Research Article

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Hepatic and Systemic Effect of Non-Alcoholic Fatty Liver Disease Severity in Obese and Non-Obese Indian Patients

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Abstract

Background: Non-alcoholic fatty liver disease (NAFLD) is now predominant globally due to increased sedentary lifestyle and obesity. Recently, high prevalence of NAFLD also has been documented in non-obese individuals with increased risk of cirrhosis and hepatocellular carcinoma. The systemic and hepatic manifestations of NAFLD severity in obese and non-obese Indian patients are not clear.

Methods: The clinically diagnosed NAFLD patients (n=54, non-obese and obese) were assessed for liver injury and hepatic fat content by histopathology, Fibroscan and MRS. Liver biopsy and hepatic venous sampling were performed by trans-jugular approach and mRNA expression was assessed by real-time PCR.

Result: High liver fat content (LFC, 20.4 ± 10.4%, 16 ± 11.5% and 9.34 ± 15.4%) and increased abdominal obesity (WHR, 1.03 ± 0.06, 0.97 ± 0.05 and 0.93 ± 0.06) was observed in both obese and non-obese NAFLD patients as compare to disease control. Histopathological examination of liver indicated increased fibrosis (grade ≥ 1) in both obese (76%) and non-obese (64%) group. Significant increased levels of LBP, MDA and adipokines levels (p < 0.001) were observed in hepatic and systemic circulation of obese and non-obese groups than healthy and diseased controls. A positive correlation of biomarkers for liver injury was found between hepatic and systemic circulation. Hepatic gene expression of adipokines and cytokines also corroborated this trend among groups.

Conclusion: The extent of liver injury is quite high in both non-obese and obese NAFLD patients. The drivers of injury in these patients are due to hepatic fat and SIBO induced endotoxin mediated up-regulation of proinflammatory adipocytokines and oxidant stress in liver.

Keywords: NAFLD, Obesity, Insulin resistance, Adipokine, Cytokines, Oxidative stress

Introduction

Non-alcoholic fatty liver disease (NAFLD) is now

pandemic, with a population prevalence ranging from 10-35% [1]. The NAFLD patients are primarily obese

associated with insulin resistance (IR) [2]. Recently, increased incidences of NAFLD were reported in non-obese or lean individuals (with normal BMI) in population of Asian origin [3,4]. In rural India, non-obese NAFLD has been documented in patients those are neither overweight nor centrally obese [4]. Lean NAFLD also has been described from other parts of the world [5]. Lean NAFLD like obese patients also develops problematic sequels like steato-hepatitis, fibrotic disease, cirrhosis and HCC [6].

It has also been documented that small intestinal bacterial overgrowth (SIBO), an association with NAFLD may be more prevalent among the Asians [4,7]. The SIBO induced endotoxins, which reaches liver through portal circulation might be inducing pro-inflammatory cytokines in liver from Kupffer's cell or Hepatic Stellate Cell (HSC) or sinusoidal endothelial cells [8]. The source of proinflammatory cytokines in obese patients have been documented to be the macrophages in peripheral adipose tissues [9] which lean NAFLD patients do not possess. The hepatic milieu of non-obese NAFLD patient may be different than obese NAFLD patient and the biomarkers for severity of NAFLD can be better represented in hepatic than systemic circulation. The adipose tissue of obese patients exerts substantial systemic effect and also NAFLD severity. Progression from Non-alcoholic Fatty Liver (NAFL) to Non-alcoholic Steatohepatitis (NASH) even though unclear, is thought to be induced by secondary hits such as proinflammatory adipo-cytokines, lipotoxicity, or oxidative stress [10]. In our earlier reported pilot study, significantly high levels of IL-6 cytokine were observed among lean NAFLD patients in comparison to controls (CHB and obese NAFLD) possibly due to SIBO induced endotoxins [11]. Whether oxidative stress, antioxidants and adipokines have similar association in lean as in obese NAFLD is also not clear.

Therefore, the present study was under taken to assess differences in hepatic fat content and clinical spectrum of NAFLD by Magnetic Resonance Spectroscopy (MRS), USG, fibroscan and histopathological examination in obese and non-obese NAFLD patients. The extent of liver injury was correlated with circulatory biomarkers of oxidant stress, antioxidants, proinflammatory adipo-cytokines and endotoxin induced lipopolysaccharide binding protein (LBP) in hepatic and systemic circulation. The hepatic gene expression of LBP and proinflammatory cytokines was confirmed in transjugular liver biopsy tissue. Anthropometric parameters, insulin resistance and SIBO were compared in lean and obese NAFLD patients.

Methods

Patients and definitions

Institute ethics committee approval (IEC/NP-87/2012, RP13/2012). This prospective observational study was carried out in tertiary care setting at the Department of Gastroenterology, All India Institute of Medical Sciences, New Delhi, India during November 2012 to January 2015 after obtaining institute ethics committee approval. All consecutive NAFLD patients and chronic hepatitis B (CHB) as disease control attending liver clinics were recruited as per inclusion and exclusion criteria. The included NAFLD patients of age > 18 years were initially screened by ultrasonography (USG) and confirmed by liver histology (involvement of > 5% of hepatocytes by steatosis and NASH was defined by NAS-CRN score $\geq$ 5) [12]. The CHB patients with minimum 1.5 times elevated ALT and without any evidence of fatty liver on histology, were included as disease controls. Healthy individuals matched for age, sex and BMI with normal liver function tests and without any evidence of fatty liver (on USG), diabetes mellitus or alcohol consumption or any other comorbid diseases, were included as healthy controls.

The exclusion criteria for patients were use of known fatty liver causing drug, anti-tuberculosis drugs and patients with diabetes mellitus, alcoholics or pregnant females. In addition, the patients with other etiologies of chronic liver disease such as hepatitis C, Wilson's disease, autoimmune liver disease, alpha 1 anti-trypsin deficiency and hemochromatosis were also excluded.

Patient evaluation and clinical investigations

The clinical, demographic and anthropometric (weight, height, waist and hip circumferences) were recorded. As per WHO recommendations, BMI cut off $\geq$ 25 kg/m² for Asian populations was considered as criteria for obesity [13]. The abdominal obesity was determined using recommended cut off for waist circumference (> 90 cm for male and > 85cm for female) and the waist hip ratio (> 0. 90 for male and > 0. 85 for female). The hepatic fat content of NAFLD and CHB patients were assessed by magnetic resonance spectroscopy [14]. Ultrasonography of abdomen and liver in NAFLD patients were carried out as per standard protocol [15]. Overnight fasting blood samples were collected for biochemical investigations. Insulin resistance (HOMA-IR) was determined by taking consideration into fasting serum insulin and fasting plasma glucoses [16]. Hypertension, Type 2 diabetes mellitus, and dyslipidemia were defined as per accepted WHO criteria [17].

Glucose hydrogen breath test (GHBT)

For GHBT, breath hydrogen in parts per million (ppm) was estimated every 15 min for 2 hour following ingestion of 70 g glucose (dissolved in 200 ml water). An increase in breath hydrogen by at least 12 ppm above the basal level or an average value of basal breath hydrogen ≥ 20 ppm following glucose administration was considered positive for SIBO [18].

Hepatic venous sampling and trans-jugular liver biopsies

The hepatic venous blood sampling was performed in NAFLD patients and CHB controls through trans-jugular approach [19]. The trans-jugular biopsy samples in parts were both formalin-fixed and snap frozen for histopathological examinations and assessed for hepatic gene expression.

Enzyme Linked Immunoassay (ELISA)

Detection of LBP and leptin in serum was done by sandwich ELISA; whereas detection of other adipokines (Visfatin, Resistin and Adiponectin) was carried out by competitive ELISA using commercial kit as per manufacturer's instructions (Ray biotech Inc, Norcross, USA).

Oxidant and antioxidant measurement

Lipid peroxidation was assessed colorimetrically by measuring plasma malondialdehyde (MDA). Total antioxidant capacities were measured as ferric reducing ability of plasma (FRAP), by the methodology given by Benzie and Strain [20]. Superoxide dismutase (SOD) was estimated by the 50% inhibition of auto-oxidation of pyragallol at pH 8.0 [21]. Plasma vitamin C was measured by dinitrophenyl hydrazine method [22].

Relative gene expression

Total RNA was isolated from the liver biopsy samples by using SV Total RNA Isolation System (Promega Corporation, Madison, USA). The cDNA was prepared using Go Script Reverse transcription system (Promega Corporation, Madison, USA). Real time PCR for gene expression was carried out by using SYBR green chemistry. The primers for real time PCR were designed (Table 1) from NCBI reference sequences for genes such as *GAPDH*, *ADIPONECTIN*, *LEPTIN*, *LBP*, *IL6*, *IL1B*, *TNF α* using bioinformatics software. The relative expression of LBP, proinflammatory cytokine and adipokine genes in liver

of non-obese and obese NAFLD patients as compared to CHB disease control were analyzed by comparative Ct method using *GAPDH* gene as endogenous normalization control.

Statistical analysis

Normally distributed continuous variables were expressed as mean $\pm$ standard deviation (SD) and categorical variables were expressed as percentages. Continuous variables were compared by student *t*-test or ANOVA test and categorical variables were compared by chi-square test and *p* value of < 0.05 was considered to be significant. Associations between variables were performed using Spearman's correlation coefficients.

Result

Patient recruitment and demographic profile

Out of 140 clinically diagnosed NAFLD cases during the study period, 86 patients were excluded due to diabetes ($n=13$), cirrhosis ($n=2$), drugs (methotrexate and anti-tuberculosis drug, $n=2$) and refusal to consent ($n=69$). A total of 104 subjects were studied including 54 NAFLD patients and 50 controls (25 CHB and 25 healthy). The demographic and baseline clinical profiles of controls and patients are depicted in table 2. The BMI, waist hip ratio (WHR), LDL, serum insulin levels, HOMA-IR and ALT levels were significantly ($p<0.001$) higher in NAFLD patients as compared to both control groups (Table 2). Metabolic syndrome criteria [23] were fulfilled in 18.51% of NAFLD patients ($n=10$).

Obesity among NAFLD patients

Using BMI cut off (> 25 kg/m²) values, 53.7% (29/54) were obese and rest 46.3% (25/54) was non-obese or lean NAFLD. Abdominal obesity was documented in 72.23% ($n=39/54$) and 94.44% ($n=51/54$) cases of NAFLD patients on the basis of waist circumference and WHR, respectively. The BMI, waist circumference and the WHR values were significantly higher in NAFLD than in CHB as well as healthy controls (Table 2).

Insulin resistance, hypertension and SIBO prevalence among NAFLD, CHB and healthy control

Insulin resistance (HOMA-IR > 2.5) was observed in 21/54 (38.8%) of NAFLD, 2/25 (8%) in CHB control and none in healthy control. IR was significantly correlated with obese NAFLD ($p = 0.02$, OR=3.57, 95% CI=1.11-11.47) as evidenced by higher proportion of IR in obese ($n=15$,

Table 1: Primer sequence of housekeeping genes.

Sl.No.	Gene Name	Primer code	Primer Sequence (5'-3')	Amplicon size
1	GAPDH	299F	CATTGACCTCAACTACATGGTTTAC	124bp
		423R	CTGGAAGATGGTGATGGGATT	
2	ADIPONECTIN	345F	CAGTGTGGGATTGGAGACTTAC	104bp
		449R	ATTTACCAGTGGAGCCATCATAG	
3	LEPTIN	226F	GTCACCGGTTTGGACTTCAT	114bp
		340R	CACGTTTCTGGAAGGCATACT	
4	LBP	861F	TTCATCGTAACCACCGTTCTC	115bp
		976R	CTGGCCGTGTTGAAGACATA	
5	IL6	492F	CTAGAGTACCTCCAGAACAGATT	92bp
		584R	TGCATCTAGATTCTTGCCTTT	
6	IL1B	172F	CCTAAACAGATGAAGTGCTCCT	81bp
		253R	TGAAGCCCTTGCTGTAGTG	
7	TNF α	440F	GTAGCCCATGTTGTAGCAAAC	84bp
		524R	CTGGTTATCTCTCAGCTCCAC	

Table 2: Demographic parameters in lean and obese NAFLD and CHB Patients and Healthy subjects.

Sl. No.	Parameters	1-Non-obese (<25 BMI)	2-Obese (>25 BMI)	3-CHB	4-Healthy Control	p-value*
1	BMI	23.19 $\pm$ 2.113	28.97 $\pm$ 3.111	21.08 $\pm$ 2.482	20.19 $\pm$ 2.903	0.000
2	Waist (cm)	88.41 $\pm$ 7.308	101.34 $\pm$ 8.226	82.00 $\pm$ 8.784	79.38 $\pm$ 7.154	0.000
3	Hip (cm)	90.44 $\pm$ 5.184	97.62 $\pm$ 5.274	87.56 $\pm$ 6.035	86.13 $\pm$ 9.351	0.000
4	Waist: Hip	0.97 $\pm$ 0.05	1.03 $\pm$ 0.06	0.93 $\pm$ 0.06	0.92 $\pm$ 0.07	0.000
5	Total Bilirubin	0.59 $\pm$ 0.501	0.66 $\pm$ 0.553	0.68 $\pm$ 0.627	0.75 0.447	0.828
6	AST	45.33 $\pm$ 29.19	59.59 $\pm$ 53.50	53.88 $\pm$ 24.90	27.81 $\pm$ 7.42	0.035
7	ALT	77.74 $\pm$ 66.38	94.41 $\pm$ 91.68	81.64 $\pm$ 31.31	28.81 $\pm$ 6.76	0.012
8	ALKP	238.56 $\pm$ 60.78	214.03 $\pm$ 67.67	238.00 $\pm$ 75.98	205.94 $\pm$ 59.1	0.255
9	Total Protein	7.70 $\pm$ 0.60	7.76 $\pm$ 0.43	7.80 $\pm$ 0.40	7.63 $\pm$ 0.50	0.707
10	Albumin	4.93 $\pm$ 0.78	4.76 $\pm$ 0.57	4.84 $\pm$ 0.62	4.56 $\pm$ 0.51	0.336
11	Fasting sugar	93.37 $\pm$ 8.85	97.17 $\pm$ 8.38	85.33 $\pm$ 7.98	83.56 $\pm$ 7.43	0.000
12	PP sugar	128.15 $\pm$ 18.13	136.91 $\pm$ 14.45	113.70 $\pm$ 16.83	129.57 $\pm$ 9.82	0.004
13	Triglyceride	144.77 $\pm$ 59.85	179.00 $\pm$ 98.39	101.32 $\pm$ 39.48	127.13 $\pm$ 30.34	0.001
14	Cholesterol	181.15 $\pm$ 39.07	203.17 $\pm$ 38.22	157.68 $\pm$ 39.14	153.47 $\pm$ 9.98	0.000
15	LDL	105.38 $\pm$ 30.89	123.34 $\pm$ 31.89	94.68 $\pm$ 33.81	84.60 $\pm$ 9.78	0.000
16	HDL	41.69 $\pm$ 8.13	44.48 $\pm$ 3.47	41.09 $\pm$ 4.13	44.13 $\pm$ 2.44	0.067
17	VLDL	28.23 $\pm$ 11.53	39.97 $\pm$ 27.61	21.91 $\pm$ 10.32	27.53 $\pm$ 10.47	0.005
18	Serum Insulin	8.73 $\pm$ 5.93	13.21 $\pm$ 12.96	5.04 $\pm$ 3.45	3.50 $\pm$ 1.63	0.000
19	TLC	7637.0 $\pm$ 2238.6	7637.0 $\pm$ 2238.6	7232.0 $\pm$ 2069.7	7468.7 $\pm$ 1591.5	0.065

51.7%) than non-obese NAFLD (n=6, 24%). However, the fasting serum Insulin levels were significantly higher in both obese and non-obese NAFLD than both healthy and CHB controls (Table 2). Hypertension was observed in 8 % (n=2/25) of non-obese and 17.2% (n=5/29) of obese NAFLD patient. The SIBO prevalence was observed in

(n=6/54) 11.11% of NAFLD patients and no SIBO in CHB and healthy control ($\chi^2=8.815$, p=0.03) by GHBT. SIBO prevalence was less (4%) in non-obese than obese (17.2%) NAFLD patients and not found statistically significant (p=0.12).

Fatty liver and its severity in NAFLD patients by MRS, USG and Fibroscan

The mean liver fat content (LFC) by MRS was $20.44 \pm 10.48\%$, $16 \pm 11.52\%$ and $9.34 \pm 15.4\%$ for obese, non-obese NAFLD and CHB patients, respectively (Figure 1B). Abnormal accumulation of liver fat ($> 5.56\%$ LFC) was observed equally high in significant proportion of obese (93.34%) and non-obese (92.86%) NAFLD patients than CHB (28.5%) control (Figure 1A). Healthy controls were not subjected to MRS due to lack of detection of fatty liver by USG. Increasing spectrums of fatty liver grade were observed in obese (Grade I: 37.94%, II: 55.17% and III: 6.89%) than non-obese (Grade I: 64%, II: 28% and III: 8%) NAFLD patients ($p = 0.0001$) by USG. No fatty liver (Grade 0) was observed in 92% ($n=23/25$) and 100% ($n=25/25$) in CHB disease and healthy control (Figure 1C). In Fibroscan, higher LSM score (>7 KPa) was observed in obese (37.9%, $n=11/29$) than non-obese (16%, $n=4/25$) NAFLD patients. The fibrosis staging among groups were as follows: non-obese (F2:8% and F3:8%), obese NAFLD (F2: 13.79%, F3:24.13%) and CHB control (F2:28%, F4: 12%) ($p = 0.02$) (Figure 1D).

NAFLD severity in liver biopsy by histopathological examination

The inflammation grades which include no inflammation (NAFLD activity score, NAS < 3), mild NASH (NAS $\geq 3-4$) and confirmed NASH (NAS ≥ 5) were found in 11.11% ($n=6/54$), 48.15% ($n=26/54$) and 40.74% ($n=22/54$) cases respectively in NAFLD patients. The fatty liver with inflammation (mild and confirmatory NASH) were equally observed in non-obese (56%, $n=14/25$ and 32%, $n=8/25$) and obese (41.38%, $n=12/29$ and 48.28%, $n=14/29$) NAFLD patients (Figure 1C). In CHB controls, minimal inflammation (HAI $\geq 3-4$) was 68% ($n=17/25$) and mild inflammation (HAI $\geq 5-8$) was observed in 32% ($n=8/25$) of cases (Figure 1E).

In NAFLD patients, the stages of fibrosis as per the NASH CRN staging criteria were F0 (no fibrosis) in 29.63% ($n=16/54$), F1 (mild fibrosis) in 48.15% ($n=26/54$), F2 (moderate fibrosis) in 12.96% ($n=7/54$) and F3 (severe fibrosis) in 9.26% ($n=5/54$) cases. In non-obese, obese and CHB controls, F0 was 36% ($n=9/25$), 24.13% ($n=7/29$) and 32% ($n=8/25$) cases; F1 was 48% ($n=12/25$), 48.28% ($n=14/29$) and 44% ($n=11/25$); F2 was 4% ($n=1/25$), 20.68% ($n=6/29$) and 16% ($n=4/25$); F3 was 12% ($n=3/25$), 6.89% ($n=2/29$) and 0 cases; F4 (cirrhosis) was observed in 8% ($n=2/25$) CHB disease control only (Figure 1F).

LBP as surrogate marker for SIBO in NAFLD patient

The upper limit of normal LBP was considered 13 $\mu\text{g/ml}$ based on the value of healthy control which was reported 10 $\mu\text{g/ml}$ earlier [24]. Significantly higher LBP levels was observed among NAFLD groups (hepatic and Systemic: 25.93 ± 15.8 $\mu\text{g/ml}$, 24.07 ± 14.7 $\mu\text{g/ml}$ in non-obese and 18.23 ± 14.90 and 17.50 ± 15.87 $\mu\text{g/ml}$ in obese) than CHB (6.91 ± 7.27 and 5.34 ± 3.72 $\mu\text{g/ml}$) and healthy (systemic: 13.01 ± 5.49 $\mu\text{g/ml}$) control ($p=0.0001$). Higher prevalence of increased hepatic and systemic LBP was found in both non-obese (56%, $n=14/25$ and 60%, $n=15/25$) and obese (41.3%, $n=12/29$ and 44%, $n=13/29$) NAFLD group as compared to CHB (12%, $n=3/25$ and 0%) and healthy (systemic: 20%, $n=5/25$) control ($p = 0.009$; 0.001 in hepatic and systemic). The systemic and hepatic LBP was positively correlated ($r=0.826$ and 0.774 , $p = 0.001$ and 0.0001 in non-obese and obese NAFLD).

Comparison of hepatic and systemic oxidative stress, adipokine and cytokine level among groups

The MDA level in hepatic and peripheral blood was significantly ($p = 0.0007$) higher in NAFLD group than CHB and healthy control (Table 3). Total antioxidants (FRAP), Vitamin C level of NAFLD and CHB control was significantly less than healthy control. The SOD level was identical among groups (Table 3). The hepatic level was positively correlated with systemic level ($r=0.812$, 0.627 , 0.8 for MDA, FRAP and Vitamin C, $p=0.001$). The pro-inflammatory adipokine, leptin (both hepatic and systemic) was significantly ($p \leq 0.01$) higher in obese and non-obese NAFLD than CHB and healthy control (Table 4). Lower levels of adiponectin were observed in obese and CHB groups (hepatic: 83.11 ± 74.03 $\mu\text{g/ml}$, and systemic: 64.63 ± 75.49 $\mu\text{g/ml}$) whereas equivalent levels were observed in non-obese (175.77 ± 170.52 $\mu\text{g/ml}$) and healthy control (systemic: 173.46 ± 156.05 $\mu\text{g/ml}$) group. Increased visfatin level was observed in non-obese NAFLD than CHB and healthy control; however equivalent levels were found in obese and control group (Table 4). Increased level of cytokine TNF- α and IL-6 was found in both obese and non-obese group than and healthy control. Comparatively low level of IL-1b was observed among all the groups (Table 5).

Figure 1: Fatty Liver and disease severity in NAFLD patients as compare to CHB disease and healthy control. Percentage of non-obese, obese NAFLD and CHB patients showing hepatic fat content above cut-offs (LFC-5.56%) in MRS (A) and mean hepatic fat (B). Fatty liver grade (C) and fibrosis stage (D) in non-obese, obese NAFLD and CHB and healthy control by USG (C) and Fibroscan (D). Histopathological examination of liver for inflammation (E) and fibrosis (F) in non-obese, obese NAFLD patients and CHB control, respectively.

Hepatic gene expression of LBP, adipokine and cytokine among groups

The mRNA expression of LBP was up-regulated in 78.9% and 70% cases of non-obese and obese patients (Figure 2a and d). The mRNA expression of leptin (Figure 2b and e) was up-regulated in 68.4% and 75% cases; whereas adiponectin (Figure 2c and f) was 73.68% and 65% cases of non-obese and obese NAFLD patients, respectively. Increased expressions of pro-inflammatory cytokines were observed in 84.21% and 65% cases for IL-6 (Figure 3a and d); 89.47% and 55% cases for IL-1b (Figure 3b and e); 57.89% and 45% cases for TNF- α (Figure 3c and f) in non-obese and obese NAFLD patients, respectively.

Figure 2: Hepatic expression of LBP and adipokine gene in non-obese and obese NAFLD groups. Relative gene expression level of LBP (a, d), Adiponectin (b, e) and Leptin (c, f) genes in non-obese (upper panel) and obese (lower panel) NAFLD patients as compared to CHB disease control individuals, respectively.

Figure 3: Hepatic expression of cytokine in non-obese and obese NAFLD patients. Expression level of IL6 (a,d), IL1B (b,e) and TNF α (c,f) genes in non-obese and obese NAFLD patients as compared to CHB disease control individual respectively.

Association of IR, SIBO and LBP with lean and obese NAFLD and its correlation

Increased association of IR was observed with obese than non-obese NAFLD group (OR-3.571, 95% CI-1.11-11.48, $P=0.02$) and it was significantly correlated with BMI ($r=0.34$, $p=0.01$). No significant association SIBO and LBP (hepatic and systemic) with obese and non-obese NAFLD was observed (OR- 4.16, $p=0.186$ and OR-0.64, 0.48; $p=0.2$, 0.22). In non-obese NAFLD patients, LBP was significantly correlated with TNF- α (Hepatic: $r=0.61$, $p=0.003$; Systemic: $r=0.56$, $p=0.009$), adiponectin (Hepatic: $r=0.66$, $p=0.001$; Systemic: $r=0.53$, $p=0.01$), visfatin (Hepatic: $r=0.67$, $p=0.001$; Systemic: $r=0.8$, $p=0.001$) and MDA ($r=0.44$, $p=0.04$); whereas LBP level was significantly correlated only with Leptin ($r=0.42$, $p=0.03$). Hepatic LBP gene expression was also significantly correlated with TNF- α ($r=0.79$, $p=0.001$), IL-6 ($r=0.47$, $p=0.03$), Leptin ($r=0.59$, $p=0.007$), and adiponectin ($r=0.57$, $p=0.009$) in non-obese NAFLD group but not in obese group.

Table 3: Level of oxidative stress markers and antioxidants in hepatic and systemic circulation.

Markers	1-Non-obese n=25	2-Obese n=29	3-CHB n=25	4-Healthy n=25	ANOVA p value	Post hoc test, p value
MDA (μ M/L) Hepatic	4.21 $\pm$ 2.58	6.08 $\pm$ 3.49	3.83 $\pm$ 3.01		0.027	1 vs. 2, p=0.104 1 vs. 3, p=0.965 2 vs. 3, p=0.037
MDA(μ M/L) Systemic	3.67 $\pm$ 2.07	5.28 $\pm$ 3.43	2.92 $\pm$ 1.84	3.28 $\pm$ 1.74	0.004	1 vs. 2, p=0.113 1 vs. 3, p=0.859 1 vs. 4, p=0.994 2 vs. 3, p=0.005 2 vs. 4, p=0.023 3 vs. 4, p=0.996
FRAP(μ M/L) Hepatic	683.25 $\pm$ 134.52	683.24 $\pm$ 137.89	633.06 $\pm$ 182.80		0.495	1 vs. 2, p=1.0 1 vs. 3, p=0.647 2 vs. 3, p=0.641
FRAP (μ M/L) Systemic	666.5 $\pm$ 123.63	650.92 $\pm$ 156.93	587.05 $\pm$ 155.127	716.93 $\pm$ 152.64	0.05	1 vs. 2, p=0.999 1 vs. 3, p=0.441 1 vs. 4, p=0.798 2 vs. 3, p=0.676 2 vs. 4, p=0.525 3 vs. 4, p=0.03
Vitamin C (mg/dl) Hepatic	3.55 $\pm$ 1.70	4.05 $\pm$ 1.60	2.47 $\pm$ 1.64		0.014	1 vs. 2, p=0.716 1 vs. 3, p=0.133 2 vs. 3, p=0.013
Vitamin C (mg/dl) Systemic	3.65 $\pm$ 1.63	4.45 $\pm$ 1.39	2.05 $\pm$ 1.26	5.97 $\pm$ 0.69	0.000	1 vs. 2, p=0.295 1 vs. 3, p=0.002 1 vs. 4, p=0.0001 2 vs. 3, p=0.0001 2 vs. 4, p=0.004 3 vs. 4, p=0.0001
SOD (U/ml) Hepatic	20.22 $\pm$ 6.07	19.25 $\pm$ 6.11	16.83 $\pm$ 6.73		0.584	1 vs. 2, p=1.0 1 vs. 3, p=0.647 2 vs. 3, p=0.641
SOD (U/ml) Systemic	16.90 $\pm$ 7.41	17.96 $\pm$ 7.18	11.81 $\pm$ 6.38	15.09 $\pm$ 2.69	0.08	1 vs. 2, p=0.613 1 vs. 3, p=0.012 1 vs. 4, p=0.257 2 vs. 3, p=0.002 2 vs. 4, p=0.065 3 vs. 4, p=0.022

Discussion

The present study provided specific clinical patterns of NAFLD associated with WHO criteria of obesity and without so, and identified the limitations of established criteria of screening individuals for NAFLD in Indians. This study evaluated the influence of obese and non-obese conditions with hepatic fat content and extent of liver injury in Indian NAFLD patients. Whether systemic changes associated with NAFLD are similar to the events inside the liver was further confirmed in hepatic circulation and also by gene expression. The NAFLD patients were compared with diseased (CHB) and healthy controls. The study population was not derived from

known high risk population of NAFLD rather obtained from consecutive non-diabetic and non-alcoholic patients incidentally detected to have fatty liver by USG with various nonspecific symptoms such as dyspepsia to mimics a real life scenario.

Out of these 54 biopsy confirmed NAFLD patients, 54% had increased BMI > 25 kg/m², 72% had increased waist circumference and 94.4% had increased WHR. In Indian subjects of without T2DM or alcohol or drug history, increased WHR can be the indicators for presence of NAFLD. In the present study, all the patients with NAFLD had MRS to estimate more objectively the fat content of the liver and in about 95% of both groups of NAFLD (BMI >

Table 4: Differences in level of hepatic and systemic adipokines among NAFLD, CHB and Healthy control.

Markers	1-Non-obese n=25	2-Obese n=29	3-CHB n=25	4-Healthy n=25	ANOVA p value	Post hoc test, p value
Leptin (pg/ml) Hepatic	1277.42±1207.13	2368.04±1854.23	1042.50±1607.61		0.013	1 vs. 2, p=0.05 1 vs. 3, p=0.94 2 vs. 3, p=0.021
Leptin (pg/ml) Systemic	1475.26±1444.23	2010.45±1928.03	929.65±958.66	477.14±510.45	0.008	1 vs. 2, p=0.73 1 vs. 3, p=0.754 1 vs. 4, p=0.215 2 vs. 3, p=0.076 2 vs. 4, p=0.01 3 vs. 4, p=0.93
Adiponectin (µg/ml) Hepatic	175.77±170.52	46.28±53.28	83.11±74.03		0.001	1 vs. 2, p=0.0001 1 vs. 3, p=0.02 2 vs. 3, p=0.655
Adiponectin (µg/ml) Systemic	222.50±158.7	65.92±84.13	64.63±75.49	173.46±156.05	0.000	1 vs. 2, p=0.0001 1 vs. 3, p=0.0001 1 vs. 4, p=0.813 2 vs. 3, p=1.0 2 vs. 4, p=0.072 3 vs. 4, p=0.093
Visfatin (ng/ml) Hepatic	83.77±26.84	55.23±24.19	55.94±17.52		0.000	1 vs 2, p=0.001 1 vs 3, p=0.002 2 vs 3, p=1.0
Visfatin (ng/ml) Systemic	85.45±24.46	59.55±20.9	57.38±14.87	75.00±39.43	0.001	1 vs 3, p=0.005 1 vs 2, p=0.005 1 vs 4, p=0.841 2 vs 3, p=1.0 2 vs 4, p=0.47 3 vs 4, p=0.381

25 and < 25 kg/m²) had fat in more than 5.56% of liver tissue and 15 to 20% of the liver content were due to fat which confirmed that indeed all of them had fatty liver which was further confirmed in liver histology. The excess fat accumulation either due to obesity or abdominal obesity alone results in adipocytes hypertrophy and macrophage infiltration which disrupts normal function of adipose tissue leading to deregulation of adipokines, alteration of glucose and lipid homeostasis; and inflammatory immune responses [25]. We had observed that even with lower BMI and increased WHR, the fat content of the liver was increased. Whether, these amounts of fat are associated with increased inflammation or fibrosis and whether it can be considered as markers for disease progression need to be evaluated. For this, the patients with lower BMI and waist circumference were evaluated by histo-pathological examination. As per NASH-CRN criteria 40.74 % (n=22) of all NAFLD had NASH (NASH-CRN > 5) and 22% (n=12) had F2 and F3 fibrosis. Even though about 27.58% (n=8) of those with higher BMI (> 25 Kg/m²) had F2/F3, about 16% (n=4) NAFLD with BMI<25 had F2/ F3 fibrosis. Similarly, about 32% of the later group and

49% of the former group had NASH CRN score of > 5. The results of Fibroscan were similar to liver histology in these patients. These findings together may suggest that Indians are more prone to develop NAFLD with minimal non overt weight gain and about 30% off these patients even without any risk factors for NAFLD had evidence of progressive disease in the form of overt histological NASH. Therefore, detection of NAFL by USG in these parts of the world need further evaluation to identify the high risk NASH who progresses and need the specific management.

In these patients with NAFLD (both high and low BMI groups), IR was documented and established earlier as pathogenetic driver. Release of free fatty acids (FFAs) to circulation either due to peripheral or central obesity modulates insulin sensitivity and NAFLD pathogenesis [26]. Equivalent proportions of increased triglyceride level (44% and 41.37%) and decreased HDL level (32% and 31.03%) were observed in both non-obese and obese NAFLD patients of this study. The Insulin levels in both the group were markedly and significantly higher than

Table 5: The level of cytokines in peripheral circulation among NAFLD, CHB and Healthy control.

Cytokines	1-Non-obese n=25	2-Obese n=29	3-CHB n=25	4-Healthy n=25	ANOVA p value	Post hoc test, p value
IL-6 (pg/ml) Systemic	26.09±74.45	56.50±135.79	16.62±44.25	9.33±18.52	0.305	1 vs. 2, p=0.323 1 vs. 3, p=0.587 1 vs. 4, p=0.280 2 vs. 3, p=0.166 2 vs. 4, p=0.091 3 vs. 4, p=0.451
IL-1 (pg/ml) Systemic	6.16±12.49	1.86±3.28	1.60±4.71	0.04±0.01	0.13	1 vs. 2, p=0.080 1 vs. 3, p=0.094 1 vs. 4, p=0.018 2 vs. 3, p=0.813 2 vs. 4, p=0.008 3 vs. 4, p=0.104
TNF-α (pg/ml) Systemic	834.71±1828.65	970.84±2033.43	613.87±1575.52	102.86±197.27	0.457	1 vs. 2, p=0.798 1 vs. 3, p=0.649 1 vs. 4, p=0.052 2 vs. 3, p=0.479 2 vs. 4, p=0.039 3 vs. 4, p=0.114

both the control groups (Table 2, $P < 0.0001$). However, HOMA-IR estimation indicating IR was documented in 51.7% ($n=15$) and 24% ($n=6$) of the patients with high and low BMI respectively. In Indian patients, cut off of HOMA-IR has not been properly worked out to indicate IR which may be much lower than the present study cut off. Earlier rural population study from India [4], reported the value of mean Insulin level as 6.83 ± 3.24 and as 6.66 ± 2.16 mIU/ml in both lean NAFLD the lean control (without NAFLD) group respectively. HOMA-IR in them was 1.63 ± 1.65 and 1.41 ± 0.39 , respectively. However all the lean NAFLD group patients had mild rise in weight in the form of increased WHR. Therefore, minimal weight in Indian patients is possibly resulting central obesity and IR as indicated by higher serum Insulin levels. The present study documented additional possible pathogenetic link which might be accelerating the effect of rise of Insulin level in such patients. We documented that the frequency of SIBO in our NAFLD patients (non-obese and obese group, 4% and 17.2%) were higher than the controls. Furthermore, significantly high LBP levels (both hepatic and systemic) in NAFLD patients as compare to control was documented. These finding were further corroborated with relative expression of LBP mRNA in NAFLD liver tissues (Figure 2a and d). These finding would suggest that the gut derived liposaccharide are indeed reaching the liver and inducing LBP production in Indian patients. Increased expressions of proinflammatory adipo-cytokines like leptin, visfatin, TNF and IL6mRNA were also documented (Figures 2 and 3). There was a linear correlation between LBP mRNA and

the cytokine mRNA. Earlier studies including our center also documented increased serum levels of MDA in obesity and NAFLD [11,27,28]. Dysregulation of adipokines due to hypertrophy of adipocytes or infiltration of immune cells has been implicated in inducing chronic inflammatory state in metabolic disorders [29]. The upregulation adipokines such as leptin, resistin, visfatin, retinol-binding protein 4 (RbP4), lipocalin 2 and angiopoietin-like protein 2 (ANGPTL2) induce pro-inflammatory state and down-regulation of anti-inflammatory adipokine such as adiponectin, SFRP5 also contributes to metabolic dysfunction and inflammation [30,31]. These inflammatory adipo-cytokine, along with FFA and gut derived LPS activate inhibitory molecules such as SOCS, PTP1b and JNK to suppress insulin signaling through interaction of IRS (insulin receptor substrate) resulting in insulin resistance [32,33]. Therefore it is possible that in Indians who develop NAFLD with minimal weight gain (so called Lean NAFLD), the disease is often progressive. SIBO and endotoxemia (evidenced by increased LBP expression in liver & increase LBP in hepatic venous and systemic blood) might be inducing upregulation of proinflammatory cytokines associated with oxidative stress (also documented among NAFLD patients in this study) may be causing liver damage in such patients.

This study has few limitations. This is an observational study from single tertiary care center in urban setting, which may be associated with a referral bias. The observations of this study for non-obese NAFLD

patients are not comparable to NAFLD patients without abdominal obesity from rural setting which may have different pathogenesis. We did not use CT scan to assess the visceral and abdominal fat.

In conclusion, minimal weight gain in Indians with increased WHR (and not necessarily with increased BMI) is associated with increased liver fat accumulation, higher serum Insulin levels and progressive liver disease (fibrotic NASH and NASH CRN score > 5 in about one third of these patients). The study further documented higher frequency of SIBO in these patients than controls. The LBP expression due to gut derived LPS had shown higher level in the liver tissue, hepatic and peripheral circulation which was well correlated with increased proinflammatory cytokines in liver tissue. The hepatic and systemic levels of biomarkers were well correlated. Increased level of lipid peroxidation product and proinflammatory adipocytokines were observed in hepatic than systemic circulations. All of these factors in combination might be causing progressive liver damage in so called "Lean NAFLD".

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