Serum Levels of Oxidative Stress Markers in Patients with Type 2 Diabetes Mellitus and Non-alcoholic Steatohepatitis

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Introduction. Oxidative stress is one of the key mechanisms responsible for disease progression in non-alcoholic fatty liver disease. The aim of this study was to evaluate the serum levels of oxidative stress markers in patients with type 2 diabetes mellitus (DMT2) and non-alcoholic steatohepatitis (NASH) and test their relationships with clinical and biochemical patient characteristics, compared to patients with DMT2 without non-alcoholic fatty liver disease (NAFLD), and controls.

Materials and methods. In all, 60 consecutive patients with DMT2 and NASH, 55 with DMT2 without NAFLD, and 50 age- and gender-matched healthy subjects participated in the study. The serum levels of protein carbonyls and 8-isoprostane were determined by ELISA methods, while the serum levels of malondialdehyde (MDA) were detected by means of the spectrophotometric method. Clinical, demographic, and laboratory parameters were examined for all the subjects included in the study. Multivariate logistic regression was used to test the independent predictive factors in the relationships investigated here.

Results. Patients with DMT2 and NASH displayed significantly higher serum levels of protein carbonyls (1.112 ± 0.42 nmol/dL), MDA (6.181 ± 1.81 ng/mL), and 8-isoprostane (338.6 ± 98.5 pg/mL) compared to patients with DMT2 without NAFLD, and controls. Results of multivariate logistic regression analyses indicate that in patients with DMT2 and NASH, the serum levels of oxidative stress markers were independently and positively associated with: HbA1c, duration of diabetes, the UKPDS cardiovascular risk score (for protein carbonyls); age, LDL-cholesterol (for 8-isoprostane); and triglycerides serum levels (for MDA).

Conclusions. Our findings indicate that the process of oxidative stress tends to increase in patients with DMT2 and NASH, compared to patients with DMT2 without NAFLD, and controls. This evidence suggests that an antioxidant therapy might prove useful in the treatment of patients with DMT2 and NASH.

Key words: type 2 diabetes mellitus (DMT2), non-alcoholic steatohepatitis (NASH), markers of oxidative stress, protein oxidation, lipid peroxidation, cardiometabolic risk.

INTRODUCTION

Diabetes mellitus (DM), type 2 DM (DMT2) in particular, is one of the fastest growing chronic diseases worldwide and continues to be a source of increased disability and mortality [1]. The DMT2 epidemic continues its development in parallel with the sharp rise in obesity prevalence, sedentary lifestyle, diabetogenic, and pro-atherogenic diet [2].

Mortality from cardiovascular cause in people with diabetes reaches levels up to 75-80% worldwide, of which 50% of mortality is due to coronary artery disease (CAD), and 15% is due to stroke. Chronic microvascular complications include diabetic retinopathy, diabetic nephropathy and diabetic neuropathy. These complications are often present, even at the time of diagnosis of DMT2, with variable prevalence between studies [3].

The current epidemiological phenomenon, marked by increasing overweight status, abdominal obesity, and DMT2, is amplified by associating the growing prevalence of metabolic syndrome (MS) with that of non-alcoholic fatty liver disease (NAFLD), regarded as a “liver pandemic” with similar global expansion, often undiagnosed, and as yet without treatment [4].

Non-alcoholic steatohepatitis (NASH) – the severe form of NAFLD – was associated with the risk of rapid progression of cardiovascular disease, in several large longitudinal studies [5]. In addition,
NASH was found to be associated with subclinical atherosclerosis manifested by endothelial dysfunction, increased intima-media thickness, and atherosclerotic plaques, at both carotid and coronary levels. NASH was found associated also with the instability of atherosclerotic plaque – the “vulnerable plaque”; with the increase of calcium score of coronary arteries; with the decrease of coronary flow reserve; and with the change of myocardial energetic metabolism respectively [6].

Given that DMT2 is the largest reservoir of cardiovascular risk factors with continuous action, and given the emergence and development of NASH which adds up components of chronic systemic inflammation, oxidative stress, and metabolic disorders, a further increase of the preexistent cardiometabolic risk is well expected.

Reactive oxygen species (ROS) in excess, in pathological conditions, are generated in mitochondria, in the respiratory chain. The harmful effect of free radicals, intracellularly, is counteracted by the ability of cells to maintain the oxidative stress balance; oxidative stress occurs when the balance “pro-oxidant/antioxidant” is disturbed, and it generates the alteration of cellular structures, thus affecting lipid components, proteins, and nucleic acids [7].

The cell membranes with increased content of polyunsaturated lipid structures are oxidized due to the action of ROS, and they trigger the increase of the cellular permeability, which can lead to cell death. For this reason, the identification of these structural changes at cellular level yields important information in terms of the degree of cell damage caused by oxidative stress [8-9].

This study has sought to assess the serum levels of oxidative stress markers in patients with DMT2 and NASH by determining the protein carbonyls (a marker of protein oxidation), malondialdehyde (MDA) and 8-isoprostane (markers of lipid peroxidation); and test their links with clinical and biochemical patient characteristics, and with the UKPDS cardiovascular risk score, in this specific population.

MATERIALS AND METHODS

The population in this study included 165 participants: 115 were consecutive patients and 50 were clinically healthy subjects without personal pathological antecedents (representing the control group).

The segment of patients who were under observation at Medical Clinic IV, Cluj-Napoca, Romania, included the following study groups: group I which consisted of 60 patients with DMT2 and NASH; and group II which consisted of 55 patients with DMT2, without NAFLD (the patients included in the study were not hospitalized at the time of their inclusion in the study; they were followed over the years in our clinic). The 50 healthy subjects forming the control group (i.e, group III) were recruited from among the persons recorded in the CF Cluj-Napoca Polyclinic, Traffic Safety Service. For each subject included in the study, we recorded a detailed history of their demographics, family history and personal pathological antecedents, lifestyle, diet, exercise, smoking status, and alcohol consumption.

The clinical examination included variables such as age, sex, and anthropometric indicators (weight, height, waist circumference); in addition, the following traditional cardiovascular risk factors were recorded: the family history, the presence of hypertension, dyslipidemia, diabetes, hyperuricemia and the current medication (antidiabetic, antihypertensive, lipid-lowering, others). To assess the prevalence of the metabolic syndrome, we used the definition criteria from the 2009 Consensus (Alberti et al.) [10]. The UKPDS engine was used to assess the UKPDS cardiovascular risk score in patients with DMT2, relative to morbidity and mortality from coronary and cerebrovascular causes [11].

For the purpose of this study, participant exclusion criteria were: alcohol consumption (> 20 g/day), the presence of viral hepatitis B, C, as well as autoimmune hepatitis, hemochromatosis, chronic medication (amiodarone, tamoxifen), and other rare liver diseases and malignancies.

The diagnosis of DMT2 was established based on the American Diabetes Association (ADA) definition criteria, in due observance of fasting, and determination of fasting plasma glucose and glycated hemoglobin (HbA1c) [12-13].

For all study participants, blood samples were collected in the morning, à jeun, after 12 hours of digestive rest. Blood samples were collected for the following determinations: liver function tests (transaminase, AST, ALT, gamma GT) and the panel of the lipid profile (total cholesterol, HDL cholesterol, LDL cholesterol, triglycerides). High sensitivity C-reactive protein (hs-CRP) with normal values ranging from 1-3 mg/L was determined using the automatic analyzer Konelab 300 – ThermoElectron Corp.
The serum levels of IL-6 were determined using the ELISA sandwich kit, Human IL-6 DuoSet ELISA sandwich kit (the normal values of serum IL-6 range between 0-25 pg/mL), while the serum levels of adiponectin were determined by means of the Human Total Adiponectin/Acrp30 Quantikine ELISA Kit (the normal values of adiponectin range between 6500-12000 ng/mL). All determinations using ultrasensitive kits were performed in dual sample, following the instructions of the manufacturing company.

Determination of the serum markers of oxidative stress for protein oxidation, namely protein carbonyls, was performed using the Protein Carbonyl Colorimetric Assay Kit, produced by Cayman Chemical Company. As for lipid peroxidation, its level was determined by means of 8-Isoprostane EIA Kit, also manufactured by Cayman Chemical Company, which has measured the serum level of 8-isoprostane (8-epi-PGF 2α). The serum level of malondialdehyde (MDA), another marker of lipid peroxidation, was determined by means of the spectrophotometric method, using thiobarbituric acid; this method has been previously described by Jentzsch et al. (1996) [14].

The diagnosis of non-alcoholic fatty liver was established by liver function tests and ultrasound, and based on the features specific to the pattern for hepatic steatosis. Consistent with recent studies, we used a panel of serological markers that included adiponectin and IL-6, as a non-invasive method for the diagnosis of positive phenotype for NASH [15]. These markers were described in previous studies as featuring good accuracy and positive predictive value [16-17].

For the purpose of diagnosing the patients with NASH, we have used a combination of two biomarkers, namely increased serum levels of IL-6 above the normal limits (more than 25 pg/mL) and decreased levels of adiponectin (less than 6500 ng/mL). All patients included in group I exhibited increased serum levels of IL-6 and decreased serum levels of adiponectin, an elevation of ALT and AST serum levels, as well as the ultrasound characteristics typical for NAFLD. In a recent study, Grigorescu et al. (2012) have shown that combining two individual markers, namely IL-6 and adiponectin, results in a positive predictive value for NASH, of up to 90.4% [17].

A liver imaging examination was performed by ultrasound, after 12 hours of fasting. Following ultrasonography, hepatic steatosis was determined based on several patterns: contrast liver-kidney (absent = 1; currently = 0); hyperechogenicity of the parenchyma (“bright liver”, ranging from normal to severe), posterior deep beam attenuation (aperture clear, bright = 0; aperture dimmed = 1), and bright intrahepatic vessel walls (present = 0; absent = 1) [18].

In addition, a small number of patients with DMT2 had histologic diagnosis by liver biopsy, with the following distribution: 12 patients with steatohepatitis (those patients were included in group I), 8 with simple steatosis (those patients were excluded from this study), and 2 with normal liver histology (those patients were included in group II).

To examine the normality of distribution for quantitative variables, we used the Kolmogorov-Smirnov test. Variables with normal distribution were represented as mean ± standard deviation, those with non-normal distribution were represented as median (quartiles 1 and 3), and categorical variables (nominal) were expressed as frequency (%). Differences between the means for continuous quantitative variables were assessed using the Student’s t-test. For the variables with non-Gaussian distribution, non-parametric tests were used (Mann-Whitney U test). To compare the differences between three or more groups, we used the one-way ANOVA test for quantitative variables with normal distribution, and for those with non-normal distribution or ordinal, we used the Kruskal-Wallis test. Multivariate logistic regression was used to test the predictive independent factors. A value of $p < 0.05$ was considered statistically significant.

All participants in the study signed an informed consent form, and confidentiality was ensured. The study was conducted according to the Helsinki Declaration and was approved by the Board of Ethics of “Iuliu Hațieganu” University of Medicine and Pharmacy, Cluj-Napoca.

RESULTS

The clinical and biochemical characteristics of subjects included in the study are displayed in Table 1. There were no significant differences in key clinical characteristics such as number of subjects, age and sex among the three groups included in this study.
The distribution of study participants according to the BMI values over 25 kg/m² (overweight and obese) was as follows: 88.33% (group I), 72.72% (group II), and 62% (control group). The results show statistical significance for overweight and obesity in the patients with DMT2 and NASH (group I) vs. patients with DMT2 without NAFLD (group II) and vs. controls (group III).

The average duration of diabetes was 14.2 years in patients with DMT2 and NASH (group I), and 13.3 years in patients with DMT2 without NAFLD (group II), with no statistically significant difference between the groups (p = 0.067).

In terms of biochemical parameters, in group I, there was a slight increase in transaminases and gamma GT serum levels, statistically significant, compared to group II (p < 0.05).

The lipid panel components – increased serum levels of TG in group I vs. II and low HDL cholesterol and the Reaven ratio (TG/HDL), a marker of insulin resistance was 6.47 vs. 4.16, p < 0.05 – indicates a significantly increased atherogenic dyslipidemia in patients with DMT2 and NASH (group I) vs. patients with DMT2 without NAFLD (group II) and vs. controls (group III). LDL cholesterol was significantly increased in group I vs. group II, and each group of patients compared to the control group displayed statistical significance (p < 0.05).

For groups I and II, the values for lipid components were found outside the range of optimum values indicated for patients with DMT2. HbA1c was higher in group I (7.85 ± 6.9) vs. group II (6.95 ± 3.1) vs. controls (5.8 ± 1.5), with statistical significance between HbA1c level in group I vs. group II (p < 0.05), indicating a weaker metabolic control in patients from group I.

The UKPDS cardiovascular risk score for CAD in group I (DMT2 and NASH) was higher,
that is, 39.04% vs. 23.8% in group II (patients with DMT2 without NAFLD) \((p = 0.003)\). The risk of death from CAD was 12.4% in group I vs. 5.9% in group II \((p = 0.023)\). The risk of stroke was 25.6% in group I vs. 16.4% in group II \((p < 0.05)\), and the risk of death from stroke was 3.8% in group I vs. 1.9% in group II \((p < 0.05)\) respectively. These average values of the UKPDS cardiovascular risk score suggest that patients with DMT2 and NASH (group I) fall in the class of high cardiovascular risk, with statistical significance when compared to patients with DMT2 without NAFLD (group II).

The serum levels of oxidative stress markers were determined in all three groups. Determination of the serum levels of protein carbonyl yielded, for each group, the following values: group I (DMT2 and NASH): 1.112 ± 0.42 nmol/dL; group II (DMT2 without NAFLD): 0.508 ± 0.35 nmol/dL; group III (controls): 0.417 ± 0.38 nmol/dL (Figure 1). As shown in Figure 1, group I exhibited the highest values of protein carbonyls, with statistically significant differences between groups I and II, between groups I and III (controls), but not between groups II and III.

Determination of serum levels of malondialdehyde (MDA) yielded the following mean values: group I: 6.181 ± 1.81 ng/mL; group II: 4.258 ± 1.54 ng/mL; group III: 2.393 ± 1.75 ng/mL. When the three groups were compared, we found statistically significant differences between all groups. Group I displayed the highest (most increased) MDA values, followed by group II and the controls (group III) (Figure 2).

The assessment of 8-isoprostane serum levels, an indicator of lipid peroxidation, was performed in all three groups, and the yielded mean values were: group I: 338.6 ± 98.5 pg/mL; group II: 269.92 ± 88.7 pg/mL; group III: 195.94 ± 45.3 pg/mL. When the three groups were compared with each other, we found statistically significant differences between all of them (Figure 3).

The results of multivariate logistic regression analysis indicate that after adjusting for covariates, in group I the serum level of protein carbonyls was significantly and independently associated with the following clinical parameters: HbA1c, duration of diabetes, and UKPDS cardiovascular risk score. In group II, the serum level of protein carbonyls was positively associated solely with HbA1c and duration of diabetes (Table 2).

Regarding MDA serum levels, the results of multivariate analysis show that in group I and group II, MDA serum levels were independently associated with triglyceride serum levels (Table 2). As for 8-isoprostane serum levels, the results of multivariate analysis show that this indicator of lipid peroxidation was significantly and independently associated with age and LDL cholesterol in group I, and associated solely with age, in group II (Table 2).

![Figure 1. Serum levels of protein carbonyls in the three groups under study (p-values are also displayed, and * indicates statistically significant differences at the comparison between groups).](image-url)
Figure 2. Serum levels of MDA in the three groups under study (p-values are also displayed, and * indicates statistically significant differences at the comparison between groups).

Figure 3. Serum levels of 8-isoprostane in the three groups under study (p-values are also displayed, and * indicates statistically significant differences at the comparison between groups).

Table 2
Results of multivariate regression analyses between serum levels of oxidative stress markers and clinical and biochemical variables

<table>
<thead>
<tr>
<th>Oxidative stress marker</th>
<th>Variable</th>
<th>Group I (DMT2 and NASH)</th>
<th>Group II (DMT2)</th>
<th>Group III (Controls)</th>
</tr>
</thead>
<tbody>
<tr>
<td>protein carbonyls</td>
<td>HbA1c (%)</td>
<td>β=0.78</td>
<td>p &lt; 0.001</td>
<td>β=0.67 p &lt; 0.001</td>
</tr>
<tr>
<td></td>
<td>Duration of DMT2 (years)</td>
<td>β=0.77</td>
<td>p &lt; 0.001</td>
<td>β=0.65 p &lt; 0.001</td>
</tr>
<tr>
<td></td>
<td>UKPDS score</td>
<td>β=0.77</td>
<td>p &lt; 0.001</td>
<td>β=0.19 p = 0.21</td>
</tr>
<tr>
<td>MDA</td>
<td>Triglycerides (mg/dL)</td>
<td>β=0.345</td>
<td>p &lt; 0.001</td>
<td>β=0.332 p &lt; 0.001</td>
</tr>
<tr>
<td>8-isoprostane</td>
<td>Age (years)</td>
<td>β=0.55</td>
<td>p &lt; 0.001</td>
<td>β=0.67 p &lt; 0.001</td>
</tr>
<tr>
<td></td>
<td>LDL Cholesterol (mg/dL)</td>
<td>β=0.33</td>
<td>p &lt; 0.001</td>
<td>β=0.25 p = 0.33</td>
</tr>
</tbody>
</table>
DISCUSSION

In this study, we investigated three serum protein and lipid oxidative stress markers, and their relationships with clinical and biochemical characteristics in patients with DMT2 and NASH (group I), in patients with DMT2 without NAFLD (group II), and in a control population (group III) respectively.

Reactive oxygen species (ROS) by their effects on the lipid and protein components, and DNA by the oxidation process, trigger a range of diseases such as cancer, atherosclerosis, cardiovascular disease and inflammatory diseases [9-10].

Protein carbonylation is a common marker of protein oxidation, represented by carbonyl derivatives produced by the direct oxidation of the amino acids in the protein structure, with formation of carbonyl groups [19].

In our study, we performed the determination of protein carbonyls, finding high levels of protein carbonyls – 2 times higher in group I vs. group II, and 2.7 times higher in group I vs. controls (group III).

Another marker of oxidative stress examined in this research – MDA indicating the level of lipid peroxidation – was found increased, that is, 1.47 times higher in group I vs. group II, and 2.58 times higher in group I vs. controls. Likewise, the serum level of 8-isoprostane, another indicator of lipid peroxidation, was found increased, namely 1.25 times higher in group I vs. group II, and 1.73 times higher in group I vs. controls. The evidence that these markers of oxidative stress were found increased suggests a severe imbalance of the oxidative stress balance in group I (DMT2 and NASH), affecting protein and lipid structures, and it also reflects the degree of severity of liver disease process and/or systemic disease. Only a limited number of studies have examined and assessed the level of oxidative stress using these parameters, in this specific population. At multivariate logistic regression analysis, after adjusting for covariates, we found that protein carbonyls did correlate independently with the following variables: HbA1c, diabetes duration/length, and the UKPDS cardiovascular risk score, in patients in group I, arguing thus the role and value of these parameters as biomarkers of cardiovascular risk. For patients in group II, no correlation was maintained for the UKPDS cardiovascular risk score. At multivariate analysis, after adjusting for covariates, MDA and 8-isoprostane – indicators of lipid peroxidation – correlated independently as follows: MDA correlated with TG in groups I and II, where we found increased mean values for TG; and 8-isoprostane was found in both groups independently associated with age, and with LDL-cholesterol, only in group I (Table 2).

Patients in group I exhibited elevated levels of oxidative stress markers regarding protein and lipid peroxidation, associated with an increased level of chronic systemic inflammation, demonstrating the association of these two pathological processes in patients with DMT2 and NASH, and arguing thus that this population belongs to the class of high cardiovascular risk.

To estimate the 10-year cardiovascular risk in the patients included in our study, we used the UKPDS engine. UKPDS score indicated an average score of risk of disease by CAD of 39.04% (group I) vs. 23.8% (group II) \( (p = 0.003) \) and an average score of risk of death from coronary cause of 12.4% (group I) vs. 5.9% (group II) \( (p = 0.023) \), indicating a class of elevated cardiovascular risk in patients with DMT2 and NASH. In addition, there were also found the average score of risk of sickness by stroke, with values of 25.6% (group I) vs. 16.4% (group II) \( (p < 0.05) \) and the average score of the risk of death from stroke, with values of 3.5% (group I) vs. 1.9% (group II) \( (p < 0.05) \).

The two groups of values indicating the average risk scores for CAD and stroke, place the patients with DMT2 and NASH in the class of very high risk. The average score of cardiovascular risk found in patients from the present study was statistically significant higher in men than in women, both in terms of coronary risk and the risk of stroke, similarly to data reported in other studies [20-21].

The profile of patients with DMT2 and NASH that emerges from our study is characterized by: overweight and abdominal obesity, high prevalence of MS with the majority of its components, poor control of diabetes, increased transaminases and gamma GT, independently correlated with hs-CRP, and an elevated 10-year cardiovascular risk of illness and mortality, both from coronary disease and cerebrovascular events. In this category of patients, men exhibit a higher cardiovascular risk compared to women.

A direct link between NAFLD and atherosclerosis was suggested in several studies reporting that patients with NAFLD exhibit markers of endothelial dysfunction and an inflammatory syndrome, largely due to oxidative stress [22-23].

Besides its contributions, this study has also limitations. One of them is the cross-sectional nature of the study, which precludes a dynamic
observation of biochemical parameters of chronic systemic inflammation and oxidative stress, and how their evolution relates to cardiovascular risk factors; in the same vein, due to the cross-sectional design of the study, it is difficult to grasp the influence of antidiabetic, lipid-lowering, and anti-hypertensive medications on these parameters.

In the diagnosis of liver disease, liver biopsy was used only for a small number of patients, which is another limitation. Likewise, the small sample size of the study limits generalization and broader observations. In addressing these constraints, future research should target larger sample sizes, and adopt a longitudinal design.

Despite these limitations, our study has the merit to focus on a category of patients little studied in Romania; these patients with DMT2 and NASH are at high cardiovascular risk. This fact requires all the attention and a thorough understanding of the overall cardiometabolic risk, in order to apply an early, intensive, multifactorial, and effective clinical management.

CONCLUSIONS

In the group of patients with DMT2 and NASH, we found increased serum levels of markers of protein oxidation and lipid peroxidation, and these oxidative stress markers were associated with increased levels of chronic systemic inflammation. Stated differently, our findings indicate that the process of oxidative stress tends to increase in patients with DMT2 and NASH, compared to patients with DMT2 without NAFLD, and controls.

In patients with DMT2 and NASH, the serum levels of protein carbonyls were positively associated with HbA1c, diabetes duration, and the UKPDS cardiovascular risk score, while the serum levels of lipid peroxidation (8-isoprostan and MDA) were linked with age, LDL cholesterol, and triglycerides respectively.

This evidence suggests that an antioxidant therapy might prove beneficial in several complementary ways. First, it could be used in the treatment of NASH, in patients with DMT2. Second, in addition to optimal cardiovascular medication, it may help limiting the long term risks of morbidity and mortality due to CAD and stroke, in this segment of patients. And finally, for this specific population, these oxidative stress markers could be useful as potential prognostic markers of response to an antioxidant treatment.

Conflicts of interest: There are no conflicts of interests related to the present study.
Concluzii. Datele din studiul nostru arată că procesul de stres oxidativ tinde să crească la pacienții cu DZT2 și SHNA, comparativ cu pacienții DZT2 fără afectare hepatică, și cu populația martor. Aceste rezultate sugerează că o terapie antioxidantă s-ar putea dovedi utilă în tratamentul pacienților cu DZT2 și SHNA.

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