# Biomarkers of Inflammation in Patients with Type 2 Diabets Mellitus and Hepatic Steatosis

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*Non-alcoholic fatty liver disease (NAFLD) and diabetes are associations that commonly coexist and act synergistically in terms of worsening the liver disease or increasing the cardiovascular risk. Risk factors or associations thereof are looked for to reduce the cardiometabolic modifiable risk. Our study tracks the correlations between different biomarkers, especially the inflammatory ones, with a degree of fatty load in the liver, and thus with cardiovascular risk in diabetic patients. We included 92 subjects with type 2 diabetes (DM 2), with an average age of 60.38 ± 10.37 years, which were investigated by evaluating the lipid profile, inflammatory markers (CRP, fibrinogen), liver function and as ultrasound investigations we used: abdominal ultrasound with measurement of the degree of fatty liver load and carotid Doppler ultrasound, with the measurement of the carotid intima-media thickness (CIMT). Calculation of the correlation coefficients between the degree of liver steatosis and the parameters included in the study showed direct significant correlation between the degree of liver steatosis and CRP ($r=0.3673$, $p=0.001$), triglycerides ($r=0.3716$, $p=0.001$), ALT ($r=0.3463$, $p=0.002$) and AST ($r=0.2432$, $p<0.0001$) and a significant inverse correlation of HDL cholesterol ($r=-0.5654$, $p=0.001$). Also, we found a significant direct correlation of the triglycerides with CIMT ($r=0.4225$, $p=0.001$). Calculation of the correlation coefficient between CIMT and the degree of hepatic steatosis showed that CIMT directly correlates significantly with the degree of hepatic steatosis ($r=0.2979$, $p<0.004$). Thus, the usual biochemical parameters and the current simple ultrasonographic methods can assess the cardiovascular risk among people with type 2 diabetes and NAFLD.*

*Keywords: NAFLD, diabetes, cardiovascular risk*

Nonalcoholic fatty liver disease (NAFLD) has become the most common chronic liver disease worldwide, with a prevalence reported between 6-33% [1] or even above 50% depending on the diagnostic method and the population studied [2,3]. It includes a spectrum of liver impairment, from simple steatosis (NAFL) to nonalcoholic steatohepatitis (NASH), fibrosis and cirrhosis, which may eventually evolve to hepatocellular carcinoma. NAFLD is strongly associated with insulin resistance (metabolic syndrome) including type 2 diabetes mellitus (DM 2). Whether it precedes or it is concurrent with elements of metabolic syndrome [4], NAFLD brings along increased risk of cardiovascular morbidity and mortality [5].

In the last two decades, in addition to the classic cardiovascular risk factors (family history, dyslipidemia, hypertension, smoking, diabetes), NAFLD is considered increasingly more an independent risk factor for cardiovascular disease [6], especially in people suffering from diabetes [7].

Systemic inflammation is decisive in NAFLD pathogenesis. The adipose tissue produces proinflammatory cytokines tumor necrosis factor alpha (TNF-$\alpha$), interleukin 6 (IL-6), C-reactive protein (CRP) and IL-8 which in turn activate other inflammatory pathways. CRP can be considered an independent risk factor for NAFLD [8]. Studies have shown that CRP, as a parameter that highlights inflammation is related to the degree of hepatic steatosis [9]. Another study shows that CRP can be used as a marker of NAFLD being quite a strong predictor [10].

Carotid ultrasound, with carotid intima-media thickness (CIMT) estimation is a validated exploration method that can be performed on large groups of people and which can predict cardiovascular disease [11,12]. A strong correlation was reported between NAFLD and CIMT, proving an increase of 13% of CIMT among patients with NAFLD compared with control subjects. Also, among subjects with NAFLD, carotid plaques had an increased prevalence [13]. NAFLD is considered an independent risk factor for atherosclerosis [14].

Few studies have examined the association between biochemical values, subclinical atherosclerosis and the degrees of fatty load of the liver.

## Experimental part

### *Material and method*

The aim of the study was to evaluate the relationship between inflammatory markers and the lipid profile with the degree of fatty load of the liver and subclinical atherosclerosis in patients with DM 2. Subclinical atherosclerosis was assessed by measuring CIMT.

We conducted an observational – transversal study, which included a total of 92 subjects, both in urban and in rural areas investigated within the Clinic of Diabetes, Nutrition and Metabolic Diseases Iasi. The following inclusion criteria were established: patients with DM 2 mellitus treated with diet or oral anti-diabetic agents (OAA) without liver damage of viral nature (hepatitis B, C virus) without toxic - ethanol hepatitis, the patients being selected in the order in which they were admitted in the Diabetes Outpatient Clinic, for a period of three months. The Ethics Committee approval was obtained and all participants signed the informed consent form before the study start.

As markers of inflammation CRP and fibrinogen have been measured. CRP (fig. 1) belongs to the family of

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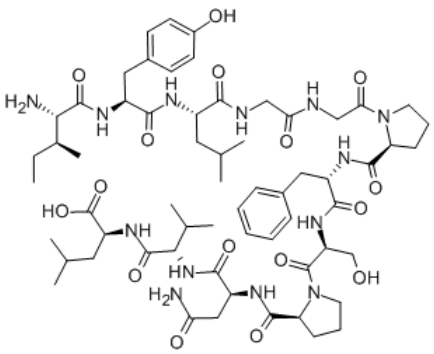

Fig. 1. CRP formula

proteins called pentraxines, including the serum amyloid component P, constituent of amyloid deposits. In human subjects CRP is an acute-phase plasma protein, which shows an increase in serum levels as a result of tissue injury or infection [15]. The structure of the CRP was analyzed by X-ray crystallography [16]. CRP is made up of a central pole around which 5 non-covalently protomers are distributed equally. The protomer contains 206 amino acid residues. The ligand binding site is located on the concave side, and is composed of two loops with two calcium ions [17, 18].

The *high-sensitive* CRP test (hs-CRP) accurately measures low levels of CRP in order to identify the low but persistent level of inflammation, thus helping in predicting the risk of developing cardiovascular disease.

The lipid profile was determined (total cholesterol, LDL-cholesterol, HDL-cholesterol, triglycerides). Triglycerides (TG) or triacylglycerols represent esters of fatty acid with a high number of carbon atoms with glycerol. Excess TG are deposited mainly in the liver. TG excess is responsible, for the most part, of liver steatosis, namely of the fatty load of liver in varying degrees. HDL-cholesterol, also called antiatherogenic lipoprotein mediates the cellular cholesterol influx. It is responsible for the reverse transport of cholesterol, being the main class of lipoproteins with antiatherogenic effect. The cholesterol released at the level of peripheral tissues returns to the liver using HDL. TG growth can disturb the lipid profile towards atherogenicity, lowering HDL-cholesterol, modifying the LDL particles size, density (LDL small and dense) and their oxidation [19].

Liver function was evaluated by determination of aspartate aminotransferase (AST), alanine amino-transferase (ALT) and gamma-glutamyl transpeptidase (GGT). Transaminases, called in the past, glutamic-pyruvic and oxalate-acetic transaminases are enzymes commonly found especially in the liver. They can be used to assess liver damage, although they are not specific only for liver diseases. GGT is an enzyme that specifically catalyzes the cleavage of gamma-glutamyl bond of glutathione and gamma glutamyl transformation in water, peptides or amino acids. In relation to cardio-metabolic diseases more data exist showing that high levels of GGT, even in the normally accepted limits are associated with increased risk of cardiovascular events, hypertension (HTA) and DM 2 [20, 21].

The degree of fatty load of the liver was evaluated by ultrasound with a probe of 3.5 MHz. Liver steatosis was divided into 4 degrees according to 5 criteria: parenchymal reflectivity, the contrast between the liver and kidney, deep beam attenuation, viewing the small vessel walls of the liver and gallbladder wall appearance. Subclinical atherosclerosis was assessed by measuring CIMT using a colour Doppler ultrasound LS 128 with linear probe HL9 / 40 / 128Z.

The statistical analysis was performed using STATISTICA software, version 7.0. $p<0.05$ was considered statistically significant. The correlations between variables were performed using *Pearson r* correlation coefficient.

## Results and discussions

From the group of 92 subjects, 44 males, 73% was represented by patients from urban areas. The average age was 60.38, with limits between 33 and 86 years. Depending on the fatty load of the liver, the group of subjects was divided into 4 groups: normal liver (9 subjects), intermediate steatosis (24), moderate steatosis (33) and severe steatosis (25). The frequency of the degrees of hepatic steatosis were not statistically significantly differentiated by gender. The proportion of those with fatty liver disease in the studied group exceeded 90%, values comparable to the research literature [22,23].

| Parameters | r coefficient | p value |
|---|---|---|
| Age | 0.01 | 0.94 |
| **PCR** | **0.3673** | **0.001** |
| Fibrinogen | -0.1962 | 0.09 |
| Total cholesterol | 0.1289 | 0.27 |
| **HDL cholesterol** | **-0.5654** | **<0.0001** |
| LDL cholesterol | 0.0136 | 0.91 |
| **TG** | **0.3716** | **0.001** |
| **ALT** | **0.3463** | **0.002** |
| **AST** | **0.2432** | **0.036** |
| GGT | 0.0268 | 0.82 |
| Total bilirubin | 0.0091 | 0.94 |
| Direct bilirubin | 0.0096 | 0.93 |
| Glycemia | 0.0335 | 0.78 |

**Table 1**
PEARSON R CORRELATION COEFFICIENT WITH THE DEGREE OF HEPATIC STEATOSIS

| Parameters | CIMT right | | CIMT left | | CIMT average | |
|---|---|---|---|---|---|---|
| | R | P | r | P | r | P |
| Age | 0.0124 | 0.92 | -0.0073 | 0.95 | 0.0026 | 0.98 |
| PCR | 0.1205 | 0.30 | 0.0486 | 0.68 | 0.1017 | 0.38 |
| Fibrinogen | -0.2146 | 0.06 | -0.0899 | 0.44 | -0.1833 | 0.11 |
| Total cholesterol | 0.0790 | 0.50 | 0.1819 | 0.12 | 0.1627 | 0.16 |
| HDL cholesterol | -0.2110 | 0.07 | -0.1379 | 0.24 | -0.2119 | 0.07 |
| LDL cholesterol | 0.0078 | 0.95 | 0.0915 | 0.43 | 0.0631 | 0.59 |
| **TG** | **0.3183** | **0.005** | **0.3686** | **0.001** | **0.4225** | **<0.0001** |
| ALT | -0.0694 | 0.55 | 0.0305 | 0.79 | -0.0211 | 0.86 |
| AST | -0.0331 | 0.78 | -0.0270 | 0.82 | -0.0367 | 0.75 |
| GGT | 0.1136 | 0.33 | 0.1217 | 0.30 | 0.1444 | 0.22 |
| Total bilirubin | 0.0346 | 0.77 | 0.0049 | 0.97 | 0.0234 | 0.84 |
| Direct bilirubin | 0.0373 | 0.75 | 0.0358 | 0.76 | 0.0448 | 0.70 |
| Glycemia | 0.1179 | 0.31 | 0.0650 | 0.58 | 0.1107 | 0.34 |

**Table 2**
PEARSON R CORRELATION COEFFICIENT WITH CIMT

Average blood glucose was not significantly different according to sex (141.44 ± 143.83 ± 32.85 in men and 34.92 in women) and did not correlate with the degree of hepatic steatosis or with CIMT value, although some literature data showed the relationship between fasting blood sugar and prevalence of NAFLD [24].

In what concerns the biomarkers of inflammation, the mean value of fibrinogen, slightly higher in men (408.02 compared to 393.4 in women) did not differ significantly by gender. Fibrinogen, as a marker of inflammation, was not correlated with any degree of hepatic steatosis or with CIMT. CRP showed significant direct correlation with the degree of hepatic steatosis. Calculation of the *Pearson r* correlation coefficients between the degree of hepatic steatosis and the parameters included in the study, showed significant direct correlations with the degree of hepatic steatosis in CRP ($r = 0.3673$, $p = 0.001$), similar to the research literature data [25,9]. The data presented above are highlighted in table 1.

Although it appeared to be higher in men, the mean levels of TG were not significantly differentiated by gender, possibly because of large inter-individual variation, particularly in men. The other parameters of the lipid profile showed no differences by gender. We found a direct significant correlation of TG with CIMT ($r=0.4225$, $p<0.0001$) (table 2). In a similar study, which included 50 subjects with DM 2 a positive correlation between TG and progression of CIMT ($r=0.838$ and $p<0.01$) and a negative correlation with HDL-cholesterol ($r=-0.689$, $p<0.01$ ) have been shown [26]. In the present study, atherogenic dyslipidemia was correlated with the degree of steatosis (the increase of plasmatic TG and the low HDL-cholesterol concentration). A low HDL-cholesterol is an independent risk factor [27]. The characteristic of HDL-cholesterol to form large lipid particles plays an essential role in preventing carotid disease [28]. For the patients in the study group, the decrease of HDL-cholesterol, in those with high degrees of hepatic steatosis may represent a decrease of the protective activity of the vascular endothelial of this cholesterol fraction. Depending on the degree of hepatic steatosis we found a significant inverse correlation with HDL-cholesterol ($r=-0.5654$, $p=0.001$). Another study on 130 diabetic patients showed that only diabetic patients with increases of the TG - HDL ratio showed a significantly increased risk of developing atherosclerosis, identifiable by measuring CIMT, by means of carotid Doppler ultrasound [29].

Most subjects (82.35%) had normal ALT values. Although the average value of ALT appeared to be higher in men (35.19 ± 30.62), the difference was not statistically proven to be significant as compared to that of women (28.15 ± 16.92), possibly because of the large inter-individual variation in male subjects (nearly twice the standard deviation). Most subjects (89.47%) had AST values within normal limits. Slightly higher in men (19.2 ± 24.86), the average AST was not significantly differentiated from that found in women (21.60 ± 10.08). The average value of AST was not significantly different depending on the area of origin (22.19 ± 14.61 in urban areas, 25.79 ± 16.41 in rural areas). Depending on the degree of hepatic steatosis we found significant direct correlations with transaminases ALT ($r=0.3463$, $p=0.002$) and AST ($r=0.2432$, $p<0.001$). These values are consistent with the research literature showing that NAFLD is the most common cause of cytolytic enzymes increase [2].

Most of the subjects (88.06%) had GGT values within normal limits. The mean value of GGT appeared significantly higher in males (39.27 ± 18.32 to 30.07 ± 17.64 in females, $p=0.02$). But no significant frequency differences of the degrees of hepatic steatosis according to GGT values were observed.

98.53% of investigated subjects had normal values of total bilirubin. The mean value of total bilirubin was not significantly differentiated by gender (0.60 in men, 0.53 in women). Neither the average value of direct bilirubin was significantly differentiated by gender (0.26 in men, 0.21 in women), possibly due to inter-individual differences (large standard deviations).

Calculation of the *Pearson r* correlation coefficients between the degree of hepatic steatosis and the

| CIMT | The degree of hepatic steatosis | |
|---|---|---|
| | R | P |
| Right CIMT | 0.1311 | 0.216 |
| **Left CIMT** | **0.3636** | **<0.0001** |
| **Average CIMT** | **0.2979** | **0.004** |

**Table 3**
THE CORRELATION BETWEEN THE DEGREE OF HEPATIC STEATOSIS AND CIMT

parameters included in the study showed direct significant correlation with the degree of hepatic steatosis in CRP (r=0.3673, p=0.001), TG (r=0.3716, p=0.001), AST (r=0.3463, p=0.002) and ALT (r=0.2432, p<0.0001) and a significant inverse correlation of HDL-cholesterol (r=-0.5654, p=0.001).

Calculation of *Pearson r* correlation coefficient between CIMT and the degree of hepatic steatosis revealed that CIMT is significantly directly correlated with the degree of hepatic steatosis (r=0.2979, p=0.004) (table 3). Similar data in the research literature have shown the association between NAFLD and endothelial dysfunction, as well as the increase of the prevalence of carotid disease [6]. The results of our study suggest that among subjects with DM 2, the relationship between NAFLD reflects mainly the atherogenic action of the metabolic syndrome, relying mainly on the atherogenic dyslipidemia, although not all the elements of this syndrome have been assessed.

The results show that the liver fat load is not only a feature of cardiovascular risk, but it can also play a key role in the development of early atherosclerosis, like other studies show [30,31].

As future perspectives we intend to continue the study with a follow-up period, in order to monitor cardiovascular events, related to the degree of liver damage and changes in the lipid profile and in markers of inflammation.

## Conclusions

Subjects with type 2 diabetes in the study show an increased incidence of NAFLD, with varying degrees of fat loading, which causes an increased cardiovascular risk. A positive correlation between the degree of steatosis, hepatic cytolysis enzymes, inflammation (CRP) and atherogenic dyslipidemia (increase in TG and decrease in HDL-cholesterol) has been established.

At the same time, the degree of hepatic steatosis has proved to be a predictor of cardiovascular risk through its direct connection with subclinical atherosclerosis assessed by CIMT.

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