NAFLD, TYPE 2 DIABETES AND CARDIOVASCULAR RISK

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Key words: hepatic steatosis, diabetes mellitus, cardiovascular risk, PNPLA3, metabolic syndrome.
I. Study Argument and Objectives

Hepatic steatosis within the non-alcoholic fatty liver disease (NAFLD) is a largely encountered condition at both a European and global level. Although many studies treat the evolution of fatty liver to non-alcoholic steatohepatitis (NASH), cirrhosis and then liver cancer, the study hereby refers especially to cardiovascular risk. This aspect is supported by the fact that the hepatic steatosis will lead the patient to potentially fatal cardiovascular diseases and, in fewer cases, to the aggravation of the hepatic condition. Thus, the vital risk for a NAFLD patient is represented by a major cardiovascular event (MI, CVA). In other words, the patient is exposed to a high risk of dying because of a major cardiovascular event and not because of the hepatic condition progressions.

The lifestyle changes (sedentary lifestyle, alimentation rich in carbon hydrates and animal fat) for many of the world populations, especially those of highly industrialized countries, led to an epidemic growth of overweight, obesity and type 2 diabetes mellitus (DM 2) during the last decades. Most of the NAFLD patients present clinical elements that can be included in the metabolic syndrome. In parallel, the DM 2 incidence is continuously increasing, while the report between metabolic conditions and NAFLD is indisputable; a significant percentage of these patients are early diagnosed with hepatic steatosis. It is well known that DM 2 leads to a major cardiovascular risk, the cause of death of these patients being the cardiovascular disease. Insulin resistance forecasts the
incidence of cardiovascular diseases and it plays a very important role in the evolution of the fatty liver patient.

Proofs in literature show that the metabolic syndrome forecasts the incidence of cardiovascular diseases, while NAFLD is considered to be a hepatic manifestation of the metabolic syndrome. Thus, we can suppose that NAFLD leads to an independent increase of cardiovascular diseases.

The presence of metabolic syndrome is associated to an increase of the carotid intima-media thickness (CIMT). Despite all these, CIMT only represent a marker of generalized atherosclerosis and it is important to see if NAFLD is significantly associated to the increased incidence of cardiovascular events, alteration of the endothelial function and increased prevalence of vulnerable coronary plaques (O’Leary, Polak, 2002).

A study with a 6y follow-up period made on a number of 2000 subjects with DM 2 showed that NAFLD was associated to double risk for cardiovascular disease, which seems to be independent from the other variables like sex, age, smoker status, diabetes duration, glycated hemoglobin (HbA1c) and low density lipoprotein cholesterol (LDLc) (Targher et al., 2007).

At the same time, especially during the last decade, more stress is placed on genetic causes, which could be the basis for different conditions. Thus, in our case, the PNPLA3 gene variations as a determining genetic factor contribute to the differences of hepatic lipids content and susceptibility for NAFLD.
Adiponutrin is expressed both at the adipocytes and hepatocytes level. This gene is one of the potential candidates attached to susceptibility for NAFLD. At this moment, LBP is used as a golden standard for NAFLD diagnosis, although it presents several risks that cannot be ignored. Although an expensive and unfeasible method from an ethical point of view, there are certain studies based on histologic diagnostic. In addition, most of the times, this invasive investigation does not bring direct benefits for the patient diagnostic, treatment and evolution. For this reason, several non-invasive diagnostic methods were proposed as an alternative to LBP, each having different specificities, sensibilities and accuracies. Thus, the goal of the last researches in the field was to evaluate the non-invasive diagnostic parameters for NAFLD and to formulate an algorithm for a better classification of the hepatic conditions spectrum.

There are several studies to evaluate rs738409 polymorphism and NAFLD risk; but there are not many studies to evaluate PNPLA3 polymorphism by a simple hepatic fat loading and hepatic steatosis levels measured by ultrasound. The use of non-invasive imagistic techniques easily accessible to detect hepatic steatosis and subclinical atherosclerosis, as well as the biochemical parameters of current use can formulate an algorithm in forecasting cardiovascular risk among the diabetic population and not only.

The object of the study was that of underlining any existing correlations between the hepatic steatosis level and subclinical atherosclerosis evaluated by CIMT, as well
as the anthropometrical coefficients and different biochemical parameters on a lot of DM 2.

The objectives of the study were the following:

- To determine the level of hepatic steatosis by ultrasound;
- To evaluate subclinical atherosclerosis by a non-invasive method, namely the Doppler carotid ultrasound with CIMT measuring;
- To compare the levels of hepatic steatosis of different anthropometrical parameters;
- To compare and set certain correlations between different biochemical parameters (lipid profile, inflammatory markers, markers of insulin resistance, laboratory tests reflecting the hepatic function, biochemical markers of cholestasis) and the levels of hepatic fat loading;
- The relation between the level of hepatic steatosis and subclinical atherosclerosis;
- The relation among the anthropometric parameters and evaluated biochemical markers and the level of subclinical atherosclerosis;
- The emphasis of the connection between a genetic marker (PNPLA3) and the levels of hepatic steatosis, respectively cardiovascular risk.

II. Material and Method

The study we made was of observational type. The study included 92 subjects evaluated for a period of 18 months in the Diabetes, Nutrition and Metabolic Diseases Clinic within the “Sf. Spiridon” Emergency Hospital of Iași. The
lot was represented by patients hospitalized within the Diabetes In-Patient Unit for a period of 60 days. Among the 130 evaluated patients, 38 did not meet the inclusion criteria. All persons included by this study accepted and signed the informed consent.

The inclusion criteria were the following:
- The subjects hospitalized within the Diabetes In-Patient Unit during the above-mentioned period
- The DM 2 patients treated with oral anti-diabetics or hygiene-dietetic regime
- Persons who signed the informed consent

The exclusion criteria were the following:
- Subjects suffering from hepatitis B or C
- Subjects under insulin therapy
- Subjects with toxic-ethylc hepatitis
- Persons who refused to participate in the study or those who did not sign the informed consent
- Patients suffering from other hepatic conditions (e.g. Wilson’s disease – declarative)

We solicited and obtained the authorization of the Research Ethics Commission of the “Grigore T. Popa” University of Medicine and Pharmacy of Iași.

Our investigations included:
- Clinical examination, anamnesis and anthropometrical evaluations
- Complete blood count
- Inflammatory markers – C-reactive protein (CRP) and fibrinogen
• Glycaemia, HbA1c, insulinaemia – with the calculation of the homeostasis model assessment (HOMA) coefficient
• Lipid profile – triglycerides, total cholesterol, high density lipoprotein cholesterol (HDLc), LDLc, non-HDLc
• Exploration of the hepatic function – aspartate transaminase (AST), alanine transaminase (ALT), gamma-glutamyl transferase (GGT), total proteins, total bilirubin, direct bilirubin
• Exploration of the renal function – urea, creatinine, serum uric acid, urinalysis
• Serum dosing of vitamin D, vitamin B12, serum iron level, total calcium, ionized calcium, magnesium
• Genetic markers – PNPLA3 dosing
• Evaluation of the hepatic fat loading by ultrasound
• Evaluation of subclinical atherosclerosis by CIMT

The database was created in Microsoft Excel, without including ID data of the subjects. The statistical analysis was made in STATISTICA, version 7.0. We considered p < 0.05 as statistically significant.

III. Results

III.1. General Data

From the lot of 92 subjects, 44 were male (47.83%) and 48 women (52.17%). According to the provenance environment, 68 subjects belong to the urban environment (73.91%) and 24 to the rural environment (26.09%). The
average age of the lot was of 60.38 ± 10.37 years, varying between the ages of 33 and 86. 9.89% of the cases were those of normal liver, while the incidence of the cases according to the level of hepatic steatosis represented 26.37% of mild steatosis, 36.26% of moderate steatosis and 27.47% of severe steatosis.

Most of the subjects presented values over the normal limit of the body mass index (BMI). Most of the patients correspond to the 1st degree obesity and overweight categories, only 11 of them presenting a 3rd degree obesity and only 5 having a normal weight. Only 9 subjects presented regular values of the abdominal circumference (AC). 75% of the subjects presented values of the blood pressure (BP) over the normal limits. 62% of the subjects presented high values of the CIMT. 9 of the investigated patients were positive for atheroma. The average value of the CRP was of 1.43 mg/l, only 25% of the investigated population being at low cardiovascular risk (CRP < 1 mg/l). As regards the atherogenic dyslipidemia markers, over half of the subjects presented high values of total cholesterol, 65% presented HDLc values under the regular limits and 50% presented hypertriglyceridemia. The metabolic syndrome criteria were met in most cases (81%). The PNPLA3 genotyping proved a 12% incidence of the GG genotype.

III.2. The Relation between the Levels of Hepatic Steatosis and Anthropometrical Coefficients

After comparing the incidence of the levels of hepatic steatosis (normal liver, mild, moderate and severe
steatosis) among the patients included by the five BMI categories (normal, overweight, 1\textsuperscript{st}, 2\textsuperscript{nd} and 3\textsuperscript{rd} degree obesity), we can notice the following aspects:

- Mild steatosis is significantly more frequent in case of 1\textsuperscript{st} degree obesity subjects (44.44\%) in comparison with the overweight (17.65\%, p = 0.01) and 3\textsuperscript{rd} degree obese (9.09\%, p = 0.02);

- Moderate steatosis is significantly more frequent in case of overweight subjects (47.06\%) in comparison with the 1\textsuperscript{st} degree obese (25.93\%, p = 0.048) and (9.09\%, p = 0.02).

The incidence of mild steatosis cases was significantly higher in case of subjects whose abdominal circumference value was over the normal value (p = 0.03).

The incidence of normal liver cases was significantly higher in case of subjects whose abdominal-gluteal index was normal (23.53\% vs. 5.63\% for those whose index was over the normal value, p = 0.001).

Seen that the incidence of the cases with steatosis were close to the three levels of hepatic steatosis and slightly higher in case of the subjects whose abdominal-gluteal index was over the normal values, we cumulated the incidences and compared the sub-group of the normal liver subjects with the sub-group of the hepatic steatosis subjects and we included the results in the following table:

By comparing the sub-group of the normal liver subjects with the sub-group of the hepatic steatosis subjects, we noticed that the incidence of normal liver cases is significantly higher in case of subjects whose abdominal-gluteal index is normal (23.53\% vs. 5.63\% for those whose index was over the normal value, p = 0.001) and the
steatosis cases frequently register an abdominal-gluteal index over the normal values (94.37% vs. 76.47% of those with a normal liver, p = 0.01).

### III.3. Levels of Hepatic Steatosis and Cardiovascular Risk

*Levels of Hepatic Steatosis and HBP*

The incidence of normal systolic blood pressure (SBP) cases was significantly higher in subjects with normal liver or mild steatosis (50% vs. 29.69% with moderate or severe steatosis, p=0.04), while the cases of moderate and severe steatosis were significantly more frequent in case of subjects whose SBP values were over the normal values (70.31%, vs. 50% in patients whose SBP values were normal, p = 0.0007). The incidence of cases with normal liver was significantly higher in case of subjects whose diastolic blood pressure (DBP) was normal, in comparison with subjects whose DBP values were over the normal values (19.51%, vs. 2.22%, p = 0.005). Taking into account the increasing tendency of the incidence of steatosis in case of patients whose DBP is over the normal values, we cumulated the incidences and compared the incidence of normal liver cases and the incidence of steatosis cases in patients whose DBP was normal or over normal. The incidence of normal liver cases was significantly higher in subjects whose DBP values were normal (19.51%, vs. 2.22% subjects with values over the normal limit, p = 0.005) and significantly more frequent cases of hepatic steatosis in patients whose
DBP values are over the normal limits (97.78%, vs. 80.49% of those with normal values, p = 0.004).

Levels of Hepatic Steatosis and CIMT

The comparison of average values of the left CIMT between different levels of hepatic affection underlined the following significant differences:

- The first notable aspect refers to the fact that the average value of the left CIMT is continuously increasing according to the level of hepatic affection: 0.84 in subjects with normal liver, 0.93 – intermediate steatosis, 1.03 – moderate steatosis, 1.05 – severe steatosis;

- In comparison with normal liver subjects, the average value of left CIMT seemed to be higher in subjects with moderate steatosis (1.03, vs. 0.84, p = 0.002) and severe steatosis (1.05, vs. 0.84, p = 0.01);

- In comparison with mild steatosis subjects, the average value of the left CIMT seemed to be higher in case of moderate steatosis subjects (1.03, vs. 0.93, p = 0.0098) and severe steatosis (1.05 ± 0.21 vs 0.93 ± 0.13, p = 0.03);

- The average values in normal liver and intermediary steatosis subjects showed no statistically significant differences: 0.84 vs. 0.93, p = 0.10;

- Similarly, the average values in subjects with moderate and severe steatosis were statistically not too different: 1.03 vs. 1.05, p = 0.76.

Thus, we also proceeded to the comparison of some average values resulting from the calculation of the average values of left and right CIMT.
The differences seemed similar to those for left CIMT, but we also noticed a statistically significant difference between mild and severe steatosis. Seeing that the average values of left CIMT in normal liver patients suffering from intermediary steatosis, on one hand and those suffering from moderate and severe steatosis, on the other hand, showed no significant differences, we compared the average values of the subjects cumulated into two groups: group 1, with normal liver or mild steatosis and group 2, with moderate or severe steatosis (table 1).

**Table 1. Differences between the Average Values of Left CIMT in Subjects with Normal Liver or Intermediary Steatosis vs Moderate and Severe Steatosis**

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Left CIMT Average</th>
<th>Std. Dev.</th>
<th>T</th>
<th>gl</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>1, normal liver or mild steatosis</td>
<td>0.906</td>
<td>0.139</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2, moderate or severe steatosis</td>
<td><strong>1.039</strong></td>
<td>0.176</td>
<td>3.682</td>
<td>89</td>
<td><strong>0.0004</strong></td>
</tr>
</tbody>
</table>

CIMT = carotid intima media thickness.

We can see that the significance of the difference between the average value of left CIMT in the subjects of the two groups appeared to be much higher (p = 0.0004). The incidence of normal liver or mild steatosis cases was significantly higher in subjects with normal left CIMT values (54.29%, vs. 25% with values over the normal limit, p = 0.003), while the incidence of moderate or severe steatosis cases was significantly higher in subjects with
values of the left CIMT over the normal limit (75%, vs. 45.71% with normal values, p = 0.003).
A number of 9 subjects (9.89% of the cases) suffered from atheroma of the carotid arteries, 8 of the 9 cases (88.89%) showing a moderate or severe steatosis level. The comparison of the incidence of these cases for the two subgroups of subjects showed that the incidence of atheroma cases was significantly higher in the group of subjects with moderate or severe steatosis (13.79% vs. 3.03% in those with normal liver or mild steatosis, p = 0.049).

Levels of Hepatic Steatosis and Inflammatory Markers

We noticed that:
- The incidence of mild steatosis cases was significantly higher in subjects with CRP values under 1 mg/l, in comparison with those whose CRP values varied between 1 and 2.99 mg/l (54.55% vs. 18.75%, p = 0.0009) and those with values of 3 mg/l and over (54.55% vs. 0%, p = 0.02);
- The incidence of severe steatosis cases was significantly higher in subjects with CRP values of 3 mg/l and over, in comparison with subjects with values under 1 mg/l (80% vs. 18.18%, p = 0.006) and those with values varying between 1 and 2.99 mg/l (80% vs. 39.06%, p = 0.04);
- The incidence of moderate steatosis cases was significantly higher in subjects with values between 1 and 2.99 mg/l, in comparison with those with values under 1 mg/l (30.06% vs. 18.18%, p = 0.04);
- The incidence of severe steatosis cases was significantly higher in subjects with values between 1 and
2.99 mg/l, in comparison with those with values under 1 mg/l (34.37% vs. 9.09%, p = 0.01). By cumulating the normal liver and mild steatosis cases and the moderate and severe steatosis cases and comparing them according to the CRP values grouped by two categories, one under 1 mg/l and one over 1 mg/l, we obtained the following results from table 2:

<table>
<thead>
<tr>
<th>CRP Values</th>
<th>Normal Liver or Mild Steatosis</th>
<th>Moderate or Severe Steatosis</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n %</td>
<td>N %</td>
<td>n %</td>
</tr>
<tr>
<td>Under mg/l (low risk)</td>
<td>16 72.73</td>
<td>6 27.27↓</td>
<td>22 100</td>
</tr>
<tr>
<td>1 – 2.99 mg/l (moderate risk)</td>
<td>17 26.56↓</td>
<td>47 73.44↓</td>
<td>64 100</td>
</tr>
<tr>
<td>3 mg/l ↑ (high risk)</td>
<td>0 0.00↓</td>
<td>5 100.00</td>
<td>5 100</td>
</tr>
<tr>
<td>Total</td>
<td>33 36.26</td>
<td>58 63.74</td>
<td>91 100</td>
</tr>
</tbody>
</table>

P 0.0001; 0.003 0.0001; 0.003; 0.0006

**Table 2. Comparison of Hepatic Steatosis Incidence according to the Normality of CRP Values**

CPR = C-reactive protein.

This way, the significant differences are better emphasized:
- The incidence of normal liver or mild steatosis cases was significantly higher in low risk subjects (with CPR values under 1 mg/l), in comparison with moderate risk subjects, with values between 1 and 2.99 mg/l (72.73% vs. 26.56%, p = 0.0001) and high risk subjects, with values of 3 mg/l and over (72.73% vs. 0%, p = 0.003);
- The incidence of moderate or severe steatosis cases was significantly higher in moderate risk subjects, with CPR values between 1 and 2.99 mg/l (73.44% vs. 27.275 low risk subjects with values under 1 mg/l, \( p = 0.0001 \)) and in high risk subjects with values of 3 mg/l and over (100% vs. 27.27 % low risk subjects, \( p = 0.006 \), and 100% vs. 73.44% moderate risk subjects, \( p = 0.003 \)).

Briefly, we can say that low risk subjects with CPR values under 1 mg/l are significantly and more frequently associated to normal liver or mild steatosis cases, while moderate and high-risk subjects are significantly and more frequently associated to moderate or severe steatosis cases. The comparison of CPR average values according to the level of hepatic steatosis showed that the CPR average value seemed significantly higher in subjects with moderate steatosis (1.72 mg/l, in comparison with 0.94 mg/l in normal liver subjects, \( p = 0.02 \); 1.72 mg/l in comparison with 0.9 mg/l in mild steatosis subjects, \( p = 0.0003 \)) and in subjects with severe steatosis (1.73 mg/l in comparison with 0.94 mg/l in normal liver subjects, \( p = 0.04 \); 1.73 mg/l in comparison with 0.9 mg/l in mild steatosis subjects, \( p = 0.0009 \)).

*Levels of Hepatic Steatosis and Atherogenic Dyslipidemia*

The incidence of mild steatosis cases was significantly higher in normal cholesterol subjects (35.9% vs. 19.23% of those with cholesterol over the normal values, \( p = 0.04 \)), while the incidence of moderate steatosis cases was significantly higher in subjects with cholesterol values over the normal limits (44.23% vs. 25.64% of those with normal cholesterol values, \( p = 0.03 \)).
We did not notice significant incidence differences per level of hepatic steatosis determined by the LDLc values. The incidence of normal liver or mild steatosis cases was significantly higher in subjects with HDLc values within the normal limits, while the incidence of moderate and severe steatosis cases was significantly higher in subjects with HDLc values under the normal limits.

Among the subjects with normal values of the HDLc we significantly meet more cases of normal liver or mild steatosis (70.97% vs. 18.33% of those with values under the normal limits, $p < 0.00001$), while among subjects with values under the normal limit, we encounter significantly more cases of moderate or severe steatosis (81.67% vs. 29.03% of those with normal values, $p < 0.00001$). Seen that the value of $\chi^2=24.5$ is very significant ($p < 0.00001$), we can say that we confirmed the hypothesis of a relation between HDLc and hepatic steatosis (fig. 1).
HDLc = **high density lipoprotein cholesterol.**

**Fig. 1. HDLc – Level of Hepatic Steatosis Correlation Diagram**

It is even more obvious that the incidence of normal liver or mild steatosis cases was significantly higher in subjects with normal values of triglycerides (53.33% vs. 19.57% in subjects with values under the normal limits, p = 0.0006), while the incidence of moderate or severe steatosis was significantly higher in subjects with triglycerides over the normal limit (80.43% vs. 46.67% in those with normal triglycerides, p = 0.0006).
III.4. Levels of Hepatic Steatosis and Metabolic Syndrome, Insulin-resistance, diabetes mellitus

Table 3. Significant Differences according to the Occurrence of Metabolic Syndrome

<table>
<thead>
<tr>
<th>Variables</th>
<th>Average</th>
<th>t</th>
<th>gl</th>
<th>p</th>
<th>Cases 1</th>
<th>Std. dev. 1</th>
<th>Cases 2</th>
<th>Std. dev. 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMI</td>
<td>32.35</td>
<td>28.48</td>
<td>2.841</td>
<td>90</td>
<td>0.006</td>
<td>74</td>
<td>18</td>
<td>5.54</td>
</tr>
<tr>
<td>AC</td>
<td>106.56</td>
<td>96.19</td>
<td>3.330</td>
<td>87</td>
<td>0.001</td>
<td>73</td>
<td>16</td>
<td>11.90</td>
</tr>
<tr>
<td>SBP</td>
<td>146.25</td>
<td>122.31</td>
<td>4.486</td>
<td>85</td>
<td>0.00002</td>
<td>71</td>
<td>16</td>
<td>19.84</td>
</tr>
<tr>
<td>DBP</td>
<td>87.10</td>
<td>79.75</td>
<td>2.691</td>
<td>85</td>
<td>0.009</td>
<td>71</td>
<td>16</td>
<td>10.15</td>
</tr>
<tr>
<td>Glycaemia</td>
<td>146.28</td>
<td>127.12</td>
<td>2.151</td>
<td>89</td>
<td>0.03</td>
<td>74</td>
<td>17</td>
<td>33.85</td>
</tr>
<tr>
<td>HDLc</td>
<td>39.66</td>
<td>46.71</td>
<td>2.515</td>
<td>89</td>
<td>0.01</td>
<td>74</td>
<td>17</td>
<td>10.22</td>
</tr>
<tr>
<td>TG</td>
<td>187.82</td>
<td>105.35</td>
<td>2.839</td>
<td>89</td>
<td>0.006</td>
<td>74</td>
<td>17</td>
<td>113.67</td>
</tr>
<tr>
<td>CRP</td>
<td>1.53</td>
<td>0.98</td>
<td>2.264</td>
<td>89</td>
<td>0.03</td>
<td>74</td>
<td>17</td>
<td>0.91</td>
</tr>
<tr>
<td>Insulinemia</td>
<td>19.36</td>
<td>12.89</td>
<td>2.775</td>
<td>76</td>
<td>0.007</td>
<td>63</td>
<td>15</td>
<td>6.39</td>
</tr>
<tr>
<td>HOMA</td>
<td>7.04</td>
<td>3.94</td>
<td>2.977</td>
<td>76</td>
<td>0.004</td>
<td>63</td>
<td>15</td>
<td>3.88</td>
</tr>
<tr>
<td>FLI</td>
<td>78.08</td>
<td>51.93</td>
<td>4.450</td>
<td>85</td>
<td>0.00003</td>
<td>72</td>
<td>15</td>
<td>20.67</td>
</tr>
<tr>
<td>CIMT</td>
<td>1.01</td>
<td>0.91</td>
<td>2.308</td>
<td>89</td>
<td>0.02</td>
<td>74</td>
<td>17</td>
<td>0.17</td>
</tr>
</tbody>
</table>

1 = with metabolic syndrome; 2 = without metabolic syndrome; AC = abdominal circumference; CIMT = carotid intima media thickness; FLI = fatty liver index; HDLc = high density lipoprotein cholesterol; HOMA = homeostasis model assessment; BMI = body mass index; BP = blood pressure.

Table 3 describes the significant correlations reported to the presence of metabolic syndrome.

The incidence of severe steatosis cases was significantly higher in subjects with metabolic syndrome (32.43% vs. 5.88% in those without metabolic syndrome, p = 0.01), while the incidence of normal liver cases was significantly higher in subjects without metabolic syndrome (23.53% vs. 6.76%, p = 0.02)
The comparison of average HOMA index among the normal liver, intermediate, moderate and severe steatosis groups of subjects showed the following:

- The average value of the HOMA index was significantly higher in severe steatosis subjects (9.13 in comparison with 3.33 in normal liver subjects, p = 0.001; 9.13 in comparison with 4.47 in mild steatosis subjects, p = 0.0004; 9.13 in comparison with 6.4 in moderate steatosis subjects, p = 0.01);

- The average value of the HOMA index was significantly higher in moderate steatosis subjects (6.4 in comparison with 3.33 in normal liver subjects, p = 0.05; 6.4 in comparison with 4.47 in mild steatosis subjects, p = 0.046).

III.5. Levels of Hepatic Steatosis and PNPLA3 rs738409 Polymorphism

The incidence of CC genotype was not significantly different for the two groups (50% for the normal liver or intermediary steatosis group, 59.09% for the moderate or severe steatosis group).

- The incidence of CG genotype was significantly higher in normal liver or intermediary steatosis group of subjects: 50% vs. 22.73% in subjects with moderate or severe steatosis, p = 0.01;

- The incidence of GG genotype was significantly higher in moderate or severe steatosis group of subjects: 18.18% vs. 0% in normal liver or intermediary steatosis subjects, p = 0.01.

Based on the results of the three comparison methods, we could say that:
- The incidence of CC, CG and GG genotypes is the same in normal liver or intermediary steatosis subjects;
- It seems like the differentiation between moderate and severe steatosis subjects is given by the significantly higher incidence of CC genotype in severe steatosis subjects (73.68% vs. 48% in moderate steatosis subjects, \( p = 0.04 \)) and of GG genotype in moderate steatosis subjects (28% vs. 5.26% in severe steatosis subjects, \( p = 0.004 \));
- It seems like the differentiation between subjects who do not suffer from steatosis and the hepatic steatosis subjects is given by the significantly higher incidence of GG genotype in (moderate or severe) steatosis subjects: 18.18% vs. 0% in subjects who are not affected by steatosis (normal liver or intermediate steatosis subjects), \( p = 0.01 \).

**IV. Discussions**

The presence of steatosis in the studied lot was of almost 90%, data corresponding to literature, where the prevalence of the hepatic condition is mentioned between 70 and 90% among DM 2 patients (Williamson et al., 2011). As regards the studied lot of patients, we noticed no statistically significant correlations according to the sex relative to the incidence of normal liver and hepatic steatosis cases, although other researchers claim that the males present a risk factor in hepatic steatosis (Vernon et al., 2011).

The level of hepatic steatosis measured by ultrasound did not prove any statistically significant correlations to the coronary disease and CVA. Still, we noticed that the average value of the *fatty liver index* (FLI) score was
significantly higher in HBP patients (76.55 vs. 64.24, \( p=0.03 \)). The FLI score is used as a non-invasive method to diagnose hepatic steatosis (Bedogni et al., 2006). Despite all these, the interpretation of this score is limited because of the inability to differentiate important aspects of the included measurements. A study that used the database of the National Health and Nutrition Examination Survey III (NHANES III) research and monitored the utility of non-invasive methods in forecasting mortality, approved that the FLI model does not forecast mortality for cardiovascular causes, unlike the NASH index (ION – which, though, was not validated yet) (Otgonsuren et al., 2014).

The hypertensive patients in our study presented significantly higher values of uric acid (5.64 vs. 4.92 mg/dl, \( p < 0.04 \)), results that are similar to other recent data from literature. For example, a study including 45,098 Korean subjects showed that in case of young males (under the age of 40) the level of uric acid was significantly associated to blood pressure, without significant correlations in the age group over 60 (Lee et al., 2015).

A research proved that obesity and insulin resistance represent important predictors of the increase of ALT level in NAFLD subjects. The above-mentioned study included 909 obese children with ages between 9 and 12. Statistically significant correlations were proved among ALT and BMI, AC, cholesterol, triglycerides, arterial pressure and insulin resistance measured by the HOMA index (Yoo et al., 2008). Our study also showed a small but statistically significant correlation between the HOMA index and ALT (\( r = 0.2329, p = 0.04 \)), without showing any correlations among insulin resistance and AST, GGT.
Hyperlipidemia, especially that caused by hyperglyceridemia is a primary risk factor for NAFLD and it even represents a consequence of NAFLD (Baldridge et al., 1995). In our study, the plasmatic level of triglycerides was significantly correlated to the hepatic steatosis levels from a statistic point of view. Even more, among all biological markers investigated, only triglycerides were correlated to CIMT.

As regards the debates on whether to include NAFLD within the metabolic syndrome, our study showed that the incidence of severe steatosis cases is significantly higher in subjects with metabolic syndrome. Abdominal obesity and metabolic syndrome lead to hepatic steatosis both by increase of transport of fatty acids to the liver and by increase of hepatic lipogenesis associated to hyperinsulinemia (Browning, Horton, 2004). At the same time, insulin resistance associated to hepatic steatosis can also aggravate the metabolic syndrome. The close association among hepatic steatosis, obesity and cardio-metabolic risk factors led to the idea that hepatic steatosis can represent a new element of the metabolic syndrome (Kotronen, Yki-Jarvinen, 2008). In our study, even among patients with obesity and metabolic syndrome, the presence of hepatic steatosis was associated to the high level of CRP. This suggests that among these high-risk populations the presence of hepatic steatosis can be a marker of the systemic inflammation level. Moreover, the combination among hepatic steatosis, obesity and metabolic syndrome was associated to a high value of the CRP in our research. Thus, we can consider that the simultaneous presence of these conditions can aggravate the inflammatory cascade leading to an increase of cardiovascular risk.
Once with the development of insulin resistance, insulin’s inhibitor effect on the glucose procedure is diminished, as well as the effect on lipogenesis (Brown, Goldstein, 2008). The animal models support the direct relationship among insulin resistance, hyperinsulinemia and hepatic steatosis (Hebbard, George, 2011). But, genetic studies suggest NAFLD per se cannot be considered to be a cause of insulin resistance, but rather a consequence of it, seen that some subjects are genetically inclined to NAFLD. Still, in our study, insulin resistance evaluated by the HOMA index was significantly correlated to the level of hepatic fat loading, despite the fact that part of the included patients were under oral anti-diabetics therapy. In our study, the level of hepatic steatosis was correlated to the CRP values, both in subjects with metabolic syndrome and without metabolic syndrome. Our results support the idea of independent association between hepatic steatosis and systemic inflammation, additional to the presence of obesity and metabolic syndrome. Our patients presented cardiovascular risk factors, high values of triglycerides and low HDLc, results that are similar to studies presented in literature (Poanta et al., 2011, Coracina et al., 2012). This type of atherogenic dyslipidemia is strongly connected to cardiovascular events (Gaziano et al., 1997). Moreover, our research proved that the presence of metabolic syndrome objectivized a significant increase of triglycerides and a significant decrease of HDLc. In our study, we noticed both a statistically significant correlation between the levels of hepatic steatosis and CIMT, as well as with regard to the presence of carotid plaques. Another research showed that subjects with
NAFLD present a high incidence of cerebrovascular disease (20% vs. 133%, \( p < 0.001 \)) (Targher et al., 2007). Other authors concluded hepatic steatosis does not represent a direct mediator of cardiovascular disease. (McKimmie et al., 2008). Thus, seen that patients with DM2 present an implicit high risk of metabolic syndrome, the presence of fatty liver confers an extra risk regarding the prevalence of carotid plaques. As regards the levels of hepatic steatosis, recent studies showed that a variant of PNPLA3 is associated to moderate - severe steatosis (Nobili et al., 2014, Shang et al., 2015). In our study, the GG genotype was associated to an increase of the hepatic fatty content. Triglycerides, cholesterol, HDLc, AST and ALT were similar among the CC, CG, GG genotypes. For this population of diabetics, our results confirm the influence of PNPLA3 polymorphism on the triglycerides content at a hepatic level. An important discovery of our research is that the association between the PNPLA3 genotypes and the metabolic syndrome elements proved no statistically significant correlations. In the sub-groups resulting from PNPLA3 genotyping, the comparison of the average values of CIMT indicate a statistical significance only between the CC and CG genotypes (\( p = 0.01 \)). The lack of associations to the elements of metabolic syndrome suggests the presence of the allele G is not connected to metabolic disorders among DM 2 subjects. The data we obtained correspond to literature studies that proved that PNPLA3 polymorphism in general population is strongly correlated to hepatic fatty content, independently from adiposity and insulin resistance (Kantartzis et al., 2009).
V. Conclusions

- The prevalence of hepatic steatosis was very high (about 90% of cases) in our population of DM 2 subjects.
- The family medical history of obesity influenced the anthropometrical coefficients and some of the biological markers of the studied patients. Thus, subjects with a family medical history for obesity presented significantly higher average values of FLI score, BMI, abdominal circumference and LDLc.
- The hypertension and stable angina personal medical history were correlated to both anthropometrical indexes and certain biological markers and subclinical atherosclerosis. Hypertensive patients presented significantly higher of the BMI, FLI score and uric acid, in comparison with the normal blood pressure patients. Subjects with stable angina presented significantly higher values of CIMT, ALT and AST.
- From the total subjects included by the study, more than a half were obese (according to the BMI values). A higher percentage of the studied lot presented values of the AC over the normal limit (about 90%).
- The AC values over the normal limit were significantly more frequent in patients with hepatic
steatosis in comparison with those with normal liver.

- The incidence of normal SBP cases was significantly higher in subjects with normal liver or mild steatosis, while the moderate and severe steatosis cases were significantly more frequent in patients with SBP values over the normal limits.
- We established a direct correlation between subclinical atherosclerosis and the level of hepatic fat loading. Thus, it was proved that the average values of CIMT are continuously increasing, according to the level of hepatic affection (especially based on the left CIMT value).
- About 10% of the evaluated subjects presented atheroma plaques at the level of carotid arteries, while 88% of the cases presented a moderate or severe steatosis. Thus, the incidence of atheroma plaque cases was significantly higher in the group of moderate or severe steatosis subjects.
- From the perspective of the CRP inflammatory marker, most of the investigated subjects were at moderate cardiovascular risk (70%). We noticed a positive correlation between the incidence of severe steatosis cases and the high values of CRP, in comparison with patients with normal values of this inflammatory marker.
- As regards the lipid profile and atherogenic dyslipidemia, we noticed a statistically direct correlation between the high level of triglycerides and hepatic fat loading and an inverse relation
between HDLc and the level of hepatic steatosis. From the studied lot, about 50% of the patients presented values of the cholesterol over the normal limit. We noticed significant differences as regards the incidence of mild steatosis cases (higher in normal cholesterol subjects), in comparison with the incidence of moderate steatosis cases (higher in high cholesterol subjects). The incidence of normal liver or mild steatosis cases was significantly higher in subjects with HDLc values within the normal limits, while the incidence of moderate and severe steatosis cases was significantly in subjects with HDLc values under the normal values. Similarly to the HDLc pattern, normal triglycerides were frequently associated to normal liver or mild steatosis, while the values over the normal limit to moderate or severe steatosis.

- The existence of metabolic syndrome was associated to a higher level of hepatic fat loading. The incidence of severe steatosis cases was significantly higher in patients with metabolic syndrome, while the incidence of normal liver cases was significantly higher in subjects without metabolic syndrome.

- Hepatic steatosis was associated to 3 of the 5 elements of metabolic syndrome (AC, triglycerides, HDLc).

- Insulin resistance was associated to the level of hepatic fat loading. The evaluation of insulin resistance objectivized: the average value of
HOMA index was significantly higher in subjects with moderate and severe steatosis in comparison with those with normal liver and mild steatosis.

- Following PNPLA3 genotyping, we noticed that the differentiation between subjects without steatosis and those with hepatic steatosis is given by the significantly higher incidence of GG genotype. The PNPLA3 genotypes were not associated to elements of metabolic syndrome or subclinical atherosclerosis (CIMT) or insulin resistance (HOMA index).
Bibliography


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