MEDICAL UNIVERSITY – PLOVDIV

FACULTY OF MEDICINE

Second Department of Internal Medicine
Section of Gastroenterology

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OXIDATIVE STRESS BIOMARKERS IN CHRONIC LIVER DISEASES, ASSOCIATED WITH INSULIN RESISTANCE

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The dissertation work is written on 141 pages and is illustrated with 31 tables, 29 figures and 5 applications. 210 literary sources were cited, of which 8 in Cyrillic and 202 in Latin.

The dissertation work was discussed and directed for protection before a Scientific Jury by an enlarged council of the Second Department of Internal Medicine, MU-Plovdiv with Protocol No 45 of 01.06.2021.

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The public protection of the dissertation work will take place on 30.06.2022 from 11:00 o'clock in a virtual environment of Zoom.
The defense materials are available in the Scientific Department, MU-Plovdiv, 15A Vassil Aprilov Blvd., and are published on the website of the University.

CONTENT

ABBREVIATIONS USED

ALAT – alanine aminotransferase
Anti HCV – antibodies against hepatitis C virus
APH – alkaline phosphatase
ASAT – aspartate aminotransferase
ATP – adenosine triphosphate
CBC – complete blood count
CV – coefficient of variation
DNA – deoxyribonucleic acid
ELISA – enzyme-linked immunosorbent assay
GPO – glutathione peroxidase
HBsAg – hepatitis B virus surface antigen
HDL-cholesterol – cholesterol in high-density lipoproteins
HOMA-IR – homeostasis model for the assessment of insulin resistance
LDL-cholesterol – cholesterol in low-density lipoproteins
MDA – malondialdehyde
Mean – arithmetic mean
RNS – reactive nitrogen species
ROS – reactive oxygen species
SD – standard deviation
SEM – standard error of the arithmetic mean
SOD – superoxide dismutase
TG – triglycerides
γ-GT – gamma-glutamyltransferase

INTRODUCTION

Despite improved diagnostic and therapeutic options, chronic liver disease remains a significant medical and socio-economic problem today. On the one hand, this is probably due to both the unclear etiopathogenetic mechanisms and its increasing prevalence. On the other hand, the transition from acute to chronic liver disease may be accompanied by nonspecific complaints or even no clinical symptoms, making it difficult to detect it in a timely manner.

It is believed that many of the pathological processes (though not all) that affect the liver are accompanied by varying degrees of oxidative stress. Unbalanced formation of reactive oxygen species because of enhanced pro-oxidant activity, suppressed antioxidant protection, or both, can affect the structure and function of various cellular components. At present, the subject of wide discussion is the enhanced oxidative processes and their relationship with some more common liver damage, leading to the development of liver fibrosis and cirrhosis. In patients with chronic liver disease, the exact mechanisms that lead to the progression of fibrosis are not yet well understood. It is thought that in chronic hepatitis it is aided by insulin resistance and related metabolic disorders and an increased risk of developing type 2 diabetes. In the process of clarification is the complicity of impaired lipid metabolism with subsequent steatosis of the liver and lipid peroxidation.

In connection with the researchers' desire to further study the role and place of oxidative stress in the pathogenesis of chronic liver disease, a variety of biomarkers are used, which can be detected in blood, urine, or other biological fluids. Their quantification provides useful information on the oxidative involvement of lipids (malondialdehyde and 4-hydroxynonenal), DNA (8-oxoguanine), arachidonic acid (isoprostanes), amino acids in proteins and other cellular structures. In this regard, in the present study, we aim to compare the changes in serum concentrations of malondialdehyde and superoxide dismutase and plasma glutathione peroxidase as markers of oxidative stress in patients with chronic liver disease and to analyze their relationship with some carbohydrate and lipid parameters. The obtained data would be useful both for a more detailed elucidation of the mechanisms of oxidative involvement in chronic liver disease and for improving and individualizing the therapeutic approach in affected individuals.

PURPOSE AND TASKS

Purpose: ***To study the role of some biomarkers of oxidative stress and their relationship to insulin resistance in patients with chronic liver disease.***

To achieve this goal, the following **tasks** have been identified:

1. To select appropriate indicators to assess the activity of oxidative stress in patients with chronic liver disease.
2. To study the serum concentrations of malondialdehyde, superoxide dismutase and plasma glutathione peroxidase in patients with chronic hepatitis and to compare with the data of the same indicators in the control group.
3. To determine the concentration of serum malondialdehyde, superoxide dismutase and plasma glutathione peroxidase in patients with liver cirrhosis and to compare with the data on these indicators in the control group.
4. To compare the indicators of oxidative stress in patients with chronic hepatitis and liver cirrhosis.
5. To study the influence of sex on metabolic indicators and indicators of oxidative stress in patients with chronic hepatitis, liver cirrhosis and in the control group.
6. To analyze the relationship between indicators of oxidative stress and metabolic indicators in patients with chronic hepatitis, liver cirrhosis and in the control group.

MATERIALS AND METHODS

1. Clinical material

1.1. Subjects of surveillance are the indicators of oxidative stress malondialdehyde, glutathione peroxidase and superoxide dismutase and insulin resistance in patients with chronic liver diseases are monitored.

1.2. Units of observation

1.2.1. Logical units of observation

- Patients with chronic liver diseases hospitalized in the Second Internal Department (Department of Gastroenterology) of Asenovgrad Hospital, Asenovgrad and in the Clinic of Gastroenterology of Kaspela University Hospital, Plovdiv. The study included two groups of patients with chronic liver diseases – one group was made up of individuals with chronic hepatitis and the other included individuals with liver cirrhosis.
- Clinically healthy adult subjects who were accepted as a basis for comparison (control group).

1.2.2. Technical units of observation – The Department of Gastroenterology of Asenovgrad Hospital, Asenovgrad and the Gastroenterology Clinic of Kaspela University Hospital, Plovdiv, where the clinical studies of patients were conducted. The serum concentrations of MDA, SOD, insulin and plasma concentration of GPO-1 are determined in the Department of Clinical Laboratory, MU-Plovdiv and the Central Clinical Laboratory of "St. George" University Hospital, Plovdiv.

1.3. Authorities of the monitoring – information on patients with chronic liver diseases and on the persons in the control group was collected for the period of 01.03.2017 to 01.02.2018 by the PhD student.

1.4. Signs of surveillance

- Anamnestic – data from the history of risk factors for the development of chronic liver disease (alcohol use, sickened acute viral hepatitis et al.).
- Clinical – data from the clinical study of patients, assessing the entire possible spectrum of discrete or prominent clinical scars of compensated or decompensated chronic liver disease.

- Clinical-laboratory – results of the clinical laboratory tests of indicators MDA, SOD, GPO-1, glucose, insulin, total cholesterol, HDL-cholesterol, triglycerides, etc. as an event of oxidative stress and insulin resistance in patients with chronic liver diseases.

1.5. Design of the study – a comparative study was conducted on some indicators of oxidative stress in patients with proven chronic liver disease associated with insulin resistance (chronic hepatitis and liver cirrhosis) and in clinically healthy individuals serving as a control group.

1.6. Clinical material, criteria for the selection of the persons from the study – a total of 84 persons aged 20 to 83 are included in the dissertation work. Of these, 55 persons are with chronic liver diseases, divided into two groups: patients with chronic hepatitis (n = 26) and patients with liver cirrhosis and portal hypertension (n = 29). Data on the etiological structure of both groups with chronic liver diseases are presented on Table 1. Patients were randomly selected.

A control group of 29 clinically healthy subjects was used for comparison. The control group is composed of persons with different professions – employees from the education system, doctors and other medical professionals, students. Medical examination and routine hematological, biochemical and urine tests have been performed to assess their health status.

Table 1. Distribution of persons from clinical groups according to the etiology of the disease

Etiological factor	Patients with chronic hepatitis (n = 26)	Patients with liver cirrhosis with portal hypertension (n = 29)
Viral hepatitis B	17	8
Viral hepatitis C	8	3
Ethylic genesis	1	13
Cardiac genesis	0	1
Metabolic genesis	0	2
Mixed genesis	0	2

The following criteria for the selection of patients from both pathological groups and healthy controls were determined in advance:

***Inclusion criteria*:**

- Patients with chronic hepatitis over 18 years of age with positive serology for HBsAg and/or Anti HCV.

- Patients with diagnosed liver cirrhosis with portal hypertension of different etiology based on conducted biological, ultrasonographic, clinical-laboratory and histological studies.
- Clinically healthy subjects over the age of 18 years of age with no evidence of alcohol, drug or drug dependence, no acute or chronic hepatobiliary or pancreatic diseases, no diabetes mellitus and insulin resistance so far, no severe cardiovascular, lung or gastrointestinal diseases, no AIDS.
- Voluntarily pre-signed informed consent form for participation in the survey.

Exclusion criteria:

- Taking antioxidant medicines at least two weeks before the study.
- Alcohol abuse at least two weeks before the study.
- Endocrine diseases – type 1 diabetes mellitus or type 2 diabetes mellitus of drug treatment, established before liver disease, hypo/hyperthyroidism, Cushing's syndrome, PCOS, etc.
- Other severe chronic diseases – kidney, lung, cardiovascular, neurological, AIDS.
- Concomitant acute infectious disease.
- Hepatocellular carcinoma.
- Pregnancy.

In order to conduct the clinical study, a positive opinion has been received from the Committee on Scientific Ethics of the Research Council at the Medical University of Plovdiv with protocol No 2/30.03.2017 on compliance of this scientific study with the standards and criteria for scientific and ethics. The voluntary participation of the persons from the surveys is certified by a pre-signed informed consent.

2. Methods used

2.1. Clinical methods

The clinical trial includes:

- Anamnestic data on risk factors for the development of chronic liver disease – alcohol use, acute viral hepatitis, other hepatotropic viral infections, risk manipulations in the past, chronic biliary diseases, concomitant pathology of various nature (vascular, metabolic), systemic administration of hepatotoxic drugs.

- Diagnostic criteria – based on a comprehensive assessment of anamnestic data, data from physical examination and the results of clinical and laboratory panels – CBC, biochemistry, ionogram, urine, virological, immunological. To assess insulin resistance, HOMA-IR = fasting glucose (mmol/l) X fasting insulin (μIU / ml) / 22.5 was calculated.
- Instrumental methods – ultrasonography and computer tomography diagnostics are routinely used, and if necessary, liver biopsy with histological examination is performed

2.2. Clinical-laboratory methods

- *Immunoenzyme method for quantitative determination of glutathione peroxidase* – a test kit Human glutathione peroxidase 1 of the company BioVendor – Laboratorni medicina a.s., Czech Republic, cat. № RAG012R.
- *Immunoenzyme method for quantitative determination of superoxide dismutase* – a Human Cu/ZnOD Platinum ELISA test kit from Affymetrix eBioscience, Austria, cat. № BMS222CE.
- *Immunoenzyme method for quantitative determination of malondialdehyde* – a test kit of the company MyBioSource, USA, cat. № MBS263626.
- *Immunoenzyme method for quantitative determination of insulin* – a test kit of the company Nova Tec immundiagnostica GmbH, Germany with cat. № DNOV111 was used.

The principle for determination of GPO, SOD, MDA, and insulin is sandwich enzyme-linked immunosorbent assay based on ELISA technique. Concentrations of these indicators were reported on a Sirio S microplate reader, SEAC, Italy

- *Other clinical-laboratory parameters used for the purpose of the dissertation:* hematological parameters were determined by analyzer Medonic (Stockholm, Sweden); biochemical parameters glucose, total protein, albumin, total and direct bilirubin, total cholesterol, HDL-cholesterol, triglycerides, AST, ALT, AF, γ-GT, electrolytes, urea, creatinine are determined on the analyzer Mindray BS 200e (China) with original reagent manufacturer programs; Coagulation assessment parameters (fibrinogen, prothrombin time) were tested on a CoaDATA 4004 coagulometer (Germany).

2.3. Virological methods – determination of HBsAg and Anti-HCV was performed.

2.4. Statistical methods

Statistical processing of the results was performed using the software product SPSS v. 19.0. The significance level of the null hypothesis is $P < 0.05$. The following *parametric methods* were used: variation and alternative analysis, t-criterion for testing hypotheses for the presence of statistically significant differences between the studied indicators in different groups, one-way analysis of variance (One-way ANOVA) when comparing more than two unknown normally distributed quantitative variables, correlation analysis (Pearson correlation coefficient, r) to assess the magnitude and strength of the relationship between normally distributed quantitative values. *Non-parametric methods* were also used: Kolmogorov-Smirnov test to check the type of distribution of quantitative indicators and Pearson's agreement criterion – $\chi 2$, Mann-Whitney U-test to compare quantitative values in two independent samples with a distribution other than normal, Kruskal-Wallis test for comparison of more than two independent samples with a different than normal distribution, non-parametric correlation test of Spearman (rho).

Graphical methods were used to visualize the results.

RESULTS

Concentrations of serum MDA, SOD, and plasma GPO in patients with chronic hepatitis and comparison with data from the same indicators in subjects from the control group

The study included 26 patients with chronic hepatitis and 29 clinically healthy individuals (controls). In both groups the relative share of women is higher: in patients with chronic hepatitis women are 57.7% (n = 15) and in controls 72.4% (n = 21). The mean age (mean ± SEM) of patients with chronic hepatitis was 49.46 ± 3.07 years, and of controls 35.62 ± 2.42 years (the difference was statistically significant, t = 3.57, P = 0.001). Data on the metabolic parameters of individuals from both groups are presented in Table 2.

Table 2. Mean metabolic parameters in patients with chronic hepatitis and control group

Group / Parameter	Chronic hepatitis (mean ± SEM)	Controls (mean ± SEM)
Total chol. (mmol/l)	4.84 ± 0.30	4.79 ± 0.14
HDL-chol. (mmol/l)	1.24 ± 0.05	1.54 ± 0.06
LDL-chol. (mmol/l)	2.93 ± 0.22	2.83 ± 0.13
TG (mmol/l)	1.44 ± 0.21	0.92 ± 0.10
Glucose (mmol/l)	5.17 ± 0.19	4.90 ± 0.13
Insulin (μIU/ml)	16.50 ± 3.81	3.99 ± 0.60
HOMA-IR	4.32 ± 1.15	0.90 ± 0.14

Compared with controls, patients with chronic hepatitis had statistically significant lower serum HDL-cholesterol (P <0.0001) and higher TG (P = 0.029), insulin (P = 0.001) and HOMA-IR (P = 0.001). The two groups did not differ in mean glucose, total cholesterol, and LDL-cholesterol (P > 0.05).

1. Serum MDA as an indicator of lipid peroxidation in patients with chronic hepatitis

The mean MDA values of patients with chronic hepatitis and the controls are presented in Table 3. Statistical analysis of the data was performed with the Mann-Whitney test. We found that patients with chronic hepatitis had significantly higher mean serum MDA concentrations than those in the control group. The mean difference in mean MDA between the two groups (mean difference ± SE difference) was 92.03 ± 13.02 nmol/ml.

Table 3. Comparison of serum MDA in patients with chronic hepatitis and in the control group

Group	Number (n)	Mean (nmol/ml)	SD (nmol/ml)	SEM (nmol/ml)	Mann-Whitney U	P
Chronic hepatitis	26	157.88	52.57	10.31	7.00	< 0.0001
Controls	29	65.85	17.31	3.22		

2. Plasma GPO and serum SOD concentrations in patients with chronic hepatitis

The data from the comparative analysis of plasma GPO and serum SOD in patients with chronic hepatitis and in the control group is presented on Table 4 and Table 5. The Mann-Whitney test was used to compare the groups.

Table 4. GPO in patients with chronic hepatitis and in the control group

Group	Number (n)	Mean (ng/ml)	SD (ng/ml)	SEM (ng/ml)	Mann-Whitney U	P
Chronic hepatitis	26	2.19	2.98	0.58	321.00	0.344
Controls	29	1.38	2.30	0.43		

Table 5. SOD in patients with chronic hepatitis and in the control group

Group	Number (n)	Mean (ng/ml)	SD (ng/ml)	SEM (ng/ml)	Mann-Whitney U	P
Chronic hepatitis	26	25.46	8.78	1.72	53.00	< 0.0001
Controls	29	128.86	123.00	22.84		

We found that patients with chronic hepatitis did not differ statistically significantly from controls on the mean concentration of GPO (Fig. 1). The obtained average difference of GPO in the persons of the two groups is 0.81 ± 0.72 ng/ml. The two groups differ statistically significantly in the concentration of SOD. Patients with chronic hepatitis have a significantly lower mean concentration of SOD compared to those in the control group (Fig. 2). The mean SOD difference between the two groups (mean difference ± SE Difference) was 103.40 ± 22.91 ng/ml.

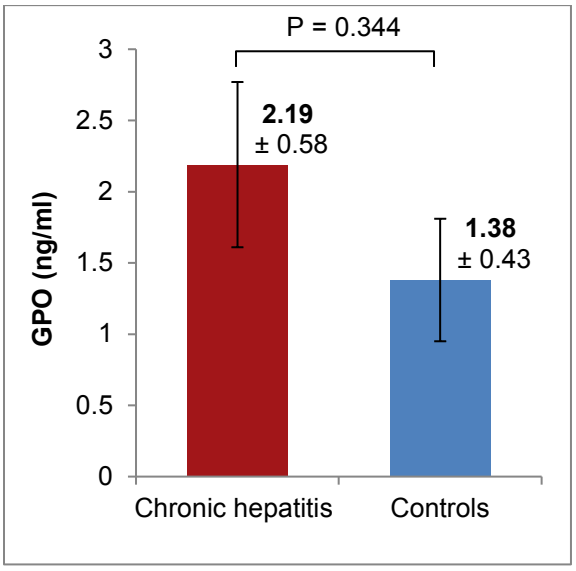

Figure 1. Mean GPO values (mean ± SEM) in patients with chronic hepatitis and in controls

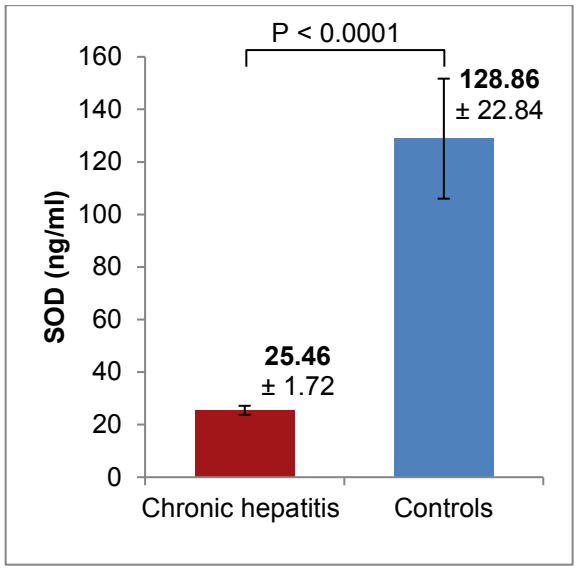

Figure 2. Mean SOD values (mean ± SEM) in patients with chronic hepatitis and in controls

Concentrations of serum MDA, SOD, and plasma GPO in patients with liver cirrhosis and comparison with data from the same indicators in subjects from the control group

The study included 29 patients with cirrhosis and 29 controls. The two groups differ in the relative share of women and men: in patients with cirrhosis, men were 79.3% (n = 23) and in controls, men were 27.6% (n = 8). The mean age of patients with cirrhosis was significantly higher than that of controls (64.45 ± 1.98 years versus 35.62 ± 2.42 years, t = 9.22, P < 0.0001). Data on the metabolic parameters of individuals from both groups are presented in Table 6.

Table 6. Mean values of metabolic parameters in patients with liver cirrhosis and in the control group

Group / Parameter	Liver cirrhosis (mean ± SEM)	Controls (mean ± SEM)
Total chol. (mmol/l)	3.32 ± 0.16	4.79 ± 0.14
HDL-chol. (mmol/l)	0.82 ± 0.06	1.54 ± 0.06
LDL-chol. (mmol/l)	2.00 ± 0.11	2.83 ± 0.13
TG (mmol/l)	1.09 ± 0.06	0.92 ± 0.10
Glucose (mmol/l)	6.11 ± 0.40	4.90 ± 0.13
Insulin (μIU/ml)	30.55 ± 4.89	3.99 ± 0.60
HOMA-IR	8.54 ± 1.69	0.90 ±0.14

Compared with controls, patients with cirrhosis had significantly lower serum total cholesterol ($P < 0.0001$), HDL-cholesterol ($P < 0.0001$) and LDL-cholesterol ($P < 0.0001$) and higher TG ($P < 0.009$), glucose ($P < 0.004$), insulin ($P < 0.0001$) and HOMA-IR ($P < 0.0001$).

1. Serum MDA as an indicator of lipid peroxidation in patients with liver cirrhosis

The mean MDA values of patients with cirrhosis and controls are presented on Table 7. It was found that the two groups differed statistically significantly in the mean serum MDA concentration. Patients with liver cirrhosis had a significantly higher mean concentration of MDA than that of controls ($P < 0.0001$). The mean difference in mean MDA between the two groups (mean difference ± SEM) was 127.89 ± 12.66 nmol/ml.

Table 7. Serum MDA in patients with liver cirrhosis and in the control group

Group	Number (n)	Mean (nmol/ml)	SD (nmol/ml)	SEM (nmol/ml)	Mann-Whitney U	P
Liver cirrhosis	29	193.74	62.93	11.69	435.00	< 0.0001
Controls	29	65.85	17.31	3.22		

2. Plasma GPO and serum SOD concentrations in patients with cirrhosis

The data from the comparative analysis of plasma GPO and serum SOD in patients with liver cirrhosis and in the control group is presented on Table 8 and Table 9.

Table 8. GPO in patients with liver cirrhosis and in the control group

Group	Number (n)	Mean (ng/ml)	SD (ng/ml)	SEM (ng/ml)	Mann-Whitney U	P
Liver cirrhosis	29	3.01	6.67	1.24	250.50	0.008
Controls	29	1.38	2.30	0.43		

Table 9. SOD in patients with liver cirrhosis and in the control group

Group	Number (n)	Mean (ng/ml)	SD (ng/ml)	SEM (ng/ml)	Mann-Whitney U	P
Liver cirrhosis	29	24.55	6.46	1.20	62.00	< 0.0001
Controls	29	128.86	123.00	22.84		

We found that patients with liver cirrhosis differed statistically significantly from the control group in plasma GPO and serum SOD levels. In patients with

liver cirrhosis, the mean concentration of GPO was significantly higher than that of subjects in the control group (Fig. 3). The mean GPO difference (mean difference ± SE difference) between the two groups was 1.63 ± 1.31 ng/ml. Patients with liver cirrhosis have a significantly lower mean SOD level than those in the control group (Fig. 4). The mean SOD difference (mean difference ± SE difference) between the two groups was 104.31 ± 22.87 ng/ml.

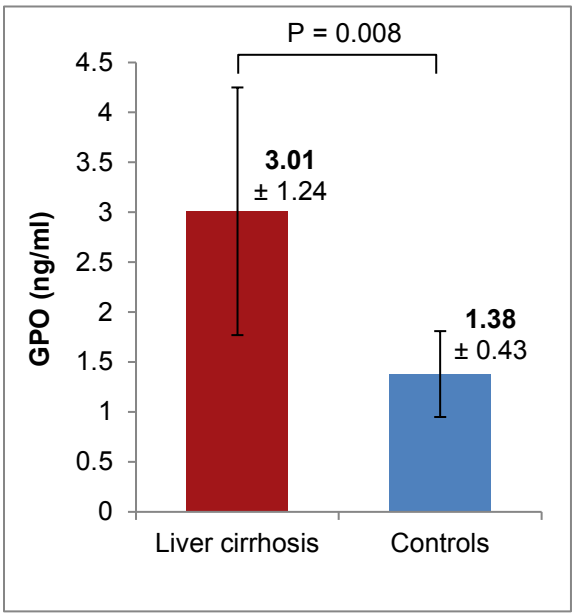

Figure 3. Mean GPO values (mean ± SEM) in patients with liver cirrhosis and in controls

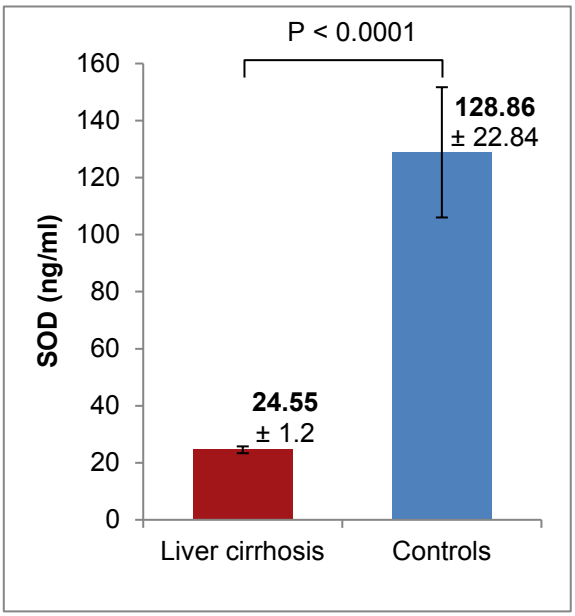

Figure 4. Mean SOD values (mean ± SEM) in patients with liver cirrhosis and in controls

Comparative study of oxidative stress in patients with chronic hepatitis and liver cirrhosis

The study included a total of 55 people, of which 26 patients with chronic hepatitis and 29 patients with cirrhosis. The two groups differ in the relative share of women and men: in patients with chronic hepatitis men are 42.3%, and in patients with liver cirrhosis men are 79.3%. The mean age (mean ± SEM) of patients with chronic hepatitis was 49.46 ± 3.07 years, and of patients with liver cirrhosis was 64.45 ± 1.98 years (the difference was statistically significant, $t = 4.19$, $P < 0.0001$).

The mean values of MDA (nmol/ml), GPO (ng/ml) and SOD (ng/ml) in patients with chronic hepatitis and liver cirrhosis are presented in Table 10. The pairwise comparisons were performed with the Mann-Whitney test.

Table 10. MDA, GPO, and SOD in patients with chronic hepatitis and liver cirrhosis

Group		Number (n)	Mean ± SEM	Mann-Whitney U	P
MDA	chronic hepatitis liver cirrhosis	26 29	157.88 ± 10.31 193.74 ± 11.69	251.00	0.034
GPO	chronic hepatitis liver cirrhosis	26 29	2.19 ± 0.58 3.01 ± 1.24	323.00	0.361
SOD	chronic hepatitis liver cirrhosis	26 29	25.46 ± 1.72 24.55 ± 1.20	367.00	0.866

A statistically significant difference was found only in the comparison of MDA in the two studied groups. Patients with liver cirrhosis have a statistically significant higher mean concentration of MDA than that of patients with chronic hepatitis (Fig. 5). The mean difference in MDA concentration between these two groups of patients (mean difference ± SE difference) was 35.85 ± 15.74 nmol/ml.

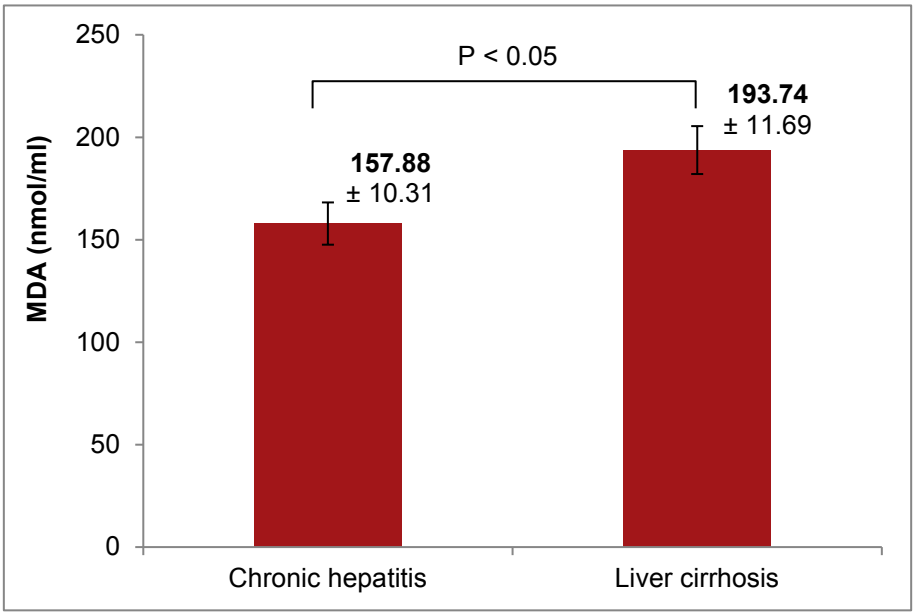

Figure 5. Mean MDA values in patients with chronic hepatitis and liver cirrhosis. Data are presented as mean ± standard error of mean

Study of the influence of gender on serum concentrations of metabolic parameters and indicators of oxidative stress in patients with chronic hepatitis, liver cirrhosis and control subjects

In the studied group of persons in general (n = 84/100%) the distribution by sex is the same: 50% are men (n = 42) and 50% are women (n = 42). However, in the separate groups, the distribution of the persons from the survey by gender is not the same (χ^2 = 16.41, P = 0.0001). Data on the intragroup distribution of the persons from the three groups included in the study by gender are presented in Table 11.

Table 11. Distribution of persons from the three groups by sex

Group	Sex	Number	% in the group	Total number /%
Chronic hepatitis	men women	11 15	42.3 57.7	26/100
Liver cirrhosis	men women	23 6	79.3 20.7	29/100
Controls	men women	8 21	27.6 72.4	29/100

In the group with *chronic hepatitis* men and women did not differ statistically significantly by age (45.18 ± 4.74 years versus 52.60 ± 3.95 years, t = 1.20, P = 0.243). Through the Student-Fisher t-test (for total cholesterol, HDL-cholesterol, LDL-cholesterol) and the Mann-Whitney U-test (for TG, glucose, insulin, HOMA-IR, MDA, SOD, GPO) we did not find statistically significant sex conditioned difference both in terms of metabolic parameters and in MDA, SOD, and GPO (P > 0.05). Data on the concentration of MDA, SOD and GPO in men and women with chronic hepatitis are presented in Fig. 6, Fig. 7, and Fig. 8.

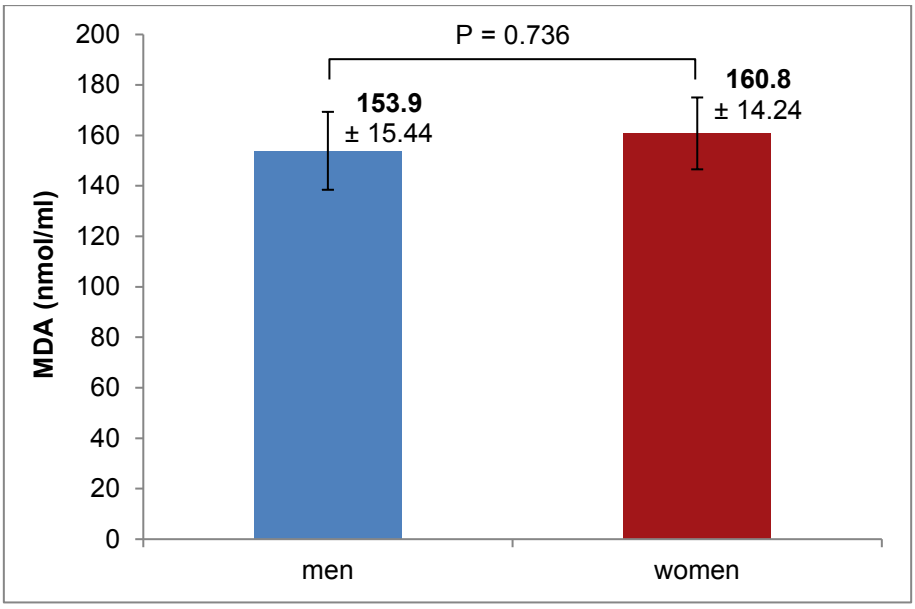

Figure 6. Mean MDA levels (mean ± SEM) in men (n = 11) and in women (n = 15) with chronic hepatitis

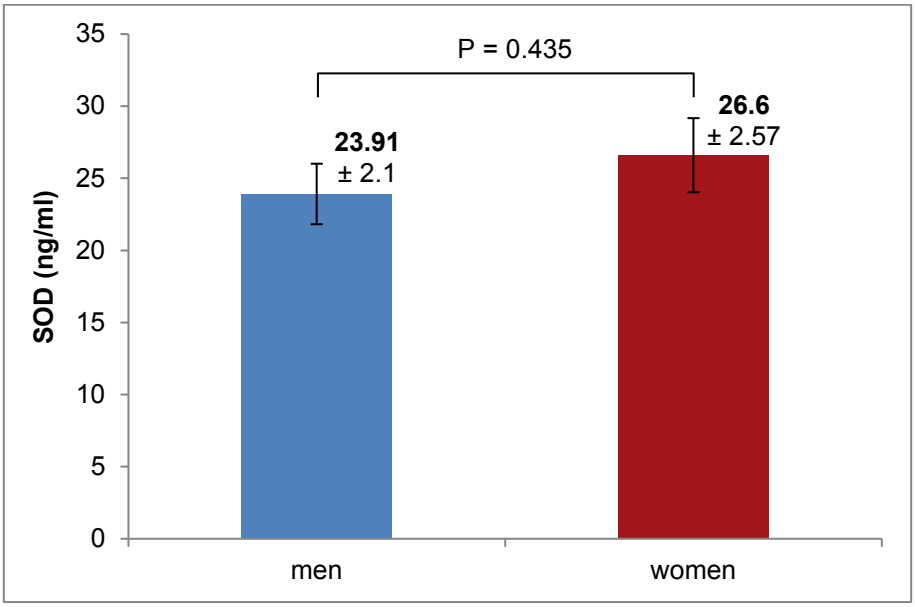

Figure 7. Mean SOD levels (mean ± SEM) in men (n = 11) and in women (n = 15) with chronic hepatitis

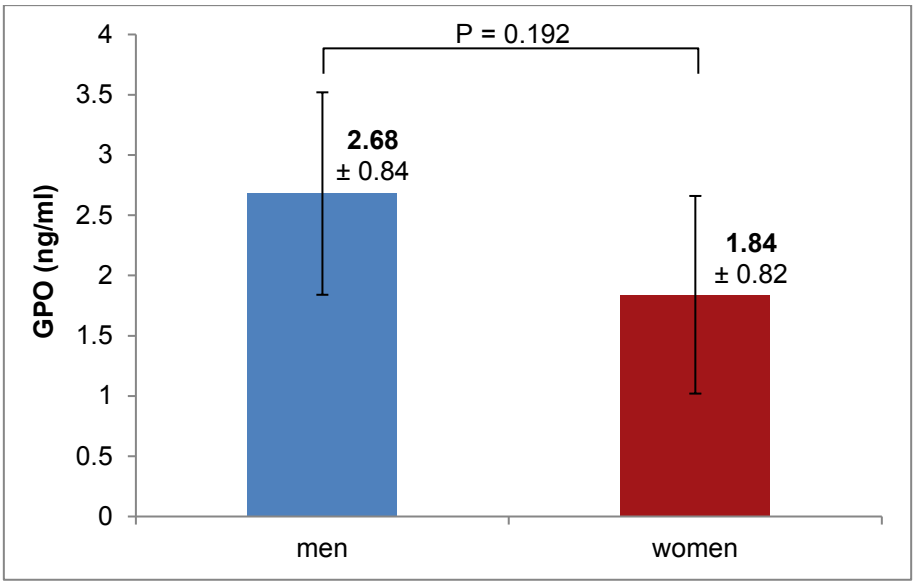

Figure 8. Mean GPO levels (mean ± SEM) in men (n = 11) and in women (n = 15) with chronic hepatitis

In patients with *liver cirrhosis*, the mean age of men was 61.96 ± 2.19 years, and of women – 74.00 ± 1.44 years (t = 4.61, P < 0.0001). In this group, the studied metabolic parameters and oxidative stress indicators did not differ between men and women, because the achieved statistical significance of the Student's t-test in independent samples and the Mann-Whitney U-test is greater than 0.05. Data on the concentration of MDA, SOD and GPO in men and women with liver cirrhosis are presented in Fig. 9, Fig. 10, and Fig. 11.

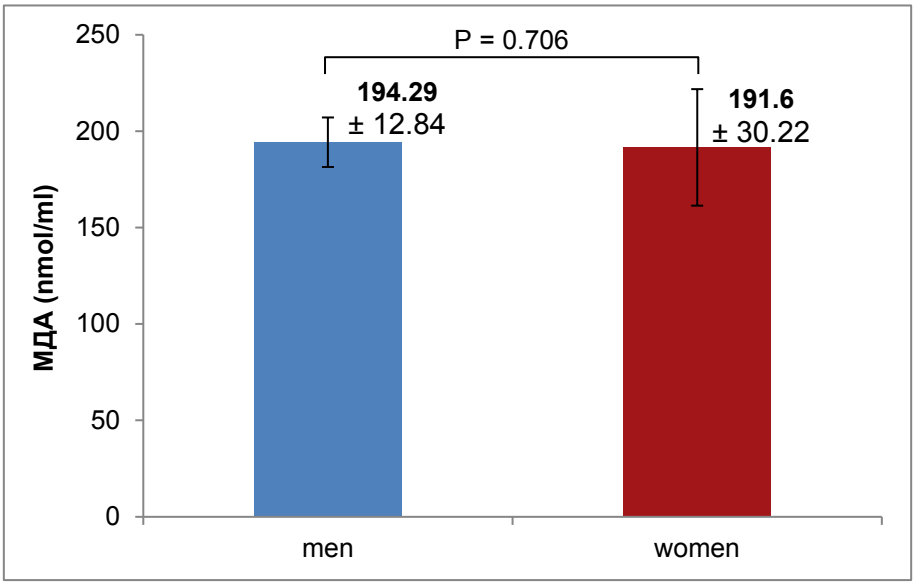

Figure 9. Mean MDA levels (mean ± SEM) in men (n = 23) and in women (n = 6) with liver cirrhosis

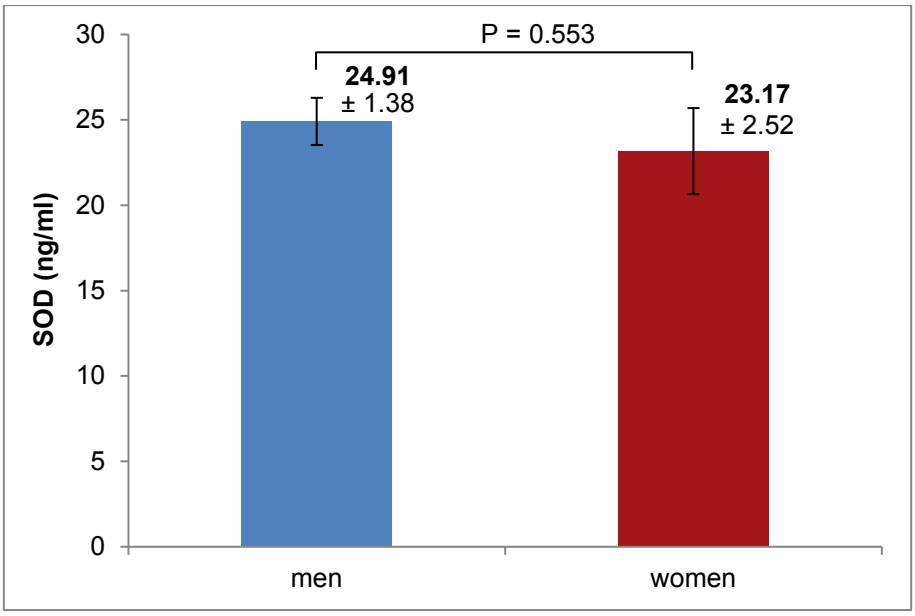

Figure 10. Mean SOD levels (mean ± SEM) in men (n = 23) and in women (n = 6) with liver cirrhosis

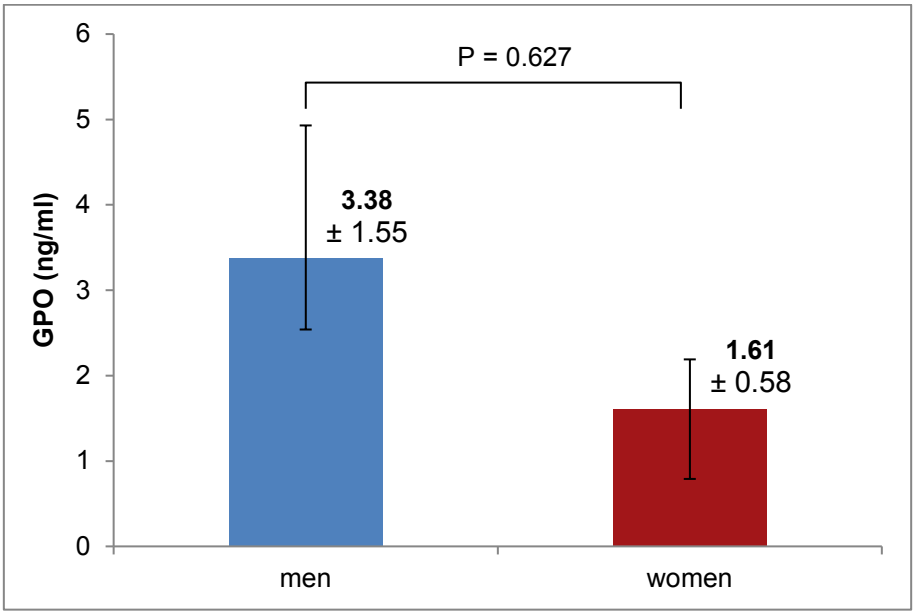

Figure 11. Mean GPO levels (mean ± SEM) in men (n = 23) and in women (n = 6) with liver cirrhosis

Men and women in the *control group* did not differ statistically significantly in age: 33.13 ± 5.15 years versus 36.57 ± 2.77 years (t = 0.59, P = 0.567). The obtained mean differences between total cholesterol, HDL-cholesterol, and LDL-cholesterol in individuals in this group did not differ statistically significant between the two sexes, P > 0.05. From the indicators analyzed by the Mann-Whitney test, only the mean value of MDA in women is

higher than that of men (70.27 ± 3.16 nmol/ml versus 54.25 ± 6.95 nmol/ml, P = 0.026). Data from the statistical analysis for the study of the influence of gender on MDA, SOD and GPO in the persons from the control group are presented in Fig. 12, Fig. 13 and Fig. 14.

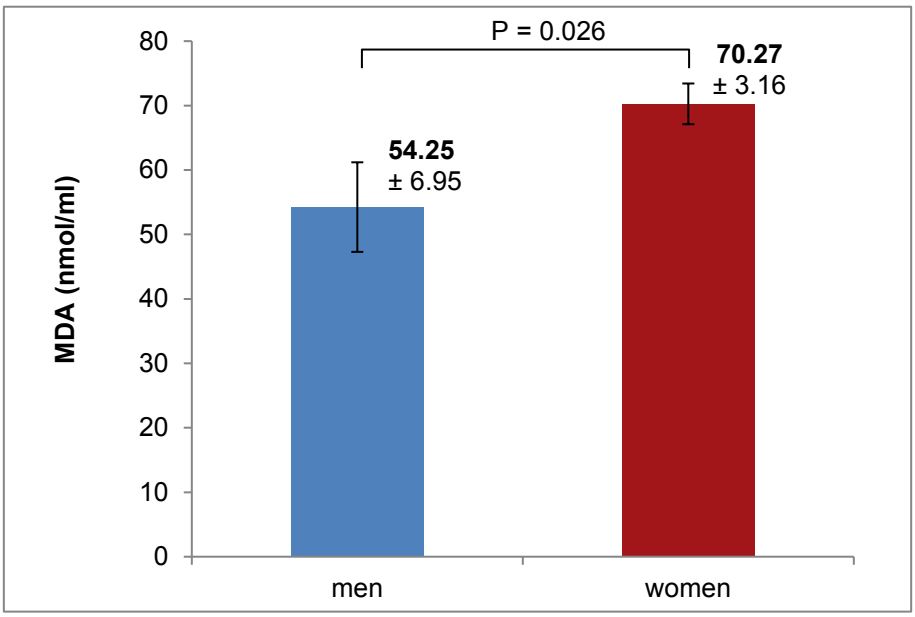

Figure 12. Mean MDA levels (mean ± SEM) in men (n = 8) and in women (n = 21) in the control group

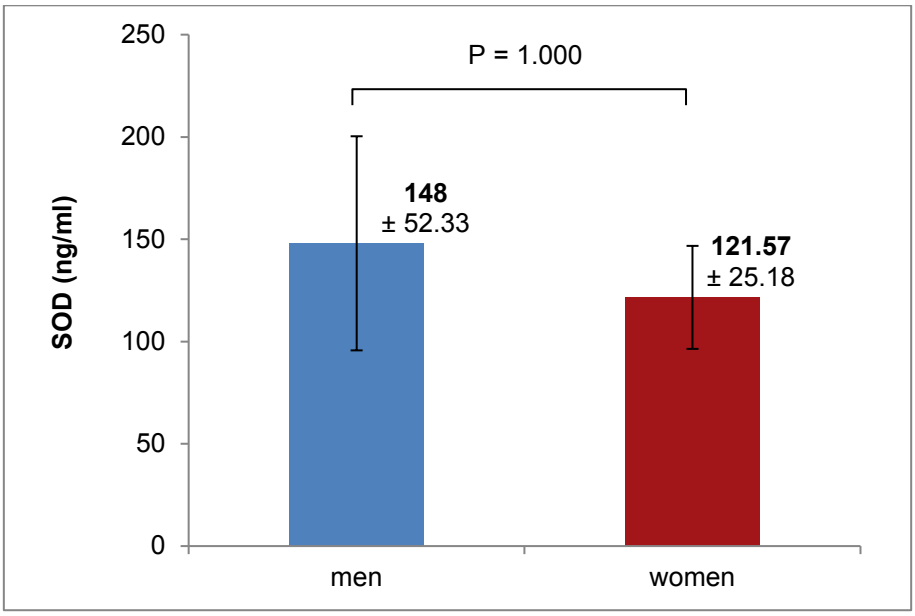

Figure 13. Mean SOD levels (mean ± SEM) in men (n = 8) and in women (n = 21) in the control group

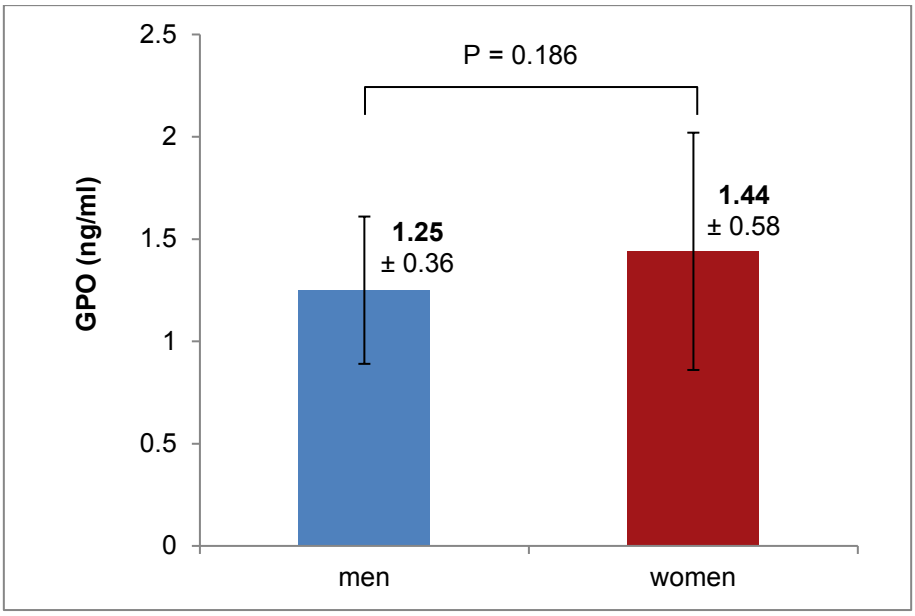

Figure 14. Mean GPO levels (mean ± SEM) in men (n = 8) and in women (n = 21) in the control group

Study of the relationship between oxidative stress indicators, as well as between oxidative stress indicators and metabolic indicators in patients with chronic hepatitis, liver cirrhosis and control subjects

Correlation analysis showed that in patients with chronic hepatitis there were no statistically significant relationships between MDA, GPO, and SOD ($P > 0.05$) and between indicators of oxidative stress on the one hand and metabolic indicators on the other ($P > 0.05$).

In patients with liver cirrhosis, a statistically significant correlation was found between SOD and TG (significant negative dependence, rho = -0.540, $P = 0.002$) (Fig. 15). In this group, no other statistically significant correlations were found between oxidative stress indicators and metabolic indicators with a distribution other than normal ($P > 0.05$) and with those with normal data distribution ($P > 0.05$).

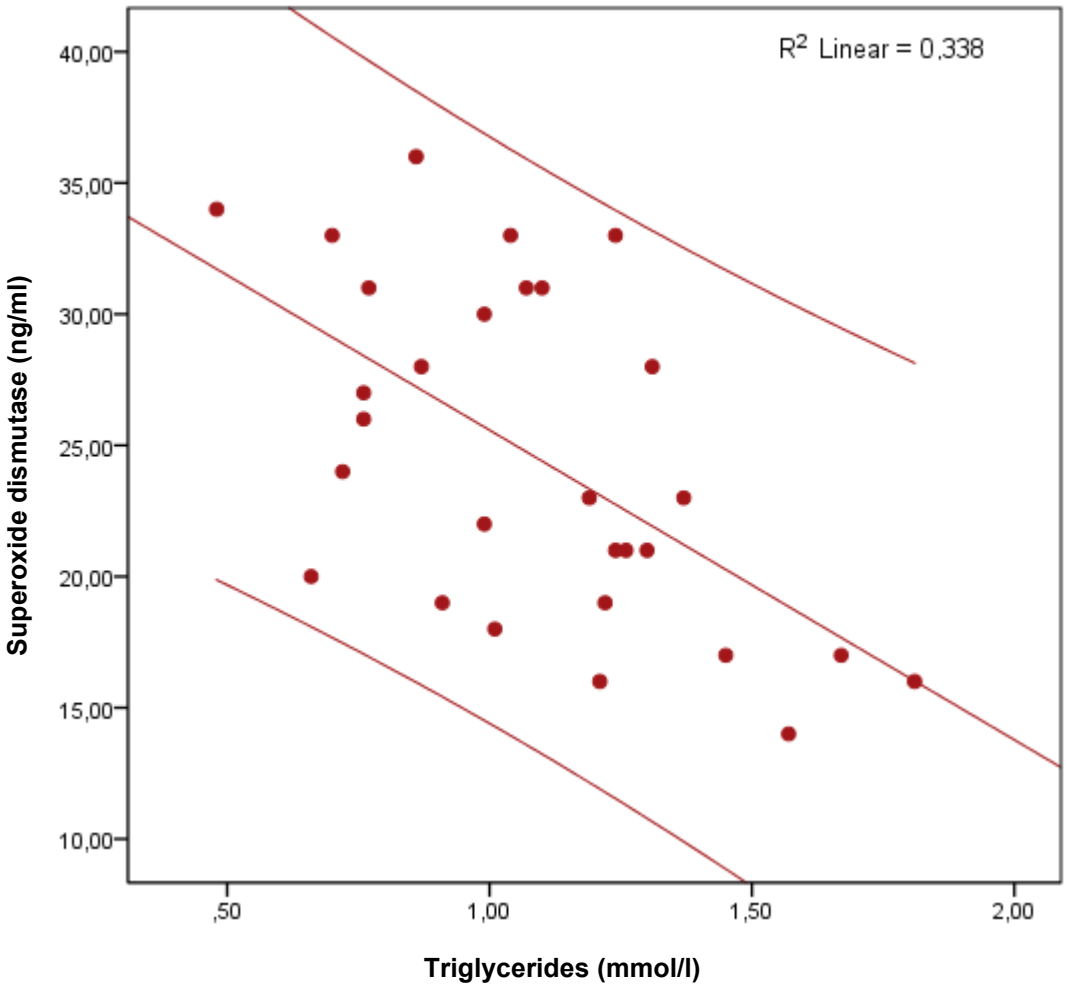

Figure 15. Negative correlation of TG with SOD in patients with liver cirrhosis

In the control group, it was found that of the metabolic parameters with normal distribution, only HDL-cholesterol correlates moderately positively with SOD (Fig. 16).

Data from the correlation analysis between the indicators of oxidative stress and the metabolic indicators with non-Gaussian distribution in the control group are presented in Table 12.

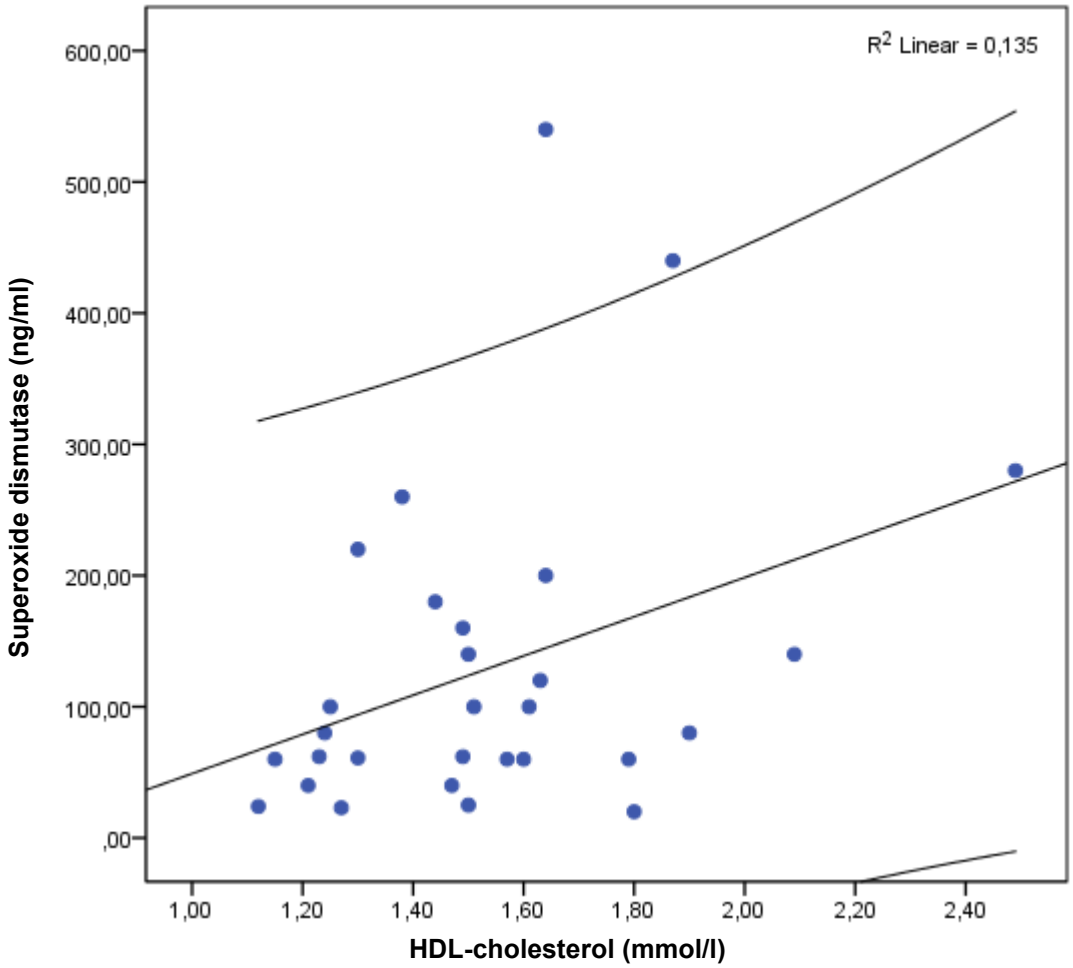

Figure 16. Positive correlation of HDL-cholesterol with SOD in control subjects (r = 0.367, P = 0.050)

Table 12. Correlations between oxidative stress and metabolic parameters with non-Gaussian distribution in control subjects

Parame-ter		MDA	SOD	GPO	TG	Glucose	Insulin	HOMA-IR
MDA	rho P		-0.367* 0.050	-0.478** 0.007	0.116 0.549	0.158 0.412	0.161 0.404	0.165 0.392
SOD	rho P	-0.367* 0.050		0.443* 0.016	-0.315 0.096	-0.410* 0.027	-0.297 0.117	-0.355 0.059
GPO	rho P	-0.478** 0.007	0.443* 0.016		0.035 0.858	-0.067 0.730	-0.016 0.934	0.003 0.987

*The correlation is significant at P < 0.05 (two-tailed test)
**The correlation is significant at P < 0.01 (two-tailed test)

A moderate negative relationship was found between MDA and SOD (Fig. 17) and between MDA and GPO (Fig. 18). SOD correlates moderately positively with GPO (Fig. 19) and moderately negatively with glucose (Fig. 20). No statistically significant correlations of MDA, SOD and GPO with triglycerides, insulin and HOMA-IR were found.

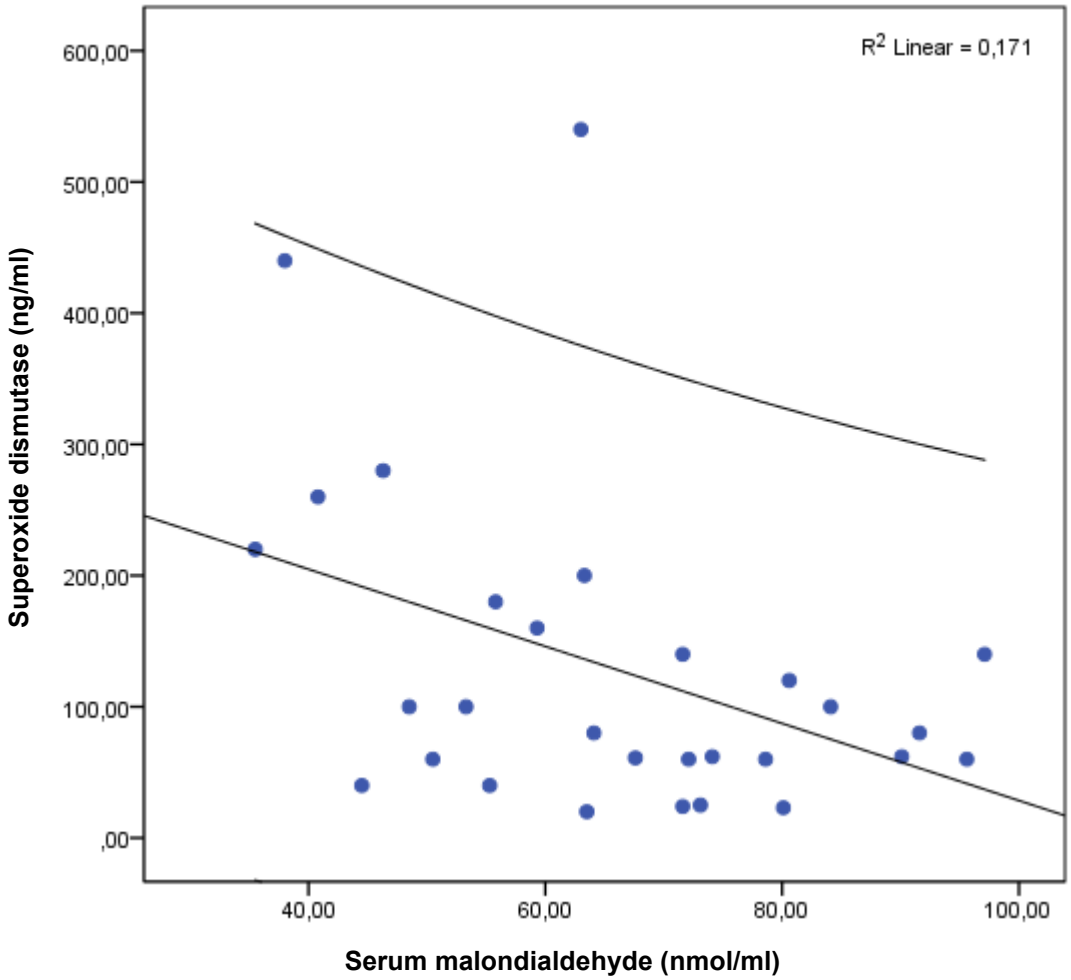

Figure 17. Negative correlation of MDA with SOD in the control group (rho = -0.367, P = 0.050)

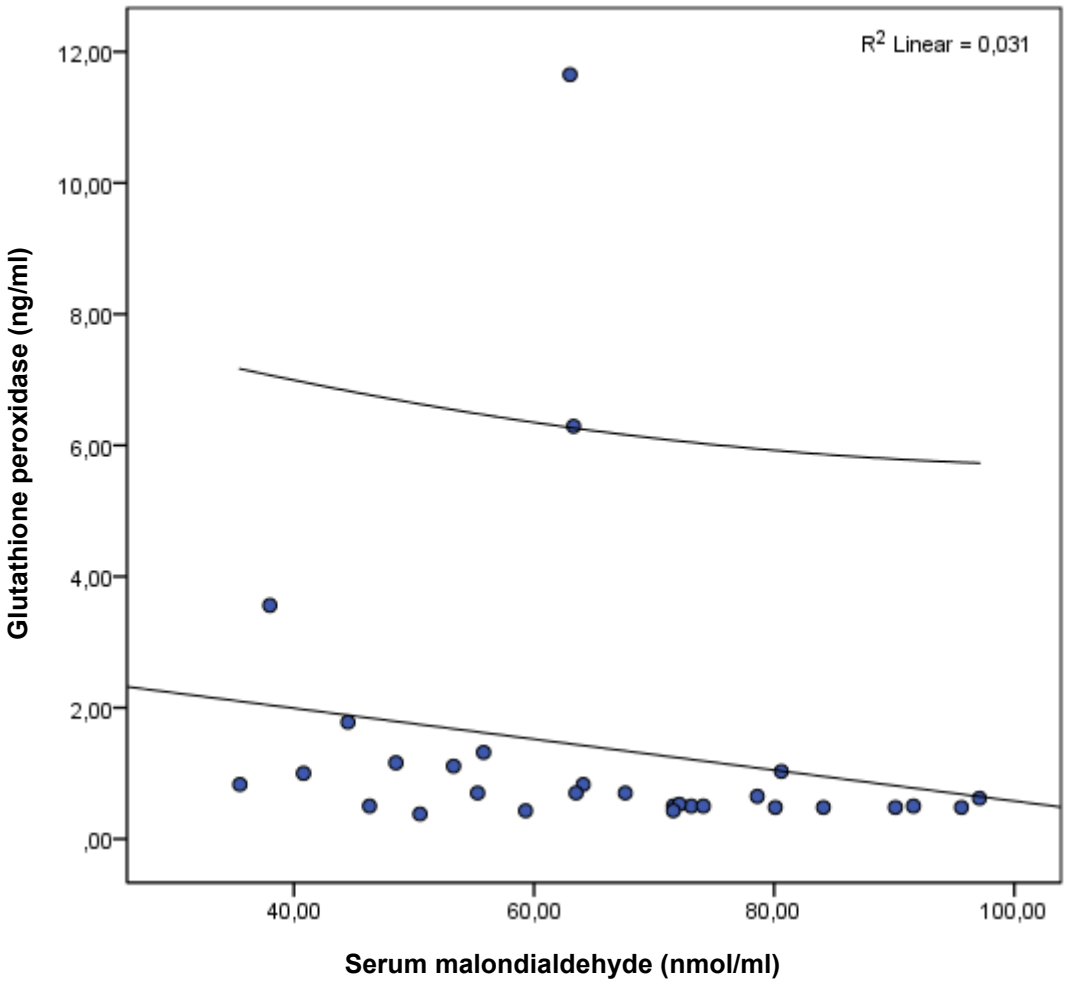

Figure 18. Negative correlation of MDA with GPO in the control group (rho = -0.478, P = 0.007)

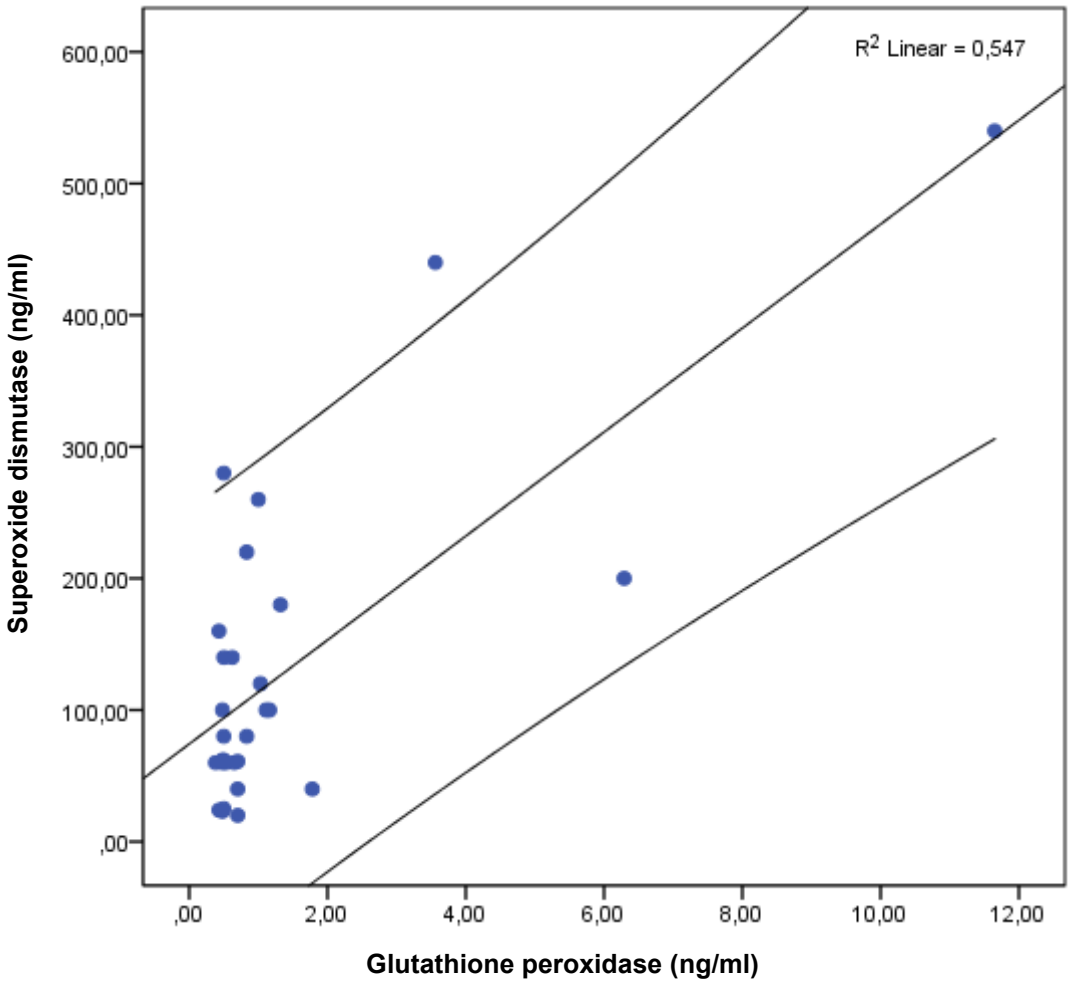

Figure 19. Positive correlation of GPO with SOD in the control group (rho = 0.443, P = 0.016)

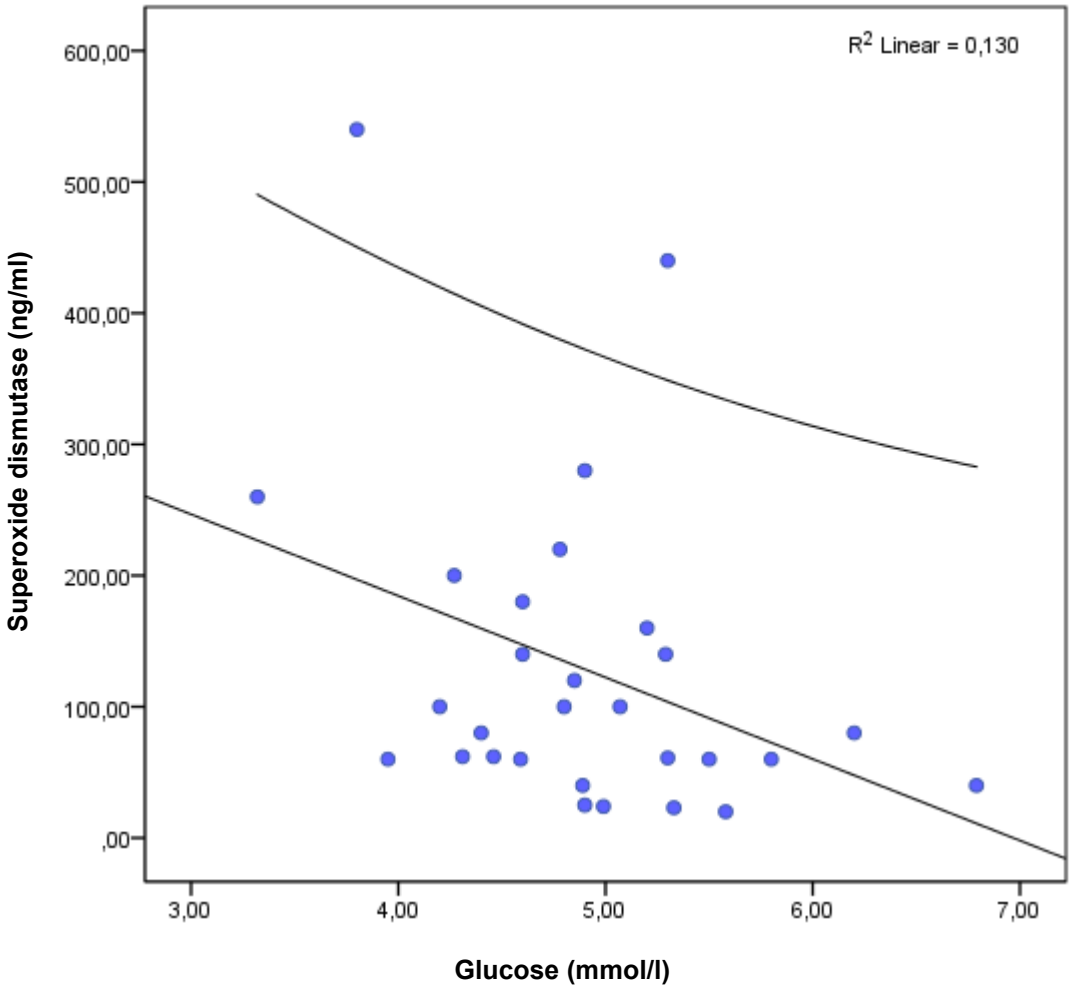

Figure 20. Negative correlation of glucose with SOD in control subjects (rho = -0.410, P = 0.027).

DISCUSSION

1. Selection of appropriate indicators to assess the activity of oxidative stress in patients with chronic liver disease

The problem of the importance of ROSs formed during chronic liver inflammation and their role in its progression has been the subject of more and more in-depth study in the last 10–15 years. It is assumed that the main role in the progression of chronic liver disease has formed during chronic inflammation ROS (Albano, 2006). High concentrations of ROS contribute to hepatocyte necrosis and/or apoptosis. However, partly due to some limitations of existing methods for assessing the state of oxidative stress in vivo in humans, there is currently no conclusive evidence for a link between oxidative stress and pathophysiological mechanisms in a few acute and chronic diseases (Dalle-Donne, 2006).

Difficulties in studying the place and role of free radicals in physiological and pathological processes stem from the various chemical reactions in which they participate and from the various processes of damage associated with their excessive accumulation. This is probably the reason why there are many complex approaches to assessing free radical activity. Scientific studies determine both the total antioxidant capacity as an expression of the cumulative effect of all antioxidants in biological samples and the concentration of individual antioxidants (Alajbeg et al., 2017). There is a wide variety of biomarkers used to assess oxidative stress, the type of biological material in which determinations are made, and methods for quantifying them. More important disadvantages of the used methods for analysis of biomarkers of oxidative and/or nitrosative stress are limited specificity of the method, the measured indicator is not a specific product of a particular ROS or RNS, insufficient sensitivity in determining the concentration of the measured indicator in healthy individuals allows the development of reference intervals, the influence of external factors on the measured concentrations, such as the lipid content of the diet, etc. (Dalle-Donne, 2006).

At present, there is still no indicator or group of indicators to be accepted as the “gold standard” in the measurement of free radicals. It is believed that the three main enzymes that protect cells from free radical overproduction are SOD, catalase, and GPO (Ighodaro et al., 2018). The results of their quantification provide information on the first level of antioxidant protection. Various indirect methods have been developed to measure serum activity or SOD concentration,

in which superoxide radicals bind to detector molecules such as cytochrome C, lucigen, pyrogallol, tetrazolium salt and others (Arauz et al., 2016). GPO can be determined by methods based on the oxidation of glutathione by GPO. For SOD and GPO there are developed commercial ELISA methods on a sandwich basis with very good characteristics of analytical reliability. The ELISA test kits used in the present work have the reproducibility of the results declared by the manufacturer in a series (intraassay reproducibility): CV ≤ 10.7% for SOD and ≤ 4.5% for GPO and in time (interassay reproducibility): CV ≤ 11.9% for SOD and ≤ 8.9% for GPO (Human Cu/Zn SOD Platinum ELISA. Product information & Manual, Human Glutathione Peroxidase 1 ELISA. Product Data Sheet).

To assess the degree of oxidative stress, it is also important to measure indirect indicators of free radical activity, such as the products of lipid peroxidation. MDA is most often used as a reliable marker for the assessment of lipid peroxidation. It is known that MDA have mutagenic, atherogenic and carcinogenic potential, which further emphasizes the need for its monitoring. The concentration of MDA in biological samples (serum, plasma or tissues) is determined by various analytical techniques. The first method for the quantification of MDA in cellular or tissue extracts and in biological fluids (serum, plasma, urine) is based on the reaction between MDA and thiobarbituric acid, in which the resulting color product can be measured spectrophotometrically or fluorimetrically (Young et al., 1991, Fogarasi et al., 2016). A significant problem with this method is its low specificity, which is due to false positive interference from sugars, biliverdin, bilirubin, albumin, sialic acid, glycoproteins, urea, and because aldehydes other than MDA react with thiobarbituric acid (Dalle-Donne, 2006, Young et al., 1991, Fogarasi et al., 2016, Marrocco et al., 2017). An important source of positive interference in these methods is hemolysis (Marrocco et al., 2017). Another group of methods for quantifying MDA includes the use of a few high-resolution techniques such as capillary electrophoresis, gas chromatography with mass spectrometric reading, liquid chromatography, high performance liquid chromatography (Young et al., 1991, Fogarasi et al., 2016, Biondi et al., 2019). Advantages of these methods are high analytical specificity, sensitivity, and reproducibility. At the same time, chromatographic methods require expensive equipment and closely profiled staff. Commercial ELISA test kits have already been developed to quantify MDA in cell or tissue lysates, serum, or plasma. They are based on a two-antibody sandwich assay, with concentrations measured on a calibration

curve. In the present work, a sandwich ELISA method with two antibodies was used – human MDA monoclonal antibody and polyclonal antibody labeled with biotin (MDA ELISA kit, Instruction for use). The intra- and interassay variation coefficient of the results in this test kit is ≤ 8% and – ≤ 12%, respectively. An important advantage of the method is that no cross-reactivity has been found between MDA and other aldehydes. The MDA concentration is measured with photometers that are affordable and easy to operate.

Conclusion: Data on the concentration of key endogenous antioxidant enzymes such as SOD and GPO, which provide the first level of cellular protection against oxidative stress by minimizing endogenous free radical formation and of MDA as an indirect indicator of increasing lipid peroxidation because of free radical damage, provide appropriate information to support the hypothesis of the role of free radicals in pathological processes.

2. Indicators of oxidative stress in patients with chronic hepatitis

According to our data, compared with controls, patients with chronic hepatitis have statistically significant higher MDA (65.85 ± 3.22 nmol/l compared to 157.88 ± 10.31 nmol/l, $P < 0.0001$) and lower SOD (128.86 ± 22.84 ng/ml versus 25.46 ± 1.72 ng/ml, $P < 0.0001$). In patients with chronic hepatitis, the MDA is about 2.4 times higher, and the SOD is about 5 times lower than in healthy controls. The mean concentration of GPO in patients with chronic hepatitis was higher than in healthy controls, but the difference did not reach statistical significance (2.19 ± 0.58 ng/ml versus 1.38 ± 0.43 ng/ml, $P = 0.344$).

Like our results, those from other studies have shown oxidative stress in patients with chronic viral B or C hepatitis, as evidenced by high MDA and compromised antioxidant status. In various studies, data on changes in individual indicators, especially with respect to antioxidant enzymes, are mixed.

Comparison with data from like our study is very difficult due to the use of different methods to determine the indicators of oxidative stress and large differences in the number of subjects, often insufficient. Like our data, statistically significant higher MDA in patients with chronic hepatitis (7 with hepatitis B infection and 18 with hepatitis C) compared to healthy controls ($P = 0.026$) were obtained from Kumar et al. (2013). Unlike us, these researchers did not differentiate between the two study groups in terms of SOD ($P = 0.127$) but found higher GPO activity in subjects with chronic hepatitis ($P = 0.006$). Our data on lower SOD are consistent with data from Yalcin et al. (2020), who in

18 patients with chronic hepatitis C (9 men and 9 women) with a mean age of 47.8 ± 3.9 years found significantly lower plasma SOD activity compared to healthy controls (2.75 ± 0.75 U/mg Prot. versus 3.76 ± 1.53 U/mg Prot., $P < 0.05$), but the activity of GPO was higher (78.25 ± 23.2 U/gHb versus 63.26 ± 6.9 U/gHb, $P < 0.05$). According to some researchers, routine use of MDA is appropriate as a useful indicator to monitor the course of chronic hepatitis C virus infection and to monitor the effect of treatment. In this regard, compared with 28 healthy controls, in 19 patients with chronic hepatitis C Levent et al. (2006) found a higher mean MDA (4.20 ± 1.47 vs. 9.28 ± 1.61, $P < 0.001$) and lower SOD (285.78 ± 96.46 vs. 213.84 ± 71.61, $P < 0.05$) and GPO (8.01 ± 1.79 vs. 6.52 ± 1.86 $P < 0.05$). After 48 weeks of treatment with pegylated interferon alfa-2b in combination with ribavirin, there was a significant decrease in MDA and an increase in SOD and GPO compared to these values in the same patients before treatment ($P < 0.001$ for all parameters).

The pathogenetic mechanisms by which hepatitis C virus infection damages cells are not fully understood (Yalcin et al., 2020). In various intracellular structures of liver cells (mitochondria, endoplasmic reticulum, peroxisomes) various reactive species (superoxide anions, hydroxyl radicals, hydrogen peroxide, nitric oxide, nitrogen dioxide, nitrous oxide, nitrate, etc.) are formed, which, under oxidative stress, either directly affect lipids, proteins, DNA, or induce damage to hepatocytes and other liver cells through fibrosis, apoptosis, or cell necrosis (Rebbani and Tsukiyama, 2016). Impaired oxidant/antioxidant ratio is often due to increased ROS and/or RNS formation and decreased antioxidant protection because of dysregulation of enzyme systems (SOD, GPO, catalase, glutathione transferase, etc.) or decreased antioxidants (albumin, bilirubin, uric acid, glutathione, transferrin, vitamin C, vitamin E, etc.). SOD is known to protect cells from the harmful effects of superoxide radicals, and GPO cleanses hydroperoxides. One possible explanation for the low concentration of SOD in chronic hepatitis is associated with either depletion of the enzyme due to accumulated non-physiological amounts of superoxide anions or impaired secretion of the enzyme by damaged hepatocytes (Yalcin et al., 2020). The change in the activity of even one of the antioxidant enzymes disturbs the balance between them and the effectiveness of their action, which is important both for the progression of the disease and for the treatment of chronic C viral hepatitis. It is believed that the addition of antioxidants to patients' complex therapy would probably be beneficial. The trend towards higher GPO values in our study could be explained by enhanced

enzyme synthesis as an adaptive response to enhanced hydrogen peroxide generation in patients with chronic hepatitis. At the same time, GPO can reduce lipid hydroperoxides, thus seeking to reduce the harmful effects of enhanced lipid peroxidation on liver cells.

Conclusion: According to our study, patients with chronic hepatitis have increased lipid peroxidation and imbalance between two of the major antioxidant enzymes – SOD and GPO. In-depth study of the pathogenesis of chronic hepatitis in the context of enhanced lipid peroxidation and antioxidant defense imbalance as an expression of oxidative stress would be important in developing rational and effective therapeutic approaches.

3. Indicators of oxidative stress in patients with liver cirrhosis

Liver cirrhosis is the final stage of chronic liver disease and often cirrhosis has a viral or alcoholic etiology. Our comparative study included patients with various etiologies of cirrhosis, with the highest relative share of people with ethylic (45%) and post-hepatitis (38%) genesis (Table 1). According to our results, patients with cirrhosis had a significantly higher mean MDA compared to controls (193.74 ± 11.69 nmol/l versus 65.85 ± 3.22 nmol/l, $P < 0.0001$). Patients with cirrhosis have about 2.9 times higher mean MDA compared to healthy controls. The two groups included in the present study differed statistically significant in the concentration of GPO and SOD. Compared with controls, patients with cirrhosis had statistically significantly higher mean plasma GPO concentrations (1.38 ± 0.43 ng/ml versus 3.01 ± 1.24 ng/ml, $P = 0.008$, Fig. 3) and lower serum SOD concentrations (128.86 ± 22.84 versus 24.55 ± 1.20 ng/ml, $P < 0.0001$, Fig. 4). GPO is about 2 times higher in patients with cirrhosis compared to healthy controls.

In the last 10–15 years, data from clinical and experimental studies on the role of oxidative stress have increased, both in the pathogenesis of liver cirrhosis and in the progression of the disease. There were reports of significantly higher serum (Galicia-Moreno et al., 2016) and plasma (Nickovic et al., 2018) MDA in patients with alcoholic cirrhosis compared to healthy controls. Czeczot et al. (2006) compared the concentration of MDA and glutathione, as well as the activity of glutathione-related enzymes (GPO, glutathione reductase, glutathione transferase) in homogenate of three types of human liver tissue – cirrhotic, neoplastic and with preserved structure (control). They found statistically significantly higher MDA, lower glutathione, lower activity of selenium-dependent GPO in cirrhotic and neoplastic tissues compared to controls.

According to these researchers, the results reported show significant changes in liver antioxidant capacity in cirrhosis and hepatocellular carcinoma, increased ROS levels and increased lipid peroxidation.

In our study, all patients had portal hypertension. A major factor in the pathogenesis of portal hypertension is increased hepatic resistance to portal blood flow. At present, the relationship between oxidative stress and portal hypertension in patients with cirrhosis is thought to be poorly understood (Wang et al. 2015) but suppressed hepatic antioxidant protection is known to be a factor that may contribute to oxidative liver damage (Clot et al., 1994). In a few other diseases with impaired blood flow, such as ischemic and hemorrhagic stroke (Valavi et al., 2016) and cardiovascular disease (Kaya et al., 2012), there is evidence of increased lipid peroxidation. According to a study by Wang et al. (2015) plasma MDA was higher in 60 subjects with post-hepatitis cirrhosis compared to 30 healthy controls (673 ± 301 nmol/l versus 288 ± 138 nmol/l, $P < 0.01$). The subdivision of patients into subgroups according to the Child-Pugh classification, the degree of esophageal varices and the width of the portal vein showed a significant increase in MDA depending on the severity of fibrosis and portal hypertension. There are studies in the scientific literature in which, like ours, patients have a mixed etiology of cirrhosis. Studies in this direction by Bhandari et al. (2008) also found statistically significantly higher serum MDA and lower erythrocyte SOD activity in patients with cirrhosis compared to healthy individuals. The subdivision of patients into subgroups according to the Child-Pugh classification shows that the Child C group has statistically significantly higher MDA and lower antioxidant markers than those in Child B class. The researchers concluded that more severe functional damage to the liver is associated with more severe oxidative stress.

Laboratory studies also support the hypothesis of the role of oxidative stress in the pathogenesis of liver cirrhosis with portal hypertension. Increased superoxide anion content was found in rat cirrhosis homogenate because of cyclooxygenase and xanthine oxidase overproduction, decreased nitric oxide content, decreased total SOD activity resulting from decreased cytoplasmic and cytoplasmic forms (Garcia-Sancho et al., 2008). The data obtained indicate the presence of oxidative stress in cirrhotic liver and support the concept that "purification" of nitric oxide from the superoxide anion is likely to be an important determinant of reduced nitric oxide bioavailability, endothelial dysfunction, and increased hepatic vascular tone in cirrhosis. Later Guillaume et al. (2013) reported that the use of recombinant human mitochondrial SOD in

cirrhotic rats reduced portal pressure without significant changes in splanchnicus blood flow, suggesting a reduction in resistance in the hepatic vessels. These data suggest the use of recombinant mitochondrial SOD as adjunctive therapy in the treatment of portal hypertension in cirrhosis. In the scientific literature is presented the modern vision on the use of antioxidants as part of the vasoprotective strategy in the treatment of portal hypertension, as well as to improve the antioxidant capacity of the liver (Vilaseca et al., 2018).

Conclusion: Significantly higher concentrations of MDA in patients with cirrhosis compared to controls are an expression of significant processes of lipid peroxidation with the formation of highly reactive toxic unsaturated aldehydes. The accumulation of lipid peroxides in patients with cirrhosis indicates their role in the disease. The higher plasma concentrations of GPO and lower serum SOD found in our study in patients with cirrhosis indicate an imbalance between these major antioxidant enzymes. The obtained significant changes in MDA, SOD and GPO in patients with cirrhosis compared to those in healthy controls confirm the presence of pronounced oxidative stress.

4. Comparison of oxidative stress indicators in patients with chronic hepatitis and liver cirrhosis

Our comparative study of oxidative stress rates between patients with chronic hepatitis and liver cirrhosis showed that the two groups differed statistically significantly in serum MDA (157.88 ± 10.31 nmol/l versus 193.74 ± 11.69 nmol/l, P = 0.034, Table 10, Fig. 5). The mean difference between the MDA concentrations of these two groups of patients (mean difference ± SE difference) was 35.85 ± 15.74 nmol/ml. MDA is higher in patients with cirrhosis than in patients with chronic hepatitis. There is a tendency for higher GPO and lower SOD in patients with cirrhosis compared to those in patients with chronic hepatitis, but the differences did not reach statistical significance.

In comparative studies assessing oxidative stress in patients with chronic hepatitis and cirrhosis, total antioxidant status (TAC) and total oxidative stress (TOS) are most determined. For example, according to a study by Bolukbas et al. (2005) the groups with chronic hepatitis B (n = 33) and cirrhosis (n = 12) did not differ in TAC (P > 0.05) and total peroxide concentration (P > 0.05). Another more recent and extensive study also compared TOC, TAC, and oxidative stress index (IOC = TOC / TAC) in 145 patients with chronic hepatitis B, 101 patients with cirrhosis, and 50 patients with hepatocellular carcinoma (Feng et al. 2017). There was no statistically significant difference in TAC

when compared patients with chronic hepatitis and cirrhosis (P = 0.152). The two groups differ statistically significantly in TOC (P = 0.021) and in IOC (P = 0.038). However, compared to patients with hepatocellular carcinoma, the TAC of patients with chronic hepatitis and cirrhosis was significantly higher (P < 0.001 and P = 0.001, respectively).

The data from these studies are useful because they give a general idea of the presence of oxidative stress in the studied patients. However, more specific information on changes in the individual factors involved in oxidative stress is provided by their quantification. It makes it possible to analyze the complex relationships between oxidants and antioxidants, as well as between different antioxidants, which is important in view of a more detailed assessment of the clinical condition of the individual patient. Research in this direction by Aksoy et al. (2003) showed significantly higher MDA in individuals with cirrhosis complicated by ascites compared to those with cirrhosis without ascites (21.10 ± 6.97 μmol/l versus 16.52 ± 5.85 μmol/l, P < 0.01). However, in contrast to our study, these researchers did not distinguish between the MDA of the cirrhosis groups with ascites and without ascites on the one hand and the group with different types of chronic hepatitis in general on the other hand (19.20 ± 5.11 μmol/l). The mean concentration of MDA in the three groups with chronic liver disease was significantly higher than in the healthy controls (P < 0.0001 for all groups). Early activation of stellate cells, which are the main source of extracellular matrix by lipid peroxides, is thought to be important in liver fibrosis.

Conclusion: The higher MDA in patients with liver cirrhosis reflects the increased lipid peroxidation processes compared to those in patients with chronic hepatitis. This indicates the presence of more pronounced oxidative stress in patients with cirrhosis, which probably results in the trend in our study towards higher serum concentrations of GPO in these patients. We assume that this is an adaptive response of GPO to neutralize the increasing amounts of hydroperoxides and to influence the increasing lipid peroxidation, striving to reduce it.

5. Influence of gender on metabolic and oxidative stress indicators in patients with chronic hepatitis, liver cirrhosis and in the control group

In the patients we studied, patients with chronic hepatitis and cirrhosis did not show a gender difference in the serum concentration of oxidative stress indicators (Figures 6, 7, 8, 9, 10 and 11) and of metabolic indicators. In the control group, the only difference we found was for serum MDA, which was statistically significant higher in women than in men (70.27 ± 3.16 nmol/ml versus 54.25 ± 6.95 nmol/ml, P = 0.026), Fig. 12.

In the scientific literature, studies examining the influence of factors of biological variation on the concentration of oxidants and antioxidants are not numerous, the results are mixed (Kowalska and Milnerowicz, 2016). According to Japanese researchers, in healthy women (n = 17) and men (n = 12) aged 61 to 80 years, plasma MDA, SOD, GPO, catalase, oxidized and reduced glutathione did not differ statistically significantly (Takahasi et al., 2013). According to Polish researchers, when comparing 74 women and 35 men aged 20 to 25 years, MDA and GPO do not differ, and SOD is statistically significantly higher in men (Kowalska and Milnerowicz, 2016). According to Mao et al. (2019) in 2223 persons (997 men and 1226 women) aged 65 to 100 years, the mean plasma activity of SOD in men (56.18 U/ml) is lower than that of women (58.50 U/ml), P < 0.001. Men and women did not differ in mean MDA concentration (P = 0.24).

Like our data on higher MDA in control women than in men, an extensive study reported the role of demographic, physical, plasma, and dietary factors in lipid peroxidation in 298 healthy individuals aged 19 years up to 78 years (Block et al., 2002). Significantly higher plasma concentrations of MDA and F2 – isoprostanes were found in women (n = 177) compared to men (n = 121), P < 0.0001. One possible explanation for the significantly higher lipid peroxidation in women, according to these researchers, is the high percentage of body fat in women, but their exact determination in this study has not been performed. Another extensive study examined the biological variation of SOD and GPO in 1863 clinically healthy individuals aged 4 to 97 years and found no gender difference in plasma activity of these parameters (Gulmouri et al., 1991). According to this study, factors such as body weight, blood pressure and menopause do not affect the enzymatic activity of these antioxidants, SOD is influenced by smoking, and GPO – by alcohol consumption.

In our study on the influence of gender, we did not find differences in metabolic and oxidative stress indicators in the two pathological groups. In a

study from 2020 (Zelber-Sagi et al.) was determined MDA in 394 people with non-alcoholic steatosis (185 women and 209 men) and found a significant difference between men and women (14.37 ± 6.50 nmol/l compared to 17.89 ± 9.79 nmol/l, respectively, $P < 0.001$). Some researchers have studied the impact of a number of other factors, such as past pregnancies, oral contraceptives, menopause, and hormone replacement therapy, on the progression of liver fibrosis in 157 women with chronic hepatitis C (Martino et al., 2004). Menopause has been shown to be an independent predictor of fibrosis progression, oral contraceptives have no significant effect on it, and past pregnancies and hormone replacement therapy have had a beneficial effect on fibrosis. It is assumed that in the long run estrogens are likely to have a protective effect on histopathological lesions in women with chronic hepatitis C.

Conclusion: In our study, we found a gender difference only in terms of MDA in the control group, which is higher in women. Men and women included in this group did not differ statistically significantly by age, but we have not analyzed the influence of other factors on oxidative stress indicators. Currently in the scientific literature there are assumptions about the influence of various factors on the indicators of oxidative stress – age, BMI, body fat percentage, smoking, cholesterol, C-reactive protein, estrogen, etc. For now, the data on the influence of gender on the indicators of oxidative stress are mixed and this does not allow to draw definitive conclusions.

6. Analysis of the relationship between oxidative stress and metabolic parameters in patients with chronic hepatitis, liver cirrhosis and the control group

Experimental (Ahmadvand et al., 2014) and clinical (Dworzański et al., 2020) studies in conditions associated with insulin resistance show the presence of altered antioxidant capacity. For the purposes of these studies, various biological samples (liver, kidney, serum, plasma, etc.) were used, there are some inconsistencies in the results obtained, which makes it difficult to compare the data. According to some studies, SOD and GPO have reduced serum activity in patients with type 2 diabetes compared to the control group without type 2 diabetes (Dworzański et al., 2020). In other studies, elevated Cu/Zn SOD and GPO were found in patients with type 1 or type 2 diabetes compared with healthy controls (Moussa, 2008, Salman, 2018). An in-depth and critical view of the most commonly used circulating biomarkers for assessing oxidative stress and the antioxidant defense system in patients with pre-diabetes, type 2 diabetes

and its complications was presented by Bigagli and Lodovici (2019). It is clear for him that there are many studies in this area, but the results are mixed, especially with regard to changes in antioxidant protection, often not taking into account the influence of certain pre-analytical factors such as potential gender differences or drug interference which have an antioxidant effect, such as statins, ß-blockers, etc. However, account is taken of the fact that the enhanced oxidative damage that accompanies hyperglycemia could be considered as a potential target in the treatment of affected patients, which would help to individualize it.

We analyzed the relationships between oxidative stress indicators on the one hand and between oxidative stress indicators and metabolic indicators on the other hand in the control group and in the groups with chronic liver disease. In the control group, MDA correlated negatively with SOD (P = 0.050) and GPO (P = 0.007), and SOD and GPO correlated positively with each other (P = 0.016). In addition, SOD correlated significantly and positively with HDL cholesterol (P = 0.050) and significantly and negatively with glucose (P = 0.027). There is a marginal significance of the relationship between SOD and HOMA-IR (P = 0.059). These relationships were not confirmed in the groups with chronic hepatitis and cirrhosis. In patients with chronic hepatitis and cirrhosis, no statistically significant correlations were found between MDA, SOD and GPO. In the group with chronic hepatitis, we did not find statistically significant correlations between oxidative stress indicators and metabolic indicators. In this group, we found a marginal significance of the relationship between SOD and insulin (P = 0.076). In the cirrhosis group, we found a significant negative correlation of SOD with triglycerides (P = 0.002) and marginal significance of the relationship between SOD and total cholesterol (P = 0.074) and between GPO and HDL-cholesterol (P = 0.063).

In our study in healthy individuals, we did not find a significant correlation between MDA and lipid parameters, but in a study by Block et al. (2002) found a positive correlation of MDA with total cholesterol and C-reactive protein in 298 healthy individuals aged 19 to 78 years, and BMI and race were associated with isoprostanes.

An extensive 2010 study analyzed data from two prospective cohort studies conducted in Scotland over a period of 42 years, involving a total of 9559 men with a mean age of 47 ± 3 years, in order to determine whether the combined effect of high alcohol consumption and high BMI increase the risk of liver disease (Hart et al., 2010). The analysis of the data showed that each of these

factors individually increases the risk of liver disease and there is an increase in the effect of their combined action. Two considerations are commented in support of this conclusion. On the one hand, obesity leads to the development of steatohepatitis by affecting hepatic insulin sensitivity. On the other hand, alcoholic fatty liver induces peripheral insulin resistance and thus promotes obesity.

Factors other than those associated with insulin resistance appear to be relevant to levels of oxidative stress in chronic liver disease. A link was found between SOD and MDA on the one hand and plasma endotoxin concentration on the other (Chen et al., 2013). A group of 94 patients with chronic hepatitis (90 with chronic hepatitis B and 4 with chronic hepatitis C) with a mean age of 35 years was studied. In 60 of them, endotoxinemia was found. SOD and MDA of persons with and without endotoxinemia were compared and patients with endotoxinemia were found to have higher MDA (6.28 ± 1.52 nmol/ml versus 4.50 ± 1.09 nmol/ml) and lower SOD (85.43 ± 23.10 NU/ml versus 120.0 ± 52.38 NU/ml), $P < 0.001$ for both indicators. A statistically significant positive correlation was found between plasma endotoxin and MDA and negative correlation with SOD, indicating an association between endotoxinemia and antioxidant capacity.

At the cellular level, ultrastructural mitochondrial lesions, decreased activity of respiratory chain complexes, and impaired ability to synthesize ATP are found in the liver tissue of patients with alcoholic and nonalcoholic liver diseases (Mansouri et al., 2018). Increased lipogenesis and suppressed β-oxidation of fatty acids lead to the accumulation of triglycerides in hepatocytes, which together with increased levels of ROS contributes to insulin resistance in patients with steatohepatitis. Mitochondrial ROS disrupts metabolic signaling pathways, which affects the development and progression of chronic liver disease. Mitochondrial stress and lesions contribute to cell death, liver fibrogenesis, inflammation, and immune responses to viral infections. Experimental in vitro studies have shown that treatment of rat hepatoma cell culture with free fatty acids increases intracellular triglycerides, induces steatosis and stress of the endoplasmic reticulum (Ota et al., 2008). Lipid-induced endoplasmic reticulum stress affects apo-B100 secretion. It changes dose-dependently, as the high lipid load suppresses it. These data were also confirmed in vivo by intravenous infusion of oleic acid in mice. The data obtained suggest that elevated lipids in the liver cause stress to the endoplasmic reticulum, limited secretion of apoB and triglycerides, which worsens steatosis.

Conclusion: Correlation analysis of data on oxidative stress and metabolic parameters in controls, as expected, showed a negative correlation of MDA with SOD and GPO, a positive correlation between the two antioxidant enzymes, and SOD correlated positively with HDL-cholesterol and negatively with glucose. Neither of these correlations was confirmed in the two groups with chronic liver disease. In the group with liver cirrhosis, there was a statistically significant negative association of SOD with triglycerides. In the groups with chronic liver disease, we did not find other significant relationships between oxidative stress indicators and metabolic indicators. We assume that probably not only the metabolic factors analyzed by us, but also other factors affect the concentration of MDA, SOD and GPO in the blood.

CONCLUSIONS

1. Elevated serum MDA and decreased SOD in patients with chronic hepatitis indicate the presence of oxidative stress, which has led to an enhanced process of lipid peroxidation and depletion of SOD. The unchanged concentration of GPO shows partially preserved antioxidant capacity in these patients.
2. In patients with cirrhosis of the liver, in addition to high MDA and low SOD, a high concentration of GPO was found. Changes in two of the major antioxidant enzymes that directly eliminate reactive oxygen species are likely to compensate for the body's response to increasing oxidative stress. However, it is not sufficient to suppress lipid peroxidation processes.
3. Patients with liver cirrhosis differ from patients with chronic hepatitis in statistically significant higher MDA. This is an expression of the more pronounced processes of lipid peroxidation in the group with liver cirrhosis.
4. In patients with chronic hepatitis and liver cirrhosis, the sex factor does not have a statistically significant effect on the concentrations of the metabolic parameters and oxidative stress indicators studied by us.
5. Gender differences were found only in the serum MDA in the control group, which is higher in women. We assume that the higher number of women included in this group is important for this result.
6. In patients with liver cirrhosis, SOD and triglycerides interact with each other in the complex process of developing insulin resistance. An expression of this interaction is the statistically significant negative correlation between serum concentrations of SOD and triglycerides.
7. In the control group, the positive correlation between SOD and GPO confirms the complex action of the two antioxidant enzymes in the disposal of free radicals.
8. The negative correlation between the two antioxidant enzymes and MDA in the control group shows the joint protective action of SOD and GPO against the effects of lipid peroxidation.
9. In the control group, the antioxidant enzyme SOD showed correlations with glucose and HDL-cholesterol. These associations have not been confirmed in patients with chronic hepatitis and liver cirrhosis.

CONTRIBUTIONS TO THE DISSERTATION

Contributions of scientific and theoretical nature

1. For the first time in our country a comprehensive clinical study on the processes of lipid peroxidation and antioxidant protection was conducted to establish the role of oxidative stress in patients with chronic liver disease of varying severity.
2. A change in the process of lipid peroxidation has been demonstrated in patients with chronic liver disease, which is more pronounced in patients with cirrhosis of the liver with portal hypertension than in patients with chronic hepatitis.
3. For the first time in Bulgaria the influence of the sex factor on the concentrations of MDA, SOD and GPO in patients with chronic liver disease and in a control group of clinically healthy individuals from the Bulgarian population was studied.
4. The conducted clinical study helps to clarify the relationship between deviations in the indicators of oxidative stress MDA, SOD and GPO and metabolic disorders in patients with chronic liver disease associated with insulin resistance.
5. For the first time in our country the connection between the indicators of oxidative stress MDA, SOD and GPO and the metabolic indicators in clinically healthy individuals from the Bulgarian population was analyzed.

Contributions of scientifically applied nature

1. The data obtained on the changes in the antioxidant enzymes SOD and GPO can serve as a basis for further in-depth studies related to the individualization of therapy in patients with chronic liver disease.
2. With the help of ELISA methods for the first time in our country the concentrations of serum MDA as a marker of lipid peroxidation and of the antioxidant enzymes SOD and GPO in patients with chronic liver disease have been studied.

List of publications and scientific communications in connection with the dissertation

I. Publications in periodicals

1. ***Dimitar Terziev.*** Relationship between antioxidant enzymes and insulin resistance in patients with chronic liver disease. 15th International Scientific Conference "Knowledge in practice", Bansko, Bulgaria, 15-17 December, 2017. Knowledge-International Journal Scientific Papers, vol. 20.4, december, 2017; pp: 1997-2002
2. ***D. Terziev***, D. Terzieva, Vl. Andonov. The role of oxidative stress in liver pathology. 16th International Scientific Conference "Knowledge without borders", 16-18.03.2018, Vranjacka banja, Serbia. Knowledge-International Journal Scientific Papers, vol. 22.5, march, 2018; pp:1403-1406
3. ***D. Terziev***, D. Terzieva, Vl. Andonov, N. Mateva. Serum concentration of some antioxidant enzymes in patients with liver cirrhosis. Scientific works of the Union of Scientists in Bulgaria-Plovdiv. Series G. Medicine, Pharmacy, Dentistry, Volume XXIV, 2020, pp. 150-153
4. ***Dimitar Terziev***, Dora Terzieva, Vladimir Andonov, Mitko Mitkov, Nonka Mateva. Association of biomarkers of oxidative stress and insulin resistance in patients with cirrhosis. Mag. "General Medicine", Issue 3/2022 (Under press)

II. Participation in national and international forums

1. ***Dimitar Terziev***, Dora Terzieva, Vl. Andonov. Serum levels of malondialdehyde in patients with chronic liver disease. The 26th meeting of the Balkan Clinical laboratory Federation. The 6th National Congress of the Macedonian Association of Medical Biochemistry and Laboratory Medicine, October 03th-05th, 2018; Skopie, Macedonia. Balkan Journal of Clinical Laboratory – XXVI, 18,1 (Scientific Program, Topic Varia): pp87; P058
2. ***D. Terziev***, D. Terzieva, V. Andonov, N. Mateva. Investigation of serum malondialdehyde in patients with chronic viral hepatitis. EuromedLab 2019-23rd IFCC-EFLM Euripean Congress of Clinical Chemistry and Laboratory Medicine, May 19-23. 2019, Barcelona, Catalonia, Spane. Clinica Chimica Acta, Special Issue/ Abstracts, vol. 493S1, June 2019; S355-S378: T394. **IF 2019: 2.615**
3. ***D. Terziev***, D. Terzieva, V. Andonov, N. Mateva. Serum levels of glutathione peroxidase and superoxide dismutase in patients with chronic viral hepatitis. EuromedLab 2019-23rd IFCC-EFLM Euripean Congress of Clinical Chemistry and Laboratory Medicine, May 19-23. 2019, Barcelona, Catalonia, Spane. Clinica Chimica Acta, Special Issue/ Abstracts, vol. 493S1, June 2019; S355-S378: T395. **IF 2019: 2.615**
4. ***D. Terziev***, Vl. Andonov, M. Mitkov, D. Terzieva, N. Mateva. Investigation of relationship between some antioxidant enzymes activity and insulin resistance in chronic liver disease patients. Folia medica 2020; vol. 62 (1):125-126