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Serum markers versus histopathological scoring for discrimination between experimental fatty liver and non-alcoholic steatohepatitis

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ABSTRACT

Non-alcoholic fatty liver disease is a multi-factorial health problem which correlates mostly with obesity and metabolic syndrome. Construction of clearer boundaries between simple steatosis and steatohepatitis is a clinical challenge that needs further examination. We aim to offer reliable non-invasive serum markers for discrimination between fatty liver and steatohepatitis thus reduce the urge for invasive histological intervention in some cases. Sixty male albino rats were used in this study, equally divided into 4 groups (15 rats/ group). Group I acted as untreated normal control. Groups II, III, IV were fed high fat diet for 4, 8, 12 weeks respectively. Histological scoring and biochemical analysis including liver enzymes, lipid profile, lipid peroxidation, serum and tissue levels of leptin, vascular endothelial growth factor, tumor necrosis factor-alpha and interleukin-6 were assessed. High fat diet caused sustained hyperglycemia, hyperinsulinemia and insulin resistance. It also induced significant histopathological and biochemical changes in liver enzymes, lipid profile and lipid peroxidation. It significantly increased both serum and tissue levels of leptin, vascular endothelial growth factor, tumor necrosis factor- alpha and interleukin-6. All tested parameters were significantly correlated with histological scoring. Suggested parameters, based on their involvement as key players in disease pathogenesis and their high correlation to histological scoring, may be useful non-invasive diagnostic tools for discrimination between fatty liver and non-alcoholic steatohepatitis.

Keywords: Non-alcoholic fatty liver diseases, fatty liver, non-alcoholic steatohepatitis, adipokines, cytokines.

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Abbreviations: AS, Aggressive steatosis; **FFA,** free fatty acids; **HFD,** high fat diet; **HOMA-IR,** homeostasis model assessment insulin resistance; **IL-6,** interleukin-6; **NAFLDs,** non-alcoholic fatty liver diseases; **NASH,** non-alcoholic steatohepatitis; **SS,** simple steatosis; **TBARS,** thiobarbituric acid reactive substances; **TNF-α,** tumor necrosis factor-α; **VEGF,** vascular endothelial growth factor.

INTRODUCTION

Non-alcoholic fatty liver diseases (NAFLDs) affect 10 to 24 percent of the general population (Lazo and Clark, 2008). They include a histological spectrum ranging from simple steatosis, manifested as fatty infiltration of the liver, to non-alcoholic steatohepatitis (NASH), characterized by steatosis with inflammation and hepatocyte necrosis (Brunt, 2004). While liver steatosis is a benign, reversible non-progressive condition NASH, on the other hand, is an irreversible progressive change which may eventually lead to liver fibrosis cirrhosis and hepatocellular carcinoma (HCC) (Day and James, 1998; James and Day, 1998).

Many questions about what triggers the transition of liver steatosis to NASH have been raised. The pathogenesis of NASH comprises two steps (Angulo, 2002). First, the healthy liver becomes steatotic, mainly due to peripheral insulin resistance, which then increase the flow of free fatty acids from adipose tissue to the liver.

Although the body develops defensive mechanisms to survive this stress, the fatty liver becomes fragile and vulnerable to any additional insults (Day, 2006). Then, a second step triggered by oxidative stress and inflammatory mediators occurs. This leads to exacerbation of insulin resistance, and eventually organelle dysfunction enriching an inflammatory environment within liver cells (Leclercq et al., 2007). Yet, despite the extensive research, the pathogenesis of NAFLDs is still a mystery; the recognised mechanisms do not fully explain disease progression patterns and the range of symptoms and physiological processes. So, the pathophysiology of NAFLDs should probably be approached as a multifactorial process (Bugianesi et al., 2005).

Obesity may be considered one of the most important causal factors for NAFLDs. Adipose tissue was considered to be inert, but it later proved to be an active metabolic organ. It also act as an endocrine unit secreting various adipocytokines including leptin, adiponectin, tumor necrosis factor-α [TNF- α] and Interleukin-6 [IL-6] (Kershaw and Flier, 2004). Leptin is an adipocyte-derived hormone which regulates food intake and energy homeostasis. Functional impairment of leptin leads to severe obesity and diabetes (Marra, 2002). TNF-α acts mainly in an autocrine/paracrine fashion in adipose tissue. IL-6, on the other hand, is an endocrine cytokine with pleiotropic action ranging from inflammation to host defence and tissue injury (Xirouchakis et al., 2009).

A link between NAFLDs progression and hepatic microvasculature changes can be observed. Angiogenesis, the formation of new blood vessels, is a key mechanism in the pathogenesis of chronic liver diseases, irrespective of their underlying aetiology (Bouloumie et al., 2002). Although multiple angiogenic factors have been identified in adipose tissue including vascular endothelial growth factor (VEGF), very little is known about the interplay between these factors in the adipose tissue environment (Carmeliet, 2005; Dvorak, 2005; Ferrara and Kerbel, 2005).

Construction of clearer boundaries between fatty liver and steatohepatitis is still a clinical challenge that needs further examination. The present study aims to offer more reliable non-invasive markers for the discrimination between pure fatty change and steatohepatitis by correlating them to histological scoring being the golden standard for NAFLDs.

MATERIALS AND METHODS

Animals

All animals received human care according to the National Institute of Health guidelines [USA]. Sixty male albino rats (2 months old) weighing from 100 to 150 g were procured from the central animal facility of the institute. They were divided in to equal 4 experimental groups (15 rats per group). All rats were provided with commercially available rat normal pellet diet and water *ad libitum*, prior to the dietary manipulation. Individual body weights and food consumption were recorded weekly.

Chemicals

All chemicals and solvents used in the study were of analytical grade and were obtained either from Sigma Aldrich chemical company unless otherwise mentioned.

Diets

Lieber-DeCarli (standard and high fat liquid) diets were used as follows:

Standard diet: [35% of energy from fat, 47% from carbohydrates, 18% from protein].

High fat diet: [71% of energy from fat, 11% from carbohydrates, 18% from protein].

The diets were given *ad libitum* or as two-thirds of the amount consumed *ad libitum* (Lieber and DeCarli, 1994; Lieber et al., 2002; Lieber et al., 2004).

Experimental procedure

1. Normal control: animals were fed standard diet for the whole period of experiment.
2. Simple steatosis [SS] group: animals were fed HFD for 4 weeks.
3. Aggressive steatosis [AS] group: animals were fed HFD for 8 weeks.
4. Non-alcoholic Steatohepatitis [NASH] Group: animals were fed HFD for 12 weeks.

Liver histopathology

Histological evaluation was performed on a lobe of the liver and portion of specimen was fixed in 10% neutral formalin for 24 h then changed to absolute ethanol for dehydration and embedded in paraffin wax. Sections were cut at 4 µm in thickness, stained with hematoxylin and eosin for histological examination. Histopathological grading was performed according to the guidelines of Brunt et al. (1999). Histological scoring system for non-alcoholic fatty liver disease [NAFLD] was adopted from Kleiner et al. (2005).

Plasma glucose and insulin levels

Glucose levels were measured using a standard Randox glucose kit. Insulin levels were measured using insulin enzyme immunoassay kit following the instructions of the manufacturer.

Index of insulin resistance

The index of insulin resistance estimated by the homeostasis model assessment [HOMA] was calculated using the following formula (Matthews et al., 1985):

$$\text{HOMA-IR} = \frac{\text{Insulin [mUI/L]} \times \text{Blood glucose [mmol/L]}}{22.4}$$

Biochemical analysis

Liver function tests (ALT, AST, GGT), lipid profile (total cholesterol,

Table 1. Body weight, food intake, plasma insulin, glucose and HOMA-IR index in different groups.

Parameter	Normal control	Simple steatosis	Aggressive steatosis	NASH
Initial Body wt (g)	104 ± 12.5	101 ± 14.7^{ns}	105 ± 15.2^{ns}	107 ± 14.8^{ns}
Final Body wt (g)	251 ± 12.7	295 ± 13.9***	373 ± 19.2***	430 ± 15.5***
Δ Body wt (g)	147 ± 11.2	194 ± 7.5***	268 ± 6.3***	323 ± 4.8***
Absolute liver wt	5.7 ± 0.4	7.6 ± 0.4**	11.4 ± 1.4***	13.9 ± 1.02***
Relative liver wt †	0.02 ± 0.002	0.03 ± 0.003**	0.03 ± 0.006***	0.03 ± 0.006***
Food intake (g/day)	32 ± 1.6	65 ± 4.9***	77 ± 5.8***	85 ± 7.9***
Insulin (mUI/L)	10.5 ± 0.7	11.2 ± 0.4*	13.9 ± 0.2**	15.2 ± 0.1***
Glucose (mmol/L)	8.2 ± 2.1	9.7 ± 3.7*	11.7 ± 3.1***	13.7 ± 3.9***
HOMA-IR index ‡	3.6 ± 0.05	5.2 ± 0.6**	7.3 ± 0.8***	9.3 ± 0.9***

Data are expressed as $\bar{X}$ ± SD of 10 rats in each group (n=10). Significant difference between groups is analyzed by t-student test, where: (*** P < 0.001, ** P < 0.01, * P < 0.05 compared to normal control)
† Relative liver wt = Absolute liver wt / Final body wt.
‡ Homeostasis Model Assessment – Insulin Resistance (HOMA-IR) index = Insulin (mIU/L) x Glucose (mmol/L) / 22.4.

LDL, HDL, triglycerides) were measured using standard Randox kits. Thiobarbituric acid reactive substances (TBARS) were measured using colorimetric kits (Cayman chemical company, USA), following the instructions of the manufacturer.

Enzyme-linked immuno sorbent assay

Leptin, VEGF (Bio-Rad Company, USA) and TNF-alpha, IL-6 (Abcam, USA) were measured in serum by rat ELISA kits following the instructions of the manufacture.

Western blotting

Leptin, VEGF, TNF-alpha, IL-6 and actin protein content in liver homogenate were also measured. Their antibodies were obtained from Abcam, USA. Up on determination of individual protein concentrations of the samples using Bradford assay (Bradford, 1976), samples were prepared for loading. Aliquots of frozen hepatic homogenates were routinely examined by sodium dodecyl sulfate polyacrylamide gel electrophoresis followed by immunoblotting.

Statistical analysis

Data were expressed as mean ± SD of 10 rats in each group. Significant difference between groups was analyzed by t-student test and correlation analysis was done using Graphpad Prism 5 statistical software.

RESULTS

Initial observations showed significant increase in food intake, final body weights and relative liver weight in HFD groups (P < 0.01) compared to normal control. Blood insulin, glucose levels and HOMA-IR indices were also significantly higher in HFD groups (P < 0.05) in groups II, III and (P < 0.001) in group IV compared to normal control (Table 1).

Histological examination showed normal hepatic lobular architecture and normal hepatocytes with granulated cytoplasm and small uniform nuclei in group I (Figure 1A). On the other hand, group II showed mild steatosis with no evidence of inflammation (Figure 1B). Group III showed massive fat accumulation (both macro- and micro-vesicular (Figure 1C). Group IV showed periportal necrosis of the hepatocytes that surround the portal area associated with massive fat accumulation (both macro and microvesicular), ballooning degeneration, Malory's bodies and inflammatory infiltrate of mononuclear inflammatory cells (Figure 1D and E).

Biochemical analysis also showed significant changes in liver enzymes, lipid profile and lipid peroxidation. Group I: did not show any significant changes in the tested parameters. Group II: showed significant changes in GGT (P < 0.05), AST, LDL, HDL (P < 0.01), total cholesterol, triglycerides and TBARS (P < 0.001) except ALT which showed non-significant change (p > 0.05). Groups III and IV: both showed significant changes (p < 0.001) in all tested parameters (Table 2).

ELISA and WB were used to measure serum and tissue levels of leptin, VEGF, TNF-α and IL-6. Leptin levels showed significant increase (P < 0.001) in both serum and tissue. VEGF levels also showed significant increase (P < 0.001) in both serum and tissue except in group II which showed non-significant increase (P > 0.05). TNF-α levels showed significant increase (P < 0.05) in group I and (P < 0.001) in groups III and IV. IL-6 levels showed significant increase (P< 0.001) in group IV, (p < 0.05) in group III and non-significant change (P > 0.05) in group II (Table 3, Figure 2).

Histopathological grading for hepatic findings revealed major changes in tested criteria (steatosis grade, affected zone, lobular inflammation grade (LIG), spotty necrosis/apoptosis (NN/A) and finally cholestasis). Group I: did not show any abnormal findings (no signs for steatosis or inflammation). Group II: showed changes in only 20% of rats manifested by mild steatosis mainly macrovesicular with no sign of inflammation, necrosis/apoptosis or cholestasis. Group III: showed

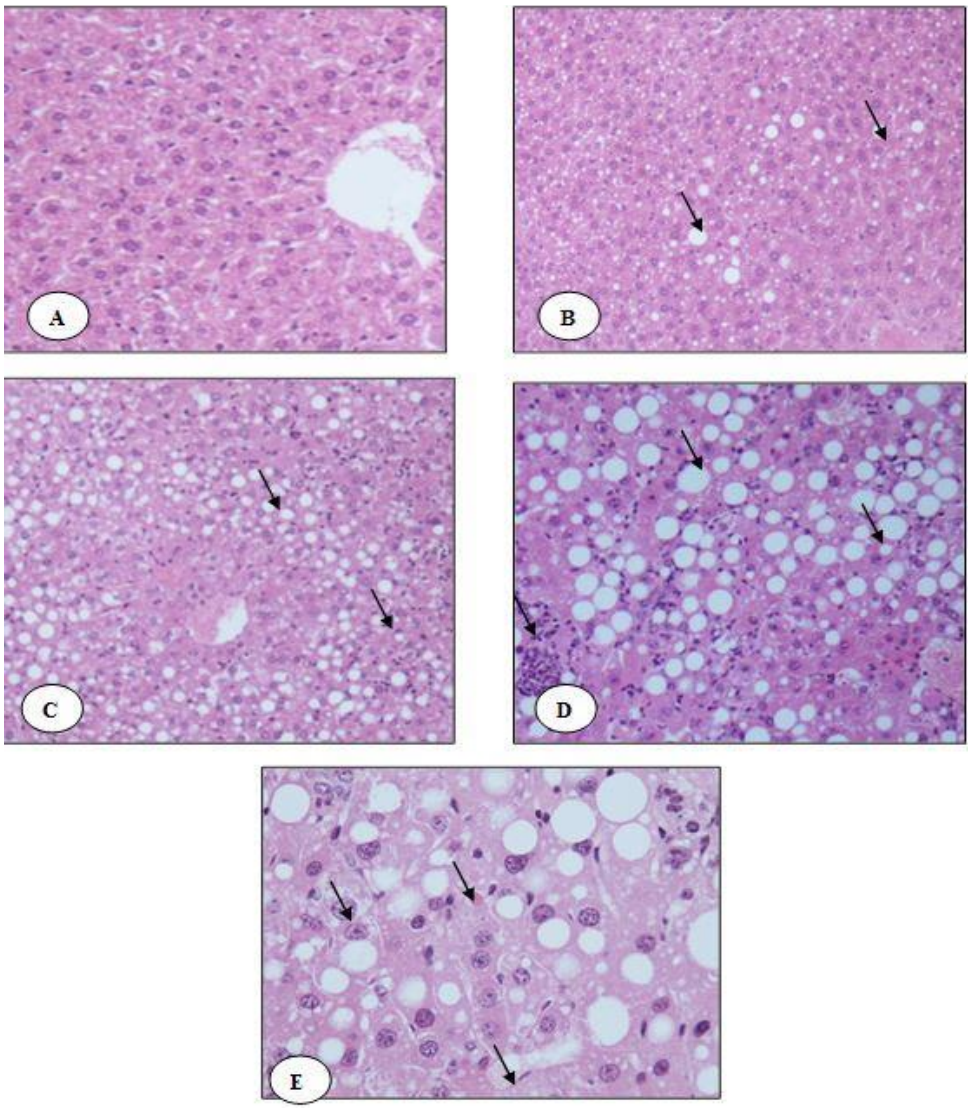

Figure 1. Representative photomicrographs of liver sections from different groups. A: Normal control: showed normal hepatic lobular architecture and normal hepatocytes with granulated cytoplasm and small uniform nuclei. B: Simple Steatosis group: Showed mild steatosis (mainly macrovesicular) with no evidence of inflammation. C: Aggressive Steatosis: Showed massive fat accumulation (both macro and micro vesicular). D and E: NASH group: showed periportal necrosis of the hepatocytes that surround the portal area associated with massive fat accumulation (both macro and micro vesicular), ballooning degeneration, Malory's bodies and inflammatory infiltration in the form of mononuclear inflammatory cell.

changes in 60% of rats manifested by moderate to severe steatosis with mild inflammation and less common necrosis/apoptosis or cholestasis. Group IV: showed typical signs of NASH in 100% of rats manifested by severe steatosis accompanied with moderate to severe inflammation with more common necrosis/apoptosis or cholestasis. Using NAFLDs scoring system (NAS) for histological scoring, groups I and II showed "NAS ≤ 2" in 100% of rats which corresponds to absence of NASH, group III showed "NAS: 3-4" in 30% of rats which corresponds to uncertain NASH, finally group IV showed "NAS ≥ 5" in 70% of rats which corresponds to definite NASH and "NAS: 3-4" in 30% of rats which corresponds to uncertain NASH (Table 4).

Correlation analysis showed that all tested parameters were strongly and positively correlated with histological scoring (NAS) ($P < 0.0001$; $r = 0.7284$, 0.8806, 0.7804 and 0.8703 for leptin, VEGF, TNF-α and IL-6, respectively (Table 5).

DISCUSSION

Non-alcoholic fatty liver disease is a multi-factorial health

Table 2. Liver enzymes, lipid profile and TBARS in different groups.

Parameter	Normal control	Simple steatosis	Aggressive steatosis	NASH
ALT (IU/L)	47.5 ± 2.9	48.3 ± 3.2 ^{ns}	122 ± 11.9***	175.03 ± 12.4***
AST (IU/L)	89.9 ± 8.5	103.5 ± 10.9**	208.4 ± 15.6***	243.4 ± 22.7***
GGT (IU/L)	21.2 ± 1.9	25.3 ± 3.8*	83.7 ± 8.4***	125.4 ± 11.1***
Total Cholesterol (mg/dl)	98.9 ± 9.8	130.1 ± 14.7***	173.3 ± 18.4***	196.5 ± 25.8***
Triglycerides (mg/dl)	97.7 ± 11.4	139.5±16.4***	195.6 ± 22.3***	226.8 ± 23.9***
LDL (mg/dl)	35.4 ± 4.7	42.9 ± 6.5**	87.3 ± 8.4***	135.2 ± 14.1***
HDL (mg/dl)	43.2 ± 3.3	36.8 ± 4.2**	22.6 ± 1.8***	14.8 ± 1.3***
$^{\$}$TBARS (µmol/L)	0.3 ± 0.04	0.6 ± 0.07***	0.9 ± 0.06***	1.5 ± 0.01***

Data are expressed as $\bar{X}$± SD of 10 rats in each group (n=10). Significant difference between groups is analyzed by t-student test, where: (*** P < 0.001, ** P < 0.01, * P < 0.05 compared to normal control)
$^{\$}$ Thiobarbituric acid reactive substances.

Table 3. Serum levels of Leptin, VEGF, TNF-α, IL-6 in different groups.

Parameter	Normal control	Simple steatosis	Aggressive steatosis	NASH
Leptin (ng/ml)	4.7 ± 0.57	9.9 ± 0.95***	16.1 ± 1.05***	18.1 ± 1.28***
VEGF (pg/ml)	88.2 ± 8.35	107.6 ± 4.48^{ns}	172.7 ± 15.89***	266.5 ± 19.27***
TNF-α (pg/ml)	15.8 ± 1.59	22.2 ± 2.73*	84.2 ± 7.36***	105.9 ± 9.94***
IL-6 (pg/ml)	42.1±2.64	51.32 ± 4.08^{ns}	66.1 ± 4.3**	179.2 ± 13.92***

Data are expressed as $\bar{X}$± SD of 10 rats in each group (n = 10). Significant difference between groups is analyzed by t-student test, where: *** P < 0.001, ** P < 0.01, * P < 0.05 compared to normal control.

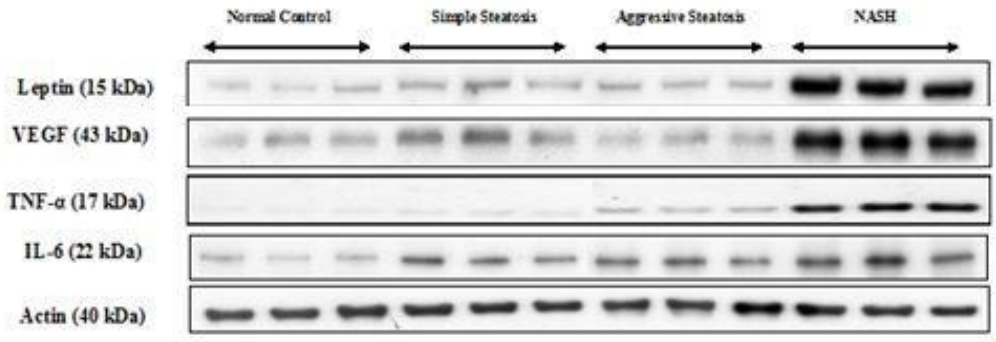

Figure 2. Western Blotting for Leptin, VEGF, TNF-α, IL-6 and Actin protein expression in the liver.

problem which correlates mostly with obesity and metabolic syndrome. It has been estimated that amongst a population of obese/diabetic individuals approximately 50 to 90% will have fatty change, 20 to 30% will progress to steatohepatitis/fibrosis and 2 to 5% will eventually become cirrhotic (Reid, 2001). $NAFLD_S$ is considered a leading cause for unexplained abnormal liver function tests which necessitates the histological evaluation for uncovering the possible explanation for such abnormalities being the golden standard till now (Angulo, 2002). Although fatty change can be reliably diagnosed by non-invasive methods, the discrimination between fatty liver and steatohepatitis can still only be made histologically because of the correlation between clinical, biochemical and histological findings is generally poor in NAFLDs patients (Ascha et al., 2010). In this paper, we aim to offer non-invasive serum markers that correlates with histological scoring, thus reduce the urge for invasive histological intervention in some cases.

To induce a reliable animal model for NASH, we selected a dietary model developed by Lieber and colleagues. This model reproduces the dominant clinical features of NASH and mimics the human condition given that NASH is intimately associated with a fat-rich life-style in obese patients (Lieber and DeCarli, 1994; Lieber et al., 2002; Lieber et al., 2004). Histopathological examination,

Table 4. Histopathological grading for liver samples.

No.	ID	Steatosis grade (0-3)[a]	Affected zones[b]	Steatosis type (Vesicular)	LIG (0-3)[c]	Hepatocyte balooning (0-2) [d]	SN/A Yes/ No [e]	Chole Yes/ No [e]	NAS (0-8)[f]
1	NC (1)	0	NA	NA	0	0	N	N	0
2	NC (2)	0	NA	NA	0	0	N	N	0
3	NC (3)	0	NA	NA	0	0	N	N	0
4	NC (4)	0	NA	NA	0	0	N	N	0
5	NC (5)	0	NA	NA	0	0	N	N	0
6	NC (6)	0	NA	NA	0	0	Y	N	0
7	NC (7)	0	NA	NA	0	0	N	N	0
8	NC (8)	0	NA	NA	0	0	N	N	0
9	NC (9)	0	NA	NA	0	0	N	N	0
10	NC (10)	0	NA	NA	0	0	N	N	0
11	SS (1)	0	NA	NA	0	0	N	N	0
12	SS (2)	2	2	Macro	0	0	N	N	2
13	SS (3)	0	NA	NA	0	0	N	N	0
14	SS (4)	0	NA	NA	0	0	N	N	0
15	SS (5)	1	2	Macro	0	0	N	N	1
16	SS (6)	0	NA	NA	0	0	Y	N	0
17	SS (7)	0	NA	NA	0	0	N	N	0
18	SS (8)	0	NA	NA	0	0	N	N	0
19	SS (9)	0	NA	NA	0	0	N	N	0
20	SS (10)	0	NA	NA	0	0	N	N	0
21	AS (1)	0	NA	NA	1	0	N	N	0
22	AS (2)	1	2	Micro Macro	1	0	N	N	2
23	AS (3)	1	2	Micro Macro	0	0	N	N	1
24	AS (4)	2	2,3	Micro Macro	1	0	N	N	3
25	AS (5)	3	1,2	Macro Micro	1	0	Y	N	4
26	AS (6)	0	NA	NA	0	0	N	N	0
27	AS (7)	0	NA	NA	1	0	N	N	1
28	AS (8)	0	NA	NA	1	0	N	N	1
29	AS (9)	1	2	Micro Macro	0	0	N	N	1
30	AS (10)	2	2,3	Micro Macro	1	0	N	N	3
31	NASH (1)	2	2,3	Macro Micro	1	0	N	Y	3

Table 4. Continues.

32	NASH (2)	3	2,3	Macro Micro	3	0	Y	Y	6
33	NASH (3)	3	2,3	Macro Micro	2	0	N	Y	5
34	NASH (4)	3	1,2,3	Macro Micro	3	0	Y	N	6
35	NASH (5)	3	2,3	Macro Micro	3	0	Y	N	6
36	NASH (6)	1	2	Micro	2	1	N	N	4
37	NASH (7)	2	2,3	Macro Micro	1	0	N	Y	3
38	NASH (8)	3	2,3	Macro Micro	3	0	Y	Y	6
39	NASH (9)	3	2,3	Macro Micro	2	0	N	Y	5
40	NASH (10)	3	1,2,3	Macro Micro	3	1	Y	N	7

LIG = Lobule Inflammation Grade, SN/A = Spotty necrosis/Apoptosis, Chole = Cholestasis.
[a] Steatosis grade: The severity of fatty change determined by estimating the proportion of hepatocytes containing fat droplets (0 (<5%) = Minimal, 1 (5-33%) = Mild, 2 (33-66%) = Moderate, 3 (> 66%) = Severe, NA = Not applicable).
[b] Affected zones: Zone 1= Peri-portal , Zone 2 = mid-zone, Zone 3 = centri-lobular
[c] Type of steatosis: when fat vesicles are large enough to distort the nucleus, the condition is known as macrovesicular steatosis; otherwise, the condition is known as microvesicular
[d] Inflammation grade: severity of lobular inflammation based on inflammatory foci per x 200 field: 0= none, 1 = 1-2, 2 = up to 4, 3 = > 4
[e] Hepatocyte ballooning: 0 = none, 1 = few ballooned cells, 2 = many cells/prominent ballooning
[f] Other tested criteria as necrosis and cholestasis were evaluated by their presence or absence (Yes/No)
[g] NAFLD Activity Score (NAS) (0–8) = calculated as sum of scores for steatosis + lobular inflammation + hepatocellular ballooning (NAS ≥ 5 (Probable or definite NASH), NAS (3-4) (Uncertain), NAS ≤ 2 (Not NASH) (19, 20).

Table 5. Pearson's correlation coefficients for leptin, TNF-α, IL-6 and VEGF versus NAS.

Parameter	Leptin	TNF-a	IL-6	VEGF
Pearson r	0.7284	0.7804	0.8703	0.8806
95% confidence interval	0.2184 to 0.9995	0.5648 to 0.9998	0.6242 to 0.9998	0.4023 to 0.9997
P value (two-tailed)	P < 0.0001	P < 0.0001	P < 0.0001	P < 0.0001
P value summary	***	***	***	***
Is the correlation significant?	Yes	Yes	Yes	Yes
R squared	0.9504	0.9782	0.9818	0.9668

grading and scoring clearly demonstrate that the ingestion of the Lieber liquid high-fat diet during 12 weeks produced all the prominent characteristics of NASH which are consistent with previous reports that illustrated typical features of steatohepatitis including hepatocellular injury (beyond simple fatty change), inflammatory infiltration, Mallory's bodies and hepatocyte ballooning besides other non-typical features like necrosis and cholestasis (Neuschwander-Tetri et al., 2010).

HFD is a well characterized rodent model that results in hyperglycaemia, insulin resistance, hyperinsulinemia, impaired glucose tolerance, defective islet compensation, and obesity (Buettner et al., 2006). Adipocytes are designated to act as a free fatty acids (FFA) storage unit and on demand supplier. Distorted adipocytokine

profile during obesity influences FFA release from adipocytes and enhances lipid delivery to the liver (Unger, 2002). Under normal conditions, lipids do not accumulate in liver cells but rather are transformed into mixed particles like very low density lipoproteins which then can be secreted in the blood stream. This scene is disrupted during the development of NAFLD which is initiated with the accumulation of circulating FFA in the liver then entering into liver cells by simple diffusion and build up in high concentrations (Bugianesi et al., 2005). This activates lipid degradation pathways (lipolysis) and suppress insulin receptor activation, as a result, hyperglycemia and secondary hyperinsulinaemia occur (Foufelle and Ferre, 2002; Leclercq et al., 2007).

Our results showed significant changes in serum and tissue levels of leptin, VEGF, TNF-α and IL-6 during progression of NASH in rats. These parameters also showed highly significant positive correlation compared to NAS, a standard histological scoring system for NAFLDs, which strongly suggests that these parameters may be used to monitor different developmental stages of NAFLDs in human NASH to limit the use of invasive histopathological diagnostic procedures. There are conflicting reports about the exact role of leptin in the pathogenesis of NAFLD. Some studies demonstrated increased leptin levels in NASH (Chitturi et al., 2002; Nakao et al., 2002), while others found no correlation between serum leptin levels and the development of NASH (Chalasani et al., 2003; Myers et al., 2008). Leptin acts mainly to limit the accumulation of fat in non-adipose tissue thus reduce lipotoxicity. When FFA supply to the liver is increased, leptin activates protective mechanisms including dephosphorylation of insulin receptor substrate 1 to prevent hepatic fat accumulation (Wang et al., 1997; Tsochatzis et al., 2009).

Interestingly, leptin has a potent angiogenic activity (McDonald and Choyke, 2003). Leptin may act by itself as a direct angiogenic factor or indirectly as potential modulator for other known angiogenic factors (Cao et al., 2001). It modulates VEGF-induced vascular activity by synergistic promotion of neovascularization *in vivo*. Leptin upregulates VEGF mRNA expression via activation of the Jak/Stat3 signalling pathway (Cao, 2007).

Although TNF-α was presented as a key factor in the development of NAFLD, its exact role remains controversial (Ma et al., 2008; Tacke et al., 2009). In adipose tissue, TNF-α acts by repressing genes involved in uptake and storage of non-esterified FFA (Koca et al., 2008). These fatty acids are thus readily available to accumulate in non adipose tissues like the liver, increasing the pool of intrahepatic FFA (Lucero et al., 2011). On the other hand, IL-6 is overexpressed in the adipose tissue of obese patients (Tilg and Moschen, 2006). Chronically elevated IL-6 levels lead to hyperinsulinaemia, impaired insulin-stimulated glucose uptake by the skeletal muscles and marked inflammation in the liver (Wieckowska et al., 2008; Marra and Bertolani, 2009). Collectively, adipose tissue may be the common origin of increased serum levels of TNF-α and IL-6 during progression of NAFLDs (Czaja, 2004). In the liver, Kupffer cells are capable of releasing large quantities of TNF-α and IL-6 directly in contact with liver cells influencing hepatic inflammation and fibrogenesis (Zhang et al., 2000; Shoelson et al., 2006; Lalor et al., 2007).

CONCLUSION

Suggested parameters, based on their involvement as key players in disease pathogenesis and their high correlation to histological staging and scoring, may be useful non-invasive diagnostic tools for discriminate between fatty liver and non-alcoholic steatohepatitis. Future work on obese patients is intended.

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