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Intima-media thickness and liver histology in obese children and adolescents with non-alcoholic fatty liver disease

Melania Manco[a,*], Giorgio Bedogni[b], Lidia Monti[c], Giuseppe Morino[d], Gianluigi Natali[c], Valerio Nobili[d]

[a] Scientific Directorate, Pediatric Hospital "Bambino Gesù", IRCCS, Piazza San Onofrio 4, 00165 Rome, Italy
[b] Clinical Epidemiology Unit, Liver Research Center, Basovizza, Trieste, Italy
[c] Department of Radiology, Pediatric Hospital "Bambino Gesù", IRCCS, Rome, Italy
[d] Liver Unit, Pediatric Hospital "Bambino Gesù", IRCCS, Rome, Italy

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ABSTRACT

Objective: To evaluate the relationship between biopsy-proven non-alcoholic fatty liver disease (NAFLD) and carotid artery intima-media thickness (CIMT) in children and adolescents.
Methods: A case–control study was performed. Cases were 31 mostly obese children and adolescents, with NAFLD detected at ultrasonography, and confirmed by liver biopsy. Controls were 49 mostly obese children matched for gender, age and BMI without NAFLD at ultrasonography and with normal levels of aminotransferases. Besides standard laboratory measurements, subjects underwent an oral glucose tolerance test to evaluate glucose tolerance and to estimate whole body insulin sensitivity (ISI).
Results: CIMT was similar in cases and controls on the right side but higher in cases on the left side. Although statistically significant, this difference is unlikely to be clinically relevant because of substantial overlap of CIMT values between cases and controls. Moreover, there was no association between CIMT and the severity of steatosis, ballooning, fibrosis, and the non-alcoholic steato-hepatitis score in cases. At multivariable analysis in the pooled sample ($n = 80$), age and the z-score of BMI but not NAFLD, gender, blood pressure and triglycerides, were associated with CIMT.
Conclusions: We found no association between CIMT and NAFLD in children and adolescents. More importantly, there was no association between histological severity and CIMT in children with NAFLD.

1. Introduction

Owing to the current epidemic of pediatric obesity, non-alcoholic fatty liver disease (NAFLD) has become the most frequently diagnosed liver disease among children and adolescents [1]. NAFLD comprises a spectrum of conditions ranging from simple steatosis to steato-hepatitis (NASH) and cirrhosis and is most commonly associated with obesity, insulin resistance and the metabolic syndrome (MS). NAFLD has been hypothesized to be an independent risk factor for cardiovascular disease [2,3]. The study of this association deserves special attention in children as the identification of its mechanisms may help to intervene before cardiovascular disease (CVD) develops.

The measurement of carotid artery intima-media thickness (CIMT) is increasingly used to evaluate the cardiovascular risk and target organ damage in children and adolescents with metabolic abnormalities [4]. So far, CIMT has been evaluated in young individuals with obesity [5], MS or its components [6,7], pre-diabetes [6], and NAFLD [8,9]. In adults with NAFLD, an association has been reported between CIMT and the severity of liver histology [10–12]. However, this association has not been studied in children and adolescents. The only two published studies that evaluated the association between NAFLD and CIMT in children used ultrasonography and elevated liver enzymes to diagnose fatty liver [8,9].

The present study aimed, therefore, at evaluating the association between liver histology and CIMT in children with biopsy-proven NAFLD. Because adults with fatty liver have reduced adiponectin levels, which might be inversely associated to histological severity [13], we investigated also the association between NAFLD and adiponectin.

2. Materials and methods

2.1. Study design and patients

Nineteen male and 12 female Caucasian children and adolescents aged 9–16 years with suspected NAFLD ("cases") were consecutively enrolled into the study at the Liver Unit of the "Bambino Gesù" Pediatric Hospital, Rome, Italy. Twenty-seven male and 22 female children without ultrasound evidence of fatty liver and

* Corresponding author. Tel.: +39 06 6859 2649; fax: +39 06 6859 2904.
E-mail address: melaniamanco@tiscali.it (M. Manco).

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with normal levels of aminotransferases were enrolled as controls. Cases and controls were matched for gender (same), age (within 1 year) and *z*-BMI [within 0.5 standard deviation scores (SDS)]. After a 1:1 matching ratio was reached between cases and controls, we continued using a 1:2 matching ratio and enrolled further 18 controls. The study protocol conformed to the guidelines of the *European Convention of Human Rights and Biomedicine for Research in Children* and was approved by the Ethics Committee of the "Bambino Gesù" Pediatric Hospital. The nature and purpose of the study were carefully explained to the parents or guardians of the children who gave their written consent to participate.

2.2. NAFLD diagnosis

NAFLD was suspected in the presence of: (1) persistently elevated serum aminotransferases and/or aminotransferases fluctuating between normal and high values with at least two high values (*i.e.* >40 U/L) during 6 months prior to enrollment); (2) diffusely hyperechogenic liver at ultrasonography (*i.e.* severe NAFLD); (3) no alcohol intake; (4) absence of HBV or HCV infection, metabolic liver disease, parenteral nutrition and use of drugs. Normal liver was defined as the absence of fatty liver and other liver abnormalities at ultrasonography. The final diagnosis of NAFLD was reached by liver biopsy as described in detail elsewhere [14]. Steatosis, lobular inflammation, ballooning and fibrosis were scored using the NASH CRN criteria [15]. Steatosis was graded as 0 = involving up to 5%; 1 = up to 33%; 2 = 33–66%; and 3 = >66% of hepatocytes. Lobular inflammation was graded as 0 = no foci; 1 = fewer than 2 foci ×200 field; 2 = 2–4 foci ×200 field; and 3 = >4 foci ×200 field. Ballooning was graded as 0 = none; 1 = few cells; and 2 = many/prominent cells. Fibrosis was graded as: 0 = no fibrosis; 1 = perisinusoidal or periportal; 2 = perisinusoidal and portal/periportal; 3 = bridging; and 4 = cirrhosis. Features of steatosis, lobular inflammation, and ballooning were combined to obtain the NAFLD activity score (NAS). Cases with NAS >5 were diagnosed as NASH and further classified as type 1, type 2 or overlap NASH [1].

2.3. Anthropometry

Weight and height were measured following standard procedures [16]. Body mass index (BMI) was calculated as weight (kg)/height (m)^2. The SDS of body mass index (BMI) were calculated using US reference values [17].

2.4. Laboratory assessment

Fasting glucose, triglycerides, cholesterol and high-density lipoprotein (HDL)-cholesterol were measured using standard laboratory methods. Insulin was measured by radioimmunoassay (MYRIA Technogenetics, Milan, Italy) with a lower limit of sensitivity of 0.3 μU/mL and an inter-assay coefficient of variation from 2.9% to 4.8%. Impaired fasting glucose or diabetes was defined as fasting glucose ≥100 mg/dL [18]; hypertriglyceridemia as fasting triglycerides ≥150 mg/dL [18]; and low HDL-cholesterol as fasting HDL <40 mg/dL in subjects under 16 years of age and HDL <40 mg/dL in males or HDL <50 mg/dL in females aged 16 years and over [18]. Oral glucose tolerance testing (OGTT) was performed with a dose of glucose of 1.75 g/kg up to a maximum of 75 g. Measurements of glucose and insulin were performed at 0 (basal), 30, 60, 90 and 120 min. Whole body insulin sensitivity was estimated from OGTT using the insulin sensitivity index (ISI) of Matsuda and DeFronzo [19].

2.5. Blood pressure

Blood pressure was measured with an aneroid sphygmomanometer (Taylor Instruments, Asheville, NC, USA) equipped with a cuff appropriate for arm size [20]. Three measurements were performed and the average of the last two was taken as the measure of blood pressure. Hypertension was defined as a value of systolic blood pressure ≥130 mmHg and/or diastolic blood pressure ≥85 mmHg [18,21]. Mean blood pressure was calculated as [2 × (diastolic blood pressure + systolic blood pressure)/3].

2.6. CIMT

Two experienced radiologists (LM & GN), who were blinded to the clinical and laboratory status of the patients, measured CIMT using a B-mode ultrasound scanner equipped with linear-array probes and a color- and power-Doppler module (Acuson Sequoia C512 with 15L8 probe, Siemens). After a 10-min rest and following standard guidelines [8], IMT was measured at the common carotid artery near the bifurcation at the far wall during end diastole. Four values were measured on each side and the maximum value was used for analysis. The agreement between the two radiologists was evaluated on 10 consecutive children.

2.7. Statistical analysis

The between-radiologist (LM *vs*. GN) and between-side (left *vs*. right) agreement in IMT measurements was evaluated using Lin's concordance correlation coefficient (CCC) [22,23]. Continuous variables are given as median and interquartile range (IQR) because of skewed distributions. IQR was calculated as the difference between the 75th and 25th percentile. The Wilcoxon–Mann–Whitney test was used for between-group comparisons of continuous variables and Fisher's exact test for those of categorical variables. The exact Jonckheere–Terpstra test for ordered alternatives (both descending and ascending) was used to test the existence of a trend between the severity of steatosis, inflammation and fibrosis. The relationship between CIMT and NAFLD was studied using a pre-specified median regression model correcting for gender, age, *z*-BMI, mean blood pressure and ISI. All predictors besides gender were modeled as continuous. We used mean blood pressure instead of systolic blood pressure and diastolic blood pressure to avoid problems of multicollinearity between the two components of blood pressure. Using the expected-value-per event rule we calculated to be able to model at best four predictors besides NAFLD status [24]. Because of heteroskedasticity, *i.e.* inconstant variance of residuals, 95% confidence intervals of regression coefficients were calculated by bootstrapping 1000 random samples of subjects without replacement [25,26]. A median regression model with bootstrapped 95% confidence intervals was also used to study the relationship between IMT and NAS coded as continuous. All statistical tests are two-tailed. Statistical significance was set to a p-value <0.05 for all tests. Statistical analysis was performed using STATA 11.0 (StataCorp, College Station, TX, USA) and StatXact and LogXact 8.0 (Cytel Inc., Cambridge, MA, USA).

3. Results

The between-radiologist agreement in CIMT measurement was quite good on both the left (CCC = 0.85, 95%CI 0.54 to 0.95, $p < 0.001$) and right side (CCC = 0.91, 95%CI 0.71 to 0.97, $p < 0.001$).

Thirty-one children (19 males and 12 females) and adolescents with severe NAFLD were compared to 49 controls (27 males and 22 females) without NAFLD after matching for gender, age and BMI. Their clinical and laboratory characteristics are given in Table 1. Because there was a good agreement between left and right CIMT in subjects without NAFLD (CCC = 0.81, 95%CI 0.70 to 0.91, $p < 0.001$), but not in those with severe NAFLD (CCC = 0.25, 95%CI −0.06 to 0.56, $p = 0.113$), we did not calculate an average value of CIMT but evaluated separately the right and left sides.

Table 1
Clinical and laboratory characteristics of children with severe NAFLD and without NAFLD.

	Severe NAFLD ($n=31$)		No NAFLD ($n=49$)		Wilcoxon–Mann–Whitney test
	Median	IQR	Median	IQR	p-value
Age (years)	13	4	12	2	0.061
Gender (M/F)	19/12[a]	–	27/22[a]	–	0.647[a]
Weight (kg)	70.0	15.4	65.3	16.0	0.236
Weight (SDS)	2.07	0.44	2.07	0.49	0.284
Height (m)	1.57	0.12	1.52	0.14	0.018
Height (SDS)	0.64	2.09	0.54	1.02	0.902
BMI (kg/m^2)	27.8	3.4	28.3	2.9	0.399
BMI (SDS)	1.96	0.32	2.10	0.22	0.013
CIMT-right (mm)	0.47	0.07	0.48	0.05	0.659
CIMT-left (mm)	0.49	0.12	0.47	0.05	0.039
Fasting glucose (mg/dL)	79	11	76	7	0.495
OGTT glucose at 120 min (mg/dL)	94	29	107	21	0.001
Insulin (μU/mL)	11	7	15	13	0.067
Insulin sensitivity index	4.1	2.1	3.1	2.7	0.148
Triglycerides (mg/dL)	120	46	79	31	0.001
Cholesterol (mg/dL)	150	33	148	36	0.590
HDL-cholesterol (mg/dL)	46	9	50	13	0.031
Systolic BP (mmHg)	116	10	112	16	0.488
Systolic BP (SDS)	0.8	1.3	0.7	1.4	0.855
Diastolic BP (mmHg)	70	12	65	12	0.005
Diastolic BP (SDS)	0.57	1.11	0.06	0.98	0.020
ALT (U/L)	79	38	20	7	0.001
AST (U/L)	68	42	22	7	0.001
Adiponectin (μg/mL)	11	2	11	3	0.116

Abbreviations—NAFLD: non-alcoholic fatty liver disease; IQR: interquartile range; SDS: standard deviation score; BMI: body mass index; CIMT: carotid intima-media thickness, OGTT: oral glucose tolerance testing; HDL: high-density lipoproteins; BP: blood pressure; ALT: alanine transaminase; AST: aspartate transaminase.
[a] Number of subjects and Fisher's exact test.

BMI ranged from 1.47 to 2.48 SDS in cases, and from 1.24 to 2.59 SDS in controls. Eighty seven percent of cases and 98% of controls were obese as defined by a BMI ≥1.64 SDS. Gender, age and BMI were similar between the two groups. Even if the SDS of BMI was slightly higher in children without NAFLD ($p=0.013$), triglycerides ($p=0.001$) and the SDS of diastolic blood pressure ($p=0.020$) were higher and HDL-cholesterol ($p=0.031$) was lower in children with severe NAFLD. Aminotransferases were higher in cases than in controls ($p=0.001$). Possibly in relation to the higher SDS of BMI, OGTT glucose at 120 min was higher in controls than in cases ($p=0.001$). However, ISI was similar in both groups. CIMT was similar on the right side but significantly higher ($p=0.039$) in children with severe NAFLD on the left side. However, the clinical relevance of this finding is doubtful because the IMT values of the two groups do largely overlap (Fig. 1). Adiponectin levels were similar in cases and controls.

The features of the liver biopsies of children with NAFLD are given in Table 2. Overall, 12 out of 31 children (39%) had NASH.

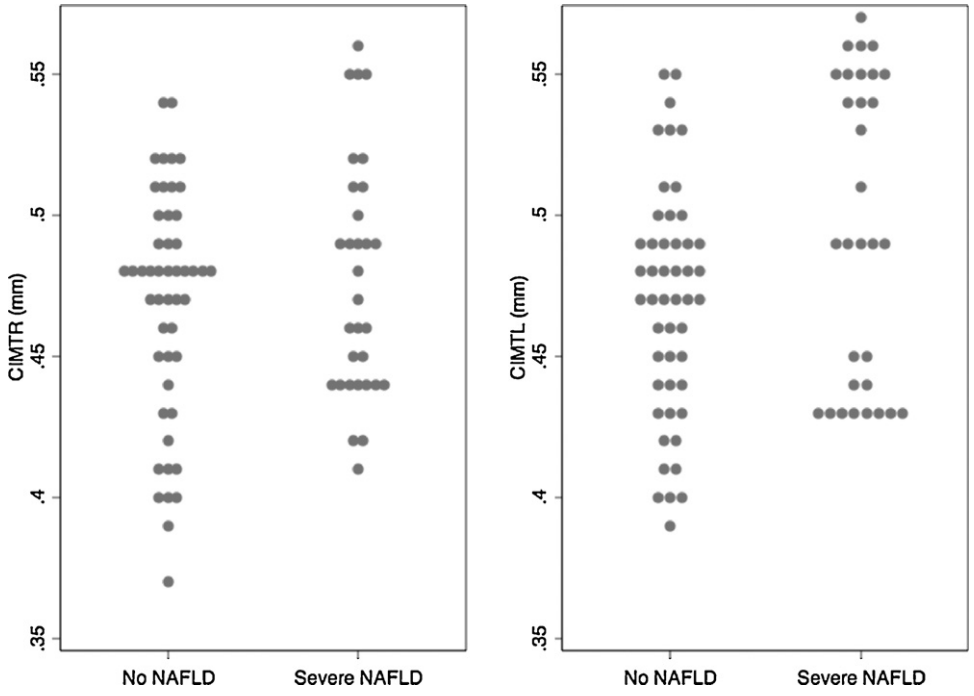

Fig. 1. Distribution of carotid intima-media thickness in cases and controls. Abbreviations—CIMTR: carotid intima-media thickness right side; CIMTL: carotid intima-media thickness left side; NAFLD: non-alcoholic fatty liver disease.

Table 2
Features of the liver biopsies of the 31 children with severe NAFLD.

	Grade or stage	*N*	%
Steatosis	0	–	–
	1	9	29
	2	13	42
	3	9	29
	Total	31	100
Inflammation	0	9	29
	1	14	45
	2	8	26
	3	0	0
	Total	31	100
Ballooning	0	16	52
	1	11	35
	2	4	13
	Total	31	100
Fibrosis	0	11	35
	1	8	26
	2	8	26
	3	4	13
	4	–	–
	Total	31	100
NAS score [median (IQR) [min – max]]		3 (3) [1–6]	
Simple NAFLD (*n*, %)		19 (61%)	
NASH (*n*, %)		12 (39%)	
NASH type (type 1/type 2/mixed)		0 (0%)/0 (0%)/31 (100%)	

Data are given as the number and percentage of subjects with the characteristic of interest. Steatosis was graded from 0 to 3; lobular inflammation from 0 to 3; ballooning from 0 to 2; fibrosis from 0 to 4 [15]. Features of steatosis, lobular inflammation, and ballooning were combined to obtain the NAFLD activity score (NAS). Cases with NAS >5 were diagnosed as NASH.

All children with NASH had overlap between type 1 and type 2 NASH.

The frequency of MS components (except waist circumference, whose measurement was not reliable in most children because of obesity) in cases with severe NAFLD and controls is given in Table 3. Low HDL-cholesterol, hypertriglyceridemia, hypertension and impaired fasting glucose/diabetes had the same frequency in the two groups. (One has however to consider the reduced power ensuing from the dichotomization of these variables so that the comparison made in Table 1 is more clinically relevant.)

The relationship between the degree of steatosis, inflammation and fibrosis and CIMT in children with NAFLD is depicted in Fig. 2. There was no evident trend between histological severity and right or left CIMT. Moreover, there was no association between NAS and IMT on both the right (regression coefficient = 0.01 (mm), 95%CI −0.01 to 0.03, $p = 0.221$) and left sides (regression coefficient = 0.01 (mm), 95%CI − 0.01 to 0.03, $p = 0.677$).

Confirming the results of univariable analysis, CIMT was not associated with NAFLD also after correction for age, gender, BMI, mean blood pressure and triglycerides (Table 4). The only predictors of CIMT were SDS of BMI and age and had the same effect size and variability on both sides.

Table 3
Frequency of selected components of the metabolic syndrome in children with severe NAFLD and without NAFLD.

	Severe NAFLD ($n = 31$)	No NAFLD ($n = 49$)	Fisher's exact test *p*-value
Low HDL-cholesterol (y/n)	4 (13%)	8 (16%)	0.758
Hypertriglyceridemia (y/n)	4 (13%)	4 (8%)	0.704
Hypertension (y/n)	5 (16%)	4 (8%)	0.298
Impaired fasting glucose or diabetes (y/n)	0 (0%)	0 (0%)	NA

Abbreviations—NAFLD: non-alcoholic fatty liver disease; y: yes; n: no.

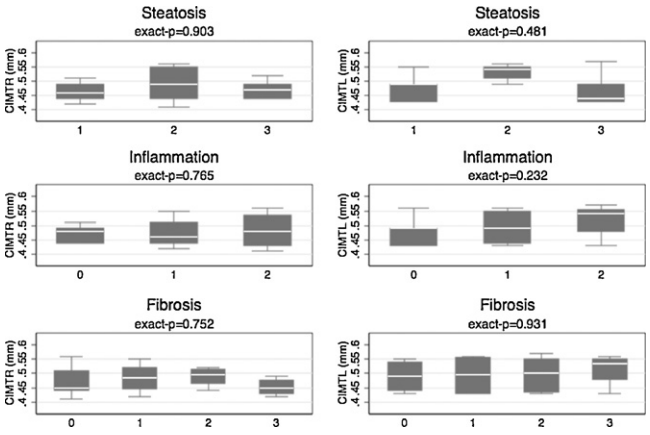

Fig. 2. Association between steatosis, inflammation, fibrosis and carotid intima-media thickness. Box-plots give the median value (white), 25th and 75th percentiles (lower and upper limits of the box) and lower and upper adjacent values (whiskers). The exact *p*-value is obtained from an exact Jonckheere–Terpstra tests for ordered alternatives (both ascending and descending). Abbreviations—CMITR: right carotid intima-media thickness; CMITL: left carotid intima-media thickness.

4. Discussion

In the present study, we measured CIMT in children with ultrasonographically detected and biopsy-confirmed NAFLD and in control children without NAFLD, matched for gender, age and BMI. Despite the fact that the median CIMT values of our children are higher than those reported as normal [4], there was no significant or clinically relevant difference in CIMT between children with and without NAFLD. More importantly, there was no association between any pattern of histological severity and CIMT in children with biopsy-proven NASH. We are aware that two limitations of the present study are the lack of normal-weight controls and the measurement of CIMT by methods different from those used in other studies.

Table 4
Multivariable median regression of carotid intima-media thickness.

	CIMTR regression coefficient (bootstrapped 95%CI)	CIMTL regression coefficient (bootstrapped 95%CI)
NAFLD (yes *vs.* no)	−0.01 [−0.04, 0.02]	0.03 [−0.01, 0.07]
Male gender (yes)	0.01 [−0.02, 0.03]	0.01 [−0.01, 0.04]
Age (years)	0.01* [0.00, 0.02]	0.01* [0.00, 0.02]
z-BMI (SDS)	0.07** [0.02, 0.12]	0.07** [0.02, 0.12]
Mean blood pressure (mmHg)	−0.00 [−0.00, 0.00]	0.00 [−0.00, 0.00]
Triglycerides (mg/dL)	−0.00 [−0.00, 0.00]	−0.00 [−0.00, 0.00]
Intercept	0.21 [−0.01, 0.43]	0.17 [−0.04, 0.38]
N	80	80

Abbreviations—CIMT: right carotid intima-media thickness; CIMTL: left carotid intima-media thickness, *z*-BMI: *z*-score of body mass index.
* $p < 0.05$.
** $p < 0.01$.

Two studies have been published so far on the association between ultrasonographically detected NAFLD and CIMT in children [8,9]. The first study was performed by Pacifico et al. on 30 normal-weight children, 33 obese children without NAFLD, and 29 obese children with NAFLD [8]. CIMT values ranged from 0.54 to 0.62 mm in obese children with NAFLD, from 0.46 to 0.52 mm in those without NAFLD, and from 0.36 to 0.43 mm in normal-weight children. On the contrary, in the present study, there was a substantial overlap of CIMT values between children with and without NAFLD (Fig. 1). The second study was performed by Demircioğlu et al. on 80 obese children and 30 normal-weight controls and found an association between fatty liver status and left CIMT measured at sites of the common carotid artery, carotid bulb and internal carotid artery [9]. In our study, left CIMT was significantly higher in children with NAFLD than in those without NAFLD but we do not consider this difference to be clinically relevant because of the substantial overlap of CIMT values between cases and controls (Fig. 1). Possible explanations for our different findings include different environmental and possibly genetic factors for the study of Demircioğlu et al. [9] and a different case-mix of patients for the study of Pacifico et al. [8]. In the latter study, obese children with NAFLD had higher values of insulin and adiponectin as compared to obese children without NAFLD and controls. Thus, these children seem to have more risk factors than those studied in the present study. Unfortunately, most of these risk factors were not evaluated by Demircioğlu et al. so that a comparison of the case-mix of this study with our study and that of Pacifico et al. is not possible.

Studies performed in normal-weight and obese adults [27,28], have shown that NAFLD is an independent predictor of CVD as estimated by the Framingham risk score. As far as the association between liver histology and CIMT is concerned, Francanzani et al. [29] reported no difference in CIMT between patients with simple NALFD and those with NASH, even if these latter had higher CIMT values than controls. They also had lower HDL and higher triglycerides, fasting glucose, insulin resistance, and frequency of MS than controls. Targher et al. measured CIMT in 85 patients with biopsy-proven NAFLD and in 160 age-, gender-, and BMI-matched healthy controls. In their study, CIMT was associated with the degree of steatosis, inflammation and fibrosis among NAFLD patients and the severity of histological features was an independent predictor of CIMT after adjustment for confounders [12]. Of course, some caution is needed when comparing adult to pediatric NASH (revised in 1). Whilst the adult pattern (type 1 NASH) is characterized by the presence of steatosis (mainly macrovesicular) with ballooning degeneration and/or perisinusoidal fibrosis in the absence of portal features, the pediatric pattern (type 2 NASH) is defined by the presence of steatosis along with portal inflammation and/or fibrosis in the absence of ballooning degeneration and perisinusoidal fibrosis. These differences in histology suggest that the liver may respond differently in the pediatric age to an insult which is not yet inveterate as in the adults. The hormonal milieu of growth might contribute to a different cross-talk between liver and adipose tissue, partly determining the histological appearance of the disease.

The present study suggests that NAFLD is not associated with increased CIMT in children and adolescents. However, whilst NAFLD may not have a direct impact on carotid atherosclerosis, it may contribute to cardiovascular disease by acting in concert with metabolic abnormalities. In agreement with this hypothesis, a recent cross-sectional study performed on Korean adults found NAFLD to be associated with CIMT only in people with the metabolic syndrome [30]. Furthermore, Reinehr et al. observed that in children with the metabolic abnormalities of MS, impaired glucose tolerance was the best predictor of a CIMT >75th percentile [6]. Giannini et al. found that in pre-pubertal children CIMT was inversely associated with whole body insulin sensitivity and glucose to insulin ratio [5]. Lastly, Toledo-Corral et al. found that in 97 overweight Latino children aged 11–18 years and at risk of diabetes, CIMT predicted the persistence of the MS over 3 years of follow up [7]; moreover, hypertension, abdominal obesity and impaired glucose tolerance were associated with increased risk for increased CIMT.

There are some possible explanations for the lack of association between NAFLD and CIMT observed in the present study. First, CIMT may not reflect all the components of the CVD risk and other parameters such as arterial stiffness and flow-mediated dilation may be more useful indexes of early vascular changes [31]. Second, a longer exposure to fatty liver may be required for children to develop higher CIMT. Longitudinal studies such as the Bogalusa Heart Study [32] and the Young Finns Study [33] found that differences as small as 0.1 mm of CIMT could be found after 20 years and over of follow up, when a combination of adverse risk factors clustered together. Moreover, genetic factors such as lipoprotein polymorphisms [34], and environmental factors, especially dietary habits, may influence the association among NAFLD, increased IMT and classical CVD risk factors. In any case, independently from NAFLD being a marker or a determinant of cardiovascular risk, treatment of NAFLD translates in reduced CVD risk, as lifestyle changes (nutritional intervention and physical exercise) are effective therapeutic approaches for both [1].

In conclusion, our study does not support the existence of an association between NAFLD and CIMT in children and adolescents, suggesting that other metabolic abnormalities might act in concert with NAFLD to promote atherosclerosis. Otherwise, it can be speculated that a longer exposure to the disease is required to determine significant differences in endothelial dysfunction.

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