World Journal of Gastroenterology

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DOI: 10.3748/wjg.v22.i21.5096

World J Gastroenterol 2016 June 7; 22(21): 5096-5103
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Observational Study

Serum adipokines might predict liver histology findings in non-alcoholic fatty liver disease

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Institutional review board statement: The study was reviewed and approved by the Kashan University of Medical Sciences Institutional Review Board.

Informed consent statement: All study participants, or their legal guardians, provided informed written consent before study enrollment.

Conflict-of-interest statement: No potential conflicts of interest relevant to this article were reported.

Data sharing statement: No additional data are available.

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Received: January 5, 2016
Peer-review started: January 5, 2016
First decision: February 18, 2016
Revised: February 25, 2016
Accepted: March 18, 2016
Article in press: March 18, 2016
Published online: June 7, 2016

Abstract

AIM: To assess significance of serum adipokines to determine the histological severity of non-alcoholic fatty liver disease.

METHODS: Patients with persistent elevation in serum aminotransferase levels and well-defined characteristics of fatty liver at ultrasound were enrolled. Individuals with a history of alcohol consumption, hepatotoxic medication, viral hepatitis or known liver disease were excluded. Liver biopsy was performed to confirm non-alcoholic liver disease (NAFLD). The degrees of liver steatosis, lobular inflammation and fibrosis were determined based on the non-alcoholic fatty liver activity score (NAS) by a single expert pathologist. Patients with a NAS of five or higher were considered to have steatohepatitis. Those with a NAS of two or lower were defined as simple fatty liver. Binary logistic regression was used to determine the independent association of adipokines with histological findings. Receiver operating characteristic (ROC) analysis was employed to determine cut-off values of serum adipokines to discriminate the grades of liver steatosis,

lobular inflammation and fibrosis.

RESULTS: Fifty-four participants aged 37.02 ± 9.82 were enrolled in the study. Higher serum levels of visfatin, IL-8, TNF-α levels were associated independently with steatosis grade of more than 33% [β = 1.08 (95%CI: 1.03-1.14), 1.04 (95%CI: 1.008-1.07), 1.04 (95%CI: 1.004-1.08), $P < 0.05$]. Elevated serum IL-6 and IL-8 levels were associated independently with advanced lobular inflammation [β = 1.4 (95%CI: 1.09-1.8), 1.07 (95%CI: 1.003-1.15), $P < 0.05$]. Similarly, higher TNF-α, resistin, and hepcidin levels were associated independently with advanced fibrosis stage [β = 1.06 (95%CI: 1.002-1.12), 19.86 (95%CI: 2.79-141.19), 560.72 (95%CI: 5.98-5255.33), $P < 0.05$]. Serum IL-8 and TNF-α values were associated independently with the NAS score, considering a NAS score of 5 as the reference value [β = 1.05 (95%CI: 1.01-1.1), 1.13 (95%CI: 1.04-1.22), $P < 0.05$].

CONCLUSION: Certain adipokines may determine the severity of NAFLD histology accurately.

Key words: Non-alcoholic fatty liver disease; Adipokine; Histology; Adiponectin; Visfatin; Resistin; Hepcidin; Interleukin; Tumor necrosis factor

Core tip: Considering the drawbacks of current assays, it seemed reasonable to find appropriate serum biomarkers to define the extent of liver damage in non-alcoholic liver disease (NAFLD). We investigated several key adipokines together with metabolic profiles and liver function tests, providing an advantage over previous studies. We concluded that serum visfatin, IL-8, TNF-α levels were associated with liver steatosis degree; serum IL-6 and IL-8 concentrations correlated with lobular inflammation grade; and TNF-α, resistin, and hepcidin levels correlated with fibrosis stage. The study suggested that certain adipokines might have better accuracy than currently used serum biomarkers to determine NAFLD histology.

Jamali R, Razavizade M, Arj A, Aarabi MH. Serum adipokines might predict liver histology findings in non-alcoholic fatty liver disease. *World J Gastroenterol* 2016; 22(21): 5096-5103 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i21/5096.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i21.5096

INTRODUCTION

Non-alcoholic liver disease (NAFLD) is a health concern worldwide. The burden of the disease is increasing because of the epidemic of obesity and the development of insulin resistance (IR) syndrome[1]. Liver function tests, metabolic profiles, liver ultrasound and clinical data are used routinely to detect the disease. Considering the limitations of these assays, liver biopsy is still the gold standard method to diagnose NAFLD[2]. However, concerns about the possible complications and invasiveness of the method have limited its application by physicians. It seems reasonable to identify appropriate serum biomarkers to diagnose and define the extent of liver damage in NAFLD. In this regard, interest in the roles of adipokines that are secreted from visceral adipose tissue (VAT) has been increasing.

NAFLD comprises a wide spectrum of liver cell injury that is induced by insulin resistance. Primarily, the accumulation of fat occurs in hepatocytes (simple fatty liver) as a consequence of hepatic insulin resistance. A growing body of evidence supports the view that adipokines modulate these metabolic processes by regulating insulin mediated glucose metabolism, fatty acid utilization and lipid accumulation of visceral tissues. At the later stages of disease, inflammatory phenomena arise that might progress to steatohepatitis and, ultimately, cirrhosis. It has been suggested that the development of steatohepatitis is a consequence of the balance between pro and anti-inflammatory effects of adipokines.

There is a paucity of literature regarding the serum threshold values and efficacy of adipokines in the diagnosis and follow-up of fatty liver patients. In this research, we evaluated certain important adipokines that were reported to be associated with NAFLD, in a cohort of biopsy-proven NAFLD patients[3-9].

The aims of this study were: (1) to evaluate the association of histological findings (steatosis, lobular inflammation and fibrosis) and serum biomarkers (including adipokines, inflammatory cytokines, liver function tests and metabolic profiles); and (2) to determine cut-off values of serum biomarkers to identify the grades of steatosis, lobular inflammation and fibrosis.

MATERIALS AND METHODS

Patient enrolment protocol

This study was conducted in the outpatient gastroenterology clinic of Shahid Beheshti general hospital, from September 2012 to September 2014. Initially, patients with persistent elevated serum aminotransferase levels and well-defined characteristics of a fatty liver *via* abdominal ultrasound (Hitachi EUB 405 apparatus equipped with a convex 3.5 MHz probe) were included (Phase 1)[1,10]. The upper normal limit of the serum aminotransferases level was considered as 40 units per liter[11]. Individuals with a history of alcohol consumption, hepatotoxic medication, viral hepatitis and known liver disease were excluded from the study (Phase 2)[1,12]. Liver biopsy was performed on the remaining patients from phase 2 to confirm diagnosis of NAFLD for final enrolment (Phase 3).

Ethical considerations

The study was conducted according to ethical standards for human experimentation (Helsinki Declaration). The ethics committee of the hospital approved the study protocol (No: 8861). The purpose of study was explained to the participants. They were enrolled in the study upon providing written informed consent.

Sample size calculation

The sample size was n = 54, considering the mean prevalence of NAFLD (P = 28%, α = 0.05, z = 1.96, and d = 0.12), according to a previous study[10].

Laboratory assays

Fasting serum samples were obtained to assess the level of adiponectin, visfatin, resistin, hepcidin, IL-6, IL-8 and tumor necrosis factor (TNF)-α by enzyme-linked immunosorbent assay (ELISA), according to the manufacturer's instructions. The following kits were used in this study: Human adiponectin and visfatin ELISA kits (Production numbers: AG-45A-0001 and AG-45A-0006 respectively; ADIPOGEN Inc., South Korea), resistin (human resistin ELISA kit, Biovendor, Czech Republic), hepcidin (Lot: RN- 24429; DEMEDITEC GmbH, Kiel-Wellsee, Germany), IL-6 (Lot: 233737; Bendered Systems GmbH, Vienna, Austria), IL-8 (Lot: ab46032; IL-8 human ELISA kit, Abcam, United States), and TNF-α (Lot: ab46087; TNF-α human ELISA kit, Abcam, United States). Fasting blood glucose, insulin, lipid profiles and liver function tests were performed as previously described[1,10-13].

Liver histology

Percutaneous liver biopsy was performed using a true cut needle (G14). A sample larger than 10 mm or with at least five portal tracts was considered acceptable for histological evaluation. Hematoxylin and Eosin (HE) and Masson's Trichrome stainings were performed to evaluate necroinflammation and fibrosis, respectively. To avoid inter-observer disagreement, a single expert pathologist who was blinded to the patient data interpreted samples. The degree of liver steatosis, lobular inflammation and fibrosis was defined based on the "non-alcoholic fatty liver activity score (NAS)"[14]. Patients with a NAS of five or higher were considered to have NASH. Those with a NAS of two or lower were defined as simple fatty liver[14].

Statistical analysis

Continuous variables were reported as the mean ± SD and categorical variables were shown as counts (percent). The Kolmogorov-Smirnov test was used to assess the distribution of serum adipokines. A χ^2 or *t*-test was applied to assess differences among groups, where appropriate. Binary logistic regression analysis using the standard model was applied to evaluate the association of independent variables (including serum adipokines and clinical data) and liver histology findings.

Hepatic steatosis severity was categorized into four degrees according to the NAS. The first two degrees (0-1) represented no and mild liver steatosis, and the next degrees (2-3) indicated moderate to severe liver steatosis. To define the risk of lower liver steatosis versus a more advanced degrees of steatosis, we considered the patients with steatosis grades of less than 33% as the "mild group". Meanwhile, those with higher degrees (2-3) were merged to form the "moderate to severe group".

The lobular inflammation range was graded from 0 to 3 by the NAS. To estimate the risk of lower lobular inflammation against more advanced grades, we labeled the individuals with lobular inflammation of less than two foci per HPF (grade 1) as the "mild group". At the same time, those with higher lobular inflammation grades (2-3) were combined to form the "moderate to severe group".

Hepatic fibrosis content was categorized into 5 stages based on NAS. The former two stages (0-1) demonstrated none/mild fibrosis and the latter stages stand for more advanced fibrosis (2-4). In order to determine the probability of lower fibrosis versus more advanced fibrosis, we labeled the subjects with perisinusoidal or periportal (stage 1) as the "mild group". Those with higher fibrosis stages (2-4) were mixed to form the "moderate to severe group".

For the regression model, liver steatosis, lobular inflammation, fibrosis stage, and NAS were employed as dependent variables; Steatosis grade of less than 33%, lobular inflammation of less than two foci per high powered field (HPF), fibrosis stage of one (perisinusoidal or periportal), and a NAS of five or higher were set as the reference groups, respectively. Standardized correlation coefficient (OR) with the 95%CI was calculated. Serum adipokines that were independently associated with the histological findings were selected for receiver operating characteristic (ROC) analysis. ROC analysis explored the serum adipokines' cut-off values and their sensitivities and specificities to discriminate higher grades of liver steatosis, lobular inflammation and fibrosis. Values with the highest sum of the sensitivity and specificity were reported as the best cut-off values. All statistical analyses were performed by SPSS, version 17 (SPSS, Chicago, United States). The probability of a difference between groups was considered statistically significant if the two-sided P value was less than 0.05.

RESULTS

Seventy participants presumed to have NAFLD were evaluated from September 2012 to September 2014 (phase 1). Reasons for leaving certain patients out of the study were patient refusal to participate in the study (n = 8), normalization of ALT during the lead-in phase (n = 6), autoimmune hepatitis (n = 1) and viral

Table 1 Clinico-demographic and laboratory data of the participants

Variable	Total	Simple fatty liver	Non-alcoholic steatohepatitis
	n = 54	*n* = 2	*n* = 28
Age (yr)	37.02 ± 9.82	27.00 ± 2.82	35.00 ± 8.47
Male gender, *n* (%)	35 (64.8)	2 (100)	17 (60.7)
Waist circumference (cm)	102.13 ± 2.69	101.00 ± 42.24	101.57 ± 2.71
Body mass index (kg/m^2)	30.55 ± 3.97	28.09 ± 7.77	29.92 ± 3.79
Diabetes mellitus present, *n* (%)	12 (22.2)	0 (100)	11 (39.3)
Metabolic syndrome present, *n* (%)	36 (66.7)	1 (50)	21 (75)
Adiponectin (mg/L)	8.14 ± 2.91	8.20 ± 2.67	7.00 ± 0.28
Visfatin (ng/mL)	19.96 ± 17.5	5.40 ± 0.84	18.34 ± 16.18
Resistin (mg/mL)	2.51 ± 1.08	1.70 ± 1.83	2.10 ± 1.04
Hepcidin (ng/mL)	64.0 ± 0.62	48.50 ± 0.38	75.00 ± 0.49
Tumor necrosis factor-alpha (pg/mL)	2.68 ± 19.32	0.96 ± 9.05	3.68 ± 20.93
Interleukin 6 (pg/mL)	7.59 ± 5.75	4.70 ± 0.24	7.41 ± 4.78
Interleukin 8 (pg/mL)	27.41 ± 24.99	13.60 ± 13.01	38.60 ± 28.21
Alanine aminotransferase (U/L)	65.91 ± 36.11	37.00 ± 0.00	82.10 ± 39.25
Aspartate aminotransferase (U/L)	42.18 ± 20.48	25.00 ± 4.24	49.67 ± 24.14
Alkaline phosphatase (U/L)	181.50 ± 76.14	144.21 ± 42.41	180.23 ± 45.22
Gamma glutamyl transpeptidase (U/L)	54.12 ± 62.55	44.40 ± 30.54	55.40 ± 33.26
Fasting blood sugar (mg/dL)	98.41 ± 14.12	98.25 ± 16.31	103.0 ± 0.00
Insulin (mU/L)	15.16 ± 13.42	10.68 ± 3.65	17.40 ± 18.00
Triglyceride (mg/dL)	150.09 ± 70.18	60.20 ± 9.70	167.57 ± 77.19
Total cholesterol (mg/dL)	177.55 ± 34.17	165.85 ± 40.65	183.77 ± 37.76
Low density lipoprotein cholesterol (mg/dL)	100.89 ± 29.03	98.75 ± 35.42	103.15 ± 32.19
High density lipoprotein cholesterol (mg/dL)	48.51 ± 9.06	55.05 ± 7.14	48.66 ± 9.67

Data are presented as mean ± SD unless otherwise noted. Patients with non-alcoholic activity score (NAS) of five or higher were considered to have non-alcoholic steatohepatitis. Those with NAS equal to two or lower were defined as simple fatty liver[14]. NAFLD: Non-alcoholic fatty liver disease.

hepatitis (*n* = 1) (phase 2). Finally, fifty-four patients with biopsy proven NAFLD were included in the study (phase 3). The clinico-demographic and laboratory data of the participants are presented in Table 1.

Participants showed NAS of 4.87 ± 1.71. The frequency of histological findings in the study population is depicted in Figure 1.

Binary logistic regression showed a positive association between serum visfatin, IL-8 and TNF-α level and the grades of steatosis. Similarly, serum IL-6 and IL-8 levels were independently associated with the degrees of lobular inflammation. Serum TNF-α, resistin and hepcidin levels were independently associated with perisinusoidal fibrosis stage. Serum IL-8 and TNF-α values were positively associated with NAS (Table 2).

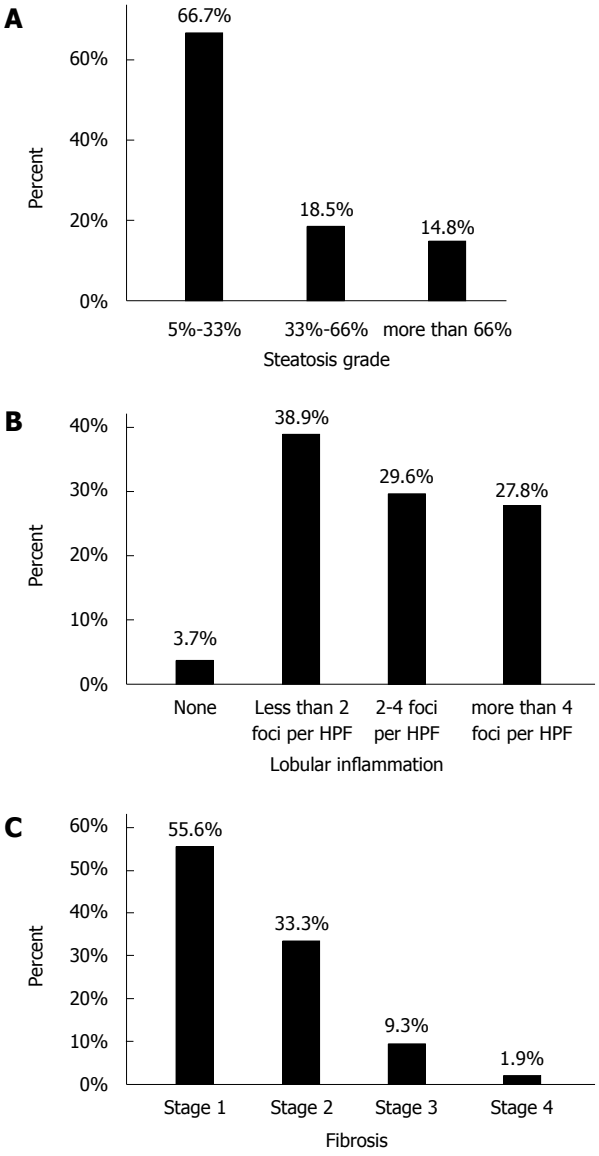

Figure 1 Frequency of histological findings in the participants. Frequency of patients with different degrees of steatosis (A), lobular inflammation grade based on foci of lobular inflammation in high power field of microscopic view (B), and fibrosis stage (C) are presented.

The ROC curves with calculated AUC (± 95%CI) to determine the best cut-off values of serum adipokines to differentiate histological groups are shown in Figure 2. The sensitivities and specificities of the cut-off values of biomarkers to identify histological groups appear in Table 3.

DISCUSSION

This study concluded that serum visfatin, IL-8 and TNF-α levels were independently associated with liver steatosis degree; serum IL-6 and IL-8 concentrations were independently associated with lobular inflammation grade; and TNF-α, resistin and hepcidin levels were independently associated with fibrosis stage in a cohort of biopsy-proven NAFLD patients. Moreover, the best cut-off values for the above-mentioned

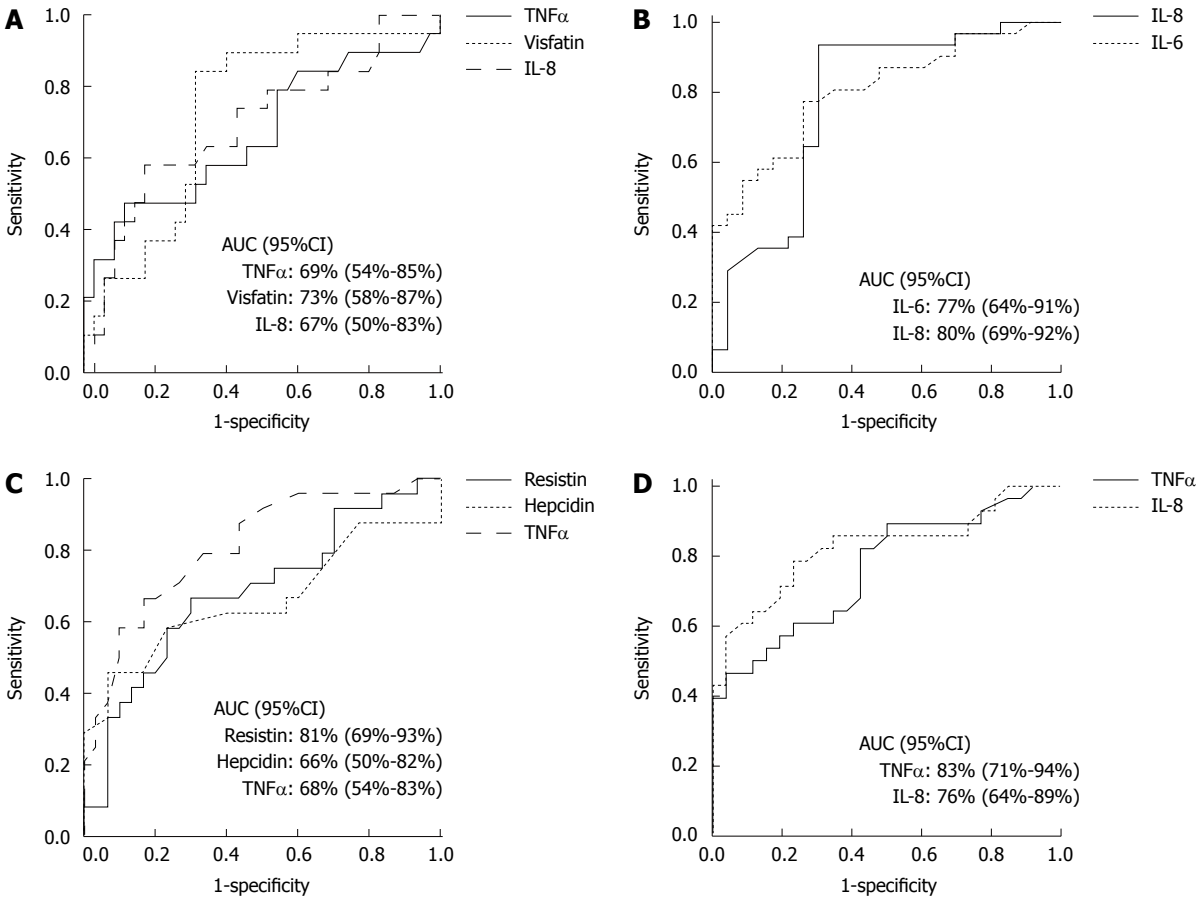

Figure 2 Receiver operating characteristic analysis to determine cut-off values of serum adipokines for differentiating histological severity. A: ROC curve of serum TNF-α, Visfatin, and IL-8 levels to differentiate steatosis degree of less than 33% from more advanced degrees of steatosis; B: ROC curve of serum IL-6 and IL-8 levels to differentiate lobular inflammation grade of less than two foci per high power field from more advanced grades of inflammation; C: ROC curve of serum Resistin, Hepcidin, and TNF-α levels to differentiate fibrosis stage of perisinusoidal or periportal from more advanced stages of fibrosis; D: ROC curve of serum TNF-α and IL-8 levels to differentiate steatohepatitis from simple fatty liver based on non-alcoholic fatty liver disease activity score. AUC: Area under curve; IL: Interleukin.

Table 2 Association between histological findings and serum adipokine levels

Adipokine	OR	95%CI	*P* value
Steatosis degree			
Visfatin	1.08	1.030-1.14	0.001
TNF-α	1.04	1.004-1.08	0.030
Interleukin 8	1.04	1.006-1.07	0.020
Lobular inflammation grade			
Interleukin 6	1.4	1.090-1.80	0.008
Interleukin 8	1.07	1.003-1.15	0.040
Fibrosis stage			
Resistin	19.86	2.790-141.19	0.003
Hepcidin	560.72	5.980-5255.33	0.006
TNF-α	1.06	1.002-1.12	0.040
NAS			
Interleukin 8	1.05	1.01-1.10	0.040
TNF-α	1.13	1.04-1.22	0.004

TNF-α: Tumor necrosis factor-alpha; NAS: Non-alcoholic fatty liver activity score.

serum adipokines were calculated to identify the liver histological findings.

The associations between certain adipokines with NAFLD were evaluated in previous reports[3-9]. We investigated several key adipokines, together with metabolic profiles and LFT, which provided an advantage over the previous studies. To improve the accuracy of the study, the cases were recruited from a cohort of biopsy-proven NAFLD patients. We used NAS for to determine the severity of the liver histology. Notably, NAS is a valid scoring system for NAFLD that differentiates the spectrum of disease with an acceptable reliability and validity[14].

Visfatin is a new adipokine with proinflammatory and metabolic properties. It is increased in IR syndrome. The expression of visfatin in VAT facilitates the maturation of preadipocyte cells to differentiated adipocytes (Paracrine effect)[3]. This fact might explain the correlation between serum visfatin levels and hepatic steatosis degree in our study. Visfatin is also associated with body fat mass in alcoholic fatty liver disease[15]. Previous studies have reported a correlation between visfatin and fibrosis stage, but not with steatosis or lobular inflammation grade in NAFLD[16]. Meanwhile, an increase in serum visfatin was shown to be associated with portal inflammation[17].

TNF-α is a pro-inflammatory cytokine and is asso-

Table 3 Best cut-off values of serum adipokine levels to differentiate histological groups according to receiver operating characteristic analysis

Adipokine	Serum concentration	Sensitivity (%)	Specificity (%)
TNF-α (pg/mL)	2.13	74	58
Visfatin (ng/mL)	13.00	84	69
Interleukin 8 (pg/mL)	24.25	58	66
Cut-off values of serum adipokine levels to differentiate lobular inflammation grade of less than 2 foci per high power field from more advanced grades of inflammation.			
Interleukin 6 (pg/mL)	3.70	94	70
Interleukin 8 (pg/mL)	13.00	77	74
Cut-off values of serum adipokine levels to differentiate perisinusoidal or periportal fibrosis from more advanced stages of fibrosis.			
Resistin (mg/mL)	1.65	79	66
Hepcidin (ng/mL)	45.00	63	60
TNF-α (pg/mL)	2.44	67	70
Cut-off values of serum adipokine levels to differentiate steatohepatitis from simple fatty liver group based on non-alcoholic fatty liver disease activity score (NAS).			
Interleukin 8 (pg/mL)	9.80	82	54
TNF-α (pg/mL)	2.44	71	81
Cut-off values of serum adipokine levels to differentiate steatosis degree of less than 33% from more advanced degrees of steatosis.			

TNF-α: Tumor necrosis factor-alpha.

ciated with hepatic IR in NAFLD[4]. It mediates the early stage of NAFLD by fat accumulation in hepatocytes. In addition, it facilitates disease progression to a more advanced stage[18]. The relationship between serum TNF-α and liver steatosis and fibrosis in our research is in line with previous observations.

IL-8 is also a pro-inflammatory cytokine that activate monocytes and attracts polymorphonuclear leukocytes to the site of inflammation[19]. It is increased in obese individuals with IR. In accordance with the literature, our results showed that serum IL-8 was associated with steatosis degree and lobular inflammation[5].

IL-6 is a liver and adipose tissue-derived proinflammatory cytokine that is implicated in hepatic and skeletal muscle IR. IL-6 is thought to act as a second hit in the pathophysiology of NAFLD, causing the progression of simple fatty liver to NASH[19]. The correlation of IL-6 with lobular inflammation grade in our study is comparable to the findings by other groups[6,20,21].

With regard to hepcidin, the circulatory level was strongly associated with fibrosis stage in our study. Nevertheless, a previous found no correlation between hepcidin and histological findings[7]. Body iron stores in NAFLD regulate hepcidin levels[22]. Therefore, it seems reasonable to adjust for patients iron storage when evaluating hepcidin levels in NAFLD patients.

Resistin is an adipokine that is considered an indicator of IR in obesity[23]. However, the pathophysiological role of resistin in NAFLD is not clear. In this study, we observed that serum resistin levels were related with fibrosis stage. On the other hand, advanced liver fibrosis was associated with reduced resistin concentration in chronic hepatitis C patients with normal body weight, glucose and lipid profiles[24]. Previous studies demonstrated a correlation between high serum resistin levels and the presence of steatosis and necroinflammation in NAFLD[8,25]. Meanwhile, another study demonstrated an association of low serum resistin levels with excessive fat accumulation in the liver[26].

Adiponectin is a well-known adipokine that regulates hepatic IR[27]. It was suggested that adiponectin might be related to steatosis grade and the severity of NAFLD; however, its definitive role remains to be addressed[28]. A decrease in serum adiponectin is the primary event in children with NAFLD before the rise of inflammatory cytokines and the development of overt diabetes[9,29]. One previous study showed that adiponectin could predict patients with higher necroinflammatory grade and fibrosis stage from those with milder histological findings[30]. Another study showed that adiponectin is related to hepatic fat content and not to necroinflammatory activity and fibrosis stage[31]. Meanwhile, our study showed no correlation between adiponectin and liver histology. This study, despite its advantages, suffers from several drawbacks: first, the study was performed in a single institution; therefore, the findings need to be generalized with caution. Second, our study was cross-sectional, which limited the interpretation of causal associations.

There is currently no defined "normal range" for serum adipokines. Moreover, adipokine levels might fluctuate over time according to the metabolic environment. These concerns might explain the differences in the results of the above-mentioned studies with our results. Further well-controlled prospective studies to determine the association of VAT-derived proteins (including proinflammatory cytokines and polypeptide hormones) and liver histological findings are recommended.

The associations of some important adipokines, together with the currently used serum biomarkers, with the liver histological findings were evaluated. Certain adipokines were independently associated with the liver histological findings. Finally, the best cut-off values of these serum adipokines were determined to detect the severity of liver steatosis, lobular inflammation and fibrosis.

In conclusion, this study suggested that certain adipokines might determine accurately the severity of NAFLD based on histological findings.

ACKNOWLEDGMENTS

We are grateful to Dr. Hassan Ehteram at the Kashan University of Medical Sciences for reviewing the liver histology samples, and to Dr. Ayat Ahmadi at the Tehran University of Medical Sciences (TUMS) for reviewing the statistical analysis. The authors would like to expand their gratitude to Drs. Vafa Rahimi

Movaghar and Arsia Jamali at TUMS for their critical review of the manuscript.

COMMENTS

Background

Non-alcoholic liver disease (NAFLD) is a health concern worldwide. The burden of disease is increasing because of an epidemic of obesity. Considering the limitations of current modalities, finding an appropriate serum biomarker to diagnose and assess the severity of liver damage in NAFLD is crucial.

Research frontiers

The roles of adipokines in the pathogenesis of NAFLD have received research interest recently. Nevertheless, there is a paucity of studies that used serum levels of adipokines in the diagnosis and follow up of NAFLD patients.

Innovations and breakthroughs

The associations between certain adipokines with NAFLD were evaluated in previous reports. The authors investigated several key adipokines together with metabolic profiles and LFT, providing an advantage over the previous studies. To improve the accuracy of the study, the cases were selected from a cohort of biopsy-proven NAFLD patients. To assess the severity of NAFLD based on histology, we applied NAS, a valid scoring system for NAFLD that categorizes the spectrum of disease with acceptable reliability and validity.

Applications

This study suggested that certain adipokines might determine accurately the presence and severity of NAFLD.

Peer-review

This manuscript evaluated the association between histological grade of NAFLD and serum biomarkers, and suggested cut-off values of the biomarkers for NASH. The authors used a direct method to define NASH, and measured various biomarkers related to adiposity and inflammation. Finally, the authors showed successfully that the indices were related to each component of NASH.

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ISSN 1007-9327