Nonalcoholic Fatty Liver Disease and Cardiovascular Risk in Children with Obesity

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Objective: Nonalcoholic fatty liver disease (NAFLD) has been recognized as an independent risk factor for cardiovascular disease in adults. It has not been established whether NAFLD is related to early atherosclerotic changes in children.

Methods: In a cross-sectional study, 78 non diabetic, non smoking children with severe obesity were evaluated for NAFLD. Proton magnetic resonance spectroscopy was used to detect liver steatosis and serum ALT was used as a surrogate marker for steatohepatitis. Carotid intima-media thickness (CIMT) and arterial wall stiffness were measured using ultrasound.

Results: Steatosis was present in 41 (53%) of subjects. Of these children, 26 out of 41 (63%) had elevated ALT levels. No differences in CIMT and arterial wall stiffness were observed between those without and with steatosis and those with steatosis plus elevated ALT levels [CIMT = 0.47 (±0.06), 0.48 (±0.06) and 0.48 (±0.07) mm, respectively; stiffness = 2.78 (±0.50), 3.00 (±0.81), and 2.90 (±0.78), respectively]. Steatosis and ALT were not correlated to CIMT (r = −0.02 and −0.14, respectively) or arterial wall stiffness (r = 0.13 and −0.11, respectively).

Conclusions: In this study, no relationship between NAFLD and early atherosclerotic changes in children was observed. An atherogenic effect of steatohepatitis (NASH) on pediatric age and long-term atherogenic consequences of simple steatosis cannot be excluded based on this study.

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Introduction

In the last two decades, nonalcoholic fatty liver disease (NAFLD) has emerged as the leading cause of liver disease in children worldwide (1). The spectrum of NAFLD ranges from simple steatosis, to steatohepatitis (NASH), to fibrosis and cirrhosis. NAFLD is strongly linked with established cardiovascular risk factors: abdominal obesity, dyslipidemia, hypertension, and insulin resistance/diabetes in both children and adults. Indeed, it is not the complications of liver disease, but cardiovascular events which are the leading cause of death in adults with NAFLD (2,3). A growing body of evidence in adults indicates that NAFLD is a risk factor for cardiovascular disease independent of the other conventional cardiovascular risk factors (4-7). Suggested pathophysiological mechanisms through which NAFLD could cause atherogenesis are the release of inflammatory cytokines from the liver, disturbances in lipoprotein metabolism, an increase in liver and whole body insulin resistance, a decrease in adiponectin, and the production of procoagulation factors (8).

Although atherosclerosis often starts in childhood or young adulthood, it has not been established whether the atherosclerotic disease risk in children and adolescents with NAFLD is increased beyond the risks associated with the conventional cardiovascular risk factors, like obesity, insulin resistance, hypertension, and smoking. Studies into the relation between the presence of NAFLD and early markers of atherosclerosis, mostly carotid vascular changes, show inconsistent findings in children (9-17). Therefore, the aim of this study was to investigate whether NAFLD is an independent risk factor for early atherosclerosis in a cohort of severely obese, non diabetic, and non smoking children.
Methods

Population
This is a cross-sectional study in children and adolescents referred between August 2008 and October 2010 to a Dutch obesity center for treatment in a lifestyle intervention program. Inclusion criteria were age from 8 to 18 years, primary obesity and a body mass index (BMI) equivalent to an adult index of ≥35 kg/m². Exclusion criteria were the presence of concomitant liver disease or diabetes, a history of smoking, (past) use of steatogenic medication or oral anti-diabetic drugs, alcohol consumption of ≥7 units/week, a history of jejunal-ileal surgery or parenteral feeding and contra-indications for MR scanning (e.g. pacemaker or claustrophobia). The study was conducted according to the Declaration of Helsinki. The study protocol was approved by the Medical Ethics Committee of the Academic Medical Center of the University of Amsterdam. Each participant and/or its guardian provided written informed consent.

Clinical assessment
Anthropometric measurements were performed at the start of the lifestyle program in the obesity center. One of two trained pediatricians conducted anthropometric measurements using a standardized protocol (all blinded for all clinical information; 11-28 years of experience). Weight and height were measured and used to calculate the age adjusted BMI standard deviation score, the BMI z score (18). Waist circumference was defined as the smallest torso circumference measured between xiphoid and umbilicus. The pubertal stages were determined by visual inspection, using Tanner’s criteria (19). Aerobic fitness was assessed by the distance run during the modified shuttle test (20). Also recorded were a family history of cardiovascular events within first- or second-degree relatives and the duration of obesity as reported by the participants or their caretakers.

After an overnight fast, venous blood was sampled for serum biochemistry studies. ALT, fat spectrum, and high-sensitive C-reactive protein (hsCRP) were measured directly after blood sampling, using standard laboratory methods by blinded and certified laboratory staff in an adjacent local hospital. In those with steatosis, an elevated serum ALT was used as a surrogate marker for steatohepatitis using a cut-off of >30 IU/L for ALT. Fasted insulin and glucose were used to calculate homeostatic model assessment of insulin resistance (HOMA-IR) as previously described (21). An oral glucose tolerance test was performed to exclude the presence of diabetes mellitus type 2. Hepatitis B and C, autoimmune hepatitis, alpha-1 antitrypsin deficiency, abetalipoproteinemia, hemochromatosis, and Wilson disease were excluded using the appropriate diagnostic tests.

Proton magnetic resonance spectroscopy
Proton magnetic resonance (1H-MR) spectroscopy is a safe and highly accurate MR technique to measure liver fat (22). MR scanning was performed in the Academic Medical Center of the University of Amsterdam from 1 month before until 2 weeks after the start of the obesity program. 1H-MR spectra were acquired using a point-resolved spectroscopy sequence (TE/TR = 38/2000 ms) in a voxel of 20 × 20 × 20 mm during free breathing on a 3.0 Tesla MR system (Philips Healthcare, Best, The Netherlands). In nine subjects, body habitus did not permit scanning on this system, so an open bore 1.0 Tesla MR scanner (Philips Healthcare, Best, The Netherlands) was used. The absolute mass concentration of liver fat was calculated as previously described by a research fellow (blinded for all clinical information; 3 years of experience) under supervision of an experienced MR physicist (blinded for all clinical information; 8 years of experience) (23). Presence of hepatic steatosis was defined as greater than 1.8% liver fat mass concentration measured with 1H-MR spectroscopy. This cut-off has been validated in the 3.0 Tesla MR system and corresponds with 5% fat containing hepatocytes at liver histology (22). The 3.0 and open bore 1.0 Tesla MR system in our center have been shown to produce similar results in a small comparative study in adults who were consecutively scanned in both apparatus (unpublished data).

Carotid ultrasound measurements
To assess carotid intima-media thickness (CIMT) and arterial wall stiffness, ultrasound scans were performed by a single trained and certified sonographer (blinded for all clinical information; 17 years of experience). An Acuson Aspen (Siemens Medical Solutions, Erlangen, Germany) equipped with a linear-array ultrasound transducer (L7, 5-12 MHz) was used. Instrument application and scanning protocols were standardized as extensively described previously (24). For CIMT measurements, in each subject three arterial segments of the right and left common carotid, carotid bulb and internal carotid were scanned. Of each of the segments scanned, a high-resolution image taken during the diastole of the artery was saved as a DICOM (Digital Imaging and Communications in Medicine) still image. CIMT was defined as the distance between the lumen-intima and media-adventitia interfaces of the far arterial wall. To assess carotid wall stiffness, bilateral M-mode traces of the distal common carotid arterial lumen for at least two cardiac cycles were obtained and saved as a DICOM still images.

For image analyses, eTRACK software (Vascular Imaging and Department of Physiology, AMC, Amsterdam, the Netherlands) was used. Image analyses were done by a single certified ultrasound analyst (blinded, 8 years of experience). The ultrasound outcome was the mean carotid IMT of the six carotid arterial wall segments per subject. The arterial wall stiffness was considered a secondary endpoint and was calculated as the natural logarithm of the following formula [SBP/DBP]/(D/D). Abbreviations: SBP, systolic blood pressure; DBP, diastolic blood pressure; D, arterial diastolic diameter; D/D, change in arterial diameter), as previously described (25).

To assess intra-sonographer reproducibility, repeat scans were assessed in 19 subjects. The observed mean absolute difference of the primary carotid ultrasound endpoint, mean CIMT, was 0.072 mm and far within the predefined intra-sonographer quality control limits of 0.10 mm. The noninvasive ultrasound measurement of CIMT is a reproducible method well correlated to histology and is predictive of myocardial infarction and stroke in adults (26).

Statistics
Results were expressed using standard descriptive statistics. Differences between groups were determined using chi-square analyses for categorical variables and t-tests for continuous variables. For comparing non-normal distributed continuous variables, log transformation was performed to obtain normality. In addition, correlation coefficients between the degree of steatosis and CIMT/stiffness and serum ALT levels and CIMT/stiffness were calculated using Pearson Correlation. The independence of an observed effect of steatosis or ALT on CIMT or stiffness would subsequently be evaluated using
We aimed to include at least 35 study subjects in each group for the primary comparison of those with steatosis versus no steatosis. Based on previous carotid ultrasound studies in this age group, we expect the SD of CIMT measurements to be 0.05 mm. Assuming a two-sided \( \alpha \) of 0.05 and a \( \beta \) of 0.2 (a power of 80%), we may observe a clinically relevant difference of 0.035 mm in CIMT between groups. All analyses were performed with PASW Statistics 18; SPSS inc. Chicago, IL, USA.

### Results

Out of 97 eligible subjects, 78 severely obese children and adolescents were included. Three subjects could not be included because no informed consent was obtained, one subject withdrew before study procedures were finished and fifteen patients met exclusion criteria: smoking (\( n = 10 \)), past use of steatogenic or oral antidiabetic drugs (\( n = 3 \)), alcohol use \( \geq 7 \) units per week (\( n = 1 \)) and magnetic sensitive implant (\( n = 1 \)).

### Characteristics of the participants

<table>
<thead>
<tr>
<th></th>
<th>All, ( n = 78 )</th>
<th>No steatosis, ( n = 37 )</th>
<th>Steatosis, ( n = 41 )</th>
<th>( p )-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age, years</strong></td>
<td>14.3 (± 2.2)</td>
<td>13.8 (± 1.8)</td>
<td>14.7 (± 2.4)</td>
<td>0.07</td>
</tr>
<tr>
<td><strong>Gender, male</strong></td>
<td>33 (42%)</td>
<td>13 (35%)</td>
<td>20 (59%)</td>
<td>0.23</td>
</tr>
<tr>
<td><strong>Ethnicity</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>European</td>
<td>60 (77%)</td>
<td>29 (78%)</td>
<td>31 (75%)</td>
<td>0.77</td>
</tr>
<tr>
<td>Middle East</td>
<td>12 (15%)</td>
<td>6 (16%)</td>
<td>6 (15%)</td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>6 (8%)</td>
<td>2 (6%)</td>
<td>4 (10%)</td>
<td></td>
</tr>
<tr>
<td><strong>Family history CVD</strong></td>
<td>44 (56%)</td>
<td>25 (67%)</td>
<td>19 (46%)</td>
<td>0.49</td>
</tr>
<tr>
<td><strong>Duration obesity, years</strong></td>
<td>7.5 (4)</td>
<td>6.5 (4)</td>
<td>9 (5)</td>
<td>0.70</td>
</tr>
<tr>
<td><strong>Anthropometry</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Puberty staged</td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>Prepubertal</td>
<td>7 (9%)</td>
<td>4 (11%)</td>
<td>3 (7%)</td>
<td>0.30e</td>
</tr>
<tr>
<td>Pubertal</td>
<td>39 (51%)</td>
<td>20 (56%)</td>
<td>19 (48%)</td>
<td></td>
</tr>
<tr>
<td>Postpubertal</td>
<td>30 (40%)</td>
<td>12 (33%)</td>
<td>18 (45%)</td>
<td></td>
</tr>
<tr>
<td><strong>BMI z score</strong></td>
<td>3.38 (± 0.34)</td>
<td>3.20 (± 0.29)</td>
<td>3.52 (± 0.34)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Waist (cm)</strong></td>
<td>105.4 (± 13.2)</td>
<td>100.2 (± 11.8)</td>
<td>110.1 (± 12.9)</td>
<td>0.001</td>
</tr>
<tr>
<td><strong>Diastolic BP (mmHg)</strong></td>
<td>80 (± 10)</td>
<td>77 (± 7)</td>
<td>83 (± 11)</td>
<td>0.12</td>
</tr>
<tr>
<td><strong>Systolic BP (mmHg)</strong></td>
<td>123 (± 14)</td>
<td>120 (± 11)</td>
<td>125 (± 15)</td>
<td>0.14</td>
</tr>
<tr>
<td><strong>10-min shuttle run (m)</strong></td>
<td>680 (320)</td>
<td>690 (310)</td>
<td>665 (378)</td>
<td>0.60</td>
</tr>
<tr>
<td><strong>Biochemistry</strong></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td><strong>ALT (IU/L)</strong></td>
<td>29 (21)</td>
<td>26 (16)</td>
<td>35 (23)</td>
<td>0.003</td>
</tr>
<tr>
<td><strong>ALT &gt; 30 IU/L</strong></td>
<td>42 (53%)</td>
<td>12 (32%)</td>
<td>25 (61%)</td>
<td>0.012</td>
</tr>
<tr>
<td><strong>hsCRP (mg/L)</strong></td>
<td>3.0 (3.5)</td>
<td>3.0 (2.8)</td>
<td>3.0 (6)</td>
<td>0.98</td>
</tr>
<tr>
<td><strong>HOMA-IR</strong></td>
<td>3.3 (3.1)</td>
<td>2.4 (2.1)</td>
<td>4.33 (3.5)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>HDL-cholesterol (mmol/L)</strong></td>
<td>1.08 (± 0.26)</td>
<td>1.04 (± 0.22)</td>
<td>1.12 (± 0.28)</td>
<td>0.17</td>
</tr>
<tr>
<td><strong>LDL-cholesterol (mmol/L)</strong></td>
<td>2.57 (± 0.72)</td>
<td>2.54 (± 0.81)</td>
<td>2.6 (± 0.65)</td>
<td>0.68</td>
</tr>
<tr>
<td><strong>Liver ^1H-MR spectroscopy</strong></td>
<td></td>
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</tr>
<tr>
<td><strong>Steatosis (%)</strong></td>
<td>5.4 (2.7)</td>
<td>2.6 (1.2)</td>
<td>14.3 (7.0)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Values are given as mean (± SD), median (interquartile range), or number (%) as appropriate. Abbreviations: CVD, cardiovascular disease; BMI, body mass index; BP, blood pressure; hsCRP, high-sensitive C-reactive protein; HOMA-IR, homeostasis model assessment of insulin resistance; ^1H-MR, proton magnetic resonance.

dComparing European versus non-European descent.
bComparing European versus non-European descent.
cFamily history positive if first- or second-degree relative has suffered a cardiovascular event.
dThree stages based on Tanner stages: prepubertal equals G/M1&P1; postpubertal equals G/M5&P5; pubertal equals all other Tanner stages.
eComparing prepubertal/pubertal versus postpubertal.
fHistological % of steatosis is depicted as recalculated from the MR determined % of steatosis (see Methods section).

Regression analysis including conventional atherosclerotic risk factors as co-variables.

We aimed to include at least 35 study subjects in each group for the primary comparison of those with steatosis versus no steatosis. Based on previous carotid ultrasound studies in this age group, we expect the SD of CIMT measurements to be 0.05 mm. Assuming a two-sided \( \alpha \) of 0.05 and a \( \beta \) of 0.2 (a power of 80%), we may observe a clinically relevant difference of 0.035 mm in CIMT between groups. All analyses were performed with PASW Statistics 18; SPSS inc. Chicago, IL, USA.

As shown in Figure 1, CIMT was not different between subjects without steatosis versus those with steatosis or steatosis plus elevated ALT [0.47 (± 0.06) vs. 0.48 (± 0.06) and 0.48 (± 0.07) mm, \( p = 0.61 \) and \( p = 0.59 \), respectively]. Also, arterial wall stiffness was not different between subjects without steatosis versus those with steatosis or steatosis plus elevated ALT [0.47 (± 0.06) vs. 0.48 (± 0.06) and 0.48 (± 0.07) mm, \( p = 0.61 \) and \( p = 0.59 \), respectively].
steatosis plus elevated ALT [2.78 (±0.50) vs 3.00 (±0.81) and 2.90 (±0.78); p = 0.18 and p = 0.59, respectively] (Figure 2).

No significant correlation was found between the (log transformed) degree of steatosis and CIMT or arterial wall stiffness (r = −0.02, p = 0.89 and r = 0.13, p = 0.26; respectively). No significant correlation was observed in those with steatosis between the level of (log transformed) ALT and CIMT or arterial wall stiffness. (r = −0.14, p = 0.44 and r = −0.11, p = 0.55; respectively). In multivariate linear regression analysis, subsequently combining the degree of steatosis and ALT with other cardiovascular risk factors (age, BMI z-score, waist circumference, insulin resistance, LDL-/HDL-cholesterol, blood pressure, gender and pubertal stage), using multiple models to prevent collinearity and overfitting, did not increase their correlation coefficient with CIMT or arterial wall stiffness.

Discussion

In this study, we observed no early atherosclerotic changes related to steatosis in a cohort of severely obese children. CIMT and arterial wall stiffness were not higher in those with steatosis despite a worse cardiovascular disease risk profile (higher BMI, waist circumference and insulin resistance). The subgroup of those with steatosis plus an elevated serum ALT, a surrogate marker for NASH, had no higher CIMT or arterial wall stiffness. The very low correlation coefficients between these parameters confirm the lack of relation between steatosis or ALT and early atherosclerotic changes in this cohort.

Previous studies examining the correlation between NAFLD and early signs of atherosclerosis have found conflicting results. Most studies only investigated CIMT as a marker of early atherosclerotic changes. Five studies found increased CIMT in those with liver steatosis (9-13) while in four studies no relation was found with CIMT (14-16) or arterial wall stiffness. Most of these studies have major methodological shortcomings; particularly the use of inaccurate diagnostic tools (mainly ultrasound) to diagnose NAFLD. (9-12,16,17) In obese children, ultrasound has been shown to have an acceptable sensitivity (85%), but a poor specificity (55%) for detecting NASH. Therefore, these studies could have included a substantial number of particularly false positive cases (27). In addition, most studies do not correct for all important cardiovascular risk factors, like insulin resistance (10,11), gender (28), pubertal stage (10,15,28) and smoking (10,15). Finally, overfitting and collinearity, two important pitfalls in multivariate analyses, are often not taken into account (9,10,13) The largest study including 132 children with ultrasound determined NAFLD found, after rigorously correcting for other cardiometabolic risk factors, that increased arterial wall stiffness was not independently associated with NAFLD. The only study using liver histology, the reference standard to diagnose NAFLD, found neither a relation with steatosis nor inflammation in the liver and CIMT (15). The present study is the second and largest pediatric study that uses 1H-MR spectroscopy to diagnose NAFLD (14). 1H-MR spectroscopy is well established as an accurate tool to measure liver fat and thus diagnose NAFLD, as pathological fat accumulation is a prerequisite for all stages of NAFLD (27,29). In addition, the 3.0 Tesla 1H-MR spectroscopy scanner setting used in this study was validated in comparison to liver histology, allowing us to determine the exact cut-off for steatosis. As mentioned in the methods section, determination of the cut-off for steatosis in the open bore 1.0 Tesla MR scanner was less extensive than for the 3.0 Tesla MR scanner. However, a limited number of participants (n = 9) were scanned in the 1.0 Tesla scanner and this will not have affected the validity of our findings. Another strength of this study is the inclusion of children with NAFLD not referred for liver disorders. This cohort is therefore a better representation for the severity of NAFLD present on population level compared to the cohorts of children referred to tertiary liver clinics that were studied in most other pediatric studies (9,10,12,13,15,16). A limitation of the present study is that 1H-MR spectroscopy accurately diagnoses liver steatosis, but not steatohepatitis. We used serum ALT as a surrogate marker of NASH in those with steatosis. No relation of NASH with CIMT and arterial wall stiffness was observed. However, this should be interpreted with caution as ALT is a poor marker of NASH. ALT levels were mostly only mildly elevated and the group of steatosis plus ALT in this study was small. Therefore, this study was underpowered to detect small absolute differences. Probably, most children
with steatosis in this cohort had simple steatosis and no NASH. Therefore, no conclusions on atherogenic effects of NASH in children can be drawn from this study. In adults it was found that the CIMT effect was more pronounced in NASH than in simple steatosis, possibly because NASH causes increased systemic inflammation (8). Another limitation of this study is its cross-sectional design, therefore it cannot be established whether prolonged presence of NAFLD can cause atherosclerotic changes in children. In this study the median duration of obesity as reported by the participants was 9 years in those with steatosis, however the duration of NAFLD in this group is unknown.

In conclusion, the present study shows no association between liver steatosis and early signs of atherosclerosis in children and adolescents. It therefore suggests that liver steatosis is not an independent risk factor for atherosclerosis at this young age. However, an atherogenic effect of NASH on pediatric age and long-term atherogenic consequences of NAFLD cannot be excluded based on this study.

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References