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Research Article

Evaluation of Pro-Inflammatory Markers IL-6 and TNF-*a* and their Correlation with Non-Alcoholic Fatty Liver Disease

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I N F O

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A B S T R A C T

Background: Non-Alcoholic Fatty Liver Disease (NAFLD) is a condition in which excess fat accumulates in the liver of a patient with no history of alcohol abuse or other causes for secondary hepatic steatosis. The pathogenesis of NAFLD and Non-Alcoholic Steatohepatitis (NASH) has not been fully elucidated. Non-Alcoholic Fatty Liver Disease (NAFLD) consists of a complex spectrum of diseases, ranging from asymptomatic steatosis with possible aminotransferase alterations to Non-Alcoholic Steato-Hepatitis (NASH), cirrhosis, and also hepatocellular carcinoma. Pro inflammatory cytokines like IL-1, IL-6 and TNF-α play a major role in the pathogenesis of NAFLD. These cytokines also play a crucial role in the development of insulin resistance, which is a key factor in the pathogenesis of NAFLD. There is limited data on the association of IL-6 and TNF-α with NAFLD from India. Hence, we aim to assess the correlation of IL-6 and TNF-α with NAFLD.

Materials and Methods: It was a cross sectional observational study which was conducted on 40 cases of NAFLD and 40 healthy controls. All relevant investigations and serum levels of IL-6 and TNF-α were measured. Statistical analysis was done using Pearson chi-square/ fisher exact test, student t-test (un-paired). Pearson correlation test was used to see the relationship between the variables.

Result: The serum levels of IL-6 and TNF-α correlated significantly with NAFLD with a p-value of <0.001. The serum levels of IL-6 showed a significant correlation with the severity of NAFLD (p<0.001), but the same was not seen with TNF-α.

Conclusion: Our study showed significant correlation of TNF-α and IL-6 with NAFLD, which suggested a proven role of these pro-inflammatory markers in the pathogenesis of this disease as shown in past studies. In future target-based therapy is new field of research.

Keywords: Non-Alcoholic Fatty Liver Disease (NAFLD), Non-Alcoholic Steatohepatitis (NASH), Interleukin-6(IL-6), Pro-Inflammatory Markers, Tumor Necrotic Factor- α (TNF-α)

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Introduction

The global prevalence of Non-Alcoholic Fatty Liver Disease (NAFLD) is on the rise, and it is one of the most common cause of asymptomatic transaminitis. As per the American Association for the Study of Liver Disease, NAFLD is defined as the presence of hepatic steatosis, either by histology or by imaging and the absence of any secondary cause of hepatic steatosis such as significant alcohol consumption, use of steatogenic medication or hereditary disorders.[1] Nowadays NAFLD is becoming the most common cause of chronic liver disease. It is because of the increasing prevalence of obesity, diabetes, heart disease, metabolic syndrome, with which NAFLD has a close proven relationship.[2] The estimated prevalence of NAFLD in the general US population is currently in the range of 20%,[3-5] and that of non-alcoholic steatohepatitis (NASH) is about 3.5-5%.[6,7] A study from coastal region of India found that 39 (24.5%) of 159 healthy attendants of patients had evidence of fatty liver on Ultrasound[8] while in another study in Mumbai the overall prevalence was found out to be 16.6%, being higher in males as compared to females.[9]

The most common underlying mechanism is believed to be insulin resistance. Pro-inflammatory markers IL-1, IL- 6 and TNF-α play major role in the pathogenesis of NAFLD by promoting both liver fibrosis and insulin resistance.[10] They activate both the Kupffer cell and the stellate cell to produce inflammation and fibrosis. They contribute to development of insulin resistance by impairing the insulin receptor signaling. Among patients with NAFLD more than 80% have isolated fatty liver disease while less than 20% have NASH. Those patients with NASH may go on to develop cirrhosis later, which subsequently leads to decompensation or development of Hepatocellular Carcinoma (HCC), hence increasing the mortality.[11] There is a paucity of data from India, so we also conducted this study to find a correlation between the level of proinflammatory markers like IL-6 and TNF-α, as they have a clear role in its pathogenesis. Moreover, it may also have a therapeutic implication in future and potential for research in terms of target-based therapy for NAFLD.

Materials and Methods

The study was conducted at department of Medicine with also involvement of department of Radiology, Gastroenterology and Biochemistry at PGIMER (Post Graduate Institute of Medical Education and Research) and Dr. RML Hospital, New Delhi from 1st November 2015 to 31st March 2017 after getting approval from Institutional Review Board (IRB) and Institutional Ethical Committee (IEC). It was a cross sectional observational type of study. Total of 40 cases of NAFLD, Diagnosed by Ultrasonography (USG) abdomen and 40 healthy controls were recruited after written informed consent. Following were the inclusion criteria; (i) patients with NAFLD diagnosed on the basis of USG abdomen (ii) age 18years to 70 years. Exclusion criteria were as follows : (i) acute or chronic Hepatitis B or C infection, (ii) Biliary disease, (iii) Chronic alcohol consumption (>20g/day),[12] (iv) Wilson's disease, (v) Hemochromatosis, (vi) chronic use of Drugs causing steatosis like Amiodarone, Tamoxifen, Steroids, Methotrexate, Zidovudine, Valproic acid, carbamazepine, estrogen within the past 3 months or for >6 months in the past 2 years, (vii) Rapid weight loss in past 6 months, (viii) Total parenteral nutrition, (ix) Chronic gingivitis, odontitis, (x) History of autoimmune diseases like rheumatoid arthritis (RA), Systemic Lupus Erythematosus (SLE), Connective Tissue Disease (CTD).

Methodology

The patients were evaluated as per the standard protocol specially concentrating on age, gender, detailed history, presence or absence of risk factors, socio-economic status. Physical examination was done which included height, weight, Body Mass Index (BMI), Waist to Hip Ratio (WHR), Blood Pressure (BP) measurement.

Fasting blood samples of all the patients were collected in pyrogen/ endotoxin free collecting tubes for the determination of biochemical tests like LFT, Lipid profile, IL-6, TNF-α, and all other relevant routine investigations. Following clotting, the samples were centrifuged at approximately 1000 rpm for 10 minutes and the serum was separated. Serum samples for IL-6 and TNF-α were aliquoted (250-500 µl) to avoid repeated freeze-thaw cycles and stored frozen at -70 °C. IL-6 was measured using Human IL-6 Enzyme Linked Immunosorbent Assay (ELISA) kit (Lot No: I60117-3) manufactured by KRISHGEN BioSystems on EVOLIS TWIN PLUS. TNF-α was measured using Human TNF-α ELISA kit (Cat. No: 950.090.096, Batch no: 1100-103) manufactured by Diaclone on EVOLIS TWIN PLUS. Diagnosis of NAFLD was done by USG. Scoring of NAFLD on USG was done according to Ultrasonographic Steatosis Score (USS)[13, 14] as follows: a. absent (score 0) steatosis is defined as normal liver echotexture b. mild (score 1) steatosis as slight and diffuse increase in fine parenchymal echoes with normal visualization of diaphragm and portal vein borders c. moderate (score 2) steatosis as moderate and diffuse increase in fine echoes with slightly impaired visualization of portal vein borders and diaphragm d. severe (score 3) steatosis as fine echoes with poor or no visualization of portal vein borders, diaphragm, and posterior portion of the right lobe. Other special biochemical investigations like, HBsAg, anti HCV and ANA also were done to rule out associated viral infections or autoimmune etiology.

Statistical Analysis

Categorical data was described as number of patient (n) and compared using Pearson chi-square/ fisher exact test. Comparison of mean values between two groups was done by using Student t-test (un-paired). Pearson correlation test

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was used to see the relationship between the variables. One-way ANOVA applied to see the mean difference between more than 2 groups. Statistical Package for Social Sciences (SPSS) version 16.0 was used for statistical analysis. 'p' value of <0.05 was considered statistically significant at 95% confidence level.

Result

Our study was conducted on 40 confirmed cases of NAFLD consisting of 22 males (55%) and 18 females (45%) and 40 controls consisting of 23 males (57%) and 17 females (43%). The mean age of cases was 56.20±7.23 years and that of control was 56.85±8.07 years. So, there wasn't any significant difference between the ages of cases and control. 21 cases (53%) were obese with BMI > 25 kg/m^2, 6 cases (15%) were overweight with BMI of 23-24.9 kg/m^2 and rest (32%) had normal BMI (18-22.9 kg/m^2). 25 controls (62%) had normal BMI (18-22.9 kg/m^2). Only 4 controls (10%) were obese and 11 controls (28%) were overweight. It was found that cases had significantly higher BMI (p<0.001) as compared to controls. Total 8 cases (20%) were showing asymptomatic transaminitis while no transaminitis observed in control group.

The baseline characteristics of cases and controls have been shown in Table 1.

Table 1.Base line characteristics of the study population

	Group	N=40	Mean	Std. deviation	t-value	p-value
Age (years)	Cases	40	56.20	7.23	0.38	0.705
	Control	40	56.85	8.07		
BMI (kg/m^2)	Cases	40	24.60	2.99	4.754	<0.001
	Control	40	21.90	2.00		
SBP (mmHg)	Cases	40	141.00	11.51	5.337	<0.001
	Control	40	129.55	7.18		
DBP (mmHg)	Cases	40	85.50	5.87	5.512	<0.001
	Control	40	78.50	5.49		
T. Bil (mg/dl)	Cases	40	0.86	0.40	1.993	0.05
	Control	40	0.73	0.18		
D. Bil (mg/dl)	Cases	40	0.26	0.20	0.9	0.371
	Control	40	0.23	0.10		
I. Bil (mg/dl)	Cases	40	0.60	0.22	2.562	0.012
	Control	40	0.50	0.14		
SGOT (U/L)	Cases	40	25.15	13.83	0.557	0.579
	Control	40	23.83	5.92		
SGPT (U/L)	Cases	40	24.55	13.03	0.067	0.947
	Control	40	24.40	5.43		
Total Chl (mg/dl)	Cases	40	272.48	31.57	13.074	<0.001
	Control	40	189.45	24.82		
TG (mg/dl)	Cases	40	234.12	26.41	13.844	<0.001
	Control	40	155.25	24.51		
WHR	Cases	40	0.93	0.25	4.22	<0.001
	Control	40	0.74	0.14		
Hb (g/dl)	Cases	40	13.19	0.76	1.494	0.139
	Control	40	13.44	0.72		
Urea (mg/dl)	Cases	40	25.38	4.89	2.333	0.022
	Control	40	28.13	5.63		
Creatinine (mg/dl)	Cases	40	0.81	0.14	0.566	0.573
	Control	40	0.82	0.14		

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Other characteristics like WHR, BP and lipid profile also had significant correlation with NAFLD as compared to controls with p-value of <0.001 each. 21 cases (52%) were hypertensive while only 6 controls (15%) were hypertensive suggesting a significant association of HTN with NAFLD with p<0.001. Central obesity as defined by WHR >0.9 for males and WHR >0.8 for females. The mean WHR of cases was 0.93±0.25 and that of control was 0.74±0.14 suggesting that WHR was significantly higher in cases (p<0.001) as compared to controls. Hence the prevalence of central obesity is more in NAFLD as compared to healthy controls. 24 cases (60%) were diabetic while only 7 controls (17%) were diabetic and the rest were non-diabetic. It was found that DM was significantly associated with NAFLD with p<0.001. The mean total cholesterol and TG values were higher in patients with NAFLD (272.48±31.57 and 234.12±26.41 respectively) as compared to controls (189.45±24.82 and 155.25 ± 24.51 respectively) with p<0.001.

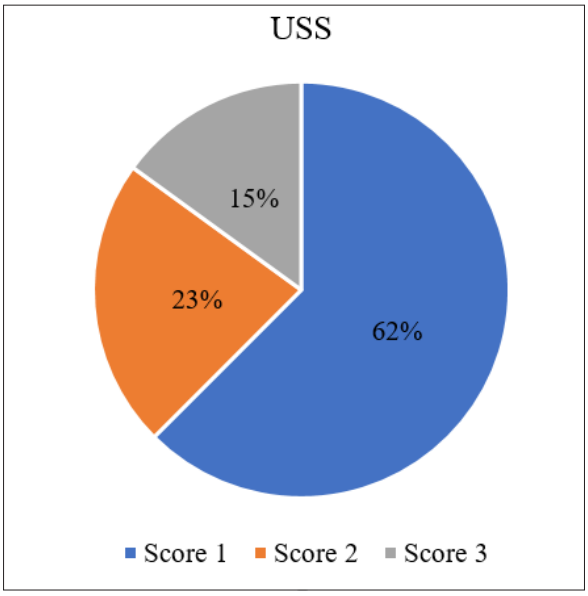
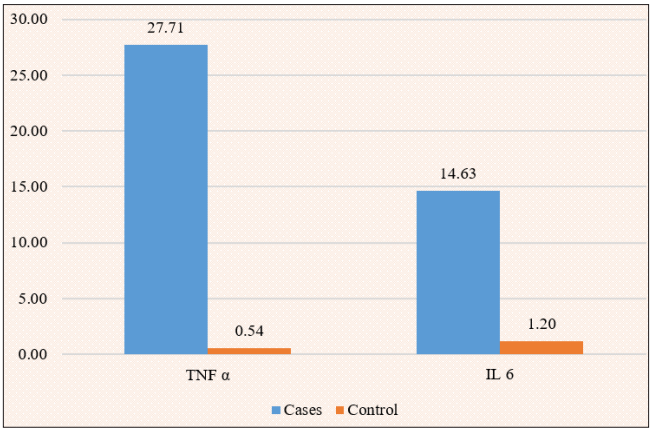


Figure 1.Ultrasonographic Steatosis Score (USS) distribution in cases

Figure 1, shows that 25 cases (62%) had USS score 1, 9 cases (23%) had score 2 and rest (15%) had score 3. All the controls had USS score 0.

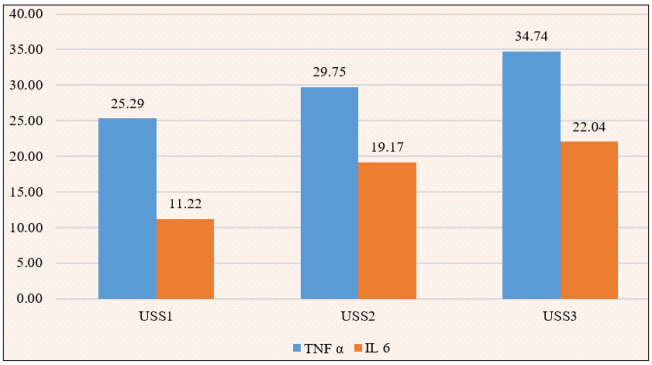
Figure 2, shows that the mean value of TNF α is 27.71±10.56, which is significantly higher (p<0.001) than in controls in which it is 0.54±0.51. Similarly, the mean value of IL-6 is significantly higher in cases, i.e. 14.63±7.5 (p<0.001) as compared to controls (1.20±0.64).

Figure 3 shows that cases with USS score 1, 2 and 3 had mean IL-6 values of 11.22±7.22, 19.17±3.98 and 22.04±1.64 respectively and mean TNF-α values of 25.29±11.45, 29.75±8.24, 34.74±5.95 respectively. The serum levels of IL-6 correlated significantly with the severity of NAFLD (p<0.001) but the same was not observed with the serum levels of TNF-α. Our study results showed that there is significant correlation of IL-6 and TNF-α with NAFLD and also significant correlation of IL-6 with the severity score (USS) of NAFLD.



(Y axis=serum levels of TNF-α and IL-6 expressed in unit pg/ml)

Figure 2.Serum levels of TNF-*α* and IL-6 in study population



(Y axis = serum levels of TNF-α and IL-6 expressed in unit pg/ml)

Figure 3.Correlation of TNF-*α* and IL-6 with the severity of NAFLD

Discussion

NAFLD is the major cause of asymptomatic abnormal liver function test and it is often associated with obesity and type 2 diabetes mellitus. One of the important pathophysiological features in NAFLD is insulin resistance which is often associated with chronic low-grade inflammation and develops as a result of numerous inflammatory mediators released from immune cells and adipocytes. Increased expression of proinflammatory cytokines like IL-1, IL-6 and TNF-α is the characteristic feature of adipose tissue inflammation. It has been seen that in fatty liver disease, the expression of these proinflammatory cytokines in adipose tissue is 100-1000 folds higher than the expression level in liver.[15]

Several previous studies have shown that TNF-α is a key factor in the development of NAFLD and NASH in humans.

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Hui JM et al.[16] quantified the serum levels of TNF-α in patients with biopsy proven NAFLD and found out that its level was significantly higher in patients with NASH as compared to controls. Chu CJ et al.[17] conducted a study with 144 patients; plasma levels of TNF-α were measured with a commercially available solid phase sandwich enzyme-linked immunosorbent assay. Mean plasma level of TNF-α was found to be significantly higher in NAFLD patients than controls (2.63±0.44 pg/mL vs. 1.56±0.10 pg/mL, p=0.016), thus concluding that TNF-α may play a role in the pathogenesis of NAFLD as shown by our study data also.

Another study conducted by Tarantino G et al.[18] showed that the NASH group had significantly higher IL-6 than the other groups (P=0.0001) included in the study. A study conducted by Kumar R et al.[19] showed that the serum levels of all the pro-inflammatory cytokines (TNF-α, IL-1, and IL-6) were significantly (P<0.001) higher among NAFLD patients compared to control groups.

At a molecular level, exposure of cells to TNF-α or elevated levels of Free Fatty Acids (FFAs) stimulated inhibitory phosphorylation of serine residues of IRS-1[20, 21] and activates IKKβ/ NF-κB and JNK pathways, two major intracellular regulators of IR. Moreover, TNF-α antagonizes adiponectin, an important insulin-sensitizing adipocytokine that signals via adiporeceptors, thus promoting insulin resistance further.[22] IL-6 signaling involves the Janus kinase - signal transducer and activator of transcription 3 (JAK - STAT3) pathway. IL-6 can induce SOCS1 and SOCS3 that link IRS to ubiquitin mediated degradation, thereby promoting insulin resistance. Proinflammatory cytokines were positively correlated with anthropometric parameters (BMI and waist circumference). The same positive correlation between TNF-α and BMI was noticed previously, also shown in our present study.

Conclusion

There has been a remarkable scientific effort to improve our understanding of the pathogenesis of NAFLD characterized by a marked activation of inflammatory cells and the up regulation of several soluble inflammatory mediators. Among several mediators, cytokines and chemokines might play a pivotal active role in NAFLD. In our study, we found correlation between WHR, BMI, diabetes mellitus and level of TNF-α and IL-6 with steatosis in NAFLD. Our study also suggested significant correlation of TNF-α and IL-6 with NAFLD and hence suggested a role of these pro-inflammatory markers in the pathogenesis of this disease. The involvement of cytokines and chemokines and their receptors in the pathogenesis of NAFLD is only partially understood and it is increasingly acknowledged that the gut microbiota might play a vital role in the disease process. Nevertheless, there is a strong association of inflammatory cytokines with NAFLD and hence future treatment strategy should focus on targeting inflammatory pathways. Proinflammatory cytokines could represent markers to evaluate changes in liver functions and severity of the disease and to predict the risk for progression. More research remains to be done to understand the complete pathophysiology and future target therapy in patients with NAFLD to prevent the morbidity and associated complications.

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Conflicts of Interest: None

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