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Factors related to non-alcoholic fatty liver disease in obese children

Obez çocuklarda alkole bağlı olmayan yağlı karaciğer hastalığı ile ilişkili faktörler

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Background/aims: *The incidence of non-alcoholic fatty liver disease has been increasing parallel to obesity in the pediatric age group. This study aimed to analyze the factors that are related to non-alcoholic fatty liver disease in obese children.* ***Methods:*** *101 obese children and 68 non-obese controls were included in the study. Liver steatosis was investigated by ultrasonography. The subjects were divided into three groups: 53 obese patients with fatty liver (Group I), 48 obese patients without steatosis (Group II), and 68 controls without steatosis (Group III). Group I was further divided into those with Grade 1 steatosis (44 patients, Group Ia) and higher grades of steatosis (9 patients, Group Ib). The relationships of body mass index, serum ALT, lipids, leptin, and insulin resistance index with steatosis were analyzed.* ***Results:*** *52.4% of obese children had fatty liver and 13.8% had high ALT levels. Additionally, all patients with elevated ALT levels were seen to have liver steatosis by ultrasonography. Leptin and insulin resistance index levels were higher in obese groups than controls; however, the difference disappeared when these levels were adjusted for body mass index. ALT levels were higher in Group I (31.5±30.2) than Group II (18.0±7.1) and Group III (14.5±5.2) ($p<0.05$). Group Ib showed higher VLDL and ALT levels than Group Ia ($p<0.05$). Multiple regression analysis revealed that body mass index was the most important determinant of liver steatosis, while body mass index and VLDL were the determinants of higher ALT levels.* ***Conclusions:*** *We suggest that body mass index and VLDL are the most important determinants of non-alcoholic fatty liver disease and elevated ALT levels in obese children. The contribution of leptin to this process could not be determined in our findings.*

Key words: Ultrasonography, non-alcoholic fatty liver disease, obesity, childhood

Amaç: *Alkole bağlı olmayan karaciğer yağlanmasının sıklığı çocukluk yaş grubunda obesiteye parallel olarak artmaktadır. Bu çalışma obez çocuklarda alkole bağlı olmayan karaciğer yağlanması ile ilişkili faktörleri belirlemek için planlandı.* ***Yöntem:*** *Çalışmaya 101 obez ve 68 obez olmayan çocuk katıldı. Karaciğerde yağlanma varlığı ultrasonografi ile araştırıldı. Olgular 3 gruba ayrıldı; 53 yağlı karaciğeri olan obez hastalar (grup I), 48 karaciğerde yağlanma olmayan obez hastalar (grup II) ve 68 karaciğerde yağlanma olmayan kontrol olgu (grup III). Grup I'de kendi içinde 2 gruba ayrıldı; grade 1 yağlanması olan (44 hasta Grup Ia), yüksek grade yağlanması olan (9 hasta, Grup Ib). Vücut kitle indeksi, serum ALT, lipidler, leptin, insülin direnci indexi ile karaciğer yağlanması arasındaki ilişki incelendi.* ***Bulgular:*** *Obez çocukların %52,4'ünde karaciğer yağlanması, %13,8'inde yüksek ALT değerleri vardı. Aynı zamanda; ALT seviyesi yüksek olan hastaların tümünde karaciğer yağlanması mevcuttu. Leptin ve insülin direnci indexi düzeyleri Grup I ve II'de Grup III'e kıyasla yüksek olmasına rağmen vücut kitle indeksi ile düzeltme yapıldığında gruplar arasında fark kalmadı. Grup I'de serum ALT düzeyleri (31.5±30.2) Grup II (18.0±7.1) ve Grup III'e (14.5±5.2) kıyasla daha yüksek idi ($p<0.05$). Grup Ib'de ise ALT ve VLDL düzeylerinin Grup Ia'ya göre daha yüksek olduğu saptandı ($p<0.05$). Çoklu regresyon analizlerinde: Yağlanma üzerinde vücut kitle indeksi belirleyici, ALT düzeylerinde ise vücut kitle indeksi ve VLDL belirleyici olarak tanımlandı.* ***Sonuç:*** *Çalışmamız vücut kitle indeksi'nin karaciğer yağlanmasında belirleyici en önemli faktör olduğunu ve VLDL ile birlikte yüksek ALT düzeylerine katkıda bulunabileceğini düşündürdü. Ancak çalışmamızda leptinin bu sürece katkısı gösterilememiştir.*

Anahtar kelimeler: Ultrasonografi, alkole bağlı olmayan yağlı karaciğer hastalığı, obezite, çocukluk çağı

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) has become a serious public health problem in childhood with the increase in overweight and obesity in the world. In the pediatric population, the incidence of NAFLD was reported to be 2.6%, but this ratio in obese children has increased to range from 20-50% (1).

NAFLD is a clinicopathological term that encompasses a disease spectrum ranging from simple hepatic steatosis to hepatic steatosis with inflam-

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mation, fibrosis, and cirrhosis (2, 3). NAFLD is usually asymptomatic in children. On physical examination, the liver might be slightly enlarged. The diagnosis of NAFLD is usually made by mild elevations in liver tests during a routine blood testing or liver ultrasonography (US) in an overweight or obese child. A mild increase in serum alanine aminotransferase (ALT) level is the most common finding in NAFLD (4-6).

The pathogenesis of NAFLD has not been well defined. The “two-hit" hypothesis was proposed to explain the pathogenesis of the disease. The “first hit” constitutes the deposition of triglycerides (TG) in the cytoplasm of the hepatocyte, whereby the first "hit" – steatosis - sensitizes the liver to a variety of second "hits", which lead to inflammation and fibrosis, the histological hallmarks of non-alcoholic steatohepatitis (NASH) (7). Mitochondrial dysfunction, exposure to cytokines (tumor necrosis factor, interleukin [IL]-1, IL-8), decreased activity in cell protective mechanism, oxidative stress, lipid peroxidation, leptin, iron and Kupffer cell dysfunction are likely to play a role in the pathogenesis of NASH as a "second hit". In addition to these factors, the genetic differences undoubtedly are important. In clinical practice, almost 90% of the patients have at least one or more of the following diseases: type 2 diabetes mellitus, obesity and hyperlipidemia (8-11).

The importance of insulin resistance (IR) in obese subjects with NAFLD was reported in many studies. Some of them have suggested that liver steatosis is a feature of IR syndromes and Syndrome X (12). However, IR was shown in non-obese subjects with NAFLD (13).

Leptin is an adipocyte-derived hormone that regulates food intake and energy expenditure through interaction with receptors in the hypothalamus (14). It is thought that leptin may contribute to or enhance hepatic steatosis by changing actions of insulin on tissues and its receptors, and it may influence the development of NASH through the regulation of inflammatory responses (15). Serum leptin levels were found elevated in several studies on patients with steatosis and/or NASH, thus it has been suggested that leptin may play a regulatory role in progression of hepatic steatosis and NASH (16).

The gold standard in diagnosis of NAFLD is liver biopsy. Liver biopsy is not usually preferred in the pediatric population because of the presence of early stages of disease in childhood, and due to its related cost and complications. Although liver US can estimate neither fibrosis nor inflammation, it has a sensitivity of 89% and a specificity of 93% for detecting histological steatosis. In the absence of liver biopsy, presumed NASH is conventionally diagnosed by US together with elevated serum ALT levels as a marker of liver inflammation and injury (3).

This study was planned to research the incidence of liver steatosis in obese children and to investigate the relationship between the presence and severity of steatosis with anthropometric measurements, hyperlipidemia, IR and leptin.

MATERIALS AND METHODS

The study included 101 patients referred to our clinics for primary obesity by physicians during the period January 2003 - March 2005. Anthropometric measurements were performed in all patients. Weight and standing height were measured with a calibrated scale and stadiometer, respectively, by standard methods. BMI was calculated as weight (kg)/height (m^2). Patients with a body mass index (BMI) of ≥95th percentile according to reference curves for Turkish children were accepted as obese (17).

A liver US was performed in all subjects for assessment of liver steatosis with a General Electric Logic 9 (MI, USA) machine, equipped with 5 MHz probes in younger children and 3 MHz in larger or markedly obese children. US was performed by the same radiologist expert on liver US who did not know the clinical course or laboratory details of the patients. The presence and severity of steatosis were assessed by the scoring system defined by Tominaga et al. (1) according to the hyperechogenicity of the liver tissue, discrepancy between liver and diaphragm and visibility of vascular structures (Grade 1, 2 and 3). The subjects were divided into groups in accordance with the presence or not of liver steatosis: Group I included 53 obese patients (27 boys, 26 girls) with liver steatosis. Group II included 48 obese patients without liver steatosis. The control group (Group III) included 68 sex-, age- and pubertal stage-matched non-obese healthy subjects without liver steatosis. Exemption from human subjects research committee review for investigations was authorized. According to US scoring: Grade 1 steatosis was determined in 44 patients and Grade 2-3 steatosis in 9 patients, defined as Group Ia and Group Ib, respectively.

Table 1. Demographic characteristics of subjects

	Group I* (n=53)	Group II** (n=48)	Group III*** (n=68)	p
Age (year) (median)	10.9±3.0	10.3±3.0	11.2±2.6	> 0.05
Sex (female/male)	26/27	26/22	34/34	> 0.05
Weight (kg)	62.8±17.3[a b]	52.4±17.1 [b]	39.0±11.8	**< 0.05**
Height (cm)	149.2±14.7	141.7±15.9	145.5±15.2	> 0.05
Body mass index (kg/m²)	27.5±3.5 [a b]	25.2 ±3.2 [b]	18.0±2.4	**< 0.05**
Pubertal stage (n)				
Prepubertal	22	21	34	> 0.05
Pubertal	31	27	34	

a: comparison with Group II, p<0.05. b: comparison with Group III, p<0.05. *Obese/US with steatosis, ** Obese/US without steatosis, ***Control

Demographic characteristics are shown in Table 1. Secondary obesity, drug use, alcohol intake, hepatitis B and hepatitis C, celiac disease and Wilson disease were excluded by history and laboratory tests in patients with fatty liver.

Biochemical tests in patients were performed in the morning after a 10-hour overnight fasting period. Levels of fasting serum glucose, insulin, leptin, ALT, total cholesterol, high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), very low-density lipoprotein (VLDL-C) and TG were measured. We used the homeostasis model assessment for IR (HOMA-IR) index as the index of IR, which was calculated as follows: HOMA IR = fasting insulin (μIU/L) X fasting glucose (mmol/L)/22.5 (18). Serum leptin levels were measured using a 125I IRMA kit (Diagnostic Systems Laboratories, Inc., Cat No. DSL-23100, TX, USA).

Statistical Analysis

The data were analyzed by *Statistical Package for Social Sciences (SPSS/PC 11.5)*. The convenience of numerical data for normal distribution was analyzed by Kolmogorov-Smirnov test. All continuous variables except age were non-normally distributed and thus underwent logarithmic transformation for statistical analysis. Clinical and laboratory data were presented as mean ± standard deviation (ranges). Comparison between groups was performed using ANOVA (post-hoc: Bonferroni), Student-t test, Mann-Whitney U test and chi-square test, where appropriate. The correlation among numerical data was analyzed by the Pearson correlation coefficient (r). To analyze the association between categorical variables, Spearman's rank correlation coefficient was used. Regression analysis was performed by multivariate or univariate logistic regression where available. A p value less than 0.05 was considered significant.

RESULTS

Demographic characteristics of the groups and laboratory results are shown in Tables 1 and 2. BMI was different between Group I, Group II and controls. Total cholesterol and TG levels were higher in Groups I and II than in controls. LDL levels were different between Group II and controls. VLDL levels showed difference between Group I and controls. HDL levels were similar between groups (p>0.05). ALT levels were found significantly increased in Group I compared to the other groups. There were differences between groups with respect to the percentages of hypercholesterolemia (total cholesterol >200 mg/dl, hypertriglyceridemia (>140 mg/dl), elevated LDL (>130 mg/dl), elevated ALT (>40 IU/L) and HOMA IR (>2.5) (Table 3). HOMA-IR (r=0.65; p<0.05) and leptin levels (r =0.82; p<0.05) in obese children were higher than in controls, but after these parameters were adjusted for BMI, this difference disappeared (p>0.05).

Table 2. Biochemical characteristics of groups

	Group I* (n=53)	Group II** (n=48)	Group III*** (n=68)	p
Triglyceride (mg/dl)	111.7±50.6 [b]	101.5±63.8 [b]	70.2±21.4	< 0.05
Cholesterol (mg/dl)	172.7±47.9 [b]	175.3±37.0 [b]	153.2±20.4	< 0.05
HDL (mg/dl)	45.7±12.6	49.3±10.3	49.3±10.4	> 0.05
LDL (mg/dl)	103.8±44.0	106.5±29.2 [b]	91.3±18.3	< 0.05
VLDL (mg/dl)	22.8±11.8 [b]	19.0±12.0	15.0± 9.8	< 0.05
ALT (IU/L)	31.5±30.2 [a b]	18.0±7.1	14.5±5.2	< 0.05
HOMA-IR	3.79±2.6 [b]	3.0±1.5 [b]	1.3±0.7	< 0.05
Leptin (ng/ml)	40.9±18.3 [a b]	31.2±15.8 [b]	8.4±5.5	< 0.05

a: comparison with Group II, p<0.05. b: comparison with Group III, p<0.05. *Obese/US with steatosis, **Obese/US without steatosis, ***Control

Table 3. Ratios of hypertriglyceridemia, hypercholesterolemia, increased LDL and ALT and HOMA-IR according to groups

	GROUP I (n=53)		GROUP II (n=48)		GROUP III (n=68)		
	n	%	n	%	n	%	p
Hypertriglyceridemia	13	24.5 [b]	7	14.6 [b]	1	1.5	< 0.05
Hypercholesterolemia	6	11.3 [b]	9	18.8 [b]	0	----	< 0.05
Elevated LDL	6	11.3 [b]	8	16.7 [b]	1	1.5	< 0.05
Elevated ALT	14	26.4 [a]	0	----	0	----	< 0.05
HOMA-IR	32	60.4 [b]	28	58.3 [b]	7	10.3	< 0.05

a: comparison with Group II, p<0.05. b: comparison with Group III, p<0.05.

When Groups Ia and Ib were compared regarding leptin, HOMA-IR, BMI, VLDL and ALT, only VLDL and ALT levels were significantly higher in Group Ib (Table 4).

To determine the factors that play a role in liver steatosis, BMI (r=0.58), TG (r=0.33), VLDL (r=0.36), leptin (r=0.57) and HOMA-IR (r=0.35) were analyzed by multiple regression method. BMI was found to be the unique parameter in the model. The same method was used for parameters related with ALT levels [BMI (r=0.42), TG (r=0.32), cholesterol (r=0.21), VLDL (r=0.36), LDL (r=0.15), leptin (r=0.36) and HOMA-IR (r=0.33), p<0.05]. BMI (β =0.34, p<0.05) and VLDL (β=0.23, p<0.05) showed a significant relationship.

Correlations between ALT and VLDL; BMI and degree of steatosis with US; and ALT and BMI are shown in Figure 1 (A, B, C), respectively.

DISCUSSION

Obesity has become a worldwide major health problem in children. Along with the rise in obesity, NAFLD is becoming responsible for the large percentage of liver disease in adults and children. NAFLD is usually diagnosed by US, enzyme analysis and liver biopsy in obese people (3).

In this study, fatty liver and degree of liver steatosis were determined by US and ALT levels. Liver biopsy was not performed in patients with NAFLD in our study since there is no proven therapy based on biopsy findings and because of the related cost and risk.

Table 4. BMI and laboratory characteristics of patients with liver steatosis

	Group Ia (n=44)	Group Ib (n=9)	p
BMI (kg/m^2)	27.5±3.5	27.8±3.4	> 0.05
Leptin (ng/ml)	39.9±18.4	45.9±17.7	> 0.05
VLDL (mg/dl)	20.7±9.3	33.0±17.2	**< 0.05**
ALT (IU/L)	22.8±11.8	73.6±52.8	**< 0.05**
HOMA-IR	3.8±2.7	3.9±2.6	> 0.05

The characteristics of NAFLD in obese children have been investigated in many studies. The frequency of elevated ALT levels was reported by Vajro et al. (20) and Tazawa et al. (19) as 10% and 24%, respectively. According to a survey study in Italy, of the 195 obese children, 55% had liver steatosis by US, 20% had elevated ALT and aspartate aminotransferase (AST) levels, and 15% had both (21). Of the 101 obese children in our study, 52.4% had liver steatosis by US and 13.8% had elevated ALT levels. Additionally, US revealed liver steatosis in all patients with elevated ALT levels. The increase in ALT and the severity of steatosis were parallel in our study group. This finding suggests that higher degree of steatosis results in an increase in inflammatory response, and subsequently, elevated ALT levels might be a predictor of the progression of steatosis stage to steatohepatitis and fibrosis stages.

The degree of steatosis was found to correlate positively with BMI, hyperlipidemia (TG, VLDL), ALT, leptin and IR indexes. After application of stepwise logistic regression analysis of these parameters that may be responsible in the pathogenesis of liver steatosis, BMI was found to be the unique and significant risk factor.

BMI values of obese patients with liver steatosis were higher than in obese patients without liver steatosis and the control group. These data were consistent with the relationship between obesity and NAFLD established in the literature (1, 4, 10).

In our study, serum leptin levels and BMI were found higher in obese patients with liver steatosis than healthy controls and obese patients without steatosis. There were no differences between groups for age, sex and puberty, which are known to affect leptin levels. However, it is reported that obese patients had higher leptin levels parallel to BMI (22-24). A number of studies have suggested that serum leptin levels increased in patients with

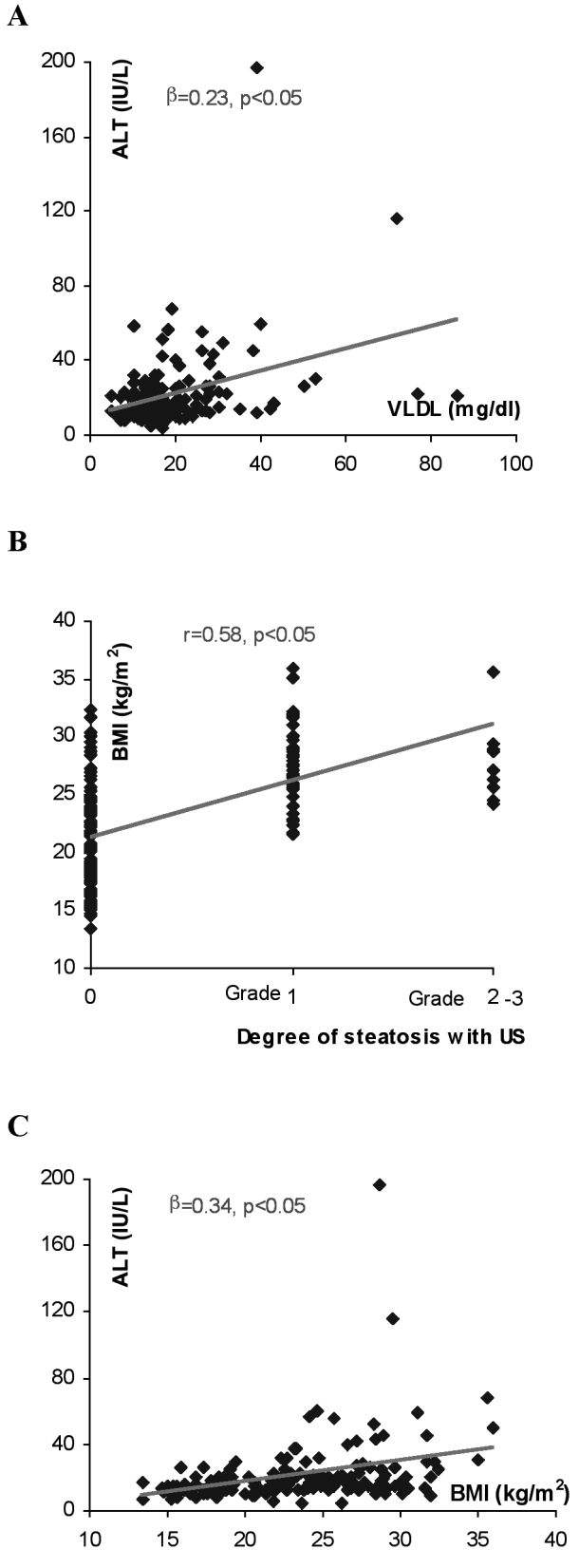

Figure 1. Correlations between ALT and VLDL; BMI and degree of steatosis with US; and ALT and BMI are shown in A, B, and C, respectively.

NAFLD and/or NASH, and this increase may be responsible in the pathogenesis of steatosis and steatohepatitis, although some studies did not support this hypothesis. Chalasani et al. (25) reported that serum leptin was not statistically different among 26 patients with biopsy-proven NAFLD and their age-, sex-, and BMI-matched controls. Furthermore, the serum leptin levels and the hepatic leptin receptor mRNA expression were similar in 5 patients with NASH and 5 patients with simple steatosis. Similar to our study, Nakao et al. (26) investigated the degree and presence of steatosis by US in patients with elevated ALT. They found that BMI, serum leptin levels, body fat ratio, and abdominal wall fat index (AFI) were higher in steatotic patients, but multiple logistic regression analysis indicated that AFI (V/S ratio) was the only independent risk factor for fatty liver in both sexes. It was shown that the main determinant of serum leptin levels in obesity was total body fat and subcutaneous fat ratio rather than BMI (27). Total body fat and subcutaneous abdominal fat were not measured in our study, but probably were responsible for the elevated leptin levels found in our steatotic patients. Although Group Ia and Group Ib patients had the same BMI, leptin levels were not different statistically.

These findings indicate that leptin is not an important factor in the pathogenesis of simple steatosis and NASH. Although previous studies have shown that leptin affects inflammation response *in vitro,* this effect was not definitely demonstrated in NASH pathogenesis in clinical trials (28). In addition, it was proposed by Serin et al. (29) that there might be a negative correlation between leptin and inflammation. Angulo et al. (30) demonstrated that the significant association between leptin and fibrosis by univariate analysis was abolished after controlling confounders including age, gender, BMI, diabetes and IR by multivariate analysis; then only age and insulin sensitivity correlated significantly with fibrosis stage. The studies performed on patients with lipodystrophy have also supported this idea. NAFLD with increased fibrosis may be seen in patients with diffuse lipodystrophy. However, there was a leptin deficiency in these patients despite the presence of IR (31). Thus, it may be proposed that leptin may not affect the development and progression of NAFLD.

All these findings together with our data have suggested that serum leptin levels in NAFLD may be related to factors that play a role in the

production and secretion of leptin, such as age, sex, puberty, total body fat mass, subcutaneous fat ratio and IR, independent of the presence and severity of NAFLD.

IR is a well-defined risk factor for obese and non-obese NAFLD patients. The positive correlation between childhood obesity and IR was demonstrated (32). Radetti et al. (33) showed a decreased insulin sensitivity in all of the obese children but no difference was found in insulin sensitivity between children with or without NAFLD. Chan et al. (34) showed a positive correlation between IR markers and presumed NASH only in male obese children. We did not find any correlation between presence and severity of steatosis with IR. Possibly, IR in obese patients may cause the progression and development of steatosis by enhancing the effects of other factors but not by itself.

The positive correlations determined between presence and severity of steatosis and BMI and also ALT levels and VLDL support the hypothesis that increased free fatty acids (FFA) influx from adipocyte to liver in obese patients may be one of responsible mechanisms in NAFLD pathogenesis.

IR in NAFLD reduces the anti-lipolytic effects of insulin on adiposities and increase in delivery of FFA from adipose tissue to liver (35). FFA are metabolized by ,-oxidation for energy production or stored as TG or sent back in VLDL to peripheral fat tissue. Also, increased intake of carbohydrates increases the synthesis of VLDL, TG and FFA. VLDL contains apolipoprotein B-100 (apoB-100), TG, cholesterol esters and phospholipids. Accumulation of fat in the liver may occur due to the excessive delivery of FFA from peripheral tissues to the liver, decreased secretion of VLDL, and increased endogenous synthesis of TG, cholesterol esters and FFA in the liver (36).

In several studies, prevention and attenuation of hepatic steatosis have been proposed as a 'function' of VLDL secretion (37). Several studies have been performed on apolipoprotein synthesis in patients with NASH and some of them have shown that the first stage of liver steatosis should arise from disturbances in synthesis and delivery of VLDL (37). In human studies, Charlton et al. (38) reported that a decrease in the hepatic synthesis of apoB-100, which is a speed-limiting step of synthesis of VLDL in patients with NASH, may be an important factor in the development of hepatic steatosis as a result of increased accumulation of TG in hepatocytes. However, this study was composed of a limited number of cases and did not specify the clinical, laboratory and histological features of patients with NASH. Koruk et al. (39) demonstrated that mean serum apoB-100 levels in the patients with biopsy-proven NASH were higher than those of the healthy volunteers. In our study, VLDL levels were found increased in the patient group that had presumptive NASH. Reduction of VLDL secretion and apoB-100 deficiency probably do not play a role in NASH pathogenesis. The excess production of VLDL may be secondary to NASH itself. As a result of increased hepatic uptake of FFA and reduction of β-oxidation of FFA in NASH, increased hepatic VLDL secretion and apoB-100 levels might arise from dealing with the excess hepatic FFA load. Nevertheless, apoB-100 levels and liver biopsy were not performed on presumptive NASH patients in our study.

The most important limiting factor of our study was that the stage of NAFLD was not determined by liver biopsy. There is no option except biopsy for determining the spectrum of disease in a patient with liver steatosis determined by US and mildly elevated liver enzymes. However, liver biopsy was not performed in patients with NAFLD in our study since there is no proven therapy based on biopsy findings and because of its related cost and risk. The absence of liver biopsy data does not allow us to confirm the presumptive NASH patients who in fact have abnormal histology. US is an operator-dependent procedure, and the numerical scoring may change with the operator. Operator bias was minimized by consulting only one radiologist. Further longitudinal studies that include liver biopsy should be required to determine the impacts of serum leptin, lipid, apoprotein, and apolipoprotein levels, IR, BMI, subcutaneous lipid profile, oxidative stress, lipid peroxidation and cytokines in NASH pathogenesis.

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