ORIGINAL ARTICLE

Influence of Visfatin on Histopathological Changes of Non-alcoholic Fatty Liver Disease

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Abstract *Background* Non-alcoholic fatty liver disease
(NAFLD) is a common liver disease. The aim of the present
study was to explore the relation of visfatin with underlying
histopathological changes of NAFLD patients. *Subjects*
A population of 55 NAFLD patients was analyzed in a cross-
sectional study. A liver biopsy was realized. Weight, basal
glucose, insulin, insulin resistance (HOMA), total choles-
terol, LDL cholesterol, HDL cholesterol, triglycerides, and
visfatin levels were measured. A bioimpedance was per-
formed. *Results and conclusions* The mean age was $42.8 \pm$
11.2 years, the mean BMI was 33.1 ± 10.2 with 37 males
(67.3%) and 18 females (32.7%). Probabilities to have;
portal inflammation increased 1.11 (CI95%:1.03–1.50) with
each increment of 1 ng/ml of visfatin concentration,
high grade of steatosis increased 1.25 (CI 95%:1.06–1.61)
with each unit of insulin concentrations, fibrosis increased
1.12 (CI 95%:1.02–1.43) with each unit of fat mass and
lobulillar inflammation increased 13.4 (CI 95%:1.3–147)
with each unit of HOMA-IR. Portal inflammation frequen-
cies were different between groups (low visfatin group
$13.07 <$ ng/ml: 37.5% versus high visfatin group
$13.07 >$ ng/ml: 62.5%; $P < 0.05$). In conclusion, several
histopathological changes in liver biopsies could be
explained by insulin concentrations, HOMA-IR, and fat

mass amount. Moreover, visfatin plasma concentrations
could predict the presence of portal inflammation in NAFLD
patients.

Keywords Adipose tissue · Non-alcoholic fatty liver
disease · Obesity · Visfatin

Introduction

Epidemiologic evidence of the rising tide of obesity and
associated pathologies has led, in the last years, to a dra-
matic increase of research on the role of adipose tissue as
an active participant in controlling the body's physiologic
and pathologic processes [1].

The current view of adipose tissue is that it functions as an
active secretory organ, sending out and responding to signals
that modulate appetite, insulin sensitivity, energy expendi-
ture, inflammation, and immunity. Visfatin was recently
identified as a protein preferentially expressed in visceral
adipose tissue, compared to subcutaneous adipose tissue [2].
It can be found in skeletal muscle, liver, bone marrow and
lymphocytes, where it was initially identified as pre-B-cell
colony-enhancing factor (PBEF). Interestingly, PBEF
expression is regulated by cytokines that promote insulin
resistance, such as tumoral necrosis factor alpha (TNF
alpha), interleukin-6 (IL-6) and lipopolysaccharide [3].
Fukuhara et al. [2] clearly suggested an endocrine role for
visfatin. It cannot be excluded that visfatin might also have a
paracrine effect on the visceral adipose tissue through its
pro-adipogenic and lipogenic actions. In fact, the overex-
pression of visfatin in a preadipocyte cell line facilities its
differentiation to mature adipocytes and promotes the
accumulation of fat through the activation of glucose
transport. Contrary to the most intuitive hypothesis, visfatin

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treatment did not promote insulin resistance, but actually exhibited insulin mimetic properties, resulting in a glucose-lowering effect. The discovery of this curious new adipokine has great potential to significantly enhance our understanding of the metabolic syndrome and its related pathologies such as non-alcoholic fatty liver disease.

Non-alcoholic fatty liver disease (NAFLD) is a common liver disease characterized by elevated serum aminotransferase levels, hepatomegaly, and accumulation of fat in liver accompanied by inflammation and necrosis resembling alcoholic hepatitis in the absence of heavy alcohol consumption [4]. It is important to discern which factors in the host metabolic milieu modulate the development of NAFLD, and, in particular, the transition from a low grade of steatosis to steatohepatitis. Perhaps, the role of visfatin on insulin actions could influence on histopathological changes of NAFLD.

Accordingly, the aim of the present study was to explore the relation of visfatin with underlying histopathological characteristics of NAFLD patients.

Subjects and Methods

Subjects

A population of 55 NAFLD patients was analyzed in a cross-sectional study. The exclusion criteria were hepatitis B, C, cytomegalovirus, Epstein-Barr infections, monogram-specific auto antibodies, alcohol consumption, diabetes mellitus, intolerance fasting glucose, medication (and diabetic drugs, blood-pressure-lowering medication, and statins) and hereditary defects (iron and copper storage diseases and alpha 1-antitrypsin deficiency). Diabetes mellitus and intolerance fasting glucose have been excluded with basal glucose after 8 h of fasting state [5]. The study was approved by an institutional ethics committee.

Procedure

All patients with a 2-week weight-stabilization period before recruitment were enrolled. A liver biopsy was realized. Weight, basal glucose, insulin, insulin resistance (HOMA), total cholesterol, LDL cholesterol, HDL cholesterol, triglycerides and visfatin blood levels were measured. A bioimpedance was performed.

Assays

Serum total cholesterol and triglyceride concentrations were determined by enzymatic colorimetric assay (Technicon Instruments, Ltd., New York), while HDL cholesterol was determined enzymatically in the supernatant after

precipitation of other lipoproteins with dextran sulphate-magnesium. LDL cholesterol was calculated using the Friedewald formula. Plasma glucose levels were determined by using an automated glucose oxidase method (Glucose Analyser 2, Beckman Instruments, Fullerton, California). Insulin was measured by enzymatic colorimetric assay (Insulin, WAKO Pure-Chemical Industries, Osaka, Japan) and the homeostasis model assessment for insulin sensitivity (HOMA) was calculated using these values [6]. Visfatin was analyzed using a commercially available ELISA kit (Phoenix Peptides, Belmont, CA). Assay sensitivity was 2 ng/ml and interassay and intraassay coefficients of variation were less than 10 and 5%, respectively.

Anthropometric Measurements

Body weight was measured to an accuracy of 0.1 kg and body mass index was computed as body weight/ (height^2). Waist (narrowest diameter between xiphoid process and iliac crest) and hip (widest diameter over greater trochanters) circumferences to derive waist-to-hip ratio (WHR) were measured, too. Tetrapolar body electrical bioimpedance was used to determine body composition [7]. An electric current of 0.8 mA and 50 kHz was produced by a calibrated signal generator (Biodynamic Model 310e, Seattle, WA, USA) and applied to the skin using adhesive electrodes placed on right-side limbs. Resistance and reactance were used to calculate total body water, fat, and fat-free mass.

Liver Biopsy

Liver tissue was stained with hematoxylin-eosin, reticulin, and Gomori trichrome stains and scored by an experienced hepathologist. All cases showed macrovesicular steatosis affecting at least 5% of hepatocytes and were classified as steatosis. In addition to steatosis, the minimum criteria for the diagnosis of steatohepatitis included the presence of lobular inflammation and either ballooning cells or perisinusoidal/pericellular fibrosis in zone 3 of the hepatic acinus. All cases were scored using the method of Brunt [8]. Steatosis was graded as follows: grade 1 (>5% and <33% of hepatocytes affected); grade 2 (33–66%); or grade 3 (>66% of hepatocytes affected). Grades 2 and 3 were combined for statistical analysis (high grade) and grade 1 (low grade). Fibrosis was assessed with the Masson trichome stain [9]. Other histological features evaluated in hematoxylin-eosin sections included lobulillar inflammation and portal inflammation.

Statistical Analysis

The results were expressed as average ± standard deviation. The distribution of variables was analyzed with

Kolmogorov-Smirnov test. Quantitative variables with normal distribution were analyzed with a two-tailed, unpaired Student's *t*-test. Non-parametric variables were analyzed with the Mann-Whitney U-test. Qualitative variables were analyzed with the Chi-square test, with Yates correction as necessary, and Fisher's test. Additionally, logistic regressions with stepwise variable selection were used to test for significant relations in histopathological lesions (steatosis, fibrosis, lobulillar inflammation, and perinusoidal) with adjustment for possible confounders. A *P*-value under 0.05 was considered statistically significant. SPSS 15.0 software (IL, USA) was used.

Results

Fifty-five patients gave informed consent and were enrolled in the study (approved by the ethical committee of our hospital). The mean age was 42.8 ± 11.2 years, the mean BMI was 33.1 ± 10.2 with 37 males (67.3%) and 18 females (32.7%).

Table 1 shows basal characteristics of patients. Table 2 shows differences between a high grade of steatosis and a low grade of steatosis. Patients with a high grade of steatosis have higher levels of insulin and HOMA than low grade steatosis.

Table 3 shows differences between patients with lobulillar inflammation versus no lobulillar inflammation. Patients with this type of inflammation have higher levels of glucose, insulin, HOMA and fat mass than patients without inflammation.

Table 4 shows differences between patients with portal inflammation versus no portal inflammation. Patients with this type of inflammation have higher levels of visfatin,

Table 1 Clinical and epidemiological characteristics of study population

Characteristics (Mean ± SD)	$n = 18$ female	$n = 37$ male	P
Age (years)	44.5 ± 14.0	40.2 ± 8.6	0.70
BMI (kg/m^2)	38.4 ± 12.1	$30.4 \pm 7.8*$	0.01
Glucose (mg/dl)	98.0 ± 10.1	97.3 ± 9.2	0.45
Total cholesterol (mg/dl)	185.7 ± 53.9	204.1 ± 55.2	0.21
LDL cholesterol (mg/dl)	129.4 ± 46.2	117.8 ± 31.2	0.18
HDL cholesterol (mg/dl)	53.6 ± 12.1	55.8 ± 17.2	0.12
Triglycerides (mg/dl)	119.7 ± 48.1	123.1 ± 49.2	0.23
Insulin (mUI/L)	12.8 ± 7.8	14.7 ± 11.2	0.67
HOMA-IR	3.1 ± 1.8	3.6 ± 2.2	0.41
Visfatin (ng/ml)	14.3 ± 8.9	14.9 ± 6.2	0.31

BMI Body mass index. *LDL* Low-density lipoprotein. *HDL* High-density lipoprotein. *HOMA-IR* (homeostasis model assessment). Insulin and HOMA-IR have non-parametric distribution (Mann-Whitney U-test used). Student's *t*-test * $P < 0.05$

Table 2 Clinical and epidemiological characteristics (low grade versus high grade of steatosis)

Characteristics (Mean ± SD)	Low grade $n = 31$	High grade $n = 24$	P
Age (years)	42.7 ± 11.1	43.1 ± 10.7	0.70
Sex (male/female)	21/10	16/8	0.81
BMI (kg/m^2)	32.6 ± 9.4	33.7 ± 11	0.91
Fat mass (kg)	22.5 ± 8.7	25.4 ± 8.2	0.12
Total cholesterol (mg/dl)	205 ± 60	196 ± 48	0.14
LDL cholesterol (mg/dl)	133.9 ± 56	123.8 ± 29	0.18
HDL cholesterol (mg/dl)	54.8 ± 13.1	55.6 ± 12.2	0.93
Glucose (mg/dl)	99.8 ± 8.1	97.2 ± 7.3	0.41
HOMA-IR	3.1 ± 1.9	$4.3 \pm 2.2*$	0.035
Insulin (mUI/l)	11.6 ± 5.7	$15.8 \pm 7.7*$	0.025
Visfatin (ng/ml)	14.1 ± 6.6	15.7 ± 7.3	0.78

BMI Body mass index. *LDL* Low-density lipoprotein. *HDL* High-density lipoprotein. *HOMA-IR* (homeostasis model assessment (glucose (mmol/l*insulin uU/ml)/22.5). Student's *t*-test used. Insulin and HOMA-IR have non-parametric distribution (Mann-Whitney U-test used). * $P < 0.05$

insulin, HOMA and fat mass than patients without inflammation.

Table 5 shows differences between patients with fibrosis versus no fibrosis. Patients with fibrosis have higher levels of insulin, HOMA BMI, and fat mass than patients without fibrosis.

Patients were divided into two groups by median visfatin value (13.07 ng/ml), group I (patients with the low values) and group II (patients with the high values). Table 6 shows the statistical differences between both groups in liver biopsy characteristics. Only portal inflammation frequencies were different between groups (low visfatin group: 37.5% versus high visfatin group: 62.5%; $P < 0.05$).

In the logistic regression with age-, sex-, fat mass- and insulin-adjusted portal inflammation, a high grade of steatosis, fibrosis and lobulillar inflammation as dependent variables, visfatin concentrations is related with portal inflammation increase 1.22 (CI95%:1.02–1.48) with each 1 ng/ml of visfatin concentration, insulin concentrations are related with a high grade of steatosis with an increase of 1.27 (CI95%:1.05–1.54) with each 1 UI/ml of insulin concentration and fat mass is related with fibrosis with an increase of 1.15 (CI95%:1.03–1.37) with each kilogram of fat mass and lobulillar inflammation with HOMA-IR 13.7 (CI95%:1. 2–156) with each unit of HOMA.

Discussion

The present study demonstrates that insulin, HOMA, visfatin, and fat mass are associated with different histopathological changes in patients with NAFLD in logistic

Table 3 Clinical and epidemiological characteristics lobulillar inflammation (no inflammation versus lobulillar inflammation)

Characteristics (Mean ± SD)	No inflammation n = 17	Lobulillar inflammation n = 38	P
Age (years)	42.1 ± 10.1	43.3 ± 11.6	0.48
Sex (male/female)	11/6	26/12	0.35
BMI (kg/m^2)	30.2 ± 7.2	34.4 ± 11.1	0.18
Fat mass (kg)	19.7 ± 8.3	26.3 ± 7.7*	0.02
Total cholesterol (mg/dl)	212.1 ± 70.2	197.2 ± 47.1	0.12
LDL cholesterol (mg/dl)	136.6 ± 59.1	126.1 ± 39.8	0.21
HDL cholesterol (mg/dl)	59.3 ± 14.2	54.5 ± 27.4	0.09
Glucose (mg/dl)	92.8 ± 9.1	97.4 ± 10.3*	0.02
HOMA-IR	2.3 ± 1.1	4.2 ± 2.3*	0.01
Insulin (mUI/l)	10.1 ± 4.1	15.1 ± 7.5*	0.01
Visfatin (ng/ml)	13.3 ± 6.1	15.3 ± 7.3	0.34

BMI Body mass index. *LDL* Low-density lipoprotein. *HDL* High-density lipoprotein. *HOMA-IR* (homeostasis model assessment (glucose (mmol/l*insulin uU/ml)/22.5). Student's *t*-test used. Insulin and HOMA-IR have non-parametric distribution (Mann- Whitney U-test used). * $P < 0.05$

Table 4 Clinical and epidemiological characteristics portal inflammation (no inflammation versus portal inflammation)

Characteristics (Mean ± SD)	No inflammation n = 45	Perinusoidal inflammation n = 10	P
Age (years)	42.3 ± 11	45.6 ± 11.6	0.27
Sex (male/female)	31/14	6/4	0.68
BMI (kg/m^2)	32.5 ± 9.5	35.8 ± 13.2*	0.04
Fat mass (kg)	23.4 ± 8.2	26.9 ± 9.4*	0.02
Total cholesterol (mg/dl)	202.1 ± 58.8	200.2 ± 45.3	0.98
LDL cholesterol (mg/dl)	129.2 ± 46.1	130.5 ± 48.2	0.78
HDL cholesterol (mg/dl)	57.2 ± 26.3	46.5 ± 13.1	0.87
Glucose (mg/dl)	99.8 ± 9.1	95.7 ± 16.2	0.44
HOMA-IR	2.8 ± 1.4	4.1 ± 2.4*	0.03
Insulin (mUI/l)	11.4 ± 5.1	14.8 ± 7*	0.04
Visfatin (ng/ml)	12.6 ± 6.2	16.3 ± 7.1*	0.04

BMI Body mass index. *LDL* Low-density lipoprotein. *HDL* High-density lipoprotein. *HOMA-IR* (homeostasis model assessment (glucose (mmol/l*insulin uU/ml)/22.5). Student's *t*-test used. Insulin and HOMA-IR have non-parametric distribution (Mann- Whitney U-test used). * $P < 0.05$

Table 5 Clinical and epidemiological characteristics liver fibrosis (no fibrosis versus fibrosis)

Characteristics (Mean ± SD)	No fibrosis n = 44	Fibrosis n = 11	P
Age (years)	41.7 ± 10.2	47.7 ± 12.6	0.56
Sex (male/female)	31/13	7/4	0.64
BMI (kg/m^2)	30.8 ± 7.3	41.9 ± 15.1*	0.01
Fat mass (kg)	22.6 ± 7.9	30.6 ± 8.2*	0.02
Total cholesterol (mg/dl)	204.1 ± 52.3	198.2 ± 68	0.23
LDL cholesterol (mg/dl)	128 ± 38	135 ± 77	0.19
HDL cholesterol (mg/dl)	54.4 ± 15.2	62.5 ± 47.3	0.23
Glucose (mg/dl)	91.8 ± 11.8	97.4 ± 10.3	0.43
HOMA-IR	3.2 ± 1.8	5.1 ± 2.9*	0.01
Insulin (mUI/l)	12.4 ± 5.7	18.1 ± 10.2*	0.02
Visfatin (ng/ml)	14.4 ± 6.3	16.1 ± 9.4	0.28

BMI Body mass index. *LDL* Low-density lipoprotein. *HDL* High-density lipoprotein. *HOMA-IR* (homeostasis model assessment (glucose (mmol/l*insulin uU/ml)/22.5). Student's *t*-test used. Insulin and HOMA-IR have non-parametric distribution (Mann- Whitney U-test used). * $P < 0.05$

Table 6 Liver histopathological changes by median visfatin

Frequencies	Low visfatin n = 27	High visfatin n = 28	P
High grade of esteatosis	45.8%	54.2%	0.45
Lobulillar inflammation	47.4%	52.6%	0.78
Portal inflammation	37.5%	62.5%*	0.03
Fibrosis	45.5%	54.5%	0.71

High frequency of portal inflammation in high visfatin group, other pathological changes without differences. Chi-square test used * $P < 0.05$

models. The novel finding of our study is the independent association of visfatin with a specific histopathological lesion of the NAFLD.

The relation of liver histopathological changes and biochemical parameters are a contradictory topic area. For example, the nature of the connection between insulin action and hepatic steatosis remains unclear [10]. In obese patients, the primary abnormality may be genetically

induced insulin resistance, with a secondary increase of serum triglyceride levels due to enhance of peripheral lipolysis. The resulting hepatic supply of fatty acids and insulin may increase triglyceride deposition in the liver [11]. In our study, steatosis and lobulillar inflammation were related with insulin and HOMA, this data confirmed the previous hypothesis.

The novel finding of our study is the relation of visfatin with portal inflammation. Visfatin was found to exert insulin-mimicking effects, such as lowering plasma glucose levels after adenoviral-mediated expression in vivo in mice [2]. Additionally, studies investigating the molecular mechanisms revealed that visfatin activates the intracellular signaling cascade for insulin, including tyrosine phosphorylation of the insulin receptor and insulin receptor substrate-1/2 (IRS1/2) as well as downstream activation of protein kinase B/Akt. Interestingly, however, visfatin activates the insulin receptor in a manner distinct from that of insulin.

The relation among visfatin levels with clinical data a contradictory area. For example, mRNA expression and circulating levels of visfatin were increased in the diabetic versus no diabetic patients [4]. One reason for this data is the high body weight in diabetic patients. Nevertheless, in other study, using waist circumference as a marker for visceral fat, showed no significant correlation with circulating visfatin. However, visfatin was reported to be more highly expressed in visceral than subcutaneous fat [4], and visceral fat may be increased in insulin-resistant patients [12], this contradictory data has no clear explanation.

It is necessary to determine contribution of visfatin originating from visceral adipose tissue to the control of global insulin sensitivity. Although the affinity of visfatin for the insulin receptors appears to be similar to insulin, its concentration in plasma is much lower of the insulin concentration under fasting conditions [13]. It remains to be established whether visfatin production is a compensatory response to tissue-specific insulin resistance or, more simply, a marker of tissue-specific inflammatory-cytokine action. Visfatin expression is regulated by cytokines that promote insulin resistance, such as tumoral necrosis factor alpha (TNF alpha) [3]. Our study shows a relation of visfatin with portal inflammation. A third hypothesis could explain this association. Firstly, visfatin could play a role such as an insulin mimetic molecule producing inflammation in the liver. This clinical relation of visfatin and insulin action is complex and unclear. Recently, Dogru et al. [14] have demonstrated that visfatin levels did not correlate with adiponectin, BMI, or HOMA in three groups of subjects (diabetes mellitus type 2, impaired glucose tolerance and controls). However, visfatin levels were higher in the diabetic group than the control. In contrast, Li et al. [15] demonstrated that visfatin levels were significantly decreased in diabetics compared to the controls and were correlated positively and significantly with BMI, waist-to-hip ratio and resistin.

Secondly, visfatin could play a direct inflammatory role. The inflammatory relations of visfatin have clear molecular explanations. For example, visfatin induces the production of IL-6 in human CD4+ monocytes [16], whereas IL6 negatively regulates visfatin gene expression in 3T3-LI adipocytes [17].

Thirdly, visfatin may be an epiphenomenon of an inflammatory state of these patients, without a direct effect on liver inflammation [18]. Our study design cannot explain causality. Further interventional studies to decrease visfatin levels [19, 20] are needed to explore histopathological improvements in liver biopsy. Moreover, visfatin levels could predict the presence of the portal inflammation, this molecule could involve a non-invasive technical to determine this pathological change.

In conclusion, several histopathological changes in liver biopsies could be explained by insulin concentrations, HOMA-IR, and and amount of fat mass. Moreover, visfatin plasma concentrations could predict the presence of portal inflammation in NAFLD patients.

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