HEPATIC STEATOSIS IN CHILDREN

CLINICAL-BIOLOGICAL CORELATIONS AND LONG TERM EVOLUTIVE IMPLICATIONS

PHD THESIS ABSTRACT

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- IASI 2013 -
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ABBREVIATIONS

AB - antibodies
ALT - alanine aminotransferase
AST - aspartate aminotransferase
AUC - area under curve
BMI - body mass index
CK18 - cytokeratin 18
D - diameter
DAMPs - damage-associated molecular patterns
DBP - diastolic blood pressure
DM - diabetes mellitus
ECM - extracellular matrix
ELISA - enzyme-linked immunosorbent assay
FBS - fetal bovine serum
FMD - flow mediated dilatation
GGT - gamma-glutamyl transpeptidase
HA - hyaluronic acid
HBP - high blood pressure
HDLc - high density lipoprotein cholesterol
HOMA-IR - Homeostasis Model Assessment for Insulin Resistance
HSCs - hepatic stellate cells
HS - hepatic steatosis
IL - interleukin
IFG - impaired fasting glucose
IMT - intima media thickness
IR - insulin resistance
ISI - insulin sensibility index
LDL - low density lipoprotein cholesterol
LPS - lipopolysaccharide
MS - metabolic syndrome
NAFLD - non-alcoholic fatty liver disease
NASH - non-alcoholic steatohepatitis
NAS score - NAFLD activity score
NF-κB - nuclear factor kappa B
PAMPs - pathogen-associated molecular patterns
PBS - phosphate buffered saline
ROS - reactive oxygen species
SBP - systolic blood pressure
SD - standard deviation
α SMA - alpha smooth muscle actin
TG - triglyceride
TGF-β - transforming growth factor beta
TLR - toll like receptor
UDCA - ursodeoxycholic acid
US - ultrasonography
VLDL - very low density lipoprotein cholesterol
WC - waist circumference

Note: the abstract shows selective references and iconography, respecting numbering and contents of the thesis.

Keywords: non-alcoholic fatty liver disease, hepatic steatosis, children, subclinical atherosclerosis, hepatic fibrosis, bacterial endotoxin.

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INTRODUCTION. RESEARCH MOTIVATION

Non-alcoholic fatty liver disease (NAFLD) is a pathological condition with increasing incidence and a major cause of hepatic related morbidity and mortality, both in adults and children (Day, 2011). In the past 30 years, interest in studying NAFLD increased progressively, the disease being in part already known, but long time underestimated, especially in the pediatric population.

NAFLD is now recognized as the main form of chronic liver disease in children, the most common cause of unexplained persistent high transaminase (Papandreou et al., 2007). NAFLD include a broad spectrum of liver disease, from simple steatosis to steatohepatitis (NASH) associated with necro-inflammation and/or fibrosis. The disease, in its more severe forms, can progress to cirrhosis, for which currently the only available therapeutic strategy is liver transplantation (Brunt et al., 2010). Effects of specific risk factors, such as obesity and sedentary lifestyle, associated with genetic predispositions lead to the development of NAFLD in children (Cohen et al., 2011).

Given the increasing prevalence of obesity and diabetes in the general population, non-alcoholic fatty liver disease has become a major public health problem (Day, 2011). In Europe it is estimated that 20-30% of adults and 3-10% of children in well-developed countries are affected by NAFLD (Vernon et al, 2011). Latest data estimated that 6 million people in the United States population has non-alcoholic steatohepatitis and about 600,000 individuals developed post NASH cirrhosis, and a percentage of 13-14% of children have NAFLD (WGO Global Guidelines 2012). Furthermore, the World Health Organization (WHO) warns that in the next 10-20 years, less developed countries and developing countries will experience the "double burden" of malnutrition and obesity coexistence in the same population, which will favor the occurrence of NAFLD (Alisi et al., 2012). Undernourished children are more susceptible to the harmful effects of a "cheap" diet (which is hyperlipidic, hyperglucidic, hypercaloric and low in nutrients), favoring the accumulation of visceral fat (Drewnowski, Darmon, 2005).

Although the etiopathogenesis of pediatric NAFLD remains incompletely known, it is assumed that a network of interactions between multiple factors promotes its development and progression (Alisi et al., 2012). Among the key factors leading to the development of hepatic steatosis are considered fat accumulation in the liver and insulin resistance. Hepatic steatosis can progress to steatohepatitis through at least three different ways: increased oxidative stress and subsequent lipid peroxidation; pro-inflammatory and pro-fibrotic cytokine production and release, with activation of signal transduction pathways; altered production of adipocytokines, strong fibrogenic stimulus as a
result of direct stimulation of stellate cells or paracrine effects on sinusoidal endothelial cells (Malaguarnera, et al., 2009).

NAFLD is associated with metabolic changes correlated with central obesity, such as elevated levels of triglycerides, hypertension, insulin resistance, which increase the risk of type II diabetes and metabolic syndrome (Schwimmer et al, 2008). NAFLD is considered as the hepatic expression of the metabolic syndrome (Khashab et al, 2008). These two diseases affect each other through a bidirectional link; detection of nonalcoholic fatty liver may be a starting point for screening other metabolic risk factors (Manco et al., 2008).

Non-alcoholic fatty liver disease is a progressive disease. The first cases of NASH in children have been reported in 1983 in obese adolescents in the absence of alcohol consumption (Moran et al., 1983). Currently, the spectrum of pediatric NAFLD disorders has been highlighted by the age of 2 years and post-NASH cirrhosis has been reported in children aged 8 (Schwimmer et al, 2005). The final stage of NASH is often a neglected cause of cryptogenic cirrhosis; progressive fibrosis can be dimmed by the worsening of steatosis and serological aspects (Schwimmer et al., 2005). The literature claim that up to 9% of children with NASH develop cirrhosis and the risk of relapse increases in transplanted graft at two years after transplantation (Feldstein et al., 2009).

NAFLD is an independent risk factor for cardiovascular disease. Hepatic steatosis and atherosclerosis have common molecular mediators, correlated with both insulin resistance and dyslipidemia, as well as with markers of subclinical inflammation, which raises the hypothesis that NAFLD may play an early role in the development and progression of atherosclerosis. NASH development is correlated with hepatic progenitor cell activation and release of proatherogenic adipokines (Lee et al, 2008).

Diagnosis of NAFLD in children is essential for understanding the origins of the disease, starting from those who are most susceptible in terms of genetic and environmental factors (Day, 2010). Due to disease progression, both in childhood and in adulthood, early diagnosis and appropriate management is important at all ages. Adults with childhood onset of NAFLD may be more likely to developed early or severe complications (Molleston et al, 2002).

The two major issues to be resolved in this disease are currently: the lack of markers for early diagnosis of the disease and the lack of effective and not harmful therapies. Identification of appropriate treatments for children and adolescents with NAFLD is an important priority for health systems worldwide. However, due to limited knowledge on the molecular pathogenesis of NAFLD, current therapeutic approaches consist of strategies designed to reduce the incidence of risk factors (obesity, dyslipidemia, insulin resistance) and/or drugs that target potentially key molecular pathways involved in the development of this disease, such as decreasing oxidative stress (Nobili, Sanyal, 2012).
The complexity of the disease (starting from the increasing prevalence to the multiple mechanisms involved in the pathogenesis, progressive nature of the disease and association of major comorbidities) associated with the importance of early diagnosis and proper management, especially in children associating risk factors, were prerequisites for choosing this theme. We considered useful to study the disease in children, in order to highlight the most frequent clinical and biological features, for a better knowledge and a rapid therapeutic and prophylactic intervention.

PERSONAL CONTRIBUTION

Chapter 6. STUDY I
ASSESMNT OF CLINICAL-BIOLOGICAL CHARACTERISTICS AND VASCULAR CHANGES IN HEPATIC STEATOSIS IN CHILDREN. THEPAPY IMPLICATION IN DISEASE EVOLUTION

The present study is an attempt of complex assessment of pediatric patients with hepatic steatosis. The study aimed the evaluation of biological and clinical correlations defining illness in children, the assessment of vascular changes associated with steatosis and the effects of therapeutic interventions in non-alcoholic fatty liver disease in children. The implications of these issues in clinical practice have been one of the study motivations.

The main objectives of the study were:

- The assessment of a group of in-patients monitored by the 2nd Pediatrics Clinic Iasi, diagnosed with hepatic steatosis;
- The analysis of the specific epidemiological features (demographic, anamnesis), for establish of risk parameters;
- The quantification of relationship between the anthropometric parameters and the presence of hepatic steatosis;
- The analysis of the biological profile of patients with hepatic steatosis;
- The assessment of correlation between the clinical and biological parameters in children with hepatic steatosis;
- The analysis of the association between hepatic steatosis and metabolic syndrome in children and the prevalence for individual components of metabolic syndrome in the study group;
- The correlation between hepatic steatosis and its severity with subclinical atherosclerosis markers;
- The assessment of the effects of treatment options on the clinical and biological parameters in children with non-alcoholic fatty liver disease.
6.2. MATERIAL AND METHODS

6.2.1. Study group

We conducted a transversal analytical study on a group of 57 patients, diagnosed and monitored in 2nd Pediatric Clinic, „Sf. Maria” Children’s Emergency Hospital Iasi between January 2010 and February 2013. Among them, 49 patients had over 2 successive admissions.

The inclusion criteria were the hyperechoic liver parenchyma appearance on ultrasound scan, associated or not with high levels of hepatic transaminases.

The exclusion criteria were represented by the secondary causes of hepatic steatosis or another chronic hepatic diseases, evaluated through anamnesis or serological tests: hepatitis B, C or infection with cytomegalovirus, Epstein-Barr virus; autoimmune or metabolic liver disease; Wilson's disease; celiac disease; alcohol consumption in adolescents; total parenteral nutrition; steatogenic drugs use. Also, the patients under the age of one were excluded, to whom the occurrence of hepatic steatosis is due mainly to secondary causes.

6.2.2. Study protocol

The patients have been evaluated at the moment of diagnosis and then periodically, at varying intervals. The regular assessment of patients involved collection of the anamnesis data, followed by the general clinical examination, laboratory tests and imaging tests, treatment recommendations. Laboratory tests included complete blood count, liver and kidney function exploration, assessment of protein, lipid and carbohydrate metabolisms, serological tests.

The study protocol followed: epidemiological data, aspects of liver ultrasound, anamnesis, anthropometric parameters, the clinical and biological parameters, the vascular ultrasound parameters in selected patients, the specific therapeutic recommendation. Secondary causes of liver injury were excluded.

6.2.3. Ethical consideration

The study was approved by the Ethics Committee of UMF "Grigore T. Popa" Iasi. The ethical rules were obeyed during the admission in the hospital. Informed consent of the patient was brought to the attention of parents and legal guardians.

6.2.4. Statistical analysis

We used a Microsoft Excel database to register the patients. Discrete numeric parameters were recodified and note those codes for analysis. In order to statistical processing the study data were used statistical software: SPSS version 19.0 for Windows, MedCalc version 11.6 software, Statistica 10 program. Descriptive statistics are reported as average ± SD if normally distributed and as median, range (minimum, maximum) for distorted distribution. Discrete variables were expressed as number and percentage.
6.3. RESULTS

6.3.1. Descriptive analysis of the study group

Descriptive analysis of the variables gender, background and age

The gender distribution of patients showed a predominance of the disease in male patients, 63% (36 patients) versus females 37% (21 patients), with a boys/girls ratio of 1.7:1.

Regarding the background of the patients, 56% of patients come from rural areas and 44% from urban areas.

The average age of the patients at initial evaluation was 9.33 ± 3.85 years, with a minimum age of 1 year and 8 months and a maximum age of 16 years and 2 months. Depending on the gender of patients, the average age in the study group was 8.78 ± 3.97 years in males and 10.27 ± 3.55 years in females, without any significant difference between genders (p = 0.164).

The distribution according to age categories showed that 8% of the patients were in the age group 1-3 years, 33% in the age group 3-10 years and 58% of patients were aged over 10 years.

The distribution of patients by age group and gender showed an increased prevalence of hepatic steatosis in males in all age groups.

Abdominal ultrasonography

In the study group, the ultrasound aspects ranged from mild to severe steatosis, depending on the intensity of the liver echogenicity and posterior beam attenuation. Depending on the steatosis severity, 35.1% had mild steatosis aspect on the ultrasound, 43.8% moderate steatosis and 21.1% severe steatosis.

During the follow-up, the liver echogenicity has improved in 32.6% of patients, with complete resolution of steatosis on ultrasound appearance at 14.28% of patients. In 2 patients, it has been noticed an increased liver reflectivity.

6.3.2. ASSESSMENT OF CLINICAL AND BIOLOGICAL FEATURES

The analytical evaluation of anamnesis features

Family history revealed the presence of familial aggregation, the risk factors for hepatic steatosis being identified within the family, at parents or siblings, at 25 patients (44%). There was a history of obesity in 30% of patients, dyslipidemia was found in the history of 12.2% children, diabetes mellitus type II in 8.5% of patients and documented hepatic steatosis in 3.5% of patients. A history of liver disease of viral etiology and Wilson disease were found in 8.7% (5) of patients.

Among perinatal history, low birth weight was found at 5 (9%) patients, while 6 (11%) of the patients were weighing more than 4000 g. In the study group, 65.7% of children received a natural or mixed nutrition for at least 2 months, and 34.3% of patients received artificial nutrition.
Symptoms and clinical examination

The general condition on admission was good in most patients (82.5%), general complaints (fever, headache, diarrhea) being found only in the initial hospitalization, at patients with intercurrent symptoms without any relationship with steatosis.

From the clinical point of view, most children with hepatic steatosis show nonspecific symptoms and signs. The main clinical parameters highlighted during assessments, reported to be associated with the presence of hepatic steatosis, are shown in Figure 6.11.

![Figure 6.11. Nonspecific clinical manifestations associated with hepatic steatosis](image)

None of the patients showed clinical signs of advanced liver disease or liver failure, such as ascites, collateral circulation, bleeding, jaundice.

Analysis of anthropometric features

Statistical characteristics of BMI and BMI Z-score of the patients at initial assessment are shown in Table 6.7.

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>95% CI</th>
<th>SD</th>
<th>SEM</th>
<th>Median</th>
<th>Minimum</th>
<th>Maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMI</td>
<td>23.22</td>
<td>21.818 - 24.636</td>
<td>5.2121</td>
<td>0.7028</td>
<td>23.629</td>
<td>15.912</td>
<td>34.876</td>
</tr>
<tr>
<td>Z Score BMI</td>
<td>1.562</td>
<td>1.314 - 1.810</td>
<td>0.9166</td>
<td>0.1236</td>
<td>1.810</td>
<td>-0.840</td>
<td>3.220</td>
</tr>
</tbody>
</table>

There were no statistically significant differences in BMI values for boys and girls, 22.74 ± 5.01 kg/m². versus 24.01 ± 5.57 kg/m² (p = 0.388).

By adjusting BMI percentiles for age and sex and BMI z-score evaluation (z score ≥ 1.6 corresponds to a BMI greater than 95th percentile) was found that 13 (23%) patients had normal weight (BMI < the 85th percentile), 10 (18%) were overweight (BMI between the 85th and the 95th percentile) and 34 (59%) were obese (BMI ≥ the 95th percentile). Among obese patients, 7 children (12.2%) had a BMI over the 99th percentile, corresponding to severe obesity.

Visceral obesity was assessed by measuring waist circumference, expressed in absolute value and percentile by age and sex, to a number of 43 patients aged more than 4 years. The average value of WC was 77.79 ± 14.36 cm. No statistical differences were recorded between boys and girls. In the study
group, a total of 24 (42.10%) patients had WC > 90th percentile, values corresponding to an abdominal obesity.

**Assessment of the hepatic parameters**

At initial assessment, hypertransaminasemia / hepatic cytolysis was present in 39 patients (68.42%), with values up to 6.5 times the upper limits of normal for ALT and 5 times the upper limits of normal for AST. Hypertransaminasemia was significantly associated with the diagnosis of hepatic steatosis ($\chi^2$=7.737, p= 0.005).

While analyzing the degree of steatosis according to the presence of hypertransaminasemia, we observed that 76% of patients with moderate steatosis and 83.3% of those with severe steatosis were found in the group with hypertransaminasemia; there were statistically significant differences compared to those with normal transaminases, p <0.01.

**Alanine aminotransferase (ALT).** The average ALT values at diagnosis was 61.11 ± 50.43 IU/L. The range was between 11 and 239 IU/L. There was a predominance of moderate elevated values, 1.5-2 times the upper limits of normal, but were also extreme values of up to 6.5 times the upper limits of normal. Initially, in the study group, 63.1% of children had ALT greater than 38 IU/L.

Depending on the degree of steatosis, the more severe steatosis was the higher the average ALT values were; there were statistically significant differences between mild steatosis compared with moderate or severe steatosis (F=7.917, p=0.001).

**Table 6.14. The ALT values according to the severity of steatosis**

<table>
<thead>
<tr>
<th>Steatosis</th>
<th>ALT (IU/L)</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>Mean</td>
<td>SD</td>
<td>Median</td>
</tr>
<tr>
<td>Mild</td>
<td>20</td>
<td>32.15</td>
<td>14.90</td>
<td>27.50</td>
</tr>
<tr>
<td>Moderate</td>
<td>25</td>
<td>67.60</td>
<td>49.21</td>
<td>55.00</td>
</tr>
<tr>
<td>Severe</td>
<td>12</td>
<td>95.83</td>
<td>57.84</td>
<td>82.00</td>
</tr>
</tbody>
</table>

**Aspartate aminotransferase (AST).** The average AST at initial assessment was 48.68 ± 32.45 IU/L, with the range between 14 and 173 IU/L. From the histogram of values it is observed a predominance of normal and slightly elevated values (< 2 times the upper limits of normal). Of the study group, 56.14% of children had values above 35 IU/L.

**Table 6.17. The AST values according to the severity of steatosis**

<table>
<thead>
<tr>
<th>Steatosis</th>
<th>AST (IU/L)</th>
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<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>Mean</td>
<td>SD</td>
<td>Median</td>
</tr>
<tr>
<td>Mild</td>
<td>20</td>
<td>36.20</td>
<td>19.12</td>
<td>29.50</td>
</tr>
<tr>
<td>Moderate</td>
<td>25</td>
<td>46.88</td>
<td>27.13</td>
<td>37.00</td>
</tr>
<tr>
<td>Severe</td>
<td>12</td>
<td>73.25</td>
<td>46.64</td>
<td>59.00</td>
</tr>
</tbody>
</table>
Related to the degree of steatosis, the more severe the steatosis was the higher the AST values were, with statistically significant differences between mild and moderate degrees of steatosis vs severe steatosis (F=5.805, p=0.005).

**Gamma-glutamyl transpeptidase (GGT).** The average GGT values in the study group was 37.59 ± 32.01 IU/L. The range of values was wide, between 10 and 190 IU/L, but the majority of patients (75.43%) had values below the 75th quartile, 40UI/L. Elevated GGT values, over 38 IU/L, were recorded in about one third of patients.

Depending on the degree of steatosis, GGT ranged from 26.45 ± 8.59 IU/L in mild steatosis vs. 34.28 ± 29.5 IU/L in moderate steatosis vs. 62.83 ± 46.39 IU/L in severe steatosis, with statistically significant differences between groups (F = 6.047, p = 0.004).

Analysis of liver parameters by gender showed higher values of ALT, AST and GGT in males versus females.

**During follow-up,** the transaminase levels remained persistently elevated in 36% of patients, fluctuated between elevated and normal in 4% of patients and normalized in 27% of patients. The persistent hypertransaminasemia was detected predominantly in patients with moderate to severe steatosis. Among patients with initially normal transaminases values, 29% had consistently normal transaminases during follow-up and in 4% of patients were recorded mild transaminases increases, but less than 1.5 times the upper limits of normal.

Analyzing in dynamic the transaminase levels in patients with initial hypertransaminasemia, we found a decrease of ALT and AST average values, both in patients who have reached the normal values (statistically significant, p <.01), as well in those who had persistently elevated liver transaminases (not statistically significant, p> 0.05) (Figure 6.26.).

![Figure 6.26. The mean values of ALT and AST in dynamic according to the evaluation time](image)

**Parameters of glucose metabolism**

The average of fasting glucose levels at diagnosis was 89.05 ± 14.78 mg/dl. The glucose values ranged between 62 and 138 mg/dl, but most patients
had values below the 75\textsuperscript{th} quartile, corresponding with the glucose value of 98 mg/dl.

Analysis of glycemic parameters according to the degree of steatosis did not revealed statistically significant differences between groups (F=0.142, p=0.868).

During follow-up, 29\% (16) of patients had at least one glucose value greater than 100 mg/dl. Of these, 14 children (25\%) had values of impaired fasting glucose and only 2 patients had fasting glucose higher of 126 mg/dl, the threshold for diabetes, confirmed by OGTT test.

The oral glucose tolerance test (OGTT) was performed in 15 patients and allowed the diagnosis of diabetes in two patients; three patients showed impaired glucose tolerance and in 10 (17.54\%) patients the OGTT was normal.

Diabetes mellitus type II was diagnosed simultaneously with the detection of liver steatosis in one patient, and the other patient was diagnosed within a year and a half after the diagnosis of hepatic steatosis.

Parameters of lipid metabolism

\textit{Total cholesterol}. The average value of total cholesterol was 175 ± 38.12 mg/dl. The range of cholesterol values was between 114 and 334 mg/dl. There were no significant differences in cholesterol levels between genders.

The correlation of total cholesterol values with the severity of steatosis showed elevated average values of cholesterol in patients with moderate steatosis, but with no statistically significant differences between different degrees of steatosis (F = 1.460, p = 0.241).

\textit{Triglycerides}. The average triglyceride value was 114.07 ± 62.48 mg/dl. The range of triglycerides values was between 43 and 378 mg/dl. Triglycerides did not vary significantly between genders.

Triglycerides were higher in moderate and severe steatosis (124.64 ± 63.44 mg/dl, respectively 136.25 ± 89.19 mg/dl) compared with mild steatosis (87.55 ± 24.81 mg/dl), p <0.05.

During follow-up, 42\% of patients have shown normal levels of cholesterol and triglyceride and in 58\% (33 patients) were present various types of dyslipidemia: 12\% had hypertriglyceridemia, 28\% hypercholesterolemia and 18\% mixed dyslipidemia. Persistent hypertriglyceridemia was recorded at 6.12\% of patients, while persistent hypercholesterolemia have been found to 14.28\% of the patients. Dyslipidemia was significantly associated with hepatic steatosis (\(\chi^2 = 5.07, p <0.024\))

\textit{Lipid fractions}. Were evaluated HDL and LDL cholesterol fractions in 32 patients (56.14\%). Lower HDL cholesterol levels (<40 mg/dl) was observed in 8 patients (14\%). Elevated LDL cholesterol levels (> 130mg/dl) were found in 7
patients (12.3%). Also 4 (7%) patients had LDL cholesterol between 110-130 mg/dl, considered borderline values.

**Clinical-biological correlations**

The analysis of correlation between the presence of hypertransaminasemia and the presence of obesity in the study group did not reveal any statistical significance, p > 0.05. Thus, the presence of obesity cannot be considered a factor associated with a greater severity of liver damage, assessed by cytolysis.

Analyzing dyslipidemia according to the presence of overweight in patients with steatosis, we found that dyslipidemia was present in 61.3% of obese/overweight patients and in 46.15% of normal weight patients, without significant differences of dyslipidemia frequency between normal weight and those presenting overweight (χ² 0.431, p = 0.087, 95% CI).

Analyzing correlation between lipid parameters and liver parameters, there was a statistically significant correlation between ALT and TG, both at the initial assessment (r = 0.4819, p < 0.0001) and during follow-up (r = 0.4263, p < 0.0001).

**Metabolic Syndrome**

During the follow-up, pathological changes of at least one of the components of metabolic syndrome were found in 27 (47.3%) patients, analyzing children over 6 years, which have shown a higher risk of developing metabolic syndrome and requiring careful follow-up. Of these, the majority (70.37%) had 1 or 2 components of metabolic syndrome. Figure 6.37 presents the main components of the metabolic syndrome found in patients of the study group.

![Figure 6.37. Metabolic syndrome components found in the study group](image)

In the study group, the **metabolic syndrome** was found in 8 (14.03%) patients, representing 24.24% of children over 10 years, five boys and three girls aged between 11 and 17 years.
While comparing the characteristics of the patients with and without metabolic syndrome, there were observed high values for all parameters in patients with metabolic syndrome. The BMI values, the BMI Z score, ALT and triglycerides were significantly higher in the group with MS, p <0.01.

6.3.3. ASSESSMENT OF MORPHOLOGICAL AND FUNCTIONAL VASCULAR CHANGES IN HEPATIC STEATOSIS

We assessed the morphological and functional vascular changes by vascular ultrasound in 24 patients with hepatic steatosis. We selected from the study group overweight and obese patients with BMI > the 85th percentile. We considered as inclusion criteria for the vascular evaluation the presence of overweight, given the fact that overweight and obesity is a common risk factor associated with both hepatic steatosis and cardiovascular disease.

To assess morphological changes we measured intima-media thickness (IMT) to the common carotid artery, bilaterally. To assess endothelial dysfunction we tracked flow mediated dilatation (FMD) – the percentage variation in brachial artery diameter after an ischemic stimulus.

Patients’ characteristics at the time of vascular evaluation

Based on clinical and ultrasonographic evaluation, 7 patients (29.1%) were classified as having mild steatosis, 10 children (41.66%) moderate steatosis and 7 children (29.1%) severe steatosis. Evaluation of liver function revealed hypertransaminasemia in 9 (37.5%) patients.

Obesity was found in 80% of children. Waist circumference was greater than the 90th percentile in 15 (62.5%) patients. Hypertension was found in 3 patients (12.5%).

Insulin values were within the normal levels for age in all patients, without detectable hyperinsulinemia, with an average value of 12.42 ± 4.99 μU/ml. However, insulin resistance evaluated by HOMA-IR score greater than 3.02 was found in 6 children (25%). HDL cholesterol levels were significantly negative correlated with the degree of insulin resistance (p = 0.0001). Also, HOMA-IR was positively correlated with BMI and WC (p <0.001).

Assessment of vascular morphological changes

IMT’s statistical parameters are shown in Table 6.36. The median values of left IMT was higher than the median of right IMT, but without statistical significance (p = 0.13).

<table>
<thead>
<tr>
<th></th>
<th>Median</th>
<th>95% CI</th>
<th>Min</th>
<th>Max</th>
<th>25 - 75 P</th>
<th>Normal Distr.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Right IMT(mm)</td>
<td>0.495</td>
<td>0.466 - 0.524</td>
<td>0.384</td>
<td>0.991</td>
<td>0.464 - 0.554</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Left IMT(mm)</td>
<td>0.524</td>
<td>0.492 - 0.611</td>
<td>0.405</td>
<td>1.016</td>
<td>0.485 - 0.639</td>
<td>0.0005</td>
</tr>
</tbody>
</table>
Right and left IMT values have increased progressively with the severity of steatosis, with statistically significant differences between categories of steatosis, p <0.05 (Table 6.37).

**Table 6.37. Left and right IMT values depending on the severity of steatosis**

<table>
<thead>
<tr>
<th>Steatosis</th>
<th>Mild Median</th>
<th>Min</th>
<th>Max</th>
<th>Moderate Median</th>
<th>Min</th>
<th>Max</th>
<th>Severe Median</th>
<th>Min</th>
<th>Max</th>
</tr>
</thead>
<tbody>
<tr>
<td>Right IMT (mm)</td>
<td>0.462</td>
<td>0.384</td>
<td>0.513</td>
<td>0.500</td>
<td>0.420</td>
<td>0.595</td>
<td>0.613</td>
<td>0.465</td>
<td>0.991</td>
</tr>
<tr>
<td>Left IMT (mm)</td>
<td>0.475</td>
<td>0.405</td>
<td>0.558</td>
<td>0.512</td>
<td>0.467</td>
<td>0.663</td>
<td>0.637</td>
<td>0.484</td>
<td>1.016</td>
</tr>
</tbody>
</table>

Patients with metabolic syndrome showed higher values of IMT bilaterally, over the median of the group, values closed to the pathological levels in adults (e.g., 0.991 mm, 1.016 mm).

**Statistical correlations**

We performed correlation analysis between IMT values and various clinical and biological parameters. Among the clinical parameters, there was a positive correlation of right and left IMT values with BMI ($r = 0.506$, respectively $r = 0.454$, p <0.05) and diastolic blood pressure ($r = 0.509$, respectively, $r = 0.458$, p <0.05). Also between the right IMT and WC there was a statistically significant correlation ($r = 0.511$, p = 0.010). Age, sex, and SBP were not associated with IMT.

Among the biological parameters, the right and left IMT has been positively correlated with ALT values ($r = 0.439$, respectively $r = 0.467$, p <0.05), but not with GGT. The left IMT was correlated with AST values ($r = 0.425$, p <0.05). Correlation analysis revealed a statistically significant positive correlation between right and left IMT and HOMA-IR ($r = 0.603$, p = 0.001, respectively, $r = 0.530$, p = 0.007), insulinemia ($r = 0.581$, respectively $r = 0.469$, p <0.05). There was no correlation between blood glucose and IMT values.

There was a moderate negative correlation for the HDL cholesterol values with the right IMT ($r = -0.581$, p = 0.003) and a low significant correlation with the left IMT ($r = 0.415$, p = 0.044). The right IMT was correlated with triglycerides values ($r = 0.402$, p <0.5). Correlation of IMT with total cholesterol levels and LDL cholesterol was not statistically significant.

**Assessment of endothelial dysfunction (DE)**

In order to assess FMD, we measured the basal diameter (absolute value of systolic brachial artery diameter at baseline) and post reactive-hyperemia diameter (systolic diameter of the brachial artery after ischemic stimulus) and we calculated the absolute variation in diameter (D) and FMD.
Table 6.41. Statistical characteristics of the evaluated parameters

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>DS</th>
<th>Median</th>
<th>Minimum</th>
<th>Maximum</th>
<th>25–75 P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline D (cm)</td>
<td>3.30</td>
<td>0.68</td>
<td>3.20</td>
<td>2.20</td>
<td>4.40</td>
<td>2.70 - 3.95</td>
</tr>
<tr>
<td>Post hyperemia D (cm)</td>
<td>3.54</td>
<td>0.69</td>
<td>3.40</td>
<td>2.30</td>
<td>4.70</td>
<td>3.00 - 4.20</td>
</tr>
<tr>
<td>D (cm)</td>
<td>0.23</td>
<td>0.08</td>
<td>0.20</td>
<td>0.10</td>
<td>0.40</td>
<td>0.20 - 0.30</td>
</tr>
<tr>
<td>FMD (%)</td>
<td>7.53</td>
<td>2.81</td>
<td>7.23</td>
<td>2.27</td>
<td>14.81</td>
<td>6.15 - 9.00</td>
</tr>
</tbody>
</table>

Depending on the severity of steatosis, the median FMD had lower values in those with severe steatosis, 5.71% (min-max range from 2.27% to 8%) compared with those with moderate steatosis, 7.36% (5.56% to 11.43%), and mild, 9.30% (from 6.67% to 14.81%), the difference being statistically significant between mild/moderate steatosis versus severe steatosis (p <0.01).

Statistical correlations
The correlation analysis showed a strong statistically significant negative correlation between the values of FMD and right and left IMT values (r = -0.588, p<0.01, respectively, r = -0.646, p<0.001).

The analysis of correlation of FMD with clinical parameters showed a significant negative correlation of FMD with BMI (r = -0.471, p = 0.02), WC (r = -0.570, p = 0.003), DBP (r = -0.441 p = 0.03). There were no correlations of FMD with age, gender, systolic blood pressure.

Analysis of FMD correlation with biological parameters showed a strong significant negative correlation of FMD with insulinemia (r = -0.693, p = 0.0002) and HOMA-IR (r = -0.608, p = 0.001). There were no correlations of FMD with glucose, triglycerides, total cholesterol, HDL, LDL cholesterol, liver transaminases levels.

6.3.4. THERAPY IMPLICATION IN DISEASE EVOLUTION
During follow-up, treatment recommendations included changes in lifestyle, consisting in diet and increased physical exercise, administration of hepatoprotective agents, omega 3 fatty acids and probiotics.

The role of diet and exercise
For overweight and obese patients, a proper diet was the main therapeutic indication. Along with this, it was also recommended the increasing of physical activity through a regular weekly exercise program.

We assessed changes in weight status and hepatic transaminases to a number of 25 patients, 76% obese and 24% overweight, for a period of time between 7 months and 2 years. In 28% of patients, compliance to dietary recommendations was absent, recording constant values or increases of BMI up to 25%. Transaminase levels have remained stable or have risen significantly in patients with important weight growth.

Following the change of lifestyle, in 72% of patients was found a decrease in body mass index. In an average period of 12.31 ± 4.09 months, the
BMI value has decreased by an average of 1.68 ± 1.12 kg/m$^2$, from 26.26 ± 4.54 kg/m$^2$ to 24.58 ± 4.60 kg/m$^2$, statistically significant, p < 0.0001. There were decreases in BMI up to 15.1% from the initial value.

Decrease in BMI was associated with a statistically significant decrease in ALT, GGT, glucose and cholesterol levels (Figure 6.49).

![Figure 6.49. Anthropometric and biological parameters versus lifestyle modification](image.png)

There was a decrease in prevalence of high levels of ALT values from 61.1% to 33.3% and elevated AST from 44.4% to 22.2%. Serum transaminases have increased above the upper limit of normal in one patient. The prevalence of hypercholesterolemia decreased from 50% to 22.2%, and hypertriglyceridemia from 27.7% to 5.5%.

During the ultrasound's assessment, liver's echogenicity revealed an improvement in steatosis in 12 patients, with complete resolution of steatosis on 5 of them.

**The benefit of ursodeoxycholic acid in steatohepatitis**

We evaluated the effect of ursodeoxycholic acid on a number of 18 children, with alanine aminotransferase ≥ 50 IU/L. Patients received UDCA 20 mg/kg/day in two divided doses, breakfast and evening, for six months.

Over the period of follow-up, there were not reported significant changes in lifestyle. BMI varied slightly from 23.02 ± 6.32 kg/m$^2$ vs. 22.71 ± 5.78 kg/m$^2$ (p = 0.12).

At the end of treatment there was a reduction in hepatic transaminases. The median (range) of absolute values of ALT was significantly improved from baseline 68.5 IU/L (50-166 IU/L) to 47 IU/L (29-104 IU/L), p < 0.001 at 6 months. The median for AST values varied significantly from 43 IU/L (29-101 IU/L) to 34 IU/L (17-75 IU/L), p < 0.001. GGT ranged from 41.5 IU/L (11-150
IU/L) vs. 22 IU/L (8-55 IU/L), p <0.05. The levels of ALT, AST and GGT reached normal levels at 22.22% (versus 0% at beginning), 61.11% (vs. 22.22%), 83.33% (against 50%) patients.

Cholesterol values have significantly decreased, from 167.5 mg/dL (114-291 mg/dl) to 154.5 mg/dL (115-239 mg/dl) (p <0.01). There were no significant differences in fasting glucose and triglyceride levels after treatment. There were no changes in the ultrasound appearance of the patients at the end of 6 months.

6.4. DISCUSSIONS

Because in the study were included patients only from a tertiary level clinic, we could not appreciate the prevalence of primary hepatic steatosis in the general population, but we could analyze some aspects of pathology that may be useful in clinical practice.

According to literature data (Schwimmer et al., 2005, Feldstein et al., 2009), in the study group, the prevalence of male gender with hepatic steatosis was higher (63%), related both to the entire group or each group of age. Male gender is known as a risk factor for NAFLD, the condition being more common in boys than in girls, with a ratio of 2:1 (Vajro et al., 2012).

Low birth weight was found in 11% of patients, emphasizing that low gestational age and low birth weight are risk factors for subsequent development of obesity, caused by a high caloric and high protein diet recommended for the weight deficit recovery, obesity being risk factor for subsequent development of hepatic steatosis (Nobili et al., 2008). In the study group two-thirds of children received natural nutrition; the obtained results do not support the idea that natural nutrition is a protective factor in the development of steatosis (Nobili et al., 2009).

It has been noticed a positive family history anamnesis for risk factors associated with hepatic steatosis in about half of patients, which supports the idea of a family predisposition to the disease. Even if the percentage was insignificant, the presence of hepatic steatosis at parents confirm this idea. Family association of obesity, insulin resistance, fatty liver and diabetes is common in children with NAFLD and should raise the suspicion of diagnosis to children who come from such families (Schwimmer et al., 2009).

Like other cohorts of children (Schwimmer et al., 2003, Nobili et al., 2006), 77% of patients were overweight or obese, which supports the idea that the primary steatosis is closely linked to the presence of child overweight. Of the study group, 23% of patients were normal weight but showed significant changes in transaminases, emphasizing that steatosis may be associated to children with no obesity, existence of other risk factors being involved. Although the association of obesity in the study group was significant, ethnic and socioeconomic differences or related to eating habits, along with conducting
the study on a group of patients selected by hospitalization may explain the lower prevalence of obesity compared with other studies (A-Kader et al., 2008, Manco et al., 2008).

Epidemiological studies have shown that non-alcoholic fatty liver disease is often revealed by elevations in ALT, AST and GGT levels (Franzese et al., 1997, Nobili et al., 2009). In agreement with literature data, while evaluating the hepatic parameters it has been revealed the presence of hypertransaminasemia at more than half of the patients at initial assessment, with persistent elevated values to a third of children.

Although liver enzymes have been described as elevated in children with NAFLD (Nobili et al., 2006, Feldstein et al., 2009), in our study 33% of children had normal transaminases at initial assessment, which subsequently remained normal or fluctuated between normal and increased values. Surprisingly, normal transaminases were found in some patients who had severe hepatic steatosis, although associated with an AST/ALT ratio > 1. This observation, in the context that the Ritis ratio > 1 seems to be a marker of advanced fibrosis (Vajro et al., 2012), raises the suspicion that in these patients there may be a more advanced form of the disease, associating postinjury hepatocellular fibrosis.

It is noticed the high prevalence of dyslipidemia, present in 58% of patients, supporting the literature data (Barshop et al, 2009). Surprisingly is the prevalence of hypercholesterolemia, found in 46% of patients. Studies from the literature reveals in particular the association of hypertriglyceridemia in patients with hepatic steatosis (Schwimmer et al., 2003, Oliviera et al., 2009).

In our study, 14% of patients, representing 24.24% of children over 10 years old, presented the criteria for metabolic syndrome. The prevalence of metabolic syndrome was lower than data reported in the literature (Manco et al., 2008), possibly due to the fact that in our study there was a higher proportion of young children in which we could not define the metabolic syndrome according to guidelines. However, 47.36% of the children had at least one pathological component of the metabolic syndrome, which emphasizes the interrelation of the defining factors of the metabolic syndrome with hepatic steatosis.

An association between hepatic steatosis and the vascular changes specific to subclinical atherosclerosis was described in adult patients and more recently in children (Brea et al., 2005, Villanova et al., 2005, Pacifico et al., 2008). In our study, the average values of IMT and FMD were similar to those reported in literature in children with NAFLD and higher than those observed in cohorts of obese children without NAFLD and healthy normal weight children (Pacifico et al., 2010, Manco et al., 2010, Gökçe et al., 2012). Depending on the severity of steatosis has been showed that IMT and FMD changes are more pronounced the severe the steatosis is. We also observed elevated IMT values.
for the pediatric age, closer to the pathological values in adults, which in the context of the small group of children with high dispersion of ages, may be interpreted as extreme values. However, higher values of IMT were observed in children who associated metabolic syndrome, with a prolonged evolution of hepatic steatosis and persistent hypertransaminasemia. Assessment of the relationship between FMD, IMT and clinical-biological parameters showed a significant correlation with BMI, WC, DBP, HDL cholesterol, HOMA-IR, emphasizing that the clinical-biological profile of patients with hepatic steatosis favors the appearance of cardiovascular disorders. The correlation with HOMA-IR supports the role of insulin resistance in atherosclerotic lesions and endothelial dysfunction (Semenkovich, 2006).

Several studies in adults and children have shown that a proper diet and an increased physical activity can lead to an improvement of hepatic parameters and histological aspects of the liver when weight loss is achieved (Tock et al., 2006, Nobili et al., 2006, Koot et al., 2011, Reinehr et al., 2009). For the monitored patients, the adherence to lifestyle changes, measured by decreased body mass index, resulted in improvement of biological parameters. Decreased hepatic transaminases was more important for children who had a weight loss greater than 5%. The improvement in BMI was associated to more than half of patients with an improvement also in liver echogenicity. This shows that the overall decrease in adiposity is accompanied by an improved of fatty liver.

Ursodeoxycholic acid, with its hepatoprotective and anti-apoptotic effects, may be beneficial in the development and progression of steatohepatitis. The literature data regarding the efficacy of UDCA in NASH are varied and discordant (Laurin et al., 1996, Leuschner et al., 2010, Ratziu et al., 2011). The administration of ursodeoxycholic acid at a dose of 20 mg/kg to the patients in the study group, led to a significant decrease in ALT levels in children with NAFLD and a significant reduction of cholesterol level. Given that in children we cannot use many of the adult therapeutic agents, therapeutic effect and safety of therapy are bases for using ursodeoxycholic acid in children with hepatic steatosis.

In conclusion, non-alcoholic fatty liver disease is more common in overweight children, which associates dyslipidemia and dysglycemia, with moderately elevated transaminase levels. But, in the same time, it can also be found in normal weight children or in the absence of other components of metabolic syndrome, and the normal transaminase levels do not exclude severe hepatic steatosis. Overweight and obese children with hepatic steatosis have an additional risk factor for the occurrence of subclinical atherosclerosis. By knowing this risk, it is beneficial to implement preventive measures and regular assessment. Moreover, prophylaxis remains topical because of the few opportunities for therapy.
7.1. MOTIVATION AND OBJECTIVES OF THE STUDY

Several studies have shown a possible role of the gut and the innate immune molecules in the transition from simple steatosis to steatohepatitis (Siebler J et al, 2008.). Particularly, a pivotal role in the pathogenesis of NAFLD appears to have the systemic endotoxemia, suggested in animal and human studies. It was reported that in patients with NASH levels of endotoxin (LPS) in plasma are significantly elevated compared to healthy individuals (Farhadi et al, 2008, Alisa, et al, 2010.). The role of bacterial endotoxin in the pathogenesis of NAFLD is confirmed by several studies in animal models (Rivera et al., 2007, Abu-Shanab, Quigley, 2010). Recently it was reported that bacterial endotoxin would have a pivotal role in inducing fibrosis, via LPS- TLR4 signaling pathways in hepatic stellate cells (Seki et al, 2007).

Based on the literature data, we initiated a study whose main purpose was to investigate the involvement of gut microbiota in the induction of fibrosis in NAFLD.

The study objectives were:
- analyzing changes in circulating levels of bacterial endotoxin and hyaluronic acid in a cohort of children and adolescents diagnosed with NAFLD;
- assessing possible correlations of bacterial endotoxin levels and hyaluronic acid with objectified histological lesions in liver biopsies;
- assessing the effect of various concentrations of LPS on the activation of hepatic stellate cell line (LX-2) cultured in vitro.

7.2. MATERIALS AND METHODS

The study was conducted between January and June 2012 in Hepato-Metabolic Department and Liver Research Unit, „Bambino Gesù“ Children’s Hospital, IRCCS, Rome, Italy. The work has resulted in two studies, one clinical and one experimental. Within the clinical study we assessed the bacterial endotoxin and hyaluronic acid levels in children's serum with NAFLD diagnosis. The experimental study has been carried out by evaluating the response a human hepatic stellate cell lines to various concentrations of bacterial endotoxin.

7.2.1 CLINICAL STUDY
Study group

This cross-sectional study included 68 children and adolescents with biopsy proven NAFLD, referred to Hepato-Metabolic Disease and Liver
Research Unit of "Bambino Gesu" Children's Hospital and Research Institute, Rome, Italy, from January 2010 and April 2012.

The inclusion criteria was the diagnosis of biopsy-proven NAFLD. Exclusion criteria were: hepatitis A, B, C, D, E or G or cytomegalovirus or Epstein-Barr virus infection; excessive alcohol intake (> 20 g/day); autoimmune or metabolic liver disease; celiac disease; Wilson’s disease; alpha-1-antitrypsin deficiency; total parenteral nutrition, use of steatogenic drugs.

The study protocol was approved by the Ethics Committee of the "Bambino Gesu" Children's Hospital. The informed consent was obtained from at least one legal guardian.

The study protocol

There were analyzed anthropometric and biological parameters. Simultaneously, it was collected a sample of serum, which was stored at -80°C, and it was performed a liver biopsy. Subsequently, hyaluronic acid (HA) was measured using a commercial ELISA kit. The concentration of bacterial endotoxin (LPS) was measured using a kit *Limulus amebocyte lysate chromogenic endpoint assay*.

Liver biopsies, which were at least 15 mm and including at least 5-6 complete portal spaces, were read by a single pathologist. The main histological features of NAFLD and NASH were marked according to the scoring system NAFLD Clinical Research Network (Kleiner et al., 2005).

7.2.2. EXPERIMENTAL STUDY

**LX2 cells (human hepatic stellate cells) cultures**

The cell line of hepatic stellate human, LX-2, was grown in monolayer, in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin G and 100 µg/ml streptomycin in a humidified atmosphere of 5% CO2 at 37 °C. The cells were cultured to 60-70% confluence and then treated with various concentrations of LPS (50 or 100 ng/ml/day). The cells were incubated at 37°C for 24 or, respectively, 48 hours after re-stimulation with the same amount of LPS.

**Immunofluorescence technique**

The cells were treated with primary and secondary antibodies, after indirect immunofluorescence technique. Briefly, the cells were incubated overnight at 4°C, with primary antibodies: monoclonal mouse anti-alpha smooth muscle actin (anti-αSMA) and polyclonal rabbit anti-NF-kB. Afterwards, they were incubated for one hour at room temperature with secondary antibody: FITC blue-conjugated goat anti-mouse IgG antibodies and Texas Red-conjugated goat anti-rabbit IgG antibodies. The nuclei were counterstained with DRAQ5™ agent for 5 minutes at room temperature. The images were acquired by confocal laser microscopy.
7.3  RESULTS

7.3.1. Clinical study

The study group consisted of 68 subjects aged 3 to 16 years, 39 male and 29 female with biopsy proven NAFLD.

7.3.1.2. Histological parameters of patients

Mild steatosis (grade 1) was found in 35.3% of subjects, while 58.8% and 5.9% of children had moderate steatosis (grade 2), respectively, severe (grade 3). Grade 1 and grade 2 inflammation was present at 67.6%, respectively, 29.4% of the patients. Inflammation was completely absent only in 3% of the studied subjects. Ballooning was absent in 66.1% of children, while 25% and 8.9% of patients presented grade 1 and, respectively, grade 2 ballooning.

In the study group, 69.1% of subjects had no fibrosis, and 27.9% of patients had mild or moderate fibrosis (F1 and F2) and about 3% had advanced fibrosis (F3). Only 19.1% of subjects had a NAFLD activity score (NAS score) ≥ 5, meeting the criteria for the diagnosis of NASH. Were considered non-NASH patients with NAS ≤ 2, accounting 29.4% and the remaining 50% of patients were considered to have borderline lesions (NAS 3-4).

7.3.1.3. Bacterial endotoxin (LPS) and hyaluronic acid circulating levels in patients with or without fibrosis

Levels of hyaluronic acid (HA) on the entire group ranged from 81.55 to 535.86 ng/ml, with an average of 232.80 ± 109.46 ng/ml. Serum LPS levels on the entire group ranged from 0.43 to 0.89 EU/ml, with an average of 0.62 ± 0.13 EU/ml.

Circulating levels of HA and LPS were significantly increased (p <0.00001) in patients with NAFLD and fibrosis (F ≥ 1) compared with those without fibrosis (F0).

Table 7.4. LPS and HA serum levels according to the degree of fibrosis

<table>
<thead>
<tr>
<th></th>
<th>F0</th>
<th></th>
<th>F≥1</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td>HA (ng/ml)</td>
<td>183.58</td>
<td>52.95</td>
<td>342.97</td>
<td>123.53</td>
</tr>
<tr>
<td>LPS (EU/ml)</td>
<td>0.58</td>
<td>0.12</td>
<td>0.73</td>
<td>0.11</td>
</tr>
</tbody>
</table>

There was a strongly statistically significant (p <0.0001) Spearman rho correlation of 0.56, between the levels of LPS and HA. Univariate logistic regression analysis showed that HA (β = 0.020, p <0.0001) and LPS (β = 10.007, p <0.001) are predictive variables for the presence of significant fibrosis on histologic evaluation.

In the ROC curve analysis, HA is predicting the fibrosis (AUC 0.863, 95% CI 0.758 to 0.935, p <0.0001). HA values greater than 285.66 ng/ml have the highest sensitivity, 76%, and a specificity of 100%.
Cut-off point analysis of endotoxin, which can predict the presence of fibrosis, showed that LPS values > 0.644 EU/ml have the highest sensitivity (81%) and specificity (78.7%) in predicting fibrosis. LPS values > 0.540 EU/ml have a sensitivity of 100% and the specificity of only 40.4%. For higher cut-off values, LPS = 0.80 EU/ml, the sensitivity decreases significantly, 28.6% and diagnostic specificity for fibrosis increases to 93.6%. In the ROC curve analysis, for the cut-off point value with the highest sensitivity and specificity, LPS has made the prediction of fibrosis (AUC 0.821, 95% CI 0.709 to 0.904, p <0.0001).

7.3.2. EXPERIMENTAL STUDY
7.3.2.2. Effect of LPS treatment for 24 hours on the induction of pro-fibrogenic phenotype and the transcriptional factor NF-kB activation in hepatic stellate cells LX-2

To investigate the potential effect of LPS on pro-fibrogenic activation of LX-2 cells, we performed the analysis of expression and intracellular localization of alpha-SMA and NF-kB. Figure 7.18 shows the changes detected between the different applied treatments.

![Image of immunofluorescence analysis](image)

Figure 7.18. Immunofluorescence analysis of HSCs treated with different doses of LPS for 24 hours. DRAQ (nuclear) staining is reported in white, while alpha-SMA and NFkB-p65 staining are reported in blue, respectively, red.
After 24 hours of treatment, the blue immunofluorescence revealed that only the treatment of LX-2 with LPS 100 ng/ml was capable to induce an increase of alpha-SMA expression even if, as highlighted by red immunofluorescence, both concentrations of LPS, 50 ng/ml and 100 ng/ml, increased the expression of NF-kB. There was no significant change in the intracellular localization of alpha-SMA and NF-kB. These results suggest that NF-kB up-regulation may be a pre-requisite for the LPS-dependent increased of alpha-SMA.

7.3.2.3. Effect of LPS treatment for 48 hours on the induction of profibrogenic phenotype and the transcriptional factor NF-kB activation in hepatic stellate cells LX-2

For a more detailed analysis of the potential effect of LPS on profibrogenic activation of LX-2 cells, we applied the same dose of LPS (50 or 100 ng/ml/day) to stimulate LX-2 for an extra 24 hours.

![Figure 7.19. Immunofluorescence analysis of HSCs treated with different doses of LPS for 48 hours. DRAQ (nuclear) staining is reported in white, while alpha-SMA and NFkB-p65 staining are reported in blue, respectively, red.](image)

After 48 hours of treatment, blue immunofluorescence revealed that both treatments with LPS were able to induce an increase of alpha-SMA and NF-kB expression. No relevant changes were found in the intracellular localization of
alpha-SMA and NF-kB. These results confirm the previous hypothesis, that NF-kB increase precedes the alpha-SMA activation, which requires more stimulation time at low LPS dosage (50 ng/ml).

7.4. DISCUSSIONS

Based on the literature data (Harte et al., 2010, Alisi et al., 2010), the primary purpose of the study was to assess whether changes in the serum levels of endogenous bacterial endotoxins can be identified in children with NAFLD and if this changes can be associated with the induction of fibrosis.

In our study we have shown that bacterial endotoxin levels are elevated in children with NAFLD associated with fibrosis. There was a significant correlation between levels of LPS, HA and histological assessed fibrosis, which may support the idea of a causal relationship between exposure to bacterial endotoxin and the presence of fibrosis in patients with NAFLD. By analyzing the ROC curves, in the study group, we have shown that bacterial endotoxin predicts fibrosis. Further studies are necessary to confirm our results and to test whether increased levels of endotoxin can be used as a marker for the presence of fibrosis.

Also, supporting the literature data (Miele et al., 2009, Nobili et al., 2010), the results of our study showed an increase in serum hyaluronic acid levels in patients with NAFLD associating fibrosis, HA making the prediction of fibrosis.

Clinical data seem to show that there is indeed an association between serum levels of LPS and fibrosis damage, revealed both by liver biopsy and by analyzing the levels of hyaluronic acid.

Based on the findings observed in patients with NAFLD, we initiated an experimental study to test the possible effect of LPS on the activation of fibrogenic phenotype in hepatic stellate cells. We studied the activation of NF-κB and α-SMA expression induced by LPS.

Previous studies have considered that the role of bacterial endotoxin in the fibrogenic process is due to its metabolic and pro-inflammatory effects predisposing the liver tissue to subsequent injuries (Lee, Friedman, 2011). Also, Kupffer cells were considered the main promoters for pro-fibrogenic transformation of HSCs through LPS-TLR4 signaling pathway, by stimulating the secretion of TGF-β (Pradere et al., 2010). Seki et al., 2007 has launched the hypothesis that HSCs has a leading role in myofibroblasts transformation after TLR4 stimulation. The activation of LPS-TLR4 signaling pathway on HSCs cause molecular changes, dependent on NF-κB (down-regulation of inhibitor pseudo-receptor of TGF-β, BAMBI, with increased sensitivity to TGF-β, chemotaxis of Kupffer cells with releasing increased amounts of TGF-β ), which favors the activation and the production of specific proteins for activated phenotype (e.g., α-SMA).
Our results show that treatment with LPS not only is the main cause of the increased expression and nuclear translocation of NF-κB in HSCs, but also causes the increasing expression of alpha-SMA, which is recognized as a marker of activation of HSCs. Particularly, we have observed that high doses of LPS (100 microM) are capable of inducing these activation markers earlier than 24 hours, whereas the lower doses of LPS (50 microM) requires at least 48 hours for increasing expression of alpha-SMA and NF-κB.

The information collected to date allow us to hypothesized that, in the liver, similar to the inflammatory normal processes, the transduction molecular system mediated by the LPS/TLR4 axis, under the action of chronic endotoxinemia, it may be responsible for mediating the typical fibrotic processes for non-alcoholic steatohepatitis, by promoting the expression of pro-inflammatory and pro-fibrotic factors associated with the disease.

Chapter 8. CONCLUSIONS

1. Non-alcoholic fatty liver disease holds an important place among the public health problems in children throughout the world; this has become evident in the context of the obesity epidemic, in relation to the other elements that define metabolic syndrome.
2. Based on the casework presented, there is a predominance of males, 63% of patients (with a boys: girls ratio of about 1.7:1), which is maintained for each age group separately.
3. Non-alcoholic fatty liver disease is found throughout childhood, but prevails in children over 10 years (58% in the study group); this suggests that the onset of puberty triggers the disease via risk factors associated with it.
4. Suggestive anamnensis data correlates the risk factors of hepatic steatosis in family history, confirmed in 44% of patients, stating familial aggregation, thus requiring a careful screening of children from such family.
5. The overweight is prevalent in children with hepatic steatosis, more than two thirds of the cases being classified according to the anthropometric criteria for age and sex as obese - 59% and overweight - 18%. Obesity was significantly associated with the presence of steatosis in the study group.
6. Hepatic steatosis is also found in normal weight children, 23% of the study group, to these the association of other risk factors, in particularly dyslipidemia, favoring the disease.
7. The clinical picture is suggestive for obesity in most patients, some of them associating markers of insulin resistance (acanthosis nigricans), to which were added fatigue, hepatomegaly, abdominal pain. In the studied group there were no cases of advanced liver disease or portal hypertension.
8. Hepatic steatosis in children is frequently associated with elevated transaminases, hepatic cytolysis being found in 68.42% of patients on the apparent onset of the disease. Transaminases values were oscillating during evolution, with significant reductions under therapy, but there were also increases correlated with diet neglect and changes of metabolic parameters. Persistently elevated values were found in 36% of patients. Transaminases were significantly higher in severe steatosis compared with mild steatosis. Correlations between cytolysis and dyslipidemic parameters show that cytolysis is the main biochemical sign of continuous inflammation in steatosis, fueled by fat burden.

9. There was a high prevalence of dysglycemia, 29% of patients, including the association of diabetes type II in evolution, at 2 children.

10. Dyslipidemia is an independent factor from obesity for developing hepatic steatosis. Dyslipidemia was found in more than half of patients, most often associated hypercholesterolemia (46% of children).

11. Careful screening of metabolic syndrome components is required in all patients with hepatic steatosis, even at young ages, for early detection of metabolic complications, this being emphasized by the existence in 47.3% of cases of at least one component of metabolic syndrome.

12. Among the comorbidities with no direct relationship with the risk factors for steatosis and metabolic syndrome, but which have influenced the quality of life of patients and raised the question of differential diagnosis, there were: asthma, gastro-esophageal reflux disease, gallstones, dysmenorrhea, ovarian cyst.

13. Hepatic steatosis is associated with an increased risk for early atherosclerotic changes. In children with hepatic steatosis it has been found elevated values of intima media thickness, which have been correlated with BMI, WC, ALT, triglycerides and insulin resistance. IMT values has grown with increasing the severity of steatosis and were higher in children with metabolic syndrome.

14. Endothelial dysfunction, a predictor of subclinical atherosclerosis, is found in patients with hepatic steatosis and it is more important in patients with severe steatosis. FMD values were negatively correlated with insulin resistance, this being a possible factor of endothelial dysfunction in hepatic steatosis.

15. Hepatic steatosis therapy has complex objectives, involving mainly a group of interventions on lifestyle. Lifestyle changes, through diet and exercise, associated with lowering body mass index, has resulted in a significant decrease in hepatic transaminases values and lipid parameters and in improving the steatosis detected by ultrasound.

16. Unfortunately, it is impossible to benefit from all therapeutic arsenal used in adults during drug therapy. The ursodeoxycholic acid represents the
therapy to which it has been reported efficiency and safety issues. Patients treated with UDCA 20 mg/kg have benefitted of a significantly decreasing of hepatic transaminases levels.

17. Bacterial endotoxin levels in children with NAFLD were significantly elevated in patients with associated fibrosis and were positively correlated with serum levels of hyaluronic acid (indirect marker of fibrosis) and histopathological aspects. Elevated levels of endotoxin could be a marker of advanced liver disease and fibrosis.

18. Evaluation of direct effects of bacterial endotoxin on hepatic stellate cells showed that LPS promotes the expression of transcription factors (NF-κB) and induces the pro-fibrogenic phenotype. These data allow us to hypothesize that the transduction system mediated by the LPS/TLR4 axis may be able, on hepatic level, of promoting the typical fibrotic processes for non-alcoholic steatohepatitis.

19. The whole study demonstrates that hepatic steatosis is an actual and challenging pathology in children, through its potential of severe morbidity in adult, and it opens new perspectives for the demonstration of potential therapeutic efficiencies.

Chapter 9. PERSPECTIVES

Based on the existing data, close supervision of children and adolescents can lead to an early diagnosis, followed by an appropriate management to prevent advanced hepatic disease and comorbidities. The screening of hepatic steatosis should be considered when identifying dyslipidemia, obesity, dysglycemia, hypertension or a family history of these risk factors in children.

Given that the data on the natural history of the disease are low, a further research by following patients a longer period and even at adulthood could provide important information on how the disease evolved, on the ability of progression or regression of steatosis, on changes associated with steatohepatitis and also on survival.

The involvement of intestinal microbiota in the pathogenesis of NAFLD can provide several lines of further clinical research. We intend to follow the peculiarities of gut microbiota in pediatric patients with steatosis, depending on environmental and phenotypic characteristics. Also, it would be very interesting to emphasize the relationship between the intestinal microbiota and the nutritional status of children with hepatic steatosis, in relation to the type of diet and precocious eating habits, in the first years of life.

Furthermore, it would be useful to assess through therapeutic interventional studies, how effective probiotics and prebiotics are in improving progression of non-alcoholic fatty liver and preventing fibrosis.
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