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Relationship between ultrasonographic liver steatosis degree and oxidative/nitrosative stress in patients diagnosed with metabolic dysfunction-associated steatotic liver disease

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ABSTRACT

Aims: Metabolic dysfunction-associated steatotic liver disease (MASLD) remains the most common chronic liver disease worldwide. It is considered to be a complication of metabolic syndrome. The main element in intra- and extrahepatic disorders in MASLD is oxidative/nitrosative stress (ONS). The relationship between the increase and decrease in these markers and the degree of liver steatosis defined sonographically has not been specifically studied before.

Methods: Patients in the MASLD spectrum were divided into 3 groups according to the degree of liver steatosis on ultrasonography (US). Patients without liver steatosis on US were taken as the control group. Nitric oxide (NO), malondialdehyde (MDA), catalase (CAT) and superoxide dismutase (SOD) were studied in the blood of these patients.

Results: Changes in the degree of liver steatosis on US and changes in the studied parameters were found to be statistically significant. In addition, the cut-off values of NO and MDA were shown to be 8.98 and 2.375, respectively, in distinguishing the healthy control group from the patient group.

Discussion: As the degree of liver steatosis increases on US, NO and MDA levels increase, while antioxidant enzymes CAT and SOD levels decrease. NO and MDA can be used to distinguish healthy and patient groups in the preliminary diagnosis of MASLD.

Conclusion: There is a significant relationship between the degree of liver steatosis on US and ONS parameters.

Keywords: Oxidative/nitrosative stress, metabolic dysfunction-associated steatotic liver disease, ultrasonography

INTRODUCTION

Metabolic dysfunction-associated steatotic liver disease (MASLD) remains the most common chronic liver disease worldwide.[1] MASLD is defined as the accumulation of triglycerides in more than 5% of hepatocytes in individuals who do not consume significant alcohol.[2] In this process, simple steatosis may be present, and it may also progress to steatohepatitis, fibrosis, cirrhosis, and hepatocellular carcinoma. Approximately one-fourth of simple steatosis develops steatohepatitis, while more than one-fourth of patients with steatohepatitis develop significant fibrosis.[3] MASLD can sometimes be a symptom of an underlying disease. However, it is not a disease in itself. And MASLD is not a single disease but encompasses a number of diseases.[4] MASLD is considered a complication of metabolic syndrome because it is associated with hypertension, obesity, insulin resistance and dyslipidemia.[2,5] MASLD does not only affect the liver. The main intra-and extrahepatic complications associated with MASLD include portal hypertension, sarcopenia, cirrhotic cardiomyopathy, hepatorenal syndrome, hepatic encephalopathy, and peripheral neuropathy.[6,7]

The main element in all these disorders is oxidative stress.[8] Under normal conditions, there are antioxidant systems that protect cells from damage by neutralizing oxidative species.[9] Free radicals are atoms or molecules that are

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unstable and reactive. There are two types of free radicals: oxygen-derived (ROS) and nitrogen-derived (RNS).[10] Oxidative/nitrosative stress (ONS) is an imbalance in favor of an increase in ROS/RNS. It is known that ONS is involved in a series of diseases, including liver diseases.[11] The most well-known of the RNS is nitric oxide (NO). Much evidence has been shown that NO plays important physiological and pathological roles in the liver. Among the antioxidant systems, superoxide dismutase (SOD) and catalase (CAT) are important enzymatic antioxidants.[10] ONS damages cellular elements and impairs their functions and contributes to the pathophysiology of many chronic diseases, including MASLD.[12,13] When reactive oxygen species increase, they can consume antioxidant molecules and inhibit antioxidant enzymes such as SOD. As a result, antioxidant systems are reduced in blood, serum, plasma and liver.[14] It has been shown that antioxidant capacity in liver cells is reduced in MASLD patients.[15]

Ultrasonography (US) is a cheap, noninvasive and easily accessible imaging method. US is the most commonly used method in the diagnosis of hepatosteatosis (82-89% sensitivity and 93% specificity). However, if hepatosteatosis is mild or the patient is obese, its sensitivity drops below 30%. Grading (Grade 1-2-3) is also performed with US. Although it was previously thought that this grading had no clinically proven importance and was only frequently used in practice to follow the disease,[16] later studies were conducted showing the correlation of this grading with liver function tests.[17]

Many relationships have been shown in the literature between MASLD and ONS, as mentioned above. This process is still a subject of research. In particular, noninvasive criteria that will contribute to the process will be even more important. In the light of this information, we wanted to shed light on a topic that has not been addressed in the literature. We investigated the relationship between the degree of ultrasonographic liver steatosis and ONS parameters.

METHODS

Ethical Approval And Informed Consent

Ethics committee approval was obtained for this study from the Kahramanmaraş Sütçü İmam University Faculty of Medicine Local Ethics Committee (Date: 13.12 2022, Decision No: 06). The study was conducted in accordance with the principles of the Declaration of Helsinki. Informed consent was obtained from patients in the patient group and control group.

Study Design

After obtaining ethics committee approval, the study was conducted prospectively. Informed consent was obtained from patients who met the inclusion criteria. Patients with exclusion criteria were not included in the study from the beginning. After the US procedure, blood samples taken from the patients were examined in the laboratory. The results were analyzed statistically.

Patients

The patient and control groups were selected from patients over the age of 18. The groups were planned to be close in number in terms of gender factor.

Ultrasound Imaging

US examination to assess the degree of liver steatosis was performed after a minimum of 8 hours of fasting. The patient was assessed in the supine position. All US examinations were performed by an experienced radiologist. All examinations were performed using a Canon Aplio a ultrasound device (Canon Medical Systems Corporation, Tokyo, Japan) with a convex probe (Multi-Frequency Slim Face Convex). The criteria we used to determine whether there is liver steatosis or to grade steatosis on US are shown in **Table 1**.

Table 1. Parameters we used to evaluate the degree of liver steatosis in US. PV; portal vein

	Degree of liver steatosis			
Sonographic parameter	**Normal**	**Grade 1**	**Grade 2**	**Grade 3**
Liver echo compared to spleen echo	Darker	Similar	Brighter	Brighter
PV wall echogenicity distinction can be made	Yes	No	No	No
Deep spaces inside the liver can be seen	Yes	Yes	No	No
Diaphragm can be seen	Yes	Yes	Yes	No

Laboratory Parameters

Blood samples were taken from the cases included in the study. These blood samples were centrifuged at 3000 g (relative centrifugal force) for 10 minutes at 4°C to separate plasma and aspirate buffy coat. Erythrocytes were washed 4 times with cold physiological saline and stored at -80°C until the day of analysis. CAT activity in erythrocytes was measured in samples using the method described by Beutler.[18] The dissociation of the H_2O_2 substrate was monitored spectrophotometrically at 240 nm. CAT activity was recorded as Ug/Hb. The method described by Fridovich was used to estimate SOD activities in erythrocytes.[19] Ug/Hb was used to express SOD activity. Lipid peroxidation level was expressed as MDA.[20] MDA levels were expressed as nmol/mL. NO levels in plasma samples were determined with a "sandwich" enzyme-linked immunosorbent assay kit (NO catalogue number MBS2540417 mybiosource elisa kit, USA) according to the manufacturer's protocol. NO levels were expressed as μmol/L.

Exclusion Criteria

Patients with known alcohol use, steatogenic drug use, hepatitis (viral, autoimmune), primary biliary cirrhosis,

alcohol-related liver disease, drug or toxin-related liver disease, liver fibrosis, hypertension, coronary atherosclerotic disease, diabetes mellitus, malignancy, and patients with hypertriglyceridemia in the blood picture were excluded from the study. In addition, patients with any space-occupying lesion in the liver during sonographic examination were excluded from the study. Also, cases with liver steatosis but with significant heterogeneity of steatosis were not included in the study to avoid grading errors.

Statistical Analysis

The conformity of quantitative variables to normal distribution was examined with the Shapiro-Wilk test. Group comparisons for variables not showing normal distribution were performed with the Kruskal-Wallis H test. Dunn-Sidak test was applied for post hoc (pairwise comparisons). Relationships between quantitative variables were examined with the Spearman correlation test. The performance of variables in diagnostic tests was examined with ROC analysis. Statistical significance was accepted as $p<0.05$. Statistical parameters were expressed as Median, (q1-q3), r (correlation coefficient). IBM SPSS version 22 (IBM SPSS for Windows version 22, IBM Corporation, Armonk, New York, United States) program was used in the evaluation of the data.

RESULTS

Analyses were conducted with 119 patients who agreed to participate in the study after the patients were excluded by the exclusion criteria. Demographic data of our patients are shown in **Table 2**.

Table 2. Demographic characteristics of our patients according to groups

Degree of liver steatosis	Number and percentage of patients	Age range	Male-female
0	28-(23.5)	19-64	15-13
1	36- (30.3)	20-61	17-19
2	31-(26.1)	19-59	17-14
3	24-(20.2)	20-63	12-12
	119-(100)	19-64	61-58

The significance study of our ONS parameters between groups is shown in **Table 3**. Each of the studied parameters was found to be statistically significantly different between the groups.

When the CAT values of the groups were examined, the median value of the Control group was 17.48 Ug/Hb, the median value of the Grade 1 group was 16.21 Ug/Hb, the median value of Grade 2 was 11.28 Ug/Hb, and the median value of Grade 3 was 9.90 Ug/Hb (**Table 3**).

When the SOD values of the groups were examined, the median value of the control group was 579.41 Ug/Hb, the median value of the Grade 1 group was 486.79 Ug/Hb, the median value of Grade 2 was 511.23 Ug/Hb, and the median value of Grade 3 was 402.03 Ug/Hb (**Table 3**).

When the MDA values of the groups were examined, the median value of the Control group was 2.24 nmol/ml, the median value of the Grade 1 group was 2.31 nmol/ml, the median value of Grade 2 was 2.56 nmol/ml, and the median value of Grade 3 was 3.98 nmol/ml (**Table 3**).

When the NO values of the groups were examined, the median value of the Control group was 8.97 U/ml, the median value of the Grade 1 group was 11.14 U/ml, the median value of Grade 2 was 14.18 U/ml, and the median value of Grade 3 was 14.78 U/ml (**Table 3**).

In addition, the evaluation results for each parameter are shown in figures (**Figure 1**). The NO variable can make a statistically significant distinction between sick and healthy individuals. The value of 8.95 is the cut-off point for sick and healthy individuals. NO can distinguish sick and healthy individuals with high sensitivity and accuracy. (**Figure 2**).

The MDA variable can make a statistically significant distinction between sick and healthy individuals. The value of 2.375 is the cut-off point for sick and healthy individuals. MDA can distinguish sick and healthy individuals with high sensitivity and specificity.

Table 3. Kruskal Wallis H test; a: 0.05; Post-Hoc: Dunn Sidak test; * the difference between the groups is statistically significant; a the difference with the control group is significant; b the difference with the grade 1 group is significant; c the difference with the grade 2 group is significant; d the difference with the grade 3 group is significant

	Group				
	Control	Grade 1	Grade 2	Grade 3	p
CAT Ug/Hb, median (Q1-Q3)	17.48 (14.07-19.35)^{c,d}	16.21 (12.09-20.12)^{c,d}	11.28 (9.04-17.13)^{a,b}	9.90 (9.31-10.40)^{a,b}	p<0.001*
SOD U/gHb, median (Q1-Q3)	579.41 (512.38-771.77)^{c,d}	486.79 (431.24-633.27)^{d}	511.23 (435.34-537.14)^{a,d}	402.03 (351.78-432.71)^{a,b,c}	p<0.001*
MDA nmol/ml, median (Q1-Q3)	2.24 (2.19-2.33)^{c,d}	2.31 (2.26-2.44)^{c,d}	2.56 (2.53-2.61)^{a,b}	3.98 (3.22-4.39)^{a,b}	p<0.001*
NO U/ml, median (Q1-Q3)	8.97 (7.69-10.68)^{b,c,d}	11.14 (10.15-14.11)^{a,d}	14.18 (11.09-15.74)^{a}	14.78 (13.59-16.47)^{a,b}	p<0.001*

CAT: Catalase, SOD: Superoxide dismutase, MDA: MDA: Malondialdehyde, NO: Nitric oxide

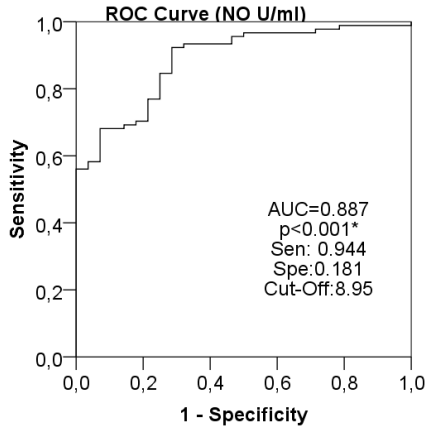

Figure 1. Differences between groups in catalase enzyme

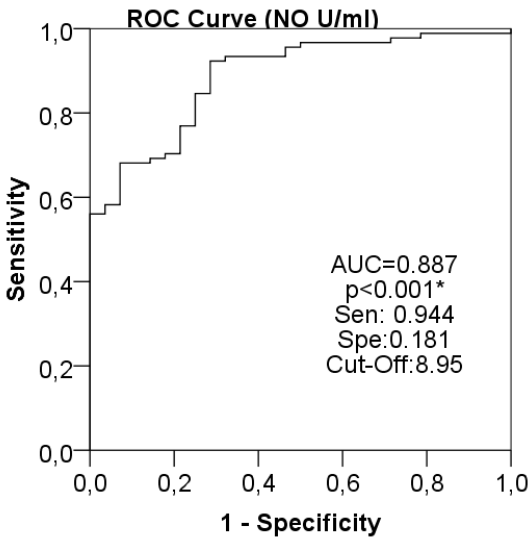

Figure 2. Determination of the cut-off point of nitric oxide values in the patient and control groups.

No statistically significant correlation was found between NO and CAT, SOD and MDA values for each group (**Table 4**).

Table 4. Spearman correlation test; a: 0.05

Group		CAT Ug/Hb		SOD U/gHb		MDA nmol/ml	
		r	p	r	p	r	p
Control	NO U/ml	-0.117	0.554	-0.077	0.696	-0.101	0.611
Grade 1	NO U/ml	-0.325	0.053	-0.147	0.392	0.086	0.616
Grade 2	NO U/ml	0.110	0.555	-0.112	0.549	-0.039	0.835
Grade 3	NO U/ml	0.220	0.302	-0.263	0.214	-0.085	0.694

DISCUSSION

In this study, multiple relationships were found between the degree of sonographic liver steatosis and the ONS parameters focused on in this study. The results obtained will be discussed below in order.

When the differences between the groups for each variable are examined, it is found that this change either decreases or increases, and it shows that the parameters are compatible with the degree of steatosis. Antioxidant enzymes CAT and SOD decrease as the degree of steatosis increases in US. NO and MDA increase as the degree of steatosis increases in US. These findings are compatible with the directions of increase and decrease in the severity of the disease defined in the literature and the directions of increase and decrease defined among ONS parameters, and previously it was defined that NO and MDA increase as the severity of the disease increases, and on the other hand, CAT and SOD decrease as the severity of the disease increases. Similarly, the findings show a change in the opposite direction as the severity of the disease decreases.[14,15,21-25] However, what makes this study special is that it is the first study to show the relationship between the degree of steatosis in US and the defined markers. As seen in **Table 3**, this relationship is very clearly seen in our study. However, a striking point is that although there is a difference between the degree of steatosis in US for each marker in general, some differences are significant between 2 groups (for example, between the control group and the degree of liver steatosis in US as grade 3), some between 3 groups and some between 4 groups. The fact that a statistically significant relationship is shown between four groups shows that the obtained data is very strong. Despite these very strong results, it is debatable why each of these does not show a difference between all groups. One reason for this is that the determination of the degree of steatosis in US is partly subjective. We believe that by resolving this situation, this difference can be shown between all groups. This may be the determination of more objective criteria for the definition of the degree of liver steatosis in US or the almost objective realization of this rating with artificial intelligence techniques through machine learning. Nevertheless, as mentioned, showing these differences between multiple groups is a great achievement and this is probably due to the richness of our exclusion criteria. We showed great care in exclusion and this care is probably reflected in our results. In addition, the results we obtained and shared in this study are suitable for use in clinical practice. This will bring significant convenience to clinicians in patient management.

This study also found values that can be used to distinguish between patient and control groups. In the literature, cut-off points for some markers for many diseases have been examined and used in diagnosis and follow-up. Finding such a cut-off point makes the job of physicians easier.

In this study, we found and show the cut-off value of NO that can be used to distinguish between patient and control groups. The NO variable can make a statistically significant distinction between patients and healthy

individuals. The value of 8.95 is the cut-off point for patients and healthy individuals. NO can distinguish patients and healthy individuals with high sensitivity and accuracy. This value revealed by our study can be used in clinical follow-up. The reasons why this value is different from zero can be questioned. One reason for this is that NO has many roles necessary for the body at low doses. On the one hand, NO is an endothelial relaxant factor. With this feature, it plays an important role in regulating blood pressure. In addition, NO attacks tumor cells, stimulates the brain and acts as a second messenger in various ways. There is a lot of evidence showing that NO plays important physiological and pathological roles in the liver.[10] Our high success in our ROC curve between groups for NO is remarkable. However, it is questionable that the results are still not 100% and it is debatable to increase these values even further. Here again, factors such as patient selection, exclusion criteria, subjectivity of sonographic parameters and number of patients may be effective.

Also, this study has shown that MDA can distinguish between patient and control groups. The MDA variable can make a statistically significant distinction between patients and healthy individuals. The value of 2.375 is the cut-off point for patients and healthy individuals. MDA can distinguish between patients and healthy individuals with high sensitivity and specificity. MDA values have been examined in the literature as a cut-off point for many diseases. However, the data obtained in this study is the first.[26-28] In this study, the value of 2.375, which can be used in clinical diagnosis and follow-up in distinguishing between the normal control group and MASLD, was introduced to the literature. In addition, its high specificity and sensitivity increase its usefulness.

Differences between variables according to groups: No statistically significant correlation was found between NO and CAT, SOD and MDA values for each group.

Limitations

The most definitive way to diagnose nonalcoholic steatohepatitis or assess the stage of fibrosis is to perform a liver biopsy.[29] A limitation of our study is that it is not based on biopsy data.

Our study only dealt with the hepatic steatosis. In this respect, it can be viewed as a narrowed specific group. This is an advantage. On the other hand, it can be considered as a limitation that it does not deal with the continuation of the spectrum.

Heterogeneous steatosis pattern, which is a version of liver steatosis, was not included in the study and was considered as an exclusion criterion: this approach was made to avoid sonographic grading errors of liver steatosis and adds strength to the study in this respect. However, it cannot analyze a group of cases within the MASLD spectrum. This is a limitation.

Not questioning dietary habits; free fatty acids induce ROS production due to high-calorie food intake, and abnormal ROS levels may mediate the progression of MASLD.[30] In our study, obesity was included as an exclusion criterion. However, not focusing on dietary habits is a limitation.

CONCLUSION

There is a significant change in ONS parameters with the change in the degree of liver steatosis on sonography. The obtained data can be further strengthened by reducing sonographic subjectivity. In addition, the cut-off values obtained for NO and MDA can be used with high accuracy in clinical practice to distinguish between groups with and without liver steatosis.

ETHICAL DECLARATIONS

Ethics Committee Approval

Ethics committee approval was obtained for this study from the Kahramanmaraş Sütçü İmam University Faculty of Medicine Local Ethics Committee (Date: 13.12.2022 Decision No: 06).

Informed Consent

All patients signed and free and informed consent form.

Referee Evaluation Process

Externally peer-reviewed.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

Financial Disclosure

The authors declared that this study has received no financial support.

Author Contributions: All of the authors declare that they have all participated in the design, execution, and analysis of the paper, and that they have approved the final version.

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