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ORIGINAL ARTICLE

Case Control Study

Increased serum soluble lectin-like oxidized low-density lipoprotein receptor-1 levels in patients with biopsy-proven nonalcoholic fatty liver disease

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Abstract

AIM: To analyze the relationship between the serum lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) levels and clinical and histopathological features of biopsy-confirmed nonalcoholic fatty liver disease (NAFLD) patients.

METHODS: Fifty-three consecutive, biopsy-proven NAFLD patients (31 males and 22 females, mean age 42.5 ± 9.6 years) and 26 age- and gender-matched, healthy controls (14 males and 12 females, mean age 39 ± 10.7 years) were included. The patients

with NAFLD were consecutive patients who had been admitted to the hepatology outpatient clinic within the last year and had been diagnosed with NAFLD as the result of liver biopsy. The healthy controls were individuals who attended the outpatient clinic for routine health control and had no known chronic illnesses. The histological evaluation was conducted according to the NAFLD activity scoring system recommended by The National Institute of Diabetes and Digestive and Kidney Diseases Nonalcoholic Steatohepatitis Clinical Research Network. The serum LOX-1 levels were measured using an ELISA kit (Life Science Inc. USCN. Wuhan, Catalog No. E1859Hu) in both patients and healthy controls. A receiver operating characteristic (ROC) curve analysis was used to identify the optimal cutoff value of LOX-1 and thereby distinguish between patients with nonalcoholic steatohepatitis (NASH) and healthy controls. A *P*-value < 0.05 was considered statistically significant.

RESULTS: NAFLD and healthy control groups were similar in terms of age and sex. NAFLD patients consisted of 8 patients with simple steatosis (15%), 27 with borderline NASH (51%) and 18 with definitive NASH (34%). Metabolic syndrome was found in 62.2% of the patients with NAFLD. The mean serum LOX-1 level in biopsy-proven NAFLD patients was 8.49 ± 6.43 ng/mL compared to 4.08 ± 4.32 ng/mL in healthy controls ($P = 0.001$). The LOX-1 levels were significantly different between controls, simple steatosis and NASH (borderline+definite) cases (4.08 ± 4.32 ng/mL, 6.1 ± 6.16 ng/mL, 8.92 ± 6.45 ng/mL, respectively, $P = 0.004$). When the cut-off value for the serum LOX-1 level was set at 5.35 ng/mL, and a ROC curve analysis was performed to distinguish between steatohepatitis patients and controls; the sensitivity and specificity of the serum LOX-1 level were 69.8% and 69.2%, respectively.

CONCLUSION: The serum LOX-1 levels were significantly higher in NAFLD patients than in healthy controls. Additionally, the serum LOX-1 levels could differentiate between steatohepatitis patients and healthy controls.

Key words: Insulin resistance; Liver fibrosis; Metabolic syndrome; Nonalcoholic fatty liver disease; Steatohepatitis

Core tip: Lipoprotein receptor-1 (LOX-1) is a biomarker that has been demonstrated to be related to atherosclerosis, insulin resistance, obesity and diabetes. Nonalcoholic fatty liver disease (NAFLD) is the hepatic manifestation of metabolic syndrome. To date, no studies have investigated the association between serum LOX-1 and liver inflammation in biopsy-proven NAFLD patients. In this study, we have shown that the serum LOX-1 levels are correlated with the NAFLD histology scores, which might decrease the need for performing liver biopsy in NAFLD patients.

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INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is the most common chronic liver disease worldwide, and its prevalence is continuously increasing[1,2]. The disease may present in different clinical forms. Though simple steatosis usually has a benign course, nonalcoholic steatohepatitis (NASH) may progress to liver fibrosis, cirrhosis and hepatocellular carcinoma due to ongoing necroinflammation[3,4].

Currently, liver biopsy is the gold standard for diagnosing NAFLD and NASH and for evaluating liver fibrosis[5]. However, liver biopsy is an invasive technique that is associated with several complications. Therefore, alternative non-invasive methods are under investigation for diagnosing this common disease[6].

Lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) is a protein coded by the oxidized low density lipoprotein (oxLDL) receptor-1 gene, and it is expressed by endothelial cells, vascular smooth muscle cells, macrophages, and adipocytes[7,8]. LOX-1 is a membrane glycoprotein that binds, internalizes, and degrades oxLDL. LOX-1 is activated by oxLDL and leads to endothelial dysfunction, apoptosis and atherosclerotic process via intracellular signal transduction. Additionally, LOX-1 has multiligand receptor features, and the defined ligands of LOX-1 are aged red blood cells, apoptotic cells, activated platelets, leukocytes, bacteria, phosphatidyl serine, advanced glycation endproducts, C reactive protein, and heat shock protein 70[9].

In addition to its role in the process of atherosclerotic, LOX-1 is active in inflammatory processes. Proinflammatory cytokines that are shown to upregulate LOX-1, such as transforming growth factor-beta, interleukin-6, interleukin-1α, interleukin-1β, and tumor necrosis factor-α, are also involved in the pathogenesis of NAFLD[9,10]. Furthermore, recent studies have reported an increase in the LOX-1 levels in diabetes mellitus, metabolic syndrome, and coronary artery disease[11,12].

In this study, we investigated the relationship between LOX-1 and histopathological changes and inflammation as well as clinical and biochemical parameters in liver biopsy-proven NAFLD (simple steatosis

and borderline and definitive NASH) patients. To address these questions, we evaluated the extracellular soluble component of LOX-1 in NAFLD cases and healthy controls.

MATERIALS AND METHODS

Study subjects

A total of 53 patients diagnosed with NAFLD (31 males and 22 females, mean age 42.5 ± 9.6 years) and 26 healthy control subjects (14 males and 12 females, mean age 39 ± 10.7 years) were included in the study. The patients with NAFLD were consecutive patients who had been admitted to the hepatology outpatient clinic within the last year and had been diagnosed with NAFLD based on liver biopsy. The healthy controls were individuals who attended the outpatient clinic for routine health control and had no known chronic illnesses. All participants in the study were recruited from Department of Gastroenterology of Istanbul Medeniyet University Göztepe Education and Research Hospital. To avoid selection bias for recruiting the patients and controls, all participants had to be residents of Istanbul for a minimum of 5 years as a prerequisite. The study was reviewed and approved by Istanbul Medeniyet University Goztepe Education and Research Hospital Institutional Review Board (document No. 8-B/28.12.2010) and written informed consent was obtained from all participants.

All NAFLD patients had elevated serum ALT levels for at least 6 mo, and none of the patients had alcohol consumption greater than 20 g/d. All patients were negative for viral hepatitis serology. Hemochromatosis, Wilson's disease, autoimmune hepatitis, primary biliary cirrhosis, sclerosing cholangitis and alpha-1 antitrypsin deficiency were ruled out. All tests/procedures for the aforementioned excluded conditions were performed for research. Patients with biliary strictures and malignancies were excluded. Additionally, the patients had no history of using hepatotoxic medicines, such as estrogens, amiodarone, steroids, tamoxifen, methotrexate, valproic acid and herbal drugs. The data regarding the hepatotoxic drug history were obtained from both the patient's medical records and their interviews. The data for the hepatotoxic drug history were obtained by interview for controls. All serological and biochemical tests of the participants were performed in the same laboratory, the Central laboratory of Göztepe Education and Research Hospital. All individuals in the control group were healthy on physical examination and had normal liver parenchyma in the sonographic liver examination. Their serological and biochemical parameters were all in the normal ranges.

Clinical and laboratory evaluations

Physical examinations, anthropometric and biochemical measurements, and body mass index (BMI) calculations were performed in all study participants. Blood pressure measurements were performed in a quiet room with a sphygmomanometer after 10 min of resting. After a 12-h overnight fasting period, blood samples, both from patients with NAFLD and controls, from the antecubital veins were collected between 8:00-9:00 am. After 2 h, which allowed the blood to clot, at room temperature, the samples were centrifuged at 1000 G for 20 min. The sera obtained from the cases were stored at -80 ℃ until further analysis.

Diabetes mellitus was diagnosed according to the American Diabetes Association criteria[13]. Metabolic syndrome was diagnosed using the Adult Treatment Panel Ⅲ criteria[14]. Homeostasis Model of Assessment - Insulin Resistance (HOMA - IR) was calculated using the following equation: insulin resistance (IR) = fasting plasma glucose (mmol/L) × fasting plasma insulin (mU/L)/22.5 (IR was accepted as normal if IR < 2.5 and present if insulin resistance IR ⩾ 2.5).

Duplicate measurements of the LOX-1 serum levels were performed using an ELISA kit (Life Science Inc. USCN. Wuhan, Catalog No. E1859Hu) according to the manufacturer's instructions. The standard curve concentrations used for the ELISA were 10 ng/mL, 5 ng/mL, 2.5 ng/mL, 1.25 ng/mL, 0.625 ng/mL, 0.312 ng/mL, and 0.156 ng/mL. The minimum detectable level of human LOX-1 is less than 0.03 ng/mL.

Histological analysis

A liver biopsy was performed under local anesthesia using a Hepafix 16-gauge needle (Braun Melsungen AG, Melsungen, Germany) with ultrasound guidance. All biopsy samples were fixed with 10% formaldehyde and then embedded in paraffin blocks. The liver specimens were stained with hematoxylin-eosin, Masson's trichrome and reticulin silver stains. An experienced hepatopathologist scored and evaluated the tissue specimens. The pathologist was blinded to all patient data. The histological evaluation was conducted according to the NAFLD activity scoring system (NAS) recommended by The National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) NASH Clinical Research Network[15]. In this scoring system, hepatic steatosis was graded from 1 to 3 according to the steatosis ratio, with 5%-33%, 33%-66% and > 66% representing scores of 1, 2 and 3, respectively. Lobular inflammation was defined as an overall assessment of all inflammation; no foci was scored as 0, < 2 foci per × 200 field scored as 1, two-four foci per × 200 field scored as 2, and more than 4 foci per × 200 field scored as 3. Ballooning scoring is defined as a score of 0 if there is no ballooning of hepatocytes, 1 if there are few ballooning hepatocytes, and 2 if there are numerous ballooning hepatocytes. Fibrosis was staged as follows: stage 0, no liver fibrosis; stage 1, perisinusoidal or periportal fibrosis; stage 2, perisinusoidal and portal/periportal fibrosis; stage 3, bridging fibrosis and stage 4, cirrhosis.

Table 1 Clinical and biochemical characteristics of healthy controls and patients with nonalcoholic fatty liver disease[1] *n* (%)

Characteristic	Control (*n* = 26)	NAFLD (*n* = 53)	Simple steatosis (*n* = 8)	NASH (borderline + definite) (*n* = 45)	*P* value[2]	*P* value[3]
Gender (males/females)	14/12	31/22	4/4	27/18	0.690	0.800
Age (yr)	39 ± 10.7	42.5 ± 9.6	42.8 ± 13.2	4 1 ± 8.6	0.190	0.310
BMI (kg/m²)	28.7 ± 5.1	31.6 ± 5.3	29.9 ± 4.31	32.07 ± 5.5	0.030	0.040
Smoking	5 (19.2)	11 (20.7)	3 (37.5)	8 (17.7)	0.900	0.080
Waist circumference (cm)	85.2 ± 7.3	102.2 ± 9.1	100.2 ± 9.9	102.6 ± 9	0.002	0.002
Diabetes Mellitus	No	11 (20.8)	3 (37.5)	8 (17.7)	0.012	0.050
Metabolic syndrome	No	33 (62.2)	3 (37.5)	30 (66.6)	< 0.001	< 0.001
Hypertension	No	12 (22.6)	3 (37.5)	9 (20)	0.008	0.010
Systolic blood pressure (mmHg)	116 ± 17	121 ± 16	115 ± 15	122 ± 17	0.310	0.270
Diastolic blood pressure (mmHg)	74 ± 12	82 ± 10	79 ± 8	82 ± 10	0.006	0.010
ESR median (min-max, mm/h)	21 (6-38)	11.5 (3-40)	16 (4-40)	11 (3-38)	0.001	0.110
CRP median (min-max, mg/L)	0.4 (0.2-1.5)	0.4 (0.1-2)	0.5 (0.1-1.4)	0.4 (0.1-2)	0.720	0.670
Hemoglobin A1c (%)	5.7 ± 0.3	5.9 ± 0.9	5.5 ± 0.4	5.9 ± 0.9	0.730	0.310
HOMA-IR median (min-max)	2 (0.6-3.2)	2.5 (0.3-11.8)	1.6 (0.3-4.2)	2.7 (0.5 ± 11.8)	0.001	0.010
AST median (min-max, U/L)	19 (9-60)	35 (16-147)	36 (19-91)	35 (16-147)	< 0.001	< 0.001
ALT median(min-max, U/L)	18 (5-50)	52 (17-196)	43 (18-97)	53 (17-196)	< 0.001	< 0.001
Total cholesterol (mmol/L)	5.15 ± 1	5.43 ± 1.39	5.46 ± 1.13	5.43 ± 1.45	0.450	0.670
HDL-cholesterol (mmol/L)	1.21 ± 0.2	1.19 ± 0.2	1.26 ± 0.2	1.16 ± 0.2	0.290	0.310
LDL-cholesterol (mmol/L)	3.52 ± 0.8	3.67 ± 0.9	3.67 ± 0.8	3.67 ± 0.9	0.520	0.770
Triglycerides (mmol/L)	1.4 ± 0.6	2.5 ± 2.12	2.2 ± 1.08	2.5 ± 2.27	0.005	0.420
Ferritin median (min-max, pmol/L)	20.7 (2.8-101)	91 (6.5-326)	141.5 (7.8-300)	88.5 (6.5-326)	< 0.001	< 0.001
LOX-1 (ng/mL)	4.08 ± 4.32	8.49 ± 6.43	6.1 ± 6.16	8.92 ± 6.45	0.001	0.004

[1]The patients with NAFLD and controls was similar according to the socioeconomic status, racial and ethnic background, and, dietary and/or physical activity habits, religion; [2]*P* value: for comparison of control and NAFLD (*t* test for continuos variables and χ^2 test for categorical, as variables); [3]*P* value: for comparison of control, simple steatosis, and NASH (One-way ANOVA for continues variables and χ^2 test for categorical variables). Normal values in laboratory tests: ESR: Erythrocyte Sedimentation Rate (0-20 mm/h); CRP: C-reactive protein (< 8 mg/L); HbA1c (4.3-5.8 proportion of total hemoglobin); total cholesterol (2.6-5.2 mmol/L); triglyceride (0.7-1.7 mmol/L); LDL cholesterol (1-3.37 mg/dL); HDL cholesterol (> 0.9 mmol/L); AST (5-32 U/L); ALT (5-38 U/L); ferritin (54-755 μg/L in males and 25-755 μg/L in females); BMI (body mass index) (18-25 kg/m²); LOX-1: Lectin-like oxidized low-density lipoprotein receptor-1; HOMA-IR and metabolic syndrome are described in the text, Diabetes Mellitus was diagnosed according to ADA 2010 criteria, NAFLD: Nonalcoholic fatty liver disease; NASH: Nonalcoholic steatohepatitis.

Histologically, the total NAS scores were calculated as a sum of the steatosis (1-3), lobular inflammation (0-3) and ballooning (0-2) scores. Based on this scoring system, patients with a total NAS score of 0-2 were diagnosed with simple steatosis, 3-4 borderline NASH, and 5 or greater definitive NASH[15].

Statistical analysis

StatMate 2.0 (GraphPad Inc., San Diego, CA, United States) was used for the power calculation of the study. The data were analyzed using SPSS 16.0 (IL United States SPSS Inc., Chicago, IL, United States). Normally distributed continuous variables are presented as the mean ± SD. Student's *t*-test was used to evaluate the difference between the independent groups. Differences in the levels of LOX-1 among the NAFLD subgroups (simple steatosis and borderline NASH+definitive NASH) and control group were determined by one-way analysis of variance (ANOVA) followed by a Bonferroni multiple-comparison post hoc test. Categorical data were analyzed using the χ^2 test. A Spearman rank correlation was used to examine the relationship between variables. A receiver operating characteristic (ROC) curve analysis was used to identify the optimal cutoff value of LOX-1 for distinguishing between patients with NASH and healthy controls. A *P*-value < 0.05 was considered statistically significant. The statistical methods of this study were reviewed by Recep Minga, biomedical statistician of İkon Research and Consultancy co.

RESULTS

The clinical and biochemical characteristics of the healthy controls and NAFLD patients are presented in Table 1. The age and gender distribution were similar in both groups. The following characteristics were significantly higher in the NAFLD patients: waist circumference, diastolic blood pressure, HOMA-IR, triglyceride level and transaminase and ferritin levels. Eight patients (15%) had simple steatosis, whereas 27 (51%) had borderline NASH and 18 (34%) were diagnosed with definite NASH. Metabolic syndrome was found in 62.2% of the patients with NAFLD.

The LOX-1 level was significantly higher in the NAFLD group compared to healthy controls (8.49 ± 6.43 ng/mL *vs* 4.08 ± 4.32 ng/mL, respectively, *P* = 0.001). The LOX-1 levels were significantly different between the control, simple steatosis and NASH (borderline + definite) cases (4.08 ± 4.32 ng/mL, 6.1 ± 6.16 ng/mL, 8.92 ± 6.45 ng/mL, respectively, *P* = 0.004). The distribution of the serum LOX-1 levels in

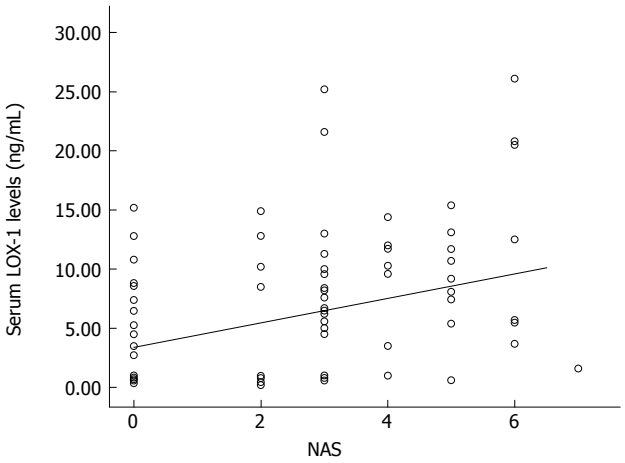

Figure 1 Distribution of serum levels of lectin-like oxidized low density lipoprotein receptor-1 in healthy controls and patients according to non-alcoholic fatty liver disease Activity Score[△]. The LOX-1 levels were significantly different between controls, simple steatosis and NASH (borderline + definite) cases (4.08 ± 4.32 ng/mL, 6.1 ± 6.16 ng/mL, 8.92 ± 6.45 ng/mL, respectively, P = 0.004). LOX-1: Lectin-like oxidized low density lipoprotein receptor-1; NAS: Non-alcoholic fatty liver disease Activity Score; [△]: All healthy controls were considered to have 0 point in NAS.

the study subgroups (controls and patients) is shown in a scatterplot figure (Figure 1). In addition, the LOX-1 serum levels were significantly higher in the NASH group than in the healthy subjects (8.92 ± 6.45 ng/mL *vs* 4.08 ± 4.32 ng/mL, P = 0.003). The LOX-1 levels were not significantly different in the NAFLD subgroups based on histological data. As a result, multiple linear regression analysis was not performed. In the NAFLD group, cases with (33) or without (20) metabolic syndrome had no significant difference in LOX-1 (7.27 ± 5.32 ng/mL *vs* 10.63 ± 7.84 ng/mL, respectively, P = 0.849).

The LOX-1 cut-off value that was used to distinguish healthy controls and NASH (borderline and definite) was 5.35 ng/mL. The area under ROC (AUROC) according to this cut-off level was 72.5% (SE = 0.06, Mann Whitney U-test, P = 0.001). With this cut-off value, the LOX-1 measurement could distinguish NASH patients from healthy controls with a sensitivity of 69.8%, specificity of 69.2%, negative predictive value of 69.6%, and positive predictive value of 69.4%.

DISCUSSION

In this study, we demonstrated, for the first time, a significant difference in the LOX-1 levels between biopsy-proven NAFLD and healthy controls. The LOX-1 levels were significantly higher in NASH patients compared with healthy controls. Therefore, LOX-1 may distinguish NASH cases from healthy subjects. There was no difference between the LOX-1 levels in the simple steatosis subgroup and the healthy controls or NASH cases.

Previous studies have reported that diabetes, obesity, and hypertension cases had high serum LOX-1 levels[9,16,17]. The elevated LOX-1 levels in NAFLD patients are in parallel with these metabolic disorders. This result may be due to insulin resistance, which is an underlying common pathophysiologic mechanism of these disorders[16,18].

In this study, the LOX-1 levels in patients with NAFLD were higher than healthy controls. Although there is a gradual increase in the progression from healthy control to simple steatosis and then to NASH (borderline + definite) subgroup cases, the only significant difference was obtained between controls and NASH patients. However, we did not find any significant difference between simple steatosis and the NASH subgroups. Lubrano *et al*[19] reported that the serum LOX-1 levels were correlated with other inflammatory markers and with the severity of coronary artery disease. Additionally, in an endothelial dysfunction study, Sakurai *et al*[20] showed that the LOX-1 level is linearly correlated with increasing oxidative stress.

The high LOX-1 levels in coronary artery disease, endothelial dysfunction and NAFLD patients suggest that inflammatory processes and oxidative stress are common pathophysiologic mechanisms. In addition, coronary artery disease and endothelial dysfunction were present in NAFLD patients[21-23]. One important result of this study is the identification of high LOX-1 levels in patients with steatohepatitis.

Liver biopsy is still the gold standard method for establishing NASH, which is the progressive form of NAFLD. However, due to several complications of this invasive procedure, a number of alternative methods and non-invasive diagnostic modalities are in development[15,24,25]. Increased LOX-1 serum levels in patients with NASH may simplify the selection of cases for differentiating between NAFLD subgroups prior to liver biopsy. However, these data must be confirmed by studies with large patient samples.

There is no relationship between the LOX-1 level and degree of fibrosis in our study. Kelly *et al*[26] reported a relationship between the LOX-1 level and renal function and fibrosis in obese and diabetic rats. Injections of anti-LOX-1 antibodies into the rats improved renal function and reduced fibrosis. In a study examining the relationship between LOX-1 and angiotensin Ⅱ (which has roles in fibrotic processes) in human coronary artery endothelial cell culture, the activation of angiotensin Ⅱ type 1 receptors was shown to increase the LOX-1 levels[27,28]. The angiotensin Ⅱ type 1 receptor is also involved in the development of liver fibrosis[29]. Although these findings show a relationship between LOX-1 and kidney fibrosis, they do not suggest a causal relationship with liver fibrosis. There is a need for large-scale studies on LOX-1 expression at the cellular level to determine the role of LOX-1 in the pathogenesis of fibrosis. Based on our data, we concluded that in the natural course of NAFLD, inflammation is associated with elevated LOX-1 levels but that fibrosis is not.

There are some limitations to this study. The first limitation is the small number of cases. Therefore, the results must be verified in additional large-scale studies. Second, the study is a cross-sectional, case-control study; therefore, it does not provide information on the pathophysiologic and causal relationship for the disease course. The measurement of only the serum LOX-1 levels and not the liver tissue levels is also a limitation. Additionally, there is no evidence available at the tissue level in patients with NAFLD. Fourth, the patients enrolled in this study were only of Turkish descent; therefore, additional research is needed to assess the role of LOX-1 in different ethnic populations. Finally, the measured and unmeasured differences between the studied groups could have accounted for the findings.

In conclusion, this study showed an association, but not casuality, between serum LOX-1 levels and both NAFLD and NASH. As a result, the serum LOX-1 levels may be a useful marker for differentiating patients with NAFLD and NASH from healthy individuals, but our results must be verified in large-scale randomized trials.

COMMENTS

Background

Nonalcoholic fatty liver disease (NAFLD) is a leading cause of chronic liver disease. It is the hepatic manifestation of metabolic syndrome. Sensitive non-invasive test is needed to diagnose patients.

Research frontiers

Serum Lipoprotein receptor-1 (LOX-1) is a novel biomarker of atherosclerosis and associated to diabetes, hypertension and metabolic syndrome. As NAFLD is related to these entities, LOX-1 might have a role in NAFLD pathogenesis.

Innovations and breakthroughs

Serum LOX-1 levels are increased in correlation with NAFLD activity scores and might improve to differentiate healthy people from definite nonalcoholic steatohepatitis (NASH).

Applications

Histopathological evaluation of the liver is needed for a definitive diagnosis of NASH. In this study serum LOX-1 was able to discriminate NASH from healthy controls. The increased levels might indicate the patients who will need histologic evaluation.

Terminology

NAFLD (non-alcoholic fatty liver) is a spectrum of chronic liver diseases where lipid accumulation exceeds 5% in the hepatocytes. Simple steatosis usually has a benign course but NASH may progress to liver fibrosis, cirrhosis and hepatocellular carcinoma due to ongoing necroinflammation. The degree of severity is determined by histopathological scoring systems.

Peer-review

The inclusion and exclusion criteria are well defined in the study. Small sample size is a limitation of this study. It might have a role in the non-invasive diagnosis of NAFLD.

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