrations were measured using a 25-OH-VitD ^{125}I RIA kit (DiaSorin, Minnesota, USA) by radioimmunoassay. Serum osteocalcin concentrations were quantified using a hOST-IRMA kit (Biosource, Neville, Belgium) by immunoradiometric assay. The rest biochemical values were detected by automatic clinical chemistry analyzer (Beckman Coulter AU5800, CA, USA).

NAFLD evaluation

Liver ultrasound examinations were performed to assess the presence of fatty liver disease. A single examiner who was blinded to the study objectives performed all imaging procedures using a high-resolution B-mode scanner (Voluson 730 Expert; GE, Milwaukee, WI, USA) equipped with a 5.0 MHz probe.

Statistical analysis

All statistical analyses were performed using SPSS version 19.0 (SPSS Inc., Chicago, IL, USA). The distribution of the data was assessed using a one-sample Kolmogorov-Smirnov test. Normally distributed continuous variables were expressed as the mean ± SD, skew-distributed continuous variables were expressed as the

Table 1. Demographic and clinical characteristics of the subjects

Characteristic	non-NAFLD	NAFLD	p
n	190	178	
Sex (n)	M: 105, F: 85	M: 168, F: 10	0.00
Age (years)	37.69 ± 10.20	42.69 ± 9.52	0.00
SBP (mmHg)	125.49 ± 14.18	135.76 ± 11.90	0.00
DBP (mmHg)	79.49 ± 9.23	83.30 ± 9.77	0.00
WC (cm)	81.81 ± 6.86	87.15 ± 7.98	0.00
BMI (kg/m^2)	23.68 ± 2.93	25.12 ± 2.28	0.00
TP (g/L)	72.78 ± 3.14	72.31 ± 3.65	0.18
ALB (g/L)	45.50 ± 2.08	45.31 ± 2.12	0.38
GLB (g/L)	27.59 ± 3.27	26.95 ± 3.15	0.06
ALT (U/L)	18.00 (14.00-24.25)	27.50 (20.00-39.00)	0.00
AST (U/L)	21.00 (18.00-25.00)	22.00 (19.75-28.00)	0.00
GGT (U/L)	16.00 (11.75-25.00)	34.00 (22.00-55.50)	0.00
ALP (U/L)	60.00 (50.00-73.25)	67.00 (58.75-82.25)	0.00
TG (mmol/L)	1.33 ± 1.15	2.14 ± 1.41	0.00
CHO (mmol/L)	4.59 ± 0.80	4.93 ± 0.85	0.00
HDL (mmol/L)	1.39 (1.10-1.64)	1.08 (0.91-1.25)	0.00
LDL (mmol/L)	2.68 ± 0.74	3.14 ± 0.79	0.00
LDLc (mmol/L)	2.51 ± 0.71	2.78 ± 0.82	0.00
UA (μmol/L)	279.88 ± 68.28	353.19 ± 63.51	0.00
CR (μmol/L)	69.68 ± 14.01	79.78 ± 13.60	0.00
UREA (mmol/L)	4.75 (4.00-5.50)	4.80 (4.20-5.80)	0.10
FPG (mmol/L)	5.15 ± 0.75	5.70 ± 1.37	0.00
HbA1c (%)	5.20 ± 0.60	5.54 ± 1.06	0.00
TBIL (μmol/L)	7.80 (5.25-11.00)	8.80 (6.38-11.60)	0.07
DBIL (μmol/L)	3.50 (2.70-4.53)	3.50 (2.80-4.20	0.84
IBIL (μmol/L)	6.30 (4.40-8.90)	6.85 (4.88-8.63)	0.26
OST (ng/ml)	17.28 ± 3.18	15.24 ± 2.50	0.00
25-OH-VitD (ng/ml)	19.31 (16.69-22.53)	14.19 (15.47-18.12)	0.00

Abbreviation: SBP, systolic blood pressure; DBP, diastolic blood pressure; WC, waist circumference; BMI, body mass index; TP, total protein; ALB, albumin; GLB, globulin; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, gamma-glutamyl transpeptidase; ALP, alkaline phosphatase; TG, triglyceride; CHO, total cholesterol; HDL, high density lipoprotein; LDL, low density lipoprotein; LDL-c, low density lipoprotein cholesterol; UA, uric acid; CR, creatinine; UREA, urea nitrogen; FPG, fasting plasma glucose; HbA1c, glycated hemoglobin A1c; TBIL, total bilirubin; DBIL, direct bilirubin; IBIL, indirect bilirubin; OST, osteocalcin; 25-OH-VitD, 25-hydroxy vitamin D.

median with interquartile range. Differences between numeric variables were tested with Student's t-test for normal distribution, or a Mann-Whitney U-test for skew distribution. Comparative analyses of categorical variables were carried out by the chi-square test. Correlations were tested with Spearman's correlation coefficient. Multivariate logistic regression models were used to estimate the odds ratios (OR) for NAFLD. A value of $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Demographic and clinical characteristics

All subjects were divided into two groups according to the ultrasound diagnosis of NAFLD. Of these subjects, 190 did not have NAFLD (non-NAFLD group), 178 had NAFLD (NAFLD group), and the demographic and clinical characteristics between the two groups were comparatively analyzed. As shown in **Table 1**, compared to the non-NAFLD group, the NAFLD group had lower levels of serum 25-OH-VitD, osteocalcin and HDL, but had higher levels of systolic blood pressure (SBP), diastolic blood pressure (DBP), WC, BMI, alanine aminotransferase (ALT), aspartate aminotransferase (AST), glutamyl transpeptidase (GGT), alkaline phosphatase (ALP), triglyceride (TG), cholesterol (CHO), high density lipoprotein (HDL), low density lipoprotein (LDL), low density lipoprotein cholesterol (LDL-c), uric acid (UA), creatinine (CR), FPG and HbA1c. Additionally, the average age of NAFLD group was older than that of non-NAFLD group. There were no significant differences in total protein (TP), albumin (ALB), globulin (GLB), urea nitrogen (UREA), total bilirubin (TBIL), direct bilirubin (DBIL) and direct bilirubin (IBIL) levels between the two groups.

Association between NAFLD and anthropometric and biochemical parameters

Spearman's correlation analysis was used to investigated the association between NAFLD and anthropometric and biochemical parameters. The results revealed that NAFLD was posi-

Table 2. Association between NAFLD and anthropometric and biochemical parameters

Variable	r	p
Age	0.25	0.00
SBP	0.37	0.00
DBP	0.24	0.00
WC	0.34	0.00
BMI	0.35	0.00
TP	-0.10	0.06
ALB	-0.03	0.57
GLB	-0.10	0.07
ALT	0.43	0.00
AST	0.19	0.00
GGT	0.49	0.00
ALP	0.25	0.00
TG	0.47	0.00
CHO	0.23	0.00
HDL	-0.43	0.00
LDL	0.31	0.00
LDLc	0.22	0.00
UA	0.49	0.00
CR	0.34	0.00
UREA	0.09	0.10
FPG	0.27	0.00
HbA1c	0.16	0.00
TBIL	0.09	0.07
DBIL	-0.01	0.84
IBIL	0.06	0.26
OST	-0.32	0.00
25-OH-VitD	-0.51	0.00

Abbreviation: SBP, systolic blood pressure; DBP, diastolic blood pressure; WC, waist circumference; BMI, body mass index; TP, total protein; ALB, albumin; GLB, globulin; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, gamma-glutamyl transpeptidase; ALP, alkaline phosphatase; TG, triglyceride; CHO, total cholesterol; HDL, high density lipoprotein; LDL, low density lipoprotein; LDL-c, low density lipoprotein cholesterol; UA, uric acid; CR, creatinine; UREA, urea nitrogen; FPG, fasting plasma glucose; HbA1c, glycated hemoglobin A1c; TBIL, total bilirubin; DBIL, direct bilirubin; IBIL, indirect bilirubin; OST, osteocalcin; 25-OH-VitD, 25-hydroxy vitamin D.

tively correlated with the age, SBP, DBP, WC, BMI, ALT, AST, GGT, ALP, TG, CHO, LDL, LDL-c, UA, CR, FPG and HbA1c ($P < 0.05$~$P < 0.01$), but it was negatively correlated with the levels of HDL, serum 25-OH-VitD and osteocalcin ($P < 0.01$) (**Table 2**).

Analysis of risk factors in NAFLD

Multiple logistic regression analysis was used to evaluate the relationship between NAFLD and risk factors. The NAFLD set as the dependent variable and the variables which has significant differences in univariate analysis (Age, Sex, SBP, DBP, WC, BMI, ALT, AST, GGT, ALP, TG, CHO, HDL, LDL, LDL-c, UA, CR, FPG, HbA1c, 25-OH-VitD and osteocalcin) set as independent variables. The results showed that the male, age, SBP, BMI, ALT, ALP, LDL and UA levels were risk factors for the development of NAFLD, but the HDL, 25-OH-VitD and osteocalcin levels were protective factors for the development of NAFLD (**Table 3**).

Discussion

To our knowledge, the present study first investigated the association of the 25-OH-VitD and osteocalcin levels with NAFLD using liver ultrasonography in the same population, at the same time. Our results showed that subjects with NAFLD have significantly higher SBP, DBP, WC, BMI, ALT, AST, GGT, ALP, TG, CHO, LDL, LDL-c, UA, CR, FPG, and HbA1c, but lower serum HDL, 25-OH-VitD and osteocalcin levels compared with those of subjects without NAFLD. Further correlation analysis revealed that NAFLD was positively correlated with the age, SBP, DBP, WC, BMI, ALT, AST, GGT, ALP, TG, CHO, LDL, LDL-c, UA, CR, FPG and HbA1c, but negatively correlated with the levels of HDL, serum 25-OH-VitD and osteocalcin. Finally, multiple logistic regression analysis demonstrated that the serum 25-OH-VitD and osteocalcin levels were protective factors for the development of NAFLD, even adjusted for age, sex and BMI.

It has been reported that NAFLD is the hepatic manifestation of the metabolic syndrome [6]. A detailed analysis of the available epidemiological data shows the major risk factors for NAFLD include obesity, hyperglycemia, insulin resistance, dyslipidemia, hypertension, age and gender [25, 26]. Both excessive BMI and visceral obesity are recognized risk factors for NAFLD. In patients with severe obesity undergoing bariatric surgery, the prevalence of NAFLD can exceed 90% and up to 5% of patients may have unsuspected cirrhosis [27, 28]. High serum triglyceride levels and low serum HDL levels are very common in patients with NAFLD. The prevalence of NAFLD in individuals with dyslipidemia attending lipid clinics was estimated to be 50% [29]. A number of studies have shown that the prevalence of NAFLD increases with age, and have reported

Table 3. Multiple logistic regression analysis of risk factors in NAFLD

Independent variables	B	S.E,	Wals	OR	95% CI	p
Sex	-1.336	0.639	4.337	0.263	0.075-0.919	0.036
Age	0.066	0.20	11.076	1.069	1.028-1.111	0.001
SBP	0.100	0.027	13.881	1.105	1.048-1.164	0.000
BMI	0.158	0.065	5.890	1.171	1.031-1.330	0.015
ALT	0.041	0.014	8.060	1.042	1.013-1.072	0.005
ALP	0.025	0.010	5.469	1.025	1.004-1.046	0.019
HDL	-2.615	0.703	13.822	0.073	0.018-0.290	0.000
UA	0.008	0.003	7.182	1.008	1.002-1.014	0.007
OST	-0.183	0.063	8.471	0.833	0.736-0.942	0.004
25-OH-VitD	-0.427	0.065	42.551	0.653	0.574-0.742	0.000

Abbreviation: SBP, systolic blood pressure; BMI, body mass index; ALT, alanine aminotransferase; ALP, alkaline phosphatase; HDL, high density lipoprotein; LDL, low density lipoprotein; UA, uric acid; OST, osteocalcin; 25-OH-VitD, 25-hydroxy vitamin D.

that male gender is also a risk factor for NAFLD [30, 31]. Moreover, hypertension, hyperglycemia and other features of metabolic syndrome have often been independently predicting progression to cirrhosis [32, 33]. In accordance with these observations, we found that there were higher levels of SBP, DBP, WC, BMI, TG, CHO, LDL, FPG, HbA1c, but lower levels of HDL in the NAFLD group. Furthermore, the average age of NAFLD group was older than that of control and most of them were male.

Vitamin D is a fat-soluble vitamin, although multiple forms of this vitamin exist, vitamin D_2 and vitamin D_3 are the two major. In the liver, vitamin D from both the skin and diet is then metabolized by 25-hydroxylase to 25-OH-VitD, which is the major circulating metabolite and the most widely used indicator of vitamin D stores [34]. Therefore, this study used 25-OH-VitD to evaluate the status of vitamin D. Vitamin D is not only essential to the regulation of the calcium and bone metabolism, but also has other extra skeleton effects. It has been reported that vitamin D was association with metabolic diseases including type 2 diabetic, dyslipidaemia, obesity and NAFLD. Targher et al. [15] found that serum 25-OH-VitD levels in NAFLD patients were significantly lower than those in the control group. Similar results were obtained by Barchetta et al. [16], they investigated that 25-OH-VitD was significantly correlated with NAFLD, and lower serum 25-OH-VitD level was an independent risk factor for NAFLD. Additionally, the the findings of a recent study by Nakano T et al. [17] suggested that serum 25-OH-VitD levels may be effective biomarkers for non-invasive diagnosis of non-alcoholic steatohepatitis (NASH, the most severe form of NAFLD) progression, and that phototherapy (a method for vitamin D supplement) may be a good complementary therapy for NASH because of its regulation of vitamin D, which provided an indirect evidence to verify the previous results. Further study of mechanism demonstrated the reason that vitamin D deficiency increased NAFLD was upregulated gene expression of hepatic inflammatory and oxidative stress [35]. Our findings are similar to these studies, the serum 25-OH-VitD levels in NAFLD group were lower than those in control group, 25-OH-VitD was negatively correlated with NAFLD and it was a protective factor for NAFLD. However, there were some opposite results, for example, Katz K et al. [18] thought NAFLD was not associated with vitamin D status in adolescents after adjustment for obesity; Li L et al. [19] also found that there was no significant association between vitamin D and NAFLD in a Chinese population. This discrepancy between studies may be attributed to differences in the protocols used in each study, such as research objects, the number of samples, diagnostic criteria of NAFLD, duration of sunshine, exercise intensity, and statistical methods.

Osteocalcin is a 49-amino acid bone matrix noncollagen protein expressed mainly by osteoblasts. It is a marker of bone formation which involved in calcium homeostasis, however, it has been recognized as a bone-derived hormone to regulate energy metabolism recently [36]. Fernández-Real et al. [20] found that circulating osteocalcin concentrations were negatively associated with blood markers of liver injury and liver disease such as ALT and AST. Recently, Yilmaz Y et al. [21] investigated that patients with biopsy-proven NAFLD had significant reductions in serum osteocalcin concentrations compared with normal subjects, which were associated with the extent of hepatocyte ballooning significantly. Aller R et al. [22] also

found that osteocalcin was associated with liver fibrosis. To date, there are two cross-sectional studies to investigate the relationship between serum osteocalcin levels and NAFLD in Chinese, but the conclusions are contradictory. Liu J et al. [23] collected the data from 1683 men in South China, they found that the levels of serum osteocalcin were lower in NAFLD participants (vs. non-NAFLD participants), and suggested that a lower serum osteocalcin levels are associated with the presence of NAFLD. Another study by Dou J et al. [24], 1558 men in Shanghai district (Eastern coastal areas of China) were recruited. Although the researchers also found that the serum osteocalcin levels were significantly lower in subjects with NAFLD than those without, they suggested that serum osteocalcin levels are not directly correlated with NAFLD. Our results keep consistent with the former one. Compare to the non-NAFLD subjects, the serum osteocalcin levels in NAFLD subjects are lower. Further analysis reveal that NAFLD is negatively correlated with the serum osteocalcin levels, and we think that osteocalcin is also a protective factor for the development of NAFLD. These conflicting results in Chinese may be due to the different district, sample size and gender of the subjects.

There are several plausible explanations for the association between osteocalcin deficiency and the development and progression of NAFLD. Inhibition of NAFLD by osteocalcin was due to reduce the serum level of triglyceride and inhibit lipid deposition in the liver [37]; osteocalcin could attenuate ER stress and rescue impaired insulin sensitivity in insulin resistance via the NF-κB signaling pathway [38]; osteocalcin suppresses development of NAFLD was also attributed to up-regulate expression of adiponectin, down-regulate expression of TNF-α gene and attenuate inflammation in liver [39].

Two limitations in our study should be considered. First, it is well known that liver biopsy is the gold standard for the diagnosis of NAFLD worldwide, we use the liver ultrasonography to detect the presence of NAFLD rather than the liver biopsy. However, the liver biopsy is not applicable to large population-based researches because of its invasive nature and risk of complications. Therefore, as an alternative, various studies have been proposed based on liver ultrasonography for screening for fatty liver in clinical and population settings because of its low cost, safety, and accessibility [40]. Second, it is a small-size cross-sectional study, which limits us to make any causal inference and follow up. Therefore, further large prospective studies are warranted. We need to follow the patients after vitamin D or osteocalcin analogy replacement to investigate whether their hepatosteatosis did reverse, and elucidate the underlying mechanisms that underpin the role of vitamin D and osteocalcin in the development of NAFLD.

In conclusion, we demonstrated that NAFLD was strongly correlated with serum 25-OH-VitD and osteocalcin, which might imply that both 25-OH-VitD and osteocalcin could be potential novel markers to assess the progression of NAFLD. In addition, our findings also partially supported the theory of bone-liver metabolism crosstalk. Further studies are needed to confirm these findings and to understand their potential mechanism.

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Disclosure of conflict of interest

None.

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