Serum Inflammatory Markers in Overweight Children and Adolescents with Non-Alcoholic Fatty Liver Disease

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ABSTRACT: Background: Obesity, a worldwide pandemic, is associated with a large variety of comorbidities, among which is non-alcoholic fatty liver disease. NAFLD is a complex disease that may eventually lead to cirrhosis, posing a high risk for the patient and thus necessitating early diagnosis and treatment.

Objectives: To evaluate the association between ultrasonographically diagnosed non-alcoholic fatty liver disease and the levels of serum inflammatory markers in obese children and adolescents.

Methods: This prospective cohort study was conducted in children and adolescents attending the endocrine obesity clinic in a tertiary care children’s hospital in 2001–2003. Blood tests and ultrasound were performed to detect the presence of fatty liver. The severity of fatty liver was determined by measuring the liver/kidney echogenicity ratio (hepatorenal index). Blood tests included complete blood count, liver enzymes, lipid profile, erythrocyte sedimentation rate, high sensitivity C-reactive protein, serum amyloid A, and the degree of erythrocyte adhesiveness/aggregation as measured in peripheral blood slides.

Results: The 30 boys and 34 girls, age 9–21 years, who participated in the study were divided into those who evidenced NAFLD on ultrasound (Group 1, n=37) and those whose liver appeared normal on ultrasound (Group 2, n=24). ESR, hs-CRP, SAA and the degree of erythrocyte adhesiveness/aggregation were compared between the groups. There was no significant association between elevated ESR, the levels of CRP, SAA and/or the degree of erythrocyte adhesiveness/aggregation and the hepatorenal index and NAFLD. The degree of erythrocyte adhesiveness/aggregation correlated with body mass index-standard deviation score in both genders (P<0.05).

Conclusions: Fatty liver itself may not be a cofactor in stimulating inflammatory markers in obese patients. Obese children diagnosed with NAFLD may have simple steatosis and their increased inflammatory markers are therefore compatible with those expected in obesity.

KEY WORDS: obesity, non-alcoholic fatty liver disease, non-alcoholic steatohepatitis, inflammatory markers, steatosis

Non-alcoholic steatohepatitis, a term coined by Ludwig et al. in 1980 [1], is one entity within a spectrum of chronic liver disease related to obesity, hyperinsulinemia, insulin resistance, and liver cell injury from free fatty acid toxicity or other oxidant stress – all related to obesity. The more inclusive term non-alcoholic fatty liver disease represents the entire range, which includes simple hepatic steatosis without inflammation, NASH, and the resulting cirrhosis. Most individuals with simple steatosis do not develop cirrhosis. In contrast, up to 20% of adults with NASH ultimately develop cirrhosis, with some eventually developing chronic liver failure and requiring liver transplantation [2]. To date, the gold standard for diagnosing NAFLD and differentiating among simple steatosis, NASH and cirrhosis is liver biopsy.

The pathogenesis of NAFLD is not completely understood. Among the factors thought to be involved are free fatty acid accumulation in the liver, hyperinsulinemia, inflammatory cytokines (such as tumor necrosis factor-alpha), mitochondrial damage, and free radicals that cause significant oxidative stress [3]. NAFLD may also be a part of the metabolic syndrome [3,4].

Few studies have explored a link between NAFLD and the levels of serum inflammatory markers. In one investigation, 18 adult patients with histologically diagnosed NASH had elevated levels of C-reactive protein and other serum inflammatory markers, compared with 16 healthy individuals [5]. The aim of the current work was to examine the association between the

N A F L D  =  non-alcoholic fatty liver disease
ESR = erythrocyte sedimentation rate
HS-CRP = high sensitivity C-reactive protein
SAA = serum amyloid A
NASH = non-alcoholic steatohepatitis
The study group consisted of 64 children and adolescents with body mass index > 85th percentile for age and gender who were attending an endocrine obesity clinic (Schneider Children Hospital) during the period 2001–2003. Excluded were those who drank alcoholic beverages or used medications that can alter liver function tests. In order to compare biochemical and inflammatory marker levels the study cohort was divided into groups: children with fatty liver grade 1 or 2 (group 1), and normal liver grade 0 (grade 2). Each group was further divided into two subgroups according to serum transaminase levels: patients with normal transaminase levels comprised the first subgroup, and those with abnormal levels the second (i.e., alanine aminotransferase, aspartate aminotransferase and/or gamma-glutamyl transpeptidase > 40 mg/L).

For each subject, BMI was calculated using the equation \[
\text{BMI} = \frac{\text{weight (kg)}}{\text{height (m)}^2},
\]
and BMI-standard deviation score was calculated using the equation \[
[(\text{Measured BMI} - \text{Expected BMI}) / \text{SD}],
\]
allowing standardization of BMI with age.

**LABORATORY STUDIES**

Blood tests included complete blood count, liver enzymes, lipid profile, glucose, insulin, glycated hemoglobin, thyroid hormones, vitamin E and inflammatory markers, which included assessment of erythrocyte sedimentation rate, high sensitivity C-reactive protein, serum amyloid A and the degree of erythrocyte aggregation as measured by slide image analysis. The latter has been described in detail elsewhere [21,22].

Elevated liver enzymes were defined as values of ALT and/or AST and/or GGT ≥ 40 mg/L. Hyperlipidemia was defined as low density lipoprotein levels > 130 mg/dl and/or high density lipoprotein-cholesterol levels < 35 mg/dl and/or triglyceride levels > 200 mg/dl. Insulin resistance was calculated by the homeostasis model assessment of insulin resistance and defined as a HOMA value > 2 [10]. HOMA levels were transformed to the natural log to normalize their distribution. Blood tests were performed to rule out any known etiology for fatty liver, including serology assays for hepatitis B and C viruses, tissue transglutaminase and ceruloplasmin level.

Serum glucose was measured by the glucose oxidase colorimetric method using an automated analyzer (Hitachi 917, Roche Diagnostics, USA), and total cholesterol, triglycerides, and HDL-cholesterol concentrations were measured by an enzymatic colorimetric method on an automated analyzer (Hitachi 904, Roche). Serum insulin concentrations were measured by an immunometric assay with the IMMULITE 2000 Analyzer (DPC, Los Angeles, CA, USA). HbA1c levels were measured by the turbidimetric inhibition immunoassay (Hitachi 911, Roche). Cross-sectional analysis was performed to evaluate the association between the biochemical laboratory values and the ultrasound results.

**ULTRASONOGRAPHIC STUDY**

An ultrasound examination was performed to identify NAFLD. All tests were performed with a single probe by one experienced radiologist, thus minimizing inter- and intra-observer variability. Semi-quantification of the severity of fatty liver using ultrasound is based on calculating the difference between the echo densities of the liver and the right kidney. Fatty liver was recognized by a bright hepatic echo pattern (hyperechogenicity) and thus easily identified as compared to the renal cortex, which has roughly similar echogenicity to normal fat-free liver. This method was found to have sensitivity and specificity of 89% and 93%, respectively [6]. The degree of fatty infiltration in the liver was quantified by using the equation: liver echo amplitude / renal echo amplitude, whereby the hepatorenal index was defined. The hepatorenal index of normal fat-free liver is about 1 In fatty liver; the severity of the liver steatosis was graded as follows: Grade I (mild fatty liver) was defined by a hepatorenal index of 1.5–2, and Grade II (severe fatty liver) by a hepatorenal index ≥ 2.

**INFLAMMATION MARKERS STUDY**

CRP and SAA were measured using particle-enhanced immunonephelometry (BN prospec system, Dade Behring, Germany). CRP was measured using a high sensitivity CRP assay (reference interval: < 1.69 mg/L in 90% and < 2.87 mg/L in 95% of healthy individuals), and SAA was measured using latex SAA (reference interval < 6.4 mg/L in healthy individuals). Blood slides were prepared from blood drawn into a syringe containing sodium citrate 3.8%, and scanned by an image analyzer. For each blood slide, several parameters were measured using the image analyzer, including vacuum ratio and erythrocyte percent. These parameters represent the degree of erythrocyte aggregation, and thus the degree of inflammation.

The study protocol was approved by the local Helsinki Committee. A signed informed consent was obtained from all the patients or their parents.
STATISTICAL ANALYSIS
All data were summarized and displayed as mean ± SD for continuous variables and as number of patients plus the percentage in each group for categorical variables. The cross-tabulations and descriptive procedures were used to produce frequencies of categorical variables and means ± SD of continuous variables. We used a logarithmic transformation for variables that have a non-normal distribution (e.g., the hs-CRP and the SAA), thus converting them to normal distribution for all statistical procedures, such as t-tests and correlations. Each result expressed as hs-CRP or SAA is a back-transformed geometric mean and standard deviation. The one-sample Kolmogorov-Smirnov test was used to test for normal distribution.

Student’s t-test for independent samples was used for all normally distributed continuous variables when comparing two categories (e.g., gender), while the one-way ANOVA was used to compare the various parameters between the groups when comparing more than two categories. The pair-wise comparison between categories was done after performing the Levene test for homogeneity of the variance: the Hochberg’s multiple comparison technique was applied when the Levene test was not significant, while the Dunnett’s T3 test was applied when it was significant. Chi-square phi and Cramer’s V statistics were used for assessing the overall significance across all the diagnosis groups for all categorical variables.

The level of significance used for all of the above analyses was two-tailed P < 0.05. The SPSS statistical package was used to perform all statistical evaluation (SSPS Inc., Chicago, IL, USA).

RESULTS
The study group consisted of 30 boys and 34 girls. Their mean age was 14.9 ± 2.8 years (range 9–21), their mean BMI was 34.6 ± 5.5, and their mean BMI-SDS 3.3 ± 1. The BMI was > 95th percentile in most of them (92%) and between the 85th and 95th percentiles in the rest. Around three-quarters (72.5%) of the patients were at advanced stages of puberty (Tanner IV–V). Familial obesity was ubiquitous, and the prevalence of type 2 diabetes and ischemic heart disease in their first- and second-degree relatives was 63% and 37%, respectively. There were no significant gender differences for age, BMI and BMI-SDS.

ULTRASOUND DATA
Twenty-seven patients had grade 0, 19 had grade I and 18 had grade II hepatic steatosis according to the ultrasonographically calculated hepatorenal index. The average BMI-SDS in the group of children with fatty liver (mild or severe) was significantly higher than the average BMI-SDS in those with normal liver [Figure 1].

BIOCHEMICAL RESULTS
Hyperlipidemia was present in 47 patients (73%); 50 (78%) had insulin resistance, and 12 (19%) had elevated liver enzymes (ALT and AST). The ALT/AST ratio was > 1 in all 12 patients whose AST and ALT values were elevated. The ALT levels averaged 24.3 ± 15.6 mg/dl, and AST 21 ± 8.9 mg/dl. The AST/ALT ratio was 0.864. Alkaline phosphatase levels were mildly elevated, consistent with the elevations found in growing adolescents. Bilirubin levels were normal in all patients and the mean leptin level was 66.2 ± 31.3 ng/ml (range 16.4–167 ng/ml). The prevalence of elevated liver enzymes between the groups with and without fatty liver was significantly different [Figure 2].

INFLAMMATORY MARKERS
The average levels were as follows: ESR 18.97 ± 14.96 mm/hr, white blood cells 8.11 ± 2.26 x 10³/µl, hemoglobin 13.15...
The true prevalence of NAFLD in the pediatric population may be underestimated. A recent retrospective review of 742 autopsies of children aged 2–19 years noted the presence of fatty liver in 13% of all subjects and in 38% of the obese children among them [16,17]. Given the high prevalence of fatty liver among obese children, it is crucial to determine the role of fatty liver in the overall morbidity of those children. Demonstrating high levels of inflammatory markers will emphasize the risk posed by fatty liver and the importance of early and aggressive treatment.

Like adults, most children are asymptomatic. In those who are symptomatic, the most common complaint is right upper quadrant pain or chronic periumbilical abdominal pain [13]. Findings on physical examination may include obesity, hepatomegaly, acanthosis nigricans, or splenomegaly (rare). Common comorbidities include hyperlipidemia, hypertension, insulin resistance and diabetes mellitus. These conditions, along with NASH, are considered part of the metabolic syndrome [13].

Mildly elevated levels of serum ALT may be found in children with NAFLD, with some having levels 10 times the

Table 1. Inflammatory markers in the study group

<table>
<thead>
<tr>
<th>Hemoglobin (mg/L)</th>
<th>Leukocytes (10⁹/L)</th>
<th>ESR (mm/hr)</th>
<th>hs-CRP (mg/L)</th>
<th>SAA (mg/L)</th>
<th>Erythrocyte percent</th>
<th>Vacuum ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>64</td>
<td>64</td>
<td>64</td>
<td>64</td>
<td>64</td>
<td>64</td>
</tr>
<tr>
<td>Average</td>
<td>13.15</td>
<td>8.11</td>
<td>18.97</td>
<td>4.2</td>
<td>6.16</td>
<td>83.29</td>
</tr>
<tr>
<td>Median</td>
<td>12.9</td>
<td>8.1</td>
<td>14.5</td>
<td>4.4</td>
<td>5.42</td>
<td>88.53</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>1.23</td>
<td>2.26</td>
<td>14.26</td>
<td>2.7</td>
<td>2.32</td>
<td>14.16</td>
</tr>
<tr>
<td>Maximum</td>
<td>17.6</td>
<td>14.3</td>
<td>68</td>
<td>46.7</td>
<td>92.7</td>
<td>99.8</td>
</tr>
<tr>
<td>Minimum</td>
<td>10.9</td>
<td>3.1</td>
<td>2</td>
<td>0.45</td>
<td>1.37</td>
<td>44.52</td>
</tr>
</tbody>
</table>

Figure 3. Body mass index (BMI) and erythrocyte percent (Erythrocyte P) was inversely correlated in females ($P < 0.05$)

DISCUSSION

Obesity is a global medical problem in the general population. The pediatric population is not spared. It is reported that around 15% of children between age 6 and 19 are above the 95th percentile of BMI. According to previous reports, there is a clear suboptimal diagnosis of obesity and its complications in children visiting clinics in Israel [10,11], emphasizing the need for early evaluation, diagnosis and treatment. Furthermore, these children are at high risk of developing cardiovascular disease in early adulthood; this risk can be assessed and treated early in their life [12]. There is a clear association between obesity and NAFLD: 85% of children with NAFLD are obese [13]. The prevalence of NAFLD, diagnosed by detecting hyper-echogenicity of the liver on ultrasound, was found to be 2.6% in normal-weight children and an alarming 22.5–52.8% in obese children [14,15]. The true prevalence of NAFLD in the pediatric population may

± 1.2 g/dl, CRP 4.2 ± 2.7 mg/L (range 0.45–46.7 mg/L), and SAA 6.2 ± 2.3 mg/L (range 1.37–92.7) [Table 1].

CORRELATION OF ESR, ELEVATED LIVER ENZYMES AND ULTRASOUND FINDINGS

There was no significant difference in ESR between subjects with normal ultrasound findings and those with ultrasound findings of fatty liver and elevated or non-elevated liver enzymes. The average ESR was 16.5 ± 11.5 mm/hr in patients with normal ultrasound findings (grade 0 hepatic steatosis), and 18.8 ± 14.1 mm/hr in patients with ultrasound findings of NAFLD (grades I and II hepatic steatosis). There was no significant difference between the two groups. Further dividing the latter group into subgroups of patients with grade I hepatic steatosis and patients with grade II hepatic steatosis also showed no significant difference in the ESR between them.

CORRELATION OF HEPATORENAL INDEX AND INFLAMMATORY MARKERS

There was no significant correlation between the hepatorenal index and the levels of the tested inflammatory markers (CRP, SAA, EP and VR).

CORRELATION OF BMI AND HISTOLOGIC INFLAMMATORY MARKERS

An inverse correlation was found between the BMI and EP in the female patients (N=34, $P < 0.05$) [Figure 3]. A direct correlation was also found between the BMI, BMI-SDS and VR in the male patients (N=30, $P < 0.05$). These findings demonstrate the correlation between BMI and the degree of inflammation as represented by the peripheral blood slides.

EP = erythrocyte percent
VR = vacuum ratio
Among obese children and adolescents, the percentage of those with elevated levels of serum transaminases ranges between 12 and 25% [13]. Serum GGT and alkaline phosphatase may be mildly elevated as well. Ultrasound evaluation of the liver in patients with NAFLD may show diffuse hyper-echogenicity compared to the kidneys. The sensitivity and specificity of ultrasound are 89% and 93% as a diagnostic tool for fatty liver, and 89% and 77% respectively to detect liver fibrosis [6]. Ultrasound has many drawbacks as a diagnostic tool because it is operator dependent, and in very obese children the subcutaneous fat can interfere with the scanning. However, in this study there was no difficulty performing the scan.

Accumulating data show high levels of serum inflammatory markers in obese individuals. Ongoing systemic inflammation in obese adults was evidenced by high levels of CRP and fibrinogen [18,19]. Similar findings were found in children [20]. To date, few studies have investigated the correlation between NAFLD and the levels of serum inflammatory markers, and all were conducted in adults [5]. The general consensus of these studies was that high serum inflammatory markers can be found in patients with NAFLD.

The inflammatory markers include CRP, fibrinogen, SAA, and inflammatory cytokines interleukin-6 and TNFα. High levels of CRP in the serum are known to be related to increased risk for cardiovascular morbidity, including the early development of atherosclerosis [21,22]. The degree of erythrocyte adhesiveness/aggregation in peripheral blood slides is another inflammation marker found to be related to obesity [24]. The correlation between the degree of erythrocyte adhesiveness/aggregation is directly related to the degree of systemic inflammation and to cardiovascular risk [25]. Given the possible relation between NAFLD and elevated levels of serum inflammatory markers, early diagnosis of NAFLD and aggressive treatment may play an important role in preventing both the progression of NAFLD into severe hepatic inflammation or cirrhosis and the early development of atherosclerosis. Furthermore, using a panel of serum inflammatory markers in conjunction with serum transaminases and ultrasound findings may replace the need for biopsy to establish the diagnosis of NASH. Treatment with statins, which act as anti-inflammatory agents in addition to lowering LDL, was recently associated with a significant reduction in CRP serum levels [23]. It therefore follows that earlier treatment with statins against the background of NAFLD may reduce even further the risk of cardiovascular morbidity.

Ultrasoundically detected fatty liver did not correlate with the levels of inflammatory markers. This may be explained by several factors: a) affected children suffer from simple fatty liver and not NASH (defined as “an inflammation of the liver”); b) obese children already have increased levels of serum inflammatory markers that may mask the small increment possibly caused by NASH itself; c) other factors, such as toxicity of triglycerides, may contribute to the development of NASH, thus weakening the correlation between “pure” inflammatory markers and NASH. In contrast, there is evidence of a significant correlation between BMI and inflammatory markers, both in serum (CRP, SAA) and in blood smears, using the image analyzer of blood smears.

It is important to emphasize that the ultrasonographic evaluation of fatty liver in this study does not differentiate well between simple steatosis and NASH. As such, further research is required, including histologic study, to correlate between the different stages of NAFLD and the levels of serum inflammatory markers in the pediatric population.

References

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Human society is organized into groups, such as those based on nationality or religion, which can lead to intergroup conflicts, with sometimes devastating consequences. Intergroup conflict engages a human behavior termed parochial altruism: For example, a soldier who fights against the enemy at risk to themselves to protect their country is a parochial altruist. De Dreu et al. have discovered a role for oxytocin, a neuropeptide produced in the hypothalamus, in regulating parochial altruism during human intergroup competition and conflict. Oxytocin is already known to play a role in trusting behavior, and naturally occurring genetic variants of the oxytocin receptor exist within the human population. Administration of oxytocin modulated defense-related aggression toward competing groups but did not affect unprovoked, hateful behavior. Thus, there may be a neurobiologic basis for intergroup conflict in humans.

Science 2010; 328: 1705
Eitan Israeli

The immune system and the gut flora

The mammalian gut is colonized by many non-pathogenic, commensal microbes. In order to prevent the body from mounting inappropriate immune responses to these microbes, plasma cells in the gut produce large amounts of immunoglobulin A (IgA) specific for commensal bacteria. Because of the difficulties of uncoupling IgA production from microbial colonization, how commensal bacteria shape the gut IgA response is not well understood. Hapfelmeier et al. devised a way to get around this problem by developing a reversible system of gut bacterial colonization in mice. Commensal-specific IgA responses were able to persist for long periods in the absence of microbial colonization and required the presence of high microbial loads in the gut for their induction. IgA responses upon bacterial re-exposure did not resemble the synergistic prime-boost effect seen in classical immunologic memory responses but rather exhibited an additive effect that matched the current bacterial content present in the gut. The body thus constantly adapts the commensal-specific immune response to the microbial species present in the gut, which contrasts with the systemic immune response that persists in the absence of pathogenic microbes.

Science 2010; 328: 1705
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Oxytocin and intergroup parochial altruist conflict

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Science 2010; 328: 1408
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“It must be borne in mind that the tragedy of life does not lie in not reaching your goal. The tragedy of life lies in having no goal to reach”

Benjamin E. Mays (1894-1984), U.S. Black minister, educator, scholar, social activist and college president. An articulate and outspoken critic of segregation before the rise of the modern civil rights movement in the United States, he was a significant mentor to civil rights leader Martin Luther King Jr.