

Carotid intima-media thickness and flow-mediated dilation in obese children with non-alcoholic fatty liver disease

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ABSTRACT

Background/Aims: This study aimed to understand the role of non-alcoholic fatty liver disease (NAFLD) in increasing the risk of atherosclerosis in obese pediatric patients.

Materials and Methods: The study included 109 obese children (age, 9-15 years) and a control group comprising 44 healthy age- and gender-matched children with normal weight. NAFLD was diagnosed using conventional ultrasound (US) examination. Both right carotid intima-media thickness (CIMT) and flow-mediated dilation (FMD) were assessed in addition to anthropometric measures and serum biochemical parameters, including lipid profile and fasting glucose and insulin levels. Homeostatic model assessment of insulin resistance (HOMA-IR) was calculated as a measure of insulin resistance.

Results: Mean age and gender distributions were similar in the obese children and control group ($p=0.168$ and $p=0.705$, respectively). Median body mass index standard deviation scores of obese children with grade II-III NAFLD were significantly higher than those of obese children without hepatosteatosis ($p<0.001$). Median total cholesterol levels were similar in all the groups ($p=0.263$). Low-density lipoprotein cholesterol and triglyceride levels increased and high-density lipoprotein cholesterol levels decreased significantly as the grade of steatosis increased ($p<0.001$, $p<0.05$, and $p=0.05$, respectively). Median alanine aminotransaminase (ALT) and HOMA-IR levels of obese children with grade II-III NAFLD were significantly higher than those of obese children without NAFLD ($p=0.01$) and obese children with grade I NAFLD ($p=0.001$). CIMT was significantly correlated with the grade of steatosis ($p<0.001$) and level of ALT ($p=0.005$). Linear regression analysis showed that the grade of hepatosteatosis had a significant effect on CIMT. FMD decreased as the grade of hepatosteatosis increased, but it did not reach a significant level.

Conclusion: The obese children with hepatosteatosis showed increased CIMT, as indicated by the grade of steatosis, compared with healthy controls and obese children without hepatosteatosis. FMD was not superior to CIMT in predicting the risk of early atherosclerosis.

Keywords: Hepatosteatosis, insulin resistance, flow-mediated dilation, carotid intima-media thickness

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is a clinicohistopathological entity associated with obesity and has recently been reported as the most common form of chronic liver disease (1). NAFLD affects 2.6%-9.8% children and adolescents; however, it affects 38%-77% obese children and adolescents (2). This potentially serious condition is strongly associated with obesity, insulin resistance (IR), hypertension, and dyslipidemia (3). Obesity and IR, which are components of metabolic

syndrome (MetS), play an important role in every stage of atherosclerosis, from initial endothelial dysfunction to final plaque rupture (4). Pathogenesis of both NAFLD and atherosclerosis share some metabolic events and markers such as IR, hyperlipidemia, oxidative stress, inflammation, and cytokines. Therefore, atherosclerotic lesions are expected to develop more frequently in obese patients with NAFLD. In adults, the severity of liver disease is associated with decreased endothelial function (5); however, limited data are available on en-

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dothelial dysfunction in pediatric patients with NAFLD. Right carotid intima-media thickness (CIMT) and brachial flow-mediated dilation (FMD) are preclinical markers of vascular health (6). The present study aimed to investigate the relationship between NAFLD and cardiovascular risk factors and increased risk of atherosclerosis in obese children with NAFLD. We hypothesized that NAFLD is associated with decreased FMD and increased CIMT, which are the early markers of atherosclerosis and which predict the risk of atherosclerosis.

MATERIALS AND METHODS

This study included 109 obese children (age, 9-15 years) and a control group comprising 44 healthy non-obese children. The study participants were recruited between January 2011 and February 2013 from the Pediatric Endocrinology and Metabolism Outpatient Clinic of Bezmialem Vakif University.

Each participant underwent a detailed physical examination, including anthropometric measurements, grade of obesity, and systolic and diastolic blood pressure (BP). Children in whom obesity was attributed to syndromes such as Prader-Willi syndrome and Laurence-Moon-Biedl syndrome and to endocrinal causes such as Cushing's syndrome or hypothyroidism were excluded from the study. In addition, children with systemic diseases (such as cystic fibrosis and inflammatory bowel disease), hepatitis, drug use, cigarette use, alcohol use, history of parenteral nutrition, and family history of hereditary hyperlipidemia or premature atherosclerosis were also excluded from the study. All the participants were screened for antibodies against hepatotropic viruses, serum ceruloplasmin, and serum α 1 antitrypsin and autoantibodies against nuclear smooth muscle and liver-kidney microsomal type-1 antigens to exclude children with hepatosteatosis who had infectious, metabolic, and autoimmune liver pathologies. Obesity was determined based on body mass index (BMI) of \geq 95th percentile for gender and age, BMI%, and BMI standard deviation score (BMI SDS) (7). Standing height was measured to the nearest 0.1 cm by using a Harpenden fixed stadiometer. Body weight (kg) was measured to the nearest 0.1 kg by using a SECA balance scale, with each subject dressed in light T-shirt and shorts. BMI was calculated by dividing body weight by height (kg/m^2). BP was measured three separate times after the children had been sitting for 10 min, and the second and third measurements were averaged. Children with systolic and/or diastolic BP of $>95^{\text{th}}$ percentile (adjusted for height, age, and gender) were diagnosed as having high BP (8).

All blood analyses were performed on fasting blood samples obtained from participants in both the study and control groups. Cholesterol, high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol, and triglycerides (TGs) were measured using a homogenous colorimetric enzyme technique (Cobas 8000; Roche). Glucose was measured using glucose oxidase technique (Advia 1800; Siemens), and insulin levels were analyzed using direct chemiluminescence technique (Siemens Centaur, USA). IR was estimated by mea-

suring fasting plasma insulin and glucose levels with HOMA-IR (insulin [mU/L] \times glucose [mmol/L]/22.5) (9). Criterion for IR was HOMA-IR value of >2.5 for prepubertal children and >4.0 for adolescents (10).

Conventional hepatic ultrasound (US) examination was performed by a radiologist by using Logiq 9 (GE Healthcare, USA) with convex transducers (frequency bandwidth, 3.5 MHz). The radiologist was blinded to the clinical and laboratory data and to the risk factors. Before the US examination, participants were asked to rest quietly in a temperature-controlled dark room for 10-15 minutes. Ultrasonographic steatosis scores were defined as follows: no hepatosteatosis (grade 0): normal liver echotexture; mild hepatosteatosis (grade 1): slight and diffuse increase in fine parenchymal echoes, with normal visualization of the diaphragm and portal vein borders; moderate hepatosteatosis (grade 2): moderate and diffuse increase in fine parenchymal echoes, with slightly impaired visualization of the diaphragm and portal vein borders; severe hepatosteatosis (grade 3): fine echoes, with poor or no visualization of the diaphragm and portal vein borders or of the posterior portion of the right lobe of the liver (11). The children were classified into the following four groups according to their ultrasonographic hepatosteatosis scores: group I, non-obese children with no hepatosteatosis (controls); group II, obese children without NAFLD; group III, obese children with grade I NAFLD; and group IV, obese children with grade II-III NAFLD.

MetS was diagnosed using a modification of National Cholesterol Education Program's Adult Treatment Panel Criteria (12). Body proportion normally changes during pubertal development and can vary among individuals, waist circumference is difficult to interpret in children. Therefore, BMI was used to determine obesity degree according to the previously defined criteria. MetS was defined as the presence of any three of the following five constituent risks: hypertension (elevated BP as systolic or diastolic BP of $\geq 95^{\text{th}}$ percentile for age and gender); low HDL cholesterol levels (below the 95th percentile for age and gender); hypertriglyceridemia (TG levels above the 95th percentile for age and gender); obesity (BMI of $\geq 95^{\text{th}}$ percentile for age and gender); and glucose impairment (determined using pediatric reference standards).

Carotid scanning was performed using B-mode US (Logiq S6; GE Healthcare, USA) with a 14-MHz linear probe by placing the patients in a supine position, with their necks extended. The probe was placed longitudinally in the anterolateral position first on the right side of the neck and then on the left side of the neck. Right CIMT was measured at 1-cm below the bifurcation. The distance between the echogenicity of the lumen-intima interface and the adventitia-media interface was recorded as the thickness of the intima-media. To prevent bias, three measurements at 3-mm intervals were taken. CIMT was calculated as the mean of three independent measurements from each side of the neck (13).

Table 1. Demographic features and clinical and laboratory data of the study and control groups

	(1) Non-obese controls (n = 44)	(2) Obese patients without NAFLD (n = 24)	(3) Obese patients with grade I NAFLD (n = 47)	(4) Obese patients with grade II-III NAFLD (n = 38)	P+
BMI SDS (median [IQR])	0.03 (0.78)	1.97 (0.47)	1.98 (0.39)	2.22 (0.37)	<0.001*
MetS (%), n	-	3 (12.5%)	16 (36.2%)	13 (34.2%)	0.098
Systolic BP (in mmHg; median [IQR])	100.0 (12.0)	110.0 (0.0)	100.0 (7.0)	110.0 (5.0)	<0.001+
Diastolic BP (in mmHg; median [IQR])	60.0 (8.0)	70.0 (8.8)	70.0 (20.0)	62.0 (20.0)	<0.001+
CIMT (in mm; median [IQR])	0.43 (0.09)	0.43 (0.06)	0.50 (0.10)	0.52 (0.10)	<0.001++ <0.01+++
FMD (in mm; median [IQR])	0.060 (0.07)	0.065 (0.08)	0.060 (0.04)	0.055 (0.04)	0.392

BMI SDS: body mass index standard deviation score; BP: blood pressure; CIMT: carotid intima-media thickness; FMD: flow-mediated dilation; IQR: interquartile range; MetS: metabolic syndrome; vs: versus

Statistical tests: Kruskal-Wallis test, Mann-Whitney U test, and Chi-square test

*1 vs 2, 3, and 4; †2 and 3 vs 4

+1 vs 2, 3, and 4; ++1 vs 3 and 4 and 2 vs 4; +++2 vs 3 and 3 vs 4

FMD of the right brachial artery was measured for each patient according to the guidelines for the US assessment of endothelial-dependent flow-mediated vasodilation of the brachial artery (14). Each patient was placed in a supine position, with the arm rested when a blood pressure cuff was placed on the forearm. Linear vascular transducer with a frequency of 10 MHz (Logiq 9) was used for imaging the brachial artery, specifically the segment of the brachial artery just above the antecubital fossa. To obtain a baseline rest image, the brachial artery was photographed using pulsed Doppler before cuff inflation, after which the cuff was inflated to 50-mmHg systolic pressure. After 5 minutes, the cuff was deflated, and the brachial artery was photographed again by using pulsed Doppler. Distance measurements for FMD analyses were taken at maximum systolic pressure.

Statistical analysis was performed using PASW Statistics v.13.0. Median and interquartile ranges were used for metric variables. Mann-Whitney *U* test was used to calculate the difference between parameters in the groups; Kruskal-Wallis test was used to compare two or more samples that are independent and have different sample size. Multiple comparisons were made using Spearman correlation. Linear regression analysis was used to evaluate the effects of risk factors on dependent variables. Categorical data were evaluated using chi-square test; $p < 0.05$ was considered statistically significant.

The study was approved by the local ethics committee, and written informed consent was obtained from the parents of all the participating children.

The study included 24 obese children (11 girls) without NAFLD, 44 obese children (20 girls) with grade I NAFLD, 38 obese children (21 girls) with grade II-III NAFLD, and 44 non-obese healthy (20 girls) children without NAFLD (controls). Median age and gender distributions were similar in the groups ($p > 0.05$). Medi-

an BMI values of obese children were significantly higher than those of controls. The median BMI value was significantly higher in obese children with grade II-III NAFLD than in obese and non-obese children without hepatosteatosis ($p < 0.001$; Table 1).

HOMA-IR values of obese children with grade II-III NAFLD were significantly higher than those of controls ($p < 0.001$), obese children without hepatosteatosis, and obese children with grade I steatosis ($p = 0.05$). The median HOMA-IR value increased as the grade of hepatosteatosis increased. Median ALT levels in obese children with grade II-III NAFLD were significantly higher than those in controls and in obese children without NAFLD and those with grade I NAFLD ($p < 0.01$). Median total cholesterol levels were similar in all the groups ($p = 0.263$). LDL cholesterol and TG levels significantly increased and HDL cholesterol levels significantly decreased as the grade of hepatosteatosis increased ($p < 0.001$, $p < 0.05$, and $p = 0.05$, respectively; Table 2).

Median systolic BP in children grade II-III NAFLD was significantly higher than that in controls and in obese children without hepatosteatosis and those with grade I hepatosteatosis ($p < 0.001$). Median diastolic BP also increased significantly as the degree of hepatosteatosis increased ($p < 0.001$). In both obese children and controls, median right CIMT increased significantly ($p < 0.001$) as the grade of steatosis increased. FMD decreased as the grade of hepatosteatosis increased but did not differ significantly between the groups. ($p = 0.392$; Table 3).

MetS was noted in 3 of 23 (12.5%) obese children without hepatosteatosis, 16 of 43 (36.2%) obese children with grade I hepatosteatosis, and 13 of 40 (34.2%) obese children with grade II-III hepatosteatosis. Occurrence of MetS increased as the grade of steatosis increased but did not reach statistical significance ($p = 0.098$; Table 3). Table 4 shows the correlations of CIMT and FMD with other anthropometric and biochemical parameters

Table 2. Biochemical characteristics of the study and control groups

	(1) Non-obese controls (n = 44)	(2) Obese patients without NAFLD (n = 24)	(3) Obese patients with grade I NAFLD (n = 47)	(4) Obese patients with grade II-III NAFLD (n = 38)	P+
HOMA-IR	1.9 (0.85)	3.24 (1.88)	3.80 (2.59)	4.19 (2.50)	<0.001*
0.05**					
Triglyceride (mg/dL)	64.0 (70.0)	83.0 (59.5)	101.0 (41.5)	102.5 (73.0)	<0.001*
Total cholesterol (mg/dL)	150.0 (33.5)	158.0 (23.0)	161.0 (27.0)	160.0 (45.0)	0.263
LDL cholesterol (mg/dL)	81.0 (32.0)	111.0 (25.0)	99.0 (34)	94.5 (44.0)	<0.05*
HDL cholesterol (mg/dL)	57.0 (32.0)	49.0 (17.0)	45.0 (18.0)	44.5 (9.0)	0.05***
ALT (U/L)	14.0 (5.5)	20.0 (29.5)	19.0 (7.5)	29.0 (13.0)	<0.01* <0.05**

HOMA-IR: homeostatic model assessment of insulin resistance; IQR: interquartile range.

Statistical tests: Kruskal-Wallis test and Mann-Whitney U test.

*1 vs 2, 3, and 4; **2 vs 4; ***1 vs 3 and 4.

Table 3. Relationship between clinical and laboratory characteristics and CIMT and FMD*

Parameters	CIMT		FMD	
	r	P	r	P
Age	0.049	0.610	0.134	0.163
BMI	0.056	0.587	-0.042	0.687
Systolic BP	0.024	0.807	-0.117	0.231
Diastolic BP	0.054	0.583	-0.100	0.308
HOMA-IR	0.068	0.531	-0.010	0.924
Total cholesterol	-0.128	0.279	-0.133	0.263
HDL cholesterol	-0.162	0.138	0.140	0.202
LDL cholesterol	-0.156	0.184	-0.094	0.428
Triglycerides	-0.009	0.930	-0.079	0.463
ALT	0.303	0.005	-0.019	0.867
MetS	-0.062	0.523	0.059	0.543
Steatosis	0.473	<0.001	-0.128	0.183

ALT: alanine aminotransferase; BMI: body mass index; BP: blood pressure; CIMT: carotid intima-media thickness; FMD: flow-mediated dilation; HDL: high-density lipoprotein; HOMA-IR: homeostatic model assessment of insulin resistance; LDL: low-density lipoprotein; MetS: metabolic syndrome

*Statistical tests: Spearman correlation analysis

obtained using Spearman correlation. HOMA-IR was not significantly correlated with CIMT and FMD. CIMT was significantly correlated with ALT levels and grade of hepatosteatosis but not with TG, LDL cholesterol, and HDL cholesterol levels. The degree of correlation between CIMT and hepatosteatosis increased significantly with an increase in the grade of steatosis.

Multiple linear regression analysis with backward elimination method showed that grade of steatosis, ALT levels, and BMI had a significant effect on increasing CIMT but had no effect on FMD (Table 4).

Table 4 Factors affecting CIMT and FMD as per linear regression analysis

	CIMT		FMD	
	β	p	β	p
BMI SDS	0.426	0.012	0.005	0.200
ALT	0.298	0.008	0.001	0.959
Grade of steatosis	0.686	<0.001	-0.009	0.738

ALT: alanine aminotransferase; BMI SDS: body mass index standard deviation score; CIMT: carotid intima-media thickness; FMD: flow-mediated dilation

DISCUSSION

Prevalence of NAFLD increases with an increase in the prevalence of overweight and obesity during childhood. Development and progression of atherosclerosis are more common in obese people with NAFLD than in healthy people and in people without NAFLD. The two markers of subclinical atherosclerosis-impaired FMD and increased CIMT-may be associated with cardiovascular outcomes in obese children.

Epidemiological data show that obesity and fatty liver are predictors of cardiovascular diseases (15). In obese children and adolescents with NAFLD, atherogenic condition is associated with the features of MetS such as abnormal values of HDL cholesterol, insulin, TGs, and BP. (6, 16-19). Approximately 90% patients with NAFLD have at least one feature of MetS, and approximately 33% patients are diagnosed as having MetS by considering NAFLD as a hepatic manifestation of MetS (20). Studies have shown that central obesity and IR are the most consistent components showing an association between MetS and NAFLD histology (13) and that impaired fasting glucose levels are the strongest indicators of carotid atherosclerosis in overweight children and adolescents (21). Our results showed that the rate of MetS increased as the severity of obesity increased. In our study, the grade of NAFLD appeared to be correlated with HOMA-IR, which is in agreement with findings of previous studies (22). Moreover, IR is more common in children

with NAFLD than in obese children without NAFLD. Mean systolic and diastolic BPs were higher in obese children with and without hepatosteatosi s than in controls; moreover, systolic BP increased as obesity and grade of steatosis increased. Median total cholesterol levels increased as the grade of steatosis increased but did not reach statistical significance. In obese children with or without hepatosteatosi s, LDL cholesterol and TG levels increased and HDL cholesterol level decreased significantly with an increase in the grade of steatosis. Recent studies have shown that the degree of atherosclerotic lesions is significantly associated with an increase in the levels of total cholesterol, LDL cholesterol, and TGs and decrease in the level of HDL cholesterol (23). Moreover, lipid ratios are markedly higher in children with hepatosteatosi s compared with children with borderline disease, indicating that severity of liver injury in children with NAFLD is strongly associated with an increased atherogenic risk (23). Abnormal CIMT is common in overweight and obese adolescents than in non-overweight and non-obese adolescents (24). However, we could not show a significant correlation of hepatosteatosi s with serum lipid levels, BP, and obesity. Lipid levels did not have a significant effect on CIMT and FMD, and there was no increasing risk of MetS in children with mild, moderate or severe hepatosteatosi s. Furthermore, we could not demonstrate a significant correlation of MetS with CIMT and FMD.

Adult obese patients with NAFLD have a high incidence of abnormal ALT levels, which is associated with the features of other MetS components (such as obesity, lipid abnormalities, and IR) (25). In obese children with NAFLD, markers of obesity and IR are independent predictors of the outcome of obesity (26). In our study, ALT levels were significantly higher in obese children with grade II-III hepatosteatosi s and played a role in increasing CIMT according to linear regression analysis. Although ALT levels are not specific or sensitive to any surrogate biomarker to indicate the severity of hepatosteatosi s, they are a widely accepted biomarker of liver fat accumulation. In our study, ALT levels were more elevated in severely obese children with grade II-III NAFLD.

Studies have shown that patients with NAFLD have higher prevalence of atherosclerosis, as shown by increased CIMT, increased number of atherosclerotic plaques, and increased plasma markers of endothelial dysfunction, and that this risk is independent of obesity and other established risk factors (27-30). In our study, obese children with NAFLD had reduced FMD compared to those without NAFLD and healthy controls; however, this did not reach statistical significance. Separate analysis of obese children showed that low FMD percentage was not significantly associated with BMI SDS, high IR, high ALT levels, or MetS and NAFLD. This result contradicts the result of a recent study by Pacifico et al., which reports that obese children with US-diagnosed NAFLD and elevated ALT levels had significantly lower FMD response and increased CIMT, which were independent of other cardiovascular risk factors and MetS, than obese

children without NAFLD (6). Thus, they concluded that obese children exhibited more functional and morphological vascular changes than lean healthy controls and that FMD response decreased independently of MetS and NAFLD (6). Results of our study were similar to those of a study by Weghuber et al., which showed that few obese children with simple hepatosteatosi s and not hepatosteatosi s appeared to have intact vascular functions and similar FMD responses (22).

Obese children with NAFLD had increased mean CIMT compared with obese children without NAFLD and healthy controls. In addition, CIMT increased as the grade of steatosis increased. Correlation analyses showed that CIMT was correlated with BMI percentage, IR, total cholesterol and ALT levels, and systolic BP. Linear regression analysis showed that CIMT was mostly affected by the grade of steatosis, BMI SDS, and ALT levels. The findings of the present study are similar to those of previous studies. Pacifico et al. (16) reported that obese patients with NAFLD had significantly increased CIMT, which was independent of anthropometric and metabolic features. Kelishadi et al. (31) reported that CIMT was strongly associated with IR and NAFLD and suggested that the liver and vessels might share common mediators. Demircioglu et al. (32) observed an association between US-detected NAFLD and CIMT measured at the common carotid artery, carotid bulb, and internal carotid artery. In contrast, Manco et al. (33) reported no significant difference in CIMT between children with and without NAFLD; however, median CIMT in obese children was greater than that in non-obese children. Moreover, they did not observe any association between CIMT and histological severity of biopsy-proven NAFLD. A population-based study (19) showed that presence of fatty liver together with BMI, waist circumference, and systolic BP was independently associated with increased CIMT. Moreover, a recent study involving a large sample size (6) reported that obese children with NAFLD had significantly higher CIMT than obese children without NAFLD, which was independent of other cardiovascular risk factors, including MetS.

The most important limitation of the present study was its cross-sectional design, which only permitted the analysis of association and not causation. In addition, some of the observed differences in parameters between the study groups may be attributed to gender, which was not well matched, and to differences in Tanner stage, which was not assessed. NAFLD was diagnosed based on US data, and the severity of liver disease was not confirmed histologically. A recent study (34) showed that US is useful for quantifying hepatosteatosi s and that its results can be strongly correlated with the grade of hepatosteatosi s obtained by liver biopsy. In addition, US is ethically relevant, easily reproducible, and not associated with any complications, which are the major drawbacks associated with liver biopsy (35). Moreover, liver biopsy is associated with sampling error (36) and intraobserver and interobserver discrepancy (37). Finally, we did not evaluate intra- or interobserver differences

in CIMT and FMD, which may affect the results of our study. To decrease intraobserver differences, we determined CIMT and FMD values as a mean of three independent measurements taken at the same time; however, taking measurements at separate time points would have been more accurate.

In conclusion, obese children with US-diagnosed NAFLD have significantly larger CIMT than non-obese healthy children and obese children without NAFLD. CIMT increased as obesity and grade of hepatosteatosis increased, thus supporting the theory that functional and morphological vascular changes might be prevalent early in obesity-related NAFLD. FMD reduced as hepatosteatosis increased but did not reach statistical significance, indicating that changes in FMD may not be as useful as changes in CIMT in predicting the risk of early onset of atherosclerosis in obese children. NAFLD, which is also associated with obesity, MetS, IR, and dyslipidemia, appears to be one of the most important factors for predicting the risk atherosclerosis. Longitudinal studies are needed to clarify the effect of NAFLD on the early onset of atherosclerosis in obese children.

Conflict of Interest: No conflict of interest was declared by the authors.

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