METABOLIC, HORMONAL, OXIDATIVE, AND INFLAMMATORY FACTORS IN PEDIATRIC OBESITY-RELATED LIVER DISEASE

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Objective  To examine the role of metabolic, hormonal, oxidative, and inflammatory factors in pediatric obesity-related liver disease.

Study design  In 50 obese children (age 7 to 14 years) with (n = 20, group 1) or without (n = 30, group 2) hypertransaminasemia and ultrasonographic liver brightness, we studied insulin resistance (fasting glucose/insulin ratio [FGIR]) and serum levels of leptin, iron, transferrin, ferritin, C-reactive protein (CRP), white blood cell (WBC) count, tumor necrosis factor (TNF)-α, interleukin (IL)-6, C282Y and H63D mutations, and erythrocytic glutathione peroxidase (GPX) activity.

Results  FGIR (6.7 ± 4.1 vs 9.2 ± 5.2; P = .02), serum ferritin (88.8 ± 36.0 vs 39.9 ± 24.0 ng/mL; P = .0001), serum CRP (5.4 ± 6.0 vs 1.1 ± 1.6 mg/dL; P = 0.004), and GPX (8.4 ± 0.9 vs 5.0 ± 0.5 U/g Hb; P = .05) were significantly higher and more frequently deranged in group 1 than in group 2. FGIR, ferritin, and CRP values were simultaneously deranged in 41% of the group 1 patients and in none of the group 2 patients (P = .098). Serum leptin, iron, and transferrin, WBC, TNF-α, IL-6, and C282Y and H63D mutations were similar in the 2 groups.

Conclusions  Insulin resistance, oxidative stress, and low-grade systemic inflammatory status are implicated in pediatric obesity-related liver disease. These findings may be useful in planning pathophysiologically based therapeutic trials for hepatopathic obese children who are unable to follow hypocaloric diets. (J Pediatr 2005;147:62-6)

Liver involvement in obese individuals has become a leading cause of liver function test abnormalities. It is classified within the abnormalities of nonalcoholic fatty liver disease (NAFLD), a condition ranging from simple hepatic steatosis (a putatively common and benign disease with an indolent course) to progressive necroinflammatory and fibrotic damage of the liver. Although liver involvement is suggested by liver brightness on ultrasonography (US) and elevated alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels, the true prevalence of NAFLD remains obscure, largely because of the variability of definition criteria, including the threshold of hypertransaminasemia itself.

NAFLD is being increasingly recognized in children. The reported prevalence of hypertransaminasemia in obese children varies between 10% and 24% to 25%, and US liver brightness has been reported to vary between 22.5% and 77%.

The reasons underlying liver involvement and disease progression in only a proportion of obese individuals remain unclear. In adults and in animal models there is evidence that increased fat deposition within the hepatocytes of obese individuals results from augmented hepatic delivery of free fatty acids. The latter is amplified by insulin resistance, which impairs suppression of lipolysis. Excessive fatty acid oxidation in the liver generates free radicals, which damage hepatocytes and induce fibrogenesis through...

ALT  Alanine aminotransferase IL  Interleukin
AST  Aspartate aminotransferase NASH  Nonalcoholic steatohepatitis
CRP  C-reactive protein TNF  Tumor necrosis factor
FGIR  Fasting glucose-to-insulin ratio US  Ultrasonography
FFA  Free fatty acid WBC  White blood cell
GPX  Glutathione peroxidase
cortisol production. Two studies conducted in children that reported that the antioxidant vitamin E induced normalization of hypertransaminasemia but did not affect ultrasonographic liver brightness also support the concept that oxidative stress plays a pathogenetic role in obesity-related liver disease. Although insulin resistance is considered pivotal to the development of fatty liver, few data have been reported in the pediatric literature. Similarly, abnormal iron handling and leptin levels are also implicated in the pathogenesis of fatty liver, but data in childhood are conflicting and scarce.

Interleukin (IL)-6 produced by fat cells induces the synthesis of C-reactive protein (CRP) by the liver; thus, obesity is associated with low-grade systemic inflammation. However, it remains to be established whether the inflammatory involvement is more severe in obese adults or children affected by liver disease.

Here we examine globally, and also in the same series of obese children, the role of metabolic, hormonal, oxidative, and inflammatory factors in pediatric obesity-related liver dysfunction.

**METHODS**

In the first stage of this study, we retrospectively recorded the anthropometric measurements and the results of liver function tests and US examinations contained in the clinical records of 256 obese (body mass index > 95th percentile) Italian children who had been monitored for at least 1 year at the pediatric obesity clinics participating in our project.

To ensure a distinct separation between individuals with and individuals without obesity-related liver disease, the study protocol was designed to evaluate only prepubertal patients (age > 6 years) who presented the following inclusion criteria: stable or increased percent of overweight due to poor compliance to a prescribed slimming diet and exercise program (n = 119), US liver brightness coupled with a persistent increase of ALT and/or AST levels > 1.5 times above normal values for age persisting for more than 6 months (hepatopathic obese patients), and normal US liver findings coupled with persistently normal ALT and AST levels (obese controls). Exclusion criteria were normalization of liver function tests after decrease in the percent overweight during follow-up, fluctuating transaminase levels reaching normal or near-normal values in the last 2 biochemical evaluations, and known causes of liver abnormalities other than obesity. Fever and respiratory tract infection during the acute phase of the disease were considered temporary exclusion criteria.

Of the 119 children identified from our pool of 256 obese children, 101 consented to a complete reassessment of anthropometric parameters, liver function tests, and a new US liver examination. Only 50 of 101 children met the more stringent inclusion criteria for the study of metabolic, hormonal, oxidative, and inflammatory factors involved in the pathogenesis of liver disease. Of these 50, 20 were hepatopathic obese children with chronic elevation of AST and/or ALT and US liver brightness (group 1). The other 30, who had persistently normal serum AST and/or ALT levels in the absence of US liver brightness at study entry, were assigned to the obese control group (group 2). All 50 patients were prepubertal, with a mean age of 9.0 ± 2.4 years; 26 were girls. None had previously been treated with hepatotoxic drugs, had undergone surgery, had received either blood or blood products, or had a history of alcohol consumption. No patient had a history of short gut syndrome, small bowel intestinal bypass, Cushing’s disease, or diabetes mellitus, which could have caused hepatic steatosis. All were asymptomatic. None had arterial hypertension. The liver was slightly enlarged but of normal consistency in 6 patients of group 1. None had splenomegaly or other stigmata of portal hypertension.

In patients with liver involvement, causes of increased transaminase levels other than obesity (eg, muscular disease, viral hepatitis B and C, autoimmune hepatitis, α1-antitrypsin deficiency, cystic fibrosis, Wilson’s disease, hemochromatosis, hereditary fructose intolerance, amino acid disorders, atypical celiac disease) were ruled out by appropriate tests.

Routine liver function tests in addition to ALT and AST (ie, alkaline phosphatase, γ-glutamyltranspeptidase, bilirubin, total protein, protein electrophoresis) were also determined. Ultrasonography of the liver was carried out as described previously. Serum CRP level, lipid levels, glucose level, glycosylated hemoglobin level, fasting insulin and glucose levels, iron status (iron levels, percent of transferrin saturation), and ferritin level were determined by standard methods.

Samples of fresh sera and plasma for determining dosage of tumor necrosis factor (TNF)-α, interleukin (IL)-6, and leptin were collected from all subjects. Blood spots were also collected on filter paper for polymerase chain reaction analysis. Lysed red blood cells were obtained from all subjects for a glutathione peroxidase (GPX) assay.

**Experimental Methods**

The fasting glucose-to-insulin ratio (FGIR) was to measure insulin resistance, with insulin resistance diagnosed when values were < 7. Blood cells eluted from filter paper were lysed and DNA was purified for polymerase chain reaction—single-strand conformation polymorphism analysis to detect the C282Y and H63D mutations causative of hereditary familial hemochromatosis (HFE), as described elsewhere. Serum concentrations of TNF-α and IL-6 were determined by enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, Minn) in accordance with the manufacturer’s instructions. A spectrophotometric reading was performed on microplates using 450-nm filters. The enzymatic activity of GPX was evaluated by an indirect colorimetric method (Bioxynet GPx-340; Oxis Research, Portland, Ore). Each sample was washed in 0.9% NaCl solution and lysed in sterilized bi-distilled frozen water. The ensuing oxidation of reduced nicotinamide adenine dinucleotide phosphate (NADPH) to NADP+ was photometrically measured by spectrophotometry (model DU 640; Beckman, Brea, Calif) at 340 nm for 180 minutes; data are expressed as U/g hemoglobin.
Table I. Demographic data, anthropometric measurements, and aminotransferase values in the 2 groups of obese subjects at study entry

<table>
<thead>
<tr>
<th></th>
<th>Group 1 (hepatopathic obese patients)</th>
<th>Group 2 (obese controls)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (M/F) (n)</td>
<td>8/12</td>
<td>16/14</td>
<td>NS</td>
</tr>
<tr>
<td>Age (years)</td>
<td>10.9 ± 3.2</td>
<td>9.3 ± 2.7</td>
<td>NS</td>
</tr>
<tr>
<td>BMI</td>
<td>27.1 ± 1.3</td>
<td>27.4 ± 1.3</td>
<td></td>
</tr>
<tr>
<td>ALT (U/L; normal, &lt; 40)</td>
<td>85.1 ± 40.9</td>
<td>23.2 ± 6.7</td>
<td>&lt; .0001</td>
</tr>
<tr>
<td>AST (U/L; normal, &lt; 40)</td>
<td>41.2 ± 18.9</td>
<td>24.8 ± 5.3</td>
<td>&lt; .0001</td>
</tr>
<tr>
<td>AST:ALT (ratio)</td>
<td>0.56 ± 0.41</td>
<td>1.05 ± 0.3</td>
<td>.002</td>
</tr>
<tr>
<td>Cholesterol (mg/dL; normal, &lt; 170)</td>
<td>153.9 ± 7.6</td>
<td>162.9 ± 8.8</td>
<td>.02</td>
</tr>
<tr>
<td>Triglycerides (mg/dL; normal, 50-200)</td>
<td>108.1 ± 12.1</td>
<td>101.9 ± 19.5</td>
<td>NS</td>
</tr>
<tr>
<td>Ultrasonographic liver brightness (n)</td>
<td>20/20</td>
<td>0/30</td>
<td>&lt; .0001</td>
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</table>

BMI, Body mass index; NS, not significant.
Mean values are reported as mean ± standard deviation.
In the hepatopathic obese patients, liver function tests other than ALT and AST were within normal limits except GGT, which was slightly increased in 3 individuals.

Informed consent was obtained from the family of each subject included in the study. The study protocol conforms to the ethical guidelines of the Declaration of Helsinki approved in 1975 and revised in 1983, and was approved by the local ethics committee.

Statistical Analysis

Differences between means were evaluated using the unpaired Student t-test. Fisher’s exact test was used to calculate the probability value for the relationship between 2 dichotomous variables (eg, patient gender). Correlation between continuous variables was evaluated by the Pearson test. The statistical significance level selected for all tests was P < .05.

RESULTS

Table I gives demographic and anthropometric measurements, as well as the results of serum lipid and liver function tests, of groups 1 and 2 at study entry. All parameters, except ALT and AST values and AST:ALT ratios, were similar in the 2 groups. In group 1, ALT levels were high in all patients; both ALT and AST were elevated in 7 patients, but in only 2 of these was AST greater than ALT.

As shown in Table II, FGIR ratios were 6.7 ± 4.1 in group 1 and 9.2 ± 5.2 in group 2 (P = .02). Values were deranged in 78% of the patients in group 1 and in and 22% of those in group 2 (P = .0036). Interestingly, FGIR values were inversely correlated with ferritin levels (r = −.6; P = .04).

DISCUSSION

Gradual weight loss and regular exercise are considered the first-line treatment for obesity-related liver disease in children and adults.8,29 However, these are often difficult to achieve, and it is not known whether this approach is beneficial in advanced disease. Innovative therapies that can modify the pathogenic mechanisms of liver damage are thus particularly urgent in obese children, whose longer life expectancy (compared with adults) makes them more prone to additional hepatotoxic noxae.

Insulin resistance is not uncommon in obese preadolescents and adolescents.37 It plays a major role in the pathogenesis of NAFLD in adults and in animal models.19 Kawasaki et al23 reported a high correlation between hyperinsulinemia and hypertransaminasemia in a group of obese prepubertal Japanese children. More recently, Schwimmer et al24 reported that impaired insulin sensitivity was nearly universal in a group of American adolescents with biopsy-proven NAFLD. Our study corroborates their findings by showing that insulin resistance is approximately 4 times more frequent already in prepubertal children affected by obesity-related liver disease compared with age-matched controls. This finding is not trivial given the beneficial effects of antihyperglycemic agents on hepatic insulin sensitivity and on liver enzyme levels obtained in a pilot study in adult nonalcoholic steatohepatitis (NASH).30

Data on the role of iron and HFE mutations as cofactors implicated in the pathogenesis and progression of NAFLD in adults are conflicting.19,31 It is now well established that despite increased ferritin levels, serum iron indices (ie, iron levels and percent transferrin saturation) and hepatic iron are rarely abnormal in adult patients with NAFLD.19,31 In our pediatric series we observed the same

Serum leptin, iron, transferrin, and transferrin saturation levels were similar in the 2 groups. Serum ferritin was 88.8 ± 36 in group 1 and 39.9 ± 24 ng/mL in group 2 (P < .0001). Values were > 100 ng/mL in 46.6% of the group 1 patients and in 4.5% of the group 2 patients (P = .0038).

Molecular analysis of the C282Y and H63D mutations of the HFE gene revealed no significant differences between the 2 groups.

CRP was significantly higher in group 1 (P = .004), and values were deranged in 64% of the group 1 patients versus 23% of the group 2 patients (P = .02). WBC, TNF-α, and IL-6 serum levels were similar in the 2 groups. TNF-α was correlated with IL-6 (r = .7; P = .005). CRP was positively correlated with IL-6 (r = .61, P = .001) and ferritin (r = .6; P = .04) values and negatively correlated with FGIR (r = −.8, P = .03). Nonetheless, IL-6 and TNF-α did not correlate with ferritin. GPX activity tended to be higher in group 1 (P = .05).

Clusters of biochemical abnormalities in the same individual were observed only in group 1. FGIR, ferritin, and CRP values were simultaneously abnormal in 41% of the group 1 patients and in none of the group 2 patients (P = .098).
pattern of normal iron indices and increased ferritin observed in adults.

No association was found between the HFE gene mutations H63D and C282Y and obesity-related liver dysfunction. The high prevalence of heterozygosity for the H63D mutation in patients and controls was consistent with that reported for the Italian normal population. Because the ferritin levels of our population correlated with CRP values, elevated ferritin should be considered an acute-phase reactant rather than a consequence of increased iron stores. Determining whether iron is indeed involved in the liver injury in our children would require liver biopsy.

Regarding the interplay between iron status and insulin sensitivity, we found a significant correlation between ferritin levels and insulin resistance. The pathophysiogenetic significance of this association (ie, insulin resistance-associated iron overload) remains unclear. High CRP values and WBC counts, which are markers of a low-grade systemic inflammatory status, have been reported in both obese adults and obese children. Their correlation with liver damage has not yet been studied in detail. Our study confirms that obese children have a moderately increased inflammatory status, as shown by slightly elevated CRP values than in normal individuals. Interestingly, the increase in CRP values was much more pronounced in children with liver involvement. As expected, CRP levels also were significantly correlated with serum levels of proinflammatory IL-6. The latter was also significantly correlated with TNF-α.

In adult studies, inflammation has also been implicated in insulin resistance and in the pathogenesis of type 2 diabetes mellitus. In our study we found a positive relationship between elevated CRP concentrations and insulin resistance, which demonstrates this association already in children.

Among the molecules involved as a second hit in NAFLD, attention has focused on leptin, a peptidic hormone prevalently derived from adipocytes. However, the available data are conflicting, probably because of the lack of appropriate controls. In our study we were unable to find differences in leptin values between the group 1 patients than in the group 2 patients. This finding is in agreement with recent findings obtained in adult NASH patients and well-matched controls.

In contrast to other pediatric series of NAFLD, our study has a prevalence of girls. Although this prevalence is not statistically significant, it would be interesting to evaluate in a larger group of patients whether the preponderance of girls affected the comparison of leptin values between groups.

Among other putative pathogenic factors that had not been extensively investigated in hepatopathic obese children, we assessed the antioxidant reserve by measuring GPX activity in red blood cells. Glutathione peroxidase detoxifies cells from peroxides and is one of the first lines of defense against free radicals. Because free radicals may form highly reactive radicals, GPX is critical in protecting cells against lipid peroxidation. In our study, GPX activity was greater in the group 1 patients than in the group 2, which probably indicates enzyme induction by toxic agents in the initial stages of liver disease. Further studies on other measures of oxidant injury or lipid peroxidation should help provide insight into the role of oxidative stress involvement in hepatopathic obese children.

In conclusion, our results show that insulin resistance and oxidative stress but not iron status are implicated in pediatric obesity-related liver disease. Moreover, our data—like those obtained in adult NASH—are not consistent with a role for leptin in the pathogenesis of pediatric obesity-related liver disease.

Increased systemic inflammation of hepatopathic obese children versus obese controls and the relationship between both CRP and ferritin with insulin resistance reported here point to the existence of a complex interplay involving low-grade inflammation and insulin sensitivity that deserves further study. Taken together, these data may be useful in the planning of innovative therapeutic approaches to liver dysfunction in obese children who are unable to follow hypocaloric diets.

Table II. Metabolic, hormonal, inflammatory, and oxidative factors studied in the 2 groups of obese subjects

<table>
<thead>
<tr>
<th>Factor</th>
<th>Group 1 (hepatopathic obese patients)</th>
<th>Group 2 (obese controls)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>FGIR (ratio; normal, M, 3-5; F, 4-8)</td>
<td>6.7 ± 4.1</td>
<td>9.2 ± 5.2</td>
<td>.02</td>
</tr>
<tr>
<td>Serum leptin (ng/mL; normal, M, 3-5; F, 4-8)</td>
<td>16.5 ± 1.7</td>
<td>16.6 ± 1.8</td>
<td>NS</td>
</tr>
<tr>
<td>Serum iron (μg/dL; normal, 16-24)</td>
<td>83.3 ± 33.0</td>
<td>73.5 ± 23.3</td>
<td>NS</td>
</tr>
<tr>
<td>Transferrin (g/L; normal, 0.95-3.85)</td>
<td>2.9 ± 0.4</td>
<td>2.8 ± 0.2</td>
<td>NS</td>
</tr>
<tr>
<td>Transferrin saturation (normal, 30%-40%)</td>
<td>21.5 ± 2.0</td>
<td>22.1 ± 1.8</td>
<td>NS</td>
</tr>
<tr>
<td>Ferritin (ng/mL; normal, 7-140)</td>
<td>88.8 ± 36.0</td>
<td>39.9 ± 24.0</td>
<td>.0001</td>
</tr>
<tr>
<td>% C282Y heterozygotes</td>
<td>0</td>
<td>0</td>
<td>NS</td>
</tr>
<tr>
<td>% H63D heterozygotes</td>
<td>30.0</td>
<td>23.0</td>
<td>NS</td>
</tr>
<tr>
<td>Serum TNF-α (pg/mL)</td>
<td>21.0 ± 8.0</td>
<td>9.0 ± 2.0</td>
<td>NS</td>
</tr>
<tr>
<td>Serum IL-6 (pg/mL)</td>
<td>2.9 ± 1.1</td>
<td>2.4 ± 0.7</td>
<td>NS</td>
</tr>
<tr>
<td>CRP (mg/dL; normal, 0-0.5)</td>
<td>5.4 ± 6.0</td>
<td>1.1 ± 1.6</td>
<td>.004</td>
</tr>
<tr>
<td>GPX activity (U/g hemoglobin)</td>
<td>8.4 ± 0.9</td>
<td>5.0 ± 0.5</td>
<td>.05</td>
</tr>
</tbody>
</table>

NS, not significant.
Mean values are reported as mean ± standard deviation.

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