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Serum levels of vaspin, obestatin, and apelin-36 in patients with nonalcoholic fatty liver disease

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Abstract

The novel adipokines vaspin, obestatin, and apelin-36 are associated with insulin resistance and the components of the metabolic syndrome. We assayed circulating levels of these molecules and examined their association with clinical, biochemical, and histologic phenotypes in patients with nonalcoholic fatty liver disease (NAFLD). Serum levels of vaspin, obestatin, and apelin-36 were assayed by enzyme-linked immunosorbent assay in 91 patients with biopsy-proven NAFLD and 81 controls. We analyzed associations between adipokines and the characteristics of patients with NAFLD using multivariable linear regression models. Univariable analysis showed that concentrations of vaspin and apelin-36 were significantly higher in patients with NAFLD than in controls, whereas no differences in obestatin levels were found. Serum vaspin levels showed a statistically significant association with C-reactive protein ($r = 0.378$, $P < .001$) and liver fibrosis scores ($r = 0.401$, $P < .001$), whereas apelin-36 levels showed a modest association with homeostasis model assessment of insulin resistance ($r = 0.204$, $P < .01$). After stepwise linear regression analysis, serum vaspin levels were the only independent predictor of liver fibrosis scores in patients with NAFLD ($\beta = 0.37$, $t = 3.99$, $P < .01$). Serum vaspin levels are raised in patients with NAFLD regardless of potential confounders and represent an independent predictor of liver fibrosis scores. These findings support further investigation of this novel adipokine in metabolic liver diseases.

1. Introduction

Nonalcoholic fatty liver disease (NAFLD), characterized by the accumulation of large droplets of triglycerides within hepatocytes in the absence of chronic alcohol consumption, is a leading cause of chronic liver disease; and its incidence is rising worldwide [1]. The term *NAFLD* is used to describe a wide spectrum of fatty liver changes ranging from simple steatosis to nonalcoholic steatohepatitis (NASH) [2]. Nonalcoholic fatty liver disease is currently considered as the hepatic manifestation of the metabolic syndrome (MetS) [3], and an association between the parameters of insulin resistance and NAFLD has been extensively reported [4-6]. These observations have led to the hypothesis that either insulin resistance plays a role in the pathogenesis and progression of liver damage, or the 2 phenomena have a common pathogenic mechanism [7].

Adipokines are fat-derived hormones and cytokines that play a central role in insulin homeostasis, as well as immunity, and are fundamental to the pathogenesis of the MetS [8]. Altered levels of classic adipokines such as leptin, adiponectin, and resistin have been repeatedly reported in patients with NAFLD [9-11]. The interplay of various adipokines may play a crucial role in the progression from fatty infiltration to inflammation and fibrosis, and the use of adipokines as noninvasive diagnostic tests in patients with NASH has generated great interest [10]. Several novel

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adipokines have been identified in recent years. Vaspin (visceral adipose tissue–derived serine protease inhibitor) is an adipokine that has been isolated from both visceral and subcutaneous white adipose tissue and displays potential insulin-sensitizing effects [12]. In a study of healthy individuals, however, serum vaspin levels did not correlate with markers of insulin sensitivity and glucose metabolism [13]. Interestingly, a recent study in patients with NAFLD has reported that serum vaspin is positively associated with hepatocyte ballooning degeneration and aminotransferase levels [14]. Obestatin (a ghrelin-associated peptide) is a 23–amino-acid peptide that is derived from posttranslational processing of the preproghrelin gene, which opposes ghrelin effects on food intake [15]. In humans, obestatin levels are decreased in obesity and seem to be negatively correlated with body mass index (BMI) and insulin resistance index [16]. Apelin is a peptide derived from a 77–amino-acid precursor, which is processed to several active molecular forms, such as apelin-36 or apelin-13 and apelin-12 in different tissues [17]. Apelin expression has been reported to be regulated by insulin, and it has been suggested to be involved in regulation of glucose homeostasis and obesity [18]. A recent clinical study has shown that plasma apelin-12 levels are higher in NAFLD patients than in healthy individuals, although these differences did not persist after adjustment for BMI and insulin resistance [19]. Of note, apelin levels were positively correlated with BMI and homeostasis model assessment (HOMA) index in subjects with NAFLD [19]. No data, however, are available in NAFLD on the levels of the apelin-36, the longest biologically active secreted form of apelin [17]. Previous studies have shown that apelin-12 and apelin-36 might play different physiologic roles in vivo [20]. Compared with smaller isoforms, apelin-36 binds to the apelin receptor APJ with higher affinity [21]. It has been previously demonstrated that apelin-36 decreases glucose-stimulated insulin secretion in mice, both in vivo and in vitro [22]. Importantly, high doses of apelin-36 have been recently shown to cause a moderate increase in glucose-stimulated insulin secretion, whereas lower concentrations of this long apelin isoform robustly reduced insulin secretion by 50% [23]. Taken together, these data suggest that apelin-36 may be involved in insulin regulation and insulin resistance.

In the present study, we assayed circulating levels of vaspin, obestatin, and apelin-36 and examined their association with clinical, biochemical, and histologic phenotypes in patients with NAFLD.

2. Subjects and methods

2.1. Ethics

The study protocol was approved by the Ethics Committee of the Marmara University School of Medicine, and all subjects gave their written informed consent to participate in the study.

2.2. Study population

In this observational case-control study, a total of 91 patients with NAFLD (43 men and 48 women; mean age, 47 ± 9 years) and 81 healthy comparison subjects (39 men and 42 women; mean age, 46 ± 11 years) were enrolled. Patients were consecutively seen at our hospital-based specialized outpatient clinics over the past 12 months. Patients with viral hepatitis, hemochromatosis, Wilson disease, autoimmune hepatitis, primary biliary cirrhosis, sclerosing cholangitis, biliary obstruction, α1 antitrypsin deficiency, ischemic cardiac or cerebrovascular disease, impaired renal function, or malignancies were carefully excluded from the present study. Subjects using estrogens, amiodarone, steroids, tamoxifen, and lipid-lowering agents were not eligible for this study. Patients with daily alcohol intake exceeding 20 g/d or previous abdominal surgery were also excluded. Eighty-one healthy age- and sex-matched volunteers were recruited as controls. All subjects included in the control group were judged to be in good health, with normal results on liver function tests and confirmed as having normal liver by ultrasound. Subjects with a consumption of alcohol exceeding 20 g/d or who were taking any medication were not included in the control group. All patients and controls were of Turkish descent.

2.3. Clinical and biochemical characterization

All subjects underwent physical examination, anthropometric measurements, and biochemical screening. Body mass index was calculated from measurements of height and weight. Diabetes mellitus was diagnosed according to American Diabetes Association criteria [24]. The MetS was diagnosed using the Adult Treatment Panel III criteria [25]. The estimate of insulin resistance was calculated using the HOMA-IR index, with the following formula: insulin resistance = fasting plasma insulin (in microunits per milliliter) × fasting plasma glucose (in millimoles per liter)/22.5 (normal if <2.5, presence of insulin resistance if ≥2.5). Twenty-four–hour urine samples were collected in a standardized manner, and all patients were advised of the collection method via a written instruction sheet. Measurement of 24-hour urinary albumin was performed in the central hospital laboratory using a BN Prospec (Dade Behring, Marburg, Germany) nephelometric system. The albumin excretion rate (expressed in milligrams per day) was then calculated. Blood pressure was measured using a mercury sphygmomanometer in a quiet room after at least 10 minutes of rest. Korotkoff 1 and 5 were taken for systolic blood pressure and diastolic blood pressure, respectively. Smoking status was assessed with a self-administered questionnaire. Routine blood chemistry analyses were performed at the central laboratory of clinical chemistry of the university hospital. Serum high-sensitivity C-reactive protein (hs-CRP) levels were measured in duplicate, in random order, and in a blinded fashion using a commercially available method (Dade Behring). The intra- and interassay

coefficients of variation for hs-CRP were 4.6% and 6.1%, respectively; and the lower detection limit was 0.19 mg/dL.

2.4. Liver histology

Ultrasonography-guided liver biopsies were performed under conscious sedation using a 16-gauge Hepafix needle (Braun Melsungen AG, Melsungen, Germany). All biopsy specimens were placed in formalin solution for fixation and embedded in paraffin blocks. Serial sections (sectioned at 4-mm intervals) were stained with hematoxylin-eosin and Masson trichrome. An experienced pathologist blinded to clinical data scored the liver biopsies according to the National Institute of Diabetes and Digestive and Kidney Diseases NASH Clinical Research Network scoring system [26]. Steatosis was scored from 0 to 3 with a 4-grade scoring system from S0 to S3: S0, no steatosis or less than 5%; S1, 5% to 33%; S2, 33% to 66%; and S3, greater than 66%. Lobular inflammation was graded as follows: stage 0, no foci; stage 1, less than 2 foci per 200× field; stage 2, 2 to 4 foci per 200× field; stage 3, more than 4 foci per 200× field. Fibrosis was staged as follows: stage 0, no fibrosis; stage 1, perisinusoidal or periportal fibrosis with 3 different patterns (1A, mild, zone 3, perisinusoidal; 1B, moderate, zone 3, perisinusoidal fibrosis; and 1C, portal/periportal fibrosis); stage 2, perisinusoidal and portal/periportal fibrosis; stage 3, bridging fibrosis; and stage 4, cirrhosis. The histologic NASH score was defined as the unweighted sum of the scores for steatosis (0-3), lobular inflammation (0-3), and ballooning (0-2), thus ranging from 0 to 8. Cases with scores of 0 to 2 were considered as having simple steatosis; on the other hand, cases with scores of 5 or greater were diagnosed as definitive NASH. Cases with activity scores of 3 and 4 were considered as borderline NASH [26].

2.5. Adipokine assays

All blood samples were collected from an antecubital vein between 8:00 and 9.00 AM after an overnight fasting. Samples were centrifuged at 2500*g* for 10 minutes, and serum aliquots were stored at −80°C until immediately before analysis. Serum vaspin levels were determined using a commercially available enzyme immunoassay kit (Alpco Diagnostics, Salem, NH) according to the manufacturer's protocol. The minimum detection limit was 0.012 ng/mL. Serum obestatin was assayed using a quantitative enzyme immunoassay kit (Yanaihara Institute, Fujinomiya-SHI Shizuoka, Japan). The minimum detection limit was 0.412 ng/mL. Apelin was quantified with the apelin-36 enzyme-linked immunosorbent assay (Phoenix Pharmaceuticals, Burlingame, CA) according to the manufacturer's instructions. The minimal detectable concentration was 0.17 ng/mL. All measurements were performed in duplicate, and the results were averaged. All samples were processed blindly to the clinical status of the participants. For all assays, the intra- and interassay coefficients of variation were less than 9% and less than 12%, respectively.

Table 1
General characteristics of the study participants

	NAFLD patients (n = 91)	Control group (n = 81)	*P* value
Sex (M/F)	43/48	39/42	NS
Age (y)	47 ± 9	46 ± 11	NS
BMI (kg/m^2)	31.3 ± 5.1	26.5 ± 3.4	<.001
Diabetes mellitus (yes/no)	22/69	0/81	<.001
MetS	33/58	0/81	<.001
HOMA-IR	3.5 (2.5-5.2)	1.6 (0.5-2.7)	<.001
Microalbuminuria (g/d)	9.5 (6.0-20.7)	–	–
Systolic blood pressure (mm Hg)	131 ± 19	125 ± 17	NS
Diastolic blood pressure (mm Hg)	84 ± 11	81 ± 11	NS
Smoking history, (never/former/current)	58/19/14	55/11/15	NS
AST (IU/L)	44 ± 19	24 ± 9	<.001
ALT (IU/L)	67 ± 33	21 ± 13	<.001
Total cholesterol (mg/dL)	222 ± 51	183 ± 6	<.01
HDL cholesterol (mg/dL)	47 ± 12	46 ± 10	NS
LDL cholesterol (mg/dL)	139 ± 46	122 ± 36	<.001
Triglycerides (mg/dL)	180 ± 87	137 ± 71	<.001
hs-CRP (mg/dL)	3.2 (2.9-5.6)	–	–
Histologic steatosis	2 (1-3)	–	–
Lobular inflammation	2 (1-3)	–	–
Ballooning	2 (1-2)	–	–
NASH score	5 (4-7)	–	–
Fibrosis	1 (0-2)	–	–

Data are presented as means and SD, counts, or medians and interquartile ranges, as appropriate. AST indicates aspartate aminotransferase; ALT, alanine aminotransferase; HDL, high-density lipoprotein; LDL, low-density lipoprotein; NS, not significant.

2.6. Data analysis

The Kolmogorov-Smirnov test was performed in all continuous variables to define the presence of normality. Gaussian variables are expressed as mean ± SD, skewed data are reported as medians and interquartile ranges, and categorical variables are expressed as counts. The Student *t* test was used to evaluate differences between the 2 study groups in normally distributed continuous variables. When normality was not confirmed, the Mann-Whitney *U* test was used. Skewed data were log-transformed for further analysis. Correlations among the study variables were tested by the Spearman correlation coefficient. Predictors of fibrosis scores in NAFLD patients were assessed using multiple stepwise linear regression analysis. All variables listed in Table 1 were entered into the model as potential predictors (independent variables). All calculations were performed using SPSS version 11.0 for Windows (SPSS, Chicago, IL). A value of $P < .01$ (2-sided) was considered as statistically significant to reduce the likelihood of type I error [27].

3. Results

The general characteristics of the study population are depicted in Table 1. The 2 study groups did not differ as

Table 2
Plasma adipokines levels in the study participants

	NAFLD patients (n = 91)	Control group (n = 81)	*P* value
Vaspin, ng/mL	0.6 (0.3-1.1)	0.4 (0.2-0.7)	<.01
Obestatin, ng/mL	3.6 (3.0-4.1)	3.6 (3.2-4.2)	NS
Apelin-36, ng/mL	1.4 (0.9-2.1)	1.1 (0.8-1.5)	<.01

Data are presented as medians and interquartile ranges.

to age, sex, systolic and diastolic blood pressure, smoking history, and high-density lipoprotein cholesterol. Between-group comparison analysis identified a number of variables to be different in patients with histology-proven NAFLD compared with controls without NAFLD, including BMI, HOMA-IR, aspartate aminotransferase, alanine aminotransferase, total cholesterol, low-density lipoprotein cholesterol, and triglycerides. The prevalence of diabetes and the MetS was higher in patients with NAFLD than in controls.

Levels of adipokines in the study participants are shown in Table 2. Obestatin levels did not differ between patients and controls, whereas both vaspin and apelin-36 levels were significantly higher in patients with biopsy-proven NAFLD than in controls (Mann-Whitney *U* test, $P < .01$).

We next assessed the association between serum adipokines and the clinical, biochemical, and histologic phenotypes of patients with NAFLD. Serum vaspin levels showed a statistically significant association with CRP ($r = 0.378$, $P < .001$) and liver fibrosis ($r = 0.401$, $P < .001$), whereas apelin-36 levels showed a modest association with HOMA-IR ($r = 0.204$, $P < .01$). Obestatin did not show any significant association with the study variables at the $P < .01$ level. There were no associations between the selected adipokines and NASH scores (data not shown), and these molecules did not discriminate between simple steatosis and definite NASH. After stepwise linear regression analysis, serum vaspin levels were the only independent predictor of liver fibrosis scores in patients with NAFLD ($\beta = 0.37$, $t = 3.99$, $P < .01$).

4. Discussion

The results of the present study indicate (*a*) that, in patients with biopsy-proven NAFLD, serum levels of vaspin and apelin-36, but not those of obestatin, are significantly higher than in normal controls and (*b*) that vaspin is a predictor of liver fibrosis, independent of potential confounders, including metabolic parameters.

Our data on raised apelin-36 levels in patients with NAFLD confirm in a larger sample of patients the pilot results obtained on apelin-12 by Ercin et al [19]. Of interest, we were also able to confirm the previously reported association between apelin and insulin resistance as assessed by the HOMA index [19]. After allowance for potential confounders, however, this result did not persist (data not shown). Taken together, these findings suggest that apelin does not seem to play a major role in the pathophysiology of NAFLD. Interestingly, the role of apelin in the regulation of insulin resistance and glucose homeostasis has been recently debated. For example, it has been shown that external administration of apelin to insulin-resistant mice is capable of stimulating glucose utilization [28]. Another study has reported that apelin knockout mice were significantly more insulin resistant than their wild-type littermates and that insulin resistance could be reversed by administration of apelin [29]. Although these studies indicate a favorable effect of apelin on insulin sensitivity in animal models, Li et al [30] showed that circulating apelin levels are increased in type 2 diabetes mellitus individuals as compared with healthy controls. Our findings, coupled with previous observations, do not point toward a major independent association of apelin with metabolic liver disease. Similarly, obestatin levels were not altered in NAFLD and did not correlate with any of the clinical, histologic, and biochemical features of our patients. These results are in contrast with previous research showing a negative correlation between obestatin and BMI as well as insulin resistance [16]. The discrepant findings may be partially explained by differences in the cohorts studied (patients with eating disorders in the study by Monteleone et al [16] vs patients with NAFLD in the present study). Further studies are needed to shed more light on the hypothesis that the dysregulation of obestatin may have a role in the pathophysiology of the MetS and related disorders.

One major finding in this study is the observation that vaspin levels are significantly increased in patients with NAFLD and represent an independent biochemical correlate of liver fibrosis scores. Recently, Kukla et al [14] have shown that circulating vaspin levels were higher in a sample of 41 patients with NAFLD and were positively associated with hepatocyte ballooning degeneration. Of note, the same authors demonstrated a trend toward higher vaspin levels according to liver fibrosis scores in patients with chronic hepatitis C [31]. The exact mechanisms underlying the positive association between vaspin levels and liver fibrosis cannot be directly inferred from the present data. However, vaspin has been identified as a novel serpin with insulin-sensitizing effects [12]. Of note, alterations of serpins that affect its structure and/or secretion and thus alter its functional levels have been shown to result in several disease conditions, including liver cirrhosis [32]. Future studies should address the relations between vaspin and transforming growth factor–β1, the master cytokine in the pathogenesis of liver fibrosis [33]. In addition, more research is needed to establish the potential role of this molecule in mediating liver and systemic inflammation. It is noteworthy that vaspin was significantly associated with CRP in our study. This finding is in keeping with pilot data showing that this adipokine may regulate immune responses and inflammation [34].

Several caveats are inherent in this study. First, although the results of this study are encouraging, the small sample size, the potential selection bias, and the single-center enrolment limit the study generalizability. Second, this was a case-control study; and thus, we are not able to elucidate the causal relationships between serum vaspin, NAFLD, and fibrosis. Eventually, a longitudinal study is needed to clarify the causal relationship between vaspin levels and liver injury. Finally, our sample included subjects of Turkish nationality; so results cannot be extrapolated to populations with different ethnic background. However, a strength of our study is that all patients with NAFLD underwent liver biopsy.

In conclusion, the current findings suggest that serum vaspin levels are raised in patients with NAFLD regardless of potential confounders and represent an independent predictor of liver fibrosis scores. These results support further investigation of this novel adipokine in metabolic liver diseases. Our report cannot assess whether the increase of vaspin observed in NAFLD may have a significance as a noninvasive method for diagnostic purposes or can show a prognostic potential in this setting. The validation of this marker in larger cohorts would be a crucial step toward its clinical use. This issue should therefore be addressed in future studies. Because no effective specific therapy is currently available for NASH, the pharmacologic manipulation of adipokines in general and vaspin in particular may be a feasible option in the future [10]. Altogether, this work adds to the growing body of literature suggesting that novel adipokines may modulate the clinical expression of NAFLD, and may open in the future a new area of investigation exploring the role of vaspin as a therapeutic target in liver diseases.

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