Original research article

Evaluation of pro-inflammatory markers-tumor necrosis factor alpha (TNF-α) and adiponectin in nonalcoholic fatty liver disease.

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Abstract

Background and Aims: Adiponectin and tumor necrosis factor (TNF-α) are adipocytokines, that have recently been found to be involved in the pathogenesis of the nonalcoholic fatty liver disease (NAFLD). The present study aimed to determine the serum level of adiponectin and TNF-α in NAFLD patients and evaluate their correlation with severity of NAFLD. **Materials and Methods**: This study was conducted on patients with a diagnosis of NAFLD, based on ultrasonography (USG) of the abdomen. Fasting serum TNF-α and adiponectin level were measured by ELISA (enzyme-linked immunosorbent assay) method along with blood test for liver biochemistry and lipid profile and clinical and anthropometric measurements in consecutive 41 NAFLD patients and 20 healthy subjects. **Results:** The serum TNF-α level was significantly higher in NAFLD patients compared to healthy subjects((17.29±5.71 vs 8.45±2.60 pg/ml, $p < 0.0001$). Similarly,serum adiponectin level was found significantly lower in NAFLD patients compared to the control group(3.29 ± 1.52 vs 8.15 ± 5.10 ug/ml, $p < 0.0001$). A significant correlation was found between TNF-α and adiponectin with an ultrasonographic grade of fatty liver.

Conclusions: Serum TNF-α level was significantly higher and significant low adiponectin level was found in NAFLD patients. TNF-α and adiponectin levels can predict the severity of NAFLD in adult patients but required a large sample for further accuracy.

Keywords: TNF-α, Adiponectin, Nonalcoholic fatty liver disease, Metabolic syndrome.

Introduction

Non-alcoholic fatty liver disease (NAFLD) is a clinicopathological condition characterized by the presence of hepatic steatosis when there are no other causes for secondary hepatic fat accumulation (eg, heavy alcohol consumption) are present. In NAFLD liver injury ranges from simple hepatic steatosis to non-alcoholic steatohepatitis (NASH) with cirrhosis. The commonest causes for NAFLD are obesity, Type 2 diabetes, metabolic syndrome, etc. Non-alcoholic fatty liver disease (NAFLD) is now one of the leading causes of chronic liver disease worldwide with prevalence as high as 30% in the general population[1].In India, the prevalence is estimated to be between 16–32%[2].This is believed to be due to the increasing industrialization along with changes in lifestyle and diet. NAFLD is now recognized as the commonest cause of altered liver tests and chronic liver disease. NAFLD is strongly associated with the features of metabolic syndrome[3,4,5].

The underlying mechanism of NAFLD pathogenesis is the two-hit model[6]. Hepatic accumulation of lipid is the first hit, related to obesity, fatty diet, and insulin resistance. The first hit sensitizing the hepatocyte for the second hit which activates an inflammatory reaction, leading to NAFLD[6].

Several bioactive proteins or adipokines, are secreted by adipose tissue that regulate hepatic and peripheral glucose and lipid metabolism. These adipokines include leptin, adiponectin, resistin, and tumor necrosis factor-alpha (TNF-α). Adipokines have many actions on insulin resistance and inflammation, they might have important roles in the pathogenesis of NAFLD[7].TNF-α is a pro-inflammatory cytokine that appears to play a crucial role in the pathogenesis of

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obesity and insulin resistance[8]. The expression of TNF-α is increased in obesity and insulin resistance in humans and is positively correlated with insulin resistance[8]. TNF-α play a major role in the pathogenesis of NAFLD by promoting both liver fibrosis and insulin resistance .Adiponectin has anti-lipogenic effects, produces insulin-sensitizing effects, and reduces fat deposition in cells[9].Low circulating levels of adiponectin are associated with several components of metabolic syndrome including visceral adiposity, hypertriglyceridemia, and insulin resistance. Adiponectin prevents lipid accumulation in nonadipocyte tissue, such as the liver because of its antilipogenic action[10].

There are limited numbers of studies using these non-invasive biomarkers and correlating them with NAFLD and its severity.The aim of this study was to measure the serum level of TNF-α and adiponectin in NAFLD patients and to evaluate their correlation with nonalcoholic fatty liver disease (NAFLD) and its severity.

Patients and methods

This study was carried out prospectively from June 2018 to May 2020, at Index medical college and hospital, Indore, M.P. We included a total of 61adults (> 18 yrs) subjects in our study. Patients were selected from those attending the outpatient clinics of the medicine department.Out of those, 41 were NAFLD patients (case), and 20 were healthy volunteers (control). Healthy volunteers were selected from the patients attending medical OPD for a routine checkup. They were age and sex matched.None of the controls had liver disease, diabetes and hypertension, dyslipidemia or other inflammatory diseases.Written informed consent was taken from all subjects and the study was approved by the local ethics committee.

NAFLD diagnosis was, based upon the patient's clinical history and examination, laboratory tests, and USG abdomen finding. Patients were excluded if they had active alcohol abuse, viral hepatitis, gastrointestinal surgery for obesity, and taking drugs like glucocorticoid and estrogen.

All patients underwent a detailed history, clinical examination, and biochemical tests. Anthropometric measurement is done in all participants, weight, height, and waist circumference was measured with the standard method and BMI was calculated by dividing weight (kg)by square of height (m^2). After overnight fasting, venous blood collected from each participant and tested for hemogram, platelet count, bilirubin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), total protein, albumin, globulin, gamma glutamyl transpeptidase (GGT), serum creatinine, lipid profile, and TSH were measured directly by an automated analyzer.Other blood investigations like HBsAg and Anti HCV were also done to rule out associated viral hepatitis.

Metabolic syndrome is defined by the Adult treatment panel III (ATPIII)[11,12] as the presence of any three of the following five traits: Abdominal obesity, defined as a waist circumference ≥102 cm (40 in) in men and ≥88 cm (35 in) in women. [waist circumference ≥90 cm in males and ≥80 cm in females in India. Serum triglycerides ≥150 mg/dL (1.7 mmol/L) or drug treatment for elevated triglycerides. Serum high-density lipoprotein (HDL) cholesterol <40 mg/dL (1 mmol/L) in men and <50 mg/dL (1.3 mmol/L) in women or drug treatment for low HDL cholesterol. Blood pressure ≥130/85 mmHg or drug treatment for elevated blood pressure. Fasting plasma glucose (FPG) ≥100 mg/dL (5.6 mmol/L) or drug treatment for elevated blood glucose.

Serum adipokine levels –

Fasting serum TNF-α and adiponectin levels were measured in all patients and the control group.

After clotting, the samples were centrifuged at approximately 3000 rpm for 10 minutes and the serum was separated. Serum samples for TNF-α and adiponectin were aliquoted (250-500 µl) to avoid repeated freeze-thaw cycles and stored frozen at -20 °C. The serum TNF-α was measured using a commercially available highly sensitive human enzyme-linked immunosorbent assay (ELISA) kit (Diaclone SAS, France) (Cat No: 950.090.096, batch no: 1100-125). The assay was performed as the manufacturer advised. The normal reference value according to the manufacturerwas below the detection level of 8 pg/ml[13].The serum adiponectin level was measured by using the Human Adiponectin Enzyme-Linked Immunosorbent Assay (ELISA) kit manufactured by Diagnostic Biochem Canada Inc.

Reference values for adiponectin[14]

Group	Mean (µg/ml)	95% confidence range (µg/ml)
BMI < 25	9.7	3.4 – 19.5
BMI 25-30	7.1	2.6 – 13.7
BMI > 30	4.5	1.8 -9.4

Ultrasound abdomen –

Diagnosis of NAFLD done by ultrasound abdomen. The degree of liver steatosis was grade from 0 (no

steatosis) as normal liver echotexture, Grade I steatosis defined as the slight and diffuse increase in echogenicity of liver parenchyma with normal visualization of the diaphragm and portal vein borders. Grade II steatosis as moderate and diffuse liver parenchymal echogenicity with slightly impaired visualization of portal vein and diaphragm. Grade III steatosis grossly increased echogenicity of the liver and no or poor visualization of portal vein and diaphragm. The steatosis grade was assessed in a blinded fashion by one radiologist without any knowledge of the patients' laboratory or clinical data[15,16].

Statistical Analysis

The statistical analysis was performed with the SPSS statistical package for windows Version 25.0. Armonk, NY: IBM Corp and Microsoft excel sheet. Results were expressed the mean and standard deviation.In the present study, for comparing the means between the two groups, the Students paired 't' test for pre-post comparison was used. For finding out the association between independent and dependent variables, Pearson's Chi-square test was applied, for finding out the statistical significance between the means of more than two groups, one-way ANOVA was used.Frequency Distribution and Independent Two-sample t-test also used. A p-value of < 0.05 was taken as statistically significant.

Results

Anthropometric and biochemical characteristics of all subjects are shown in table no 1.

Table 1: Baseline characteristic of NAFLD patients and control group

Characteristic	NAFLD pt. (41) Mean±SD	Control (20) Mean±SD	t-value	p-value	Result
AGE (years)	56.93±11.30	61.15±7.40	-1.516	0.135	Non-Sig
WAIST CIRC. (Cm)	97.68±8.41	86.05±4.55	5.766	0.000	Significant
BMI (kg/m^2)	27.88±3.75	24.35±3.61	3.489	0.000	Significant
SYSTOLIC BP (Hg)	131.10±13.53	117.60±9.50	3.997	0.000	Significant
DIASTOLIC BP (Hg)	87.32±8.27	83.05±5.61	2.079	0.042	Significant
FBS (mg/dl)	114.04±29.74	95.25±8.22	2.765	0.008	Significant
TRIGLYCERIDE (mg/dl)	180.87±97.17	130.70±31.05	2.24	0.029	Significant
HDL (mg/dl)	40.46±8.92	41.70±5.25	-0.572	0.570	Non-Sig
TNF α (pg/ml)	17.29±5.71	8.45±1.60	6.769	0.000	Significant
ADIPONECTIN (µg/dl)	3.29±1.52	8.15±5.10	-5.646	0.000	Significant
FATTY LIVER					
GRADE I	(n) 20	0			
GRADE II	(n) 15	0			
GRADE III	(n) 06	0			

Table 2: Anthropometric characteristic of NAFLD patients and control group

Variables	Sex	Case			Control			T-Test	p-value
		N	Mean	Std. Deviation	N	Mean	Std. Deviation		
Age	Male	31	56.45	11.58	16	60.69	8.16	1.303	0.199
	Female	10	58.40	10.86	4	63.00	2.94	0.816	0.430
WAIST	Male	31	97.39	8.72	16	85.13	4.53	5.250	0.000*
	Female	10	98.60	7.73	4	89.75	2.50	2.195	0.049*
BMI	Male	31	27.58	3.19	16	23.63	3.46	3.913	0.000*
	Female	10	28.80	5.22	4	27.25	2.99	0.550	0.592

As noted from table 1, the mean age of patients was 56.93±11.30 years and control were 61.15± 7.40 years. Out of 41 patients, 10 patients (24.39%) were female. Twenty patients (48.7%) were fatty liver grade I, 15 patients (36.58%) had grade II and 6 patients (14.63%) had grade III fatty liver. Out of 41 patients, twenty-two patients (53.65%) patients were diabetic and 31 patients (75.56%) also had metabolic syndrome. Twenty healthy subjects (control) had normal liver function tests and ultrasound scans.

Compared to control, NAFLD patients were obese. NAFLD patients had significantly higher BMI and waist circumference than controls (p-value< 0.05). Twenty-four (58.53%) patients had more than 25 kg/m^2, and 8 patients (19.51%) had more than 30 kg/$m^2$and 9 patients (21.95%) had less than 25 kg/m^2.This study demonstrated that patients with NAFLD had a higher level of fasting blood glucose, higher serum triglyceride level and higher BMI compared to the control group, using the students (t) test and the p-value was < .005.

Assessment of serum TNF–α level: Mean±SD of serumTNF-α was significantly higher in NAFLD patients than in the healthy controls group (17.29±5.71 vs 8.45±2.60 pg/ml, p <0.000). When we compared serum TNF–α level with a various grade of fatty liver, and control group with one-way ANOVA test, we observed that grade I, grade II and grade III and control group had (13.40±2.60, 19.00±4.82, 26.00±3.09 and 8.45±1.60 pg/ml respectively p-value 0.000).TNF–α level was statistically significant when compared within the fatty liver grade and when compared with control (One-way ANOVA, F value = 63.80, p-value = 0.000, Significant). We found a linear correlation between TNF-α level and fatty liver grade. We also observed a positive correlation between serum TNF-α level with BMI, fasting blood sugar, and serum triglyceride level.

Table 3: TNF-α and Adiponectin level in relation with fatty liver grade.

Variable	Grade I Mean ±SD	Grade II Mean ±SD	Grade III Mean ±SD	Case(n-41) Mean ±SD	Control (n.20) Mean ±SD	p-value
TNF-α	13.40±2.60	19.00±4.82	26.00±3.09	17.29±5.71	8.45±2.60	0.000
Adipo-nectin	3.05±1.39	3.87±1.64	2.67±1.36	3.29±1.52	8.15±5.10	0.000

Serum Adiponectin level.

As noted from table 3 - mean ± SD of serum adiponectin level was significantly low in NAFLD patients compared to the healthy control group (3.29 ± 1.52 vs 8.15 ± 5.10µg/ml, p< .001). Adiponectin level was inversely related to fatty liver grade.When we compared serum adiponectin level with various grade of fatty liver, and control group with one-way ANOVA test, we observed that grade I, grade II and grade III and control group had (3.05±1.39, 3.87±1.64, 2.67±1.36 and 8.15±5.10 µg/ml respectively p-value 0.000). Adiponectin level was statistically significant when compared fatty liver grade with control but when compared within fatty liver grade it was not significant (One-way ANOVA, F value = 10.694, p-value = 0.000, Significant). Serum adiponectin level was negatively correlated with fasting blood glucose, serum triglyceride level, and BMI.

Establishment of cutoff values of TNF–α and Adiponectin in NAFLD.

The receiver operating curve of TNF-α and adiponectin value was built to predict the severity of fatty liver. The best cut-off value of TNF-α obtained by the ROC curve was 11.2 pg/ml for NAFLD (AUROC 0.97, p <0.0001, with 90.24%Sensitivity, 90.05% Specificity, 100%, Accuracy, 91.80%). The best cut off value of adiponectin obtained by ROC curve was < 4.2 µg/ml for NAFLD (AUROC 0.87, p < 0.0001, with % Sensitivity, 80.49% Specificity, 85.00%, Accuracy, 80.32%). Lebensztejn DM found best cut-off value of serum TNF-α was above 2.07 pg/ml with sensitivity of 81% and specificity 67% [20]. Shimada et al. reported that the serum adiponectin was significantly lower in patients with early-stage NASH than in those with simple steatosis. Adiponectin had an AUROC of 0.765, sensitivity of 68%, and specificity of 79% for distinguishing early-stage NASH, using a cutoff value of ≤4.0 µg/mL and cut off value is similar to our study[17]. Other study done by Lucero D. et al. found cut off value for adiponectin < 5.0 µg/ml for grade II and grade III fatty liver[18]. Cut off value for TNF-α and adiponectin may differ from other study because of different company kit may be used.

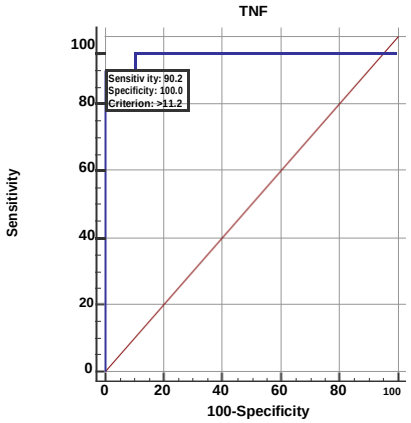

Figure 1: ROC curve of Serum TNF- α for predicting the fatty liver grade.

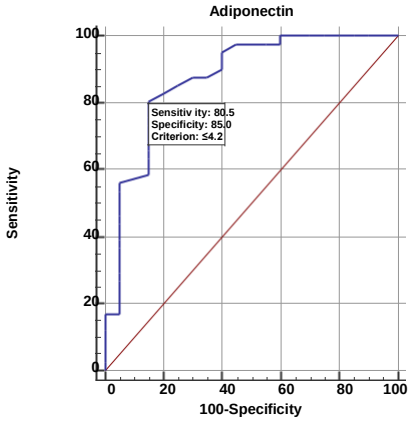

Figure 2: ROC curve of serum level of Adiponectin for predicting the fatty liver severity

Discussion

Nonalcoholic fatty liver disease is emerging as the most common liver disease globally[19]. NAFLD patients are at risk not only for liver-related morbidity and mortality but also for the increased cardiovascular disease risk and increased incidence of diabetes mellitus on long-term follow-up[20]. There is an increased expression of a proinflammatory mediators like adipokines. As adipokines have many actions on insulin resistance and inflammation, they might have important roles in the pathogenesis of NAFLD[6]. The main aim of this study was to examine the correlation between the adipokine levels and grade of fatty liver steatosis in ultrasonographic evaluation. However gold standard method to detect the fatty liver is liver biopsy. As liver biopsy is costly and invasive we used ultrasound to identify and grade the fatty liver. USG is non-invasive technique and easily available, low cost and can be repeated if required. USG abdomen has sufficient specificity and sensitivity to compare with liver biopsy[21].

We have found a significantly higher serum level of TNF-α in NAFLD patients than in healthy control and correlated with USG grade of liver steatosis. Similarly,a study done by Kugelmas et al.[22] and Bahcecioglu et al.[23] on biopsy-proven NAFLD patients, found that plasma TNF-α level was significantly higher in the patient of Nonalcoholic steatohepatitis (NASH) compared to control. J Khurana et al study showed significant correlation of TNF-α (high serum level) and IL-6 with NAFLD, which suggested a proven role of these pro-inflammatory markers in pathogenesis of this disease.[24]Hue et al[25] found that the TNF α level was higher for the NASH subjects compared with controls matched for obesity but was similar between the subjects with NASH and those with simple steatosis.Lebensztejn DM et al[26], done a study on 32 children of fatty liver diagnosed with USG abdomen and found a significant correlation between TNF-α level and ultrasonographic grade of the liver of steatosis. Obese children with NAFLD had significantly higher serum concentrations of TNF-α and TNF-α receptor compared to control. But the level of serum adiponectin was not significantly different in NAFLD and control group. Another study by Khura J et al[27]on 40 NAFLD patients and 40 healthy control patients found serum level TNF-α and IL6 correlated significantly with NAFLD with a p-value of <0.001. But no significant correlation was found with the severity of NAFLD.

Our study demonstrated that patient with NAFLD had a significantly lower level of serum adiponectin compared to the control group and a similar result was found in other studies Bugianesi et al. [28], Paggano et al,[29] and Sargin et al.[30] In one study the levels of plasma adiponectin were significantly higher in females than in males, this observation was supported by Yamamoto et al.[31] who stated that adiponectin concentration may be gender-dependent and found higher in female compared to male. Our study does not show any gender base difference in serum adiponectin level. Serum Adiponectin level was negatively correlated with BMI, fasting blood sugar, and serum triglyceride level in the present study and study done by Bugianesi et al. [28], Paggano et al,[29].

Our study found that NAFLD patients had a higher level of fasting blood glucose, dyslipidemia, and higher BMI. Serum TNF-α level was higher in patients who have higher BMI, higher fasting blood glucose, and hypertriglyceridemia. Moon YS et al [32], and Winkler G

et al [33] have found a similar linear correlation of serum TNF-α and BMI, fasting blood glucose, and hypertriglyceridemia.

Similarly, a negative correlation was found between adiponectin and BMI, fasting blood glucose, dyslipidemia in our study.

Our study has few limitations, first, adipokines were measured at a single point of time, a small sample size,and liver histology was not done.

Conclusions

Our study suggests that TNF-α level was significantly higher in NAFLD patients and its level increased as fatty liver grade increases and higher TNF-α level if NAFLD is associated with metabolic syndrome. Serum adiponectin level was found significantly low in NAFLD patients.We have found a new cut-off value for TNF-α was 11.2pg/ml and adiponectin was 4.2µg/ml. These adipokines could represent markers to evaluate NAFLD pathogenesis and severity of the disease. TNF-α and adiponectin levels can predict the severity of NAFLD in adult patients but required a large sample and liver histology for accuracy.

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Abbreviation-

BMI Body mass index
FBG Fasting blood sugar
IR Insulin resistance
MS Metabolic syndrome
NAFLD Nonalcoholic fatty liver disease
NASH Nonalcoholic steatohepatitis
TNF-α Tumor necrosis factor alpha

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