Clinical Chemistry and Metabolism **Edited by: W. März**

Mehmet Asil*, Ramazan Dertli, Murat Biyik, Ramazan Yolacan, Ozcan Erel, Salim Neselioglu, Huseyin Ataseven, Hakkı Polat and Ali Demir

Dynamic thiol-disulfide homeostasis is disturbed in patients with non-alcoholic fatty liver disease

https://doi.org/10.1515/labmed-2017-0018
Received February 27, 2017; accepted September 12, 2017; previously published online November 27, 2017

Abstract

Background: Oxidative stress has been implicated in the pathogenesis of non-alcoholic fatty liver disease (NAFLD). Plasma thiols are major defense mechanisms against oxidative stress and undergo oxidation to form disulfides under oxidative conditions. This study was conducted to investigate thiol-disulfide homeostasis in NAFLD patients.
Methods: Thirty patients with biopsy proven non-alcoholic steatohepatitis (NASH), 40 patients with simple steatosis and 50 healthy controls were included in the study. Serum total and native thiol concentrations and serum disulfide concentration were measured using the Erel and Neselioglu's method.
Results: The mean serum total thiol concentrations in the NASH, simple steatosis and control groups were 415±64 μmol/L, 447±38 μmol/L and 480±37 μmol/L, respectively ($p<0.001$). The mean serum native thiol concentrations in the NASH, simple steatosis and control groups were 378±62 μmol/L, 416±41 μmol/L and 451±36 μmol/L, respectively ($p<0.001$). The mean serum disulfide concentrations in the NASH, simple steatosis and control groups were 18.5±6.3 μmol/L, 15.5±4.8 μmol/L and 14.9±3.6 μmol/L, respectively ($p=0.005$). The native thiol/total thiol ratio was significantly lower and the disulfide/total thiol and disulfide/native thiol ratios were significantly higher in the NASH group than in the simple steatosis and control groups.
Conclusions: Thiol-disulfide homeostasis is disturbed and shifted toward disulfide side in NAFLD and NASH patients.

Keywords: disulfides; fatty liver; oxidative stress; steatohepatitis; thiols.

Introduction

Non-alcoholic fatty liver disease (NAFLD) is the most common liver disease, with increasing prevalence worldwide [1]. The estimated prevalence of NAFLD in the general population is around 20–30% [2]. NAFLD is characterized by excessive fat accumulation within the hepatocytes and it constitutes a group of clinical disorders with distinct clinical, laboratory and histological characteristics. Simple steatosis lies at one end of the NAFLD spectrum and it is associated with fatty infiltration of the liver without necroinflammation, hepatocyte damage or fibrosis. On the other hand, non-alcoholic steatohepatitis (NASH) is associated with a progressive hepatocellular inflammation and fibrosis and it can eventually progress to cirrhosis and hepatocellular carcinoma. Although several risk factors have been defined, the pathophysiological mechanisms underlying NAFLD are not thoroughly understood. Insulin resistance, genetic factors, gut microbiota, several cytokines as well as oxidative stress have been implicated to contribute to the pathogenesis of fat accumulation, necroinflammation and fibrogenesis in NAFLD patients [3–6].

Plasma thiols are a class of organosulfur compounds that contain a sulfhydryl group. The plasma thiol pool is mainly formed by albumin thiols and protein thiols [7]. Under oxidative conditions, thiols undergo oxidation to form disulfides. This reaction is one of the most important primary defense mechanisms against oxidative stress. The disulfide bonds thus formed can again be reduced to thiol

***Correspondence: Mehmet Asil,** Necmettin Erbakan University, Meram School of Medicine, Department of Internal Medicine, Division of Gastroenterology, Meram, Konya, Turkey, Phone: +905054007407, +903322236587, Fax: +903323237120, E-Mail: drmehmetasil@yahoo.com.tr
Ramazan Dertli, Murat Biyik, Huseyin Ataseven and Ali Demir: Necmettin Erbakan University, Meram School of Medicine, Department of Internal Medicine, Division of Gastroenterology, Meram, Konya, Turkey
Ramazan Yolacan and Hakkı Polat: Necmettin Erbakan University, Meram School of Medicine, Department of Internal Medicine, Meram, Konya, Turkey
Ozcan Erel and Salim Neselioglu: Department of Biochemistry, Faculty of Medicine, Yıldırım Beyazıt University, Ankara, Turkey

groups. This thiol-disulfide interconversion is strictly controlled in vitro, maintaining dynamic thiol-disulfide homeostasis. In fact, the thiol-disulfide homeostasis is thought to be the primary regulator of intracellular redox homeostasis and these reactions are of particular importance for antioxidant defense, redox regulation of cell signaling and apoptosis [8, 9]. In the past, quantitative measurement of dynamic thiol-disulfide homeostasis in the serum was challenging. Recently, Erel and Neselioglu developed a simple, automated spectrophotometric method to measure serum total thiol, native thiol and disulfide concentrations [10]. Since then many studies have been conducted using this new method and disturbances in dynamic thiol-disulfide homeostasis have been documented in several diseases [11–15]. Taking into consideration that, oxidative stress has been implicated to contribute to the pathogenesis of NAFLD, disturbances in thiol-disulfide homeostasis can also be expected to occur in NAFLD patients. Therefore, we aimed to conduct this study to investigate dynamic thiol-disulfide homeostasis in NAFLD patients.

Materials and methods

Thirty patients with NASH, 40 patients with simple steatosis and 50 healthy controls were included in the study. Written informed consents were taken from all participants. This study has been performed in accordance with the ethical standards of Declaration of Helsinki and ethical approval of the study protocol was obtained from local Committee of Ethics of Necmettin Erbakan University, Meram Faculty of Medicine. The NASH group consisted of patients with elevated liver enzymes lasting for at least 3 months and various degrees of liver steatosis diagnosed using abdominal ultrasound. Liver biopsy was performed on all patients in the NASH group and the presence of steatohepatitis was confirmed by a histologic examination. On the other hand, the simple steatosis group included patients with normal serum transaminases whose transabdominal ultrasound showed the presence of hepatosteatosis. The control group consisted of healthy people with normal liver enzymes. An abdominal ultrasound was performed again on all patients in the control group to confirm the absence of liver steatosis. Viral hepatitis markers (hepatitis B core antigen, hepatitis B surface antigen and anti-hepatitis C virus (HCV) antibody), autoimmune hepatitis markers (anti nuclear antibody, anti-smooth muscle antibody, anti-liver-kidney microsomal antibody), serum copper and ceruloplasmin levels and serum transferrin saturation were obtained from all patients in the NASH group to exclude chronic liver disease of any other etiology. On the other hand, hepatitis B surface antigen, hepatitis B core antigen and anti-HCV antibodies were obtained from all patients in the simple steatosis and control groups and the subjects with positive serological test results were excluded. Patients with liver diseases of any other etiology, patients with considerable alcohol consumption (>20 g/day for males and >10 g/day for females), patients with known malignancies, autoimmune diseases or any active infections were also excluded from the study.

From January 2016 to December 2016, 1312 patients were admitted to our gastroenterology outpatient clinic for several reasons and 64 patients fulfilling the criteria for NASH were identified and offered a liver biopsy. A liver biopsy was performed on 35 patients who agreed for the biopsy. Five patients were not included in the study due to their unwillingness and 30 patients were included in the study. During the study period 97 patients meeting the criteria for simple steatosis were identified from our outpatient clinic, and age- and sex-matched 40 patients who agreed to participate in the study were randomly included in the simple steatosis group.

Anthropometric measurements were obtained from all subjects and the weight and height of the participants were measured with calibrated scales. Body mass index (BMI) was computed using the formula: BMI = body weight/(height)^2. Blood samples for the biochemical analysis were obtained early in the morning after overnight fasting. Serum transaminases, fasting plasma glucose, bilirubin, alkaline phosphatase (ALP) and γ-glutamyltransferase (GGT) levels were measured in our hospital's laboratory using automated analyzers (Abbott Architect 16000 system, Abbott Laboratories, Abbott Park, IL, USA). Blood samples for thiol-disulfide hemostasis analysis were centrifuged at 4000 rpm/min for 5 min, serum samples were separated and transferred to freezer to be stored at −80 °C until the day of analysis. As previously mentioned dynamic thiol-disulfide homeostasis was measured using the Erel and Neselioglu's method; in this method sodium borohydride (Merck Co., Darmstadt, Germany) is added to the serum sample to reduce all dynamic disulfide bonds to functional thiols. Then, unconsumed sodium borohydride is removed from the milieu using formaldehyde and the total thiol concentration is measured using the modified Ellman's reagent (Merck Co., Darmstadt, Germany). The native thiol concentration is subtracted from the total thiol concentration and half of the obtained difference gives the disulfide bond amount. Then the disulfide/native thiol, disulfide/total thiol and native thiol/total thiol ratios are calculated arithmetically.

Statistical analyses were done using the computer software IBM SPSS Statistics for Windows, Version 19.0. (IBM Corp., Armonk, NY, USA). The one-sample Kolmogorov-Smirnov test was used to test normality. Continuous variables were expressed as mean ± standard deviation (SD). Statistical analysis to test for the significance of difference between three or more groups were done using the one-way analysis of variance (ANOVA) test and the Student's t-test was used to determine the significance of differences between the two groups. The Pearson correlation coefficient was used to test for the significance of linear dependence between two continuous variables. Receiver operating characteristic (ROC) curves were obtained for tested parameters to differentiate NASH patients from patients with simple steatosis and sensitivities and specificities were calculated. The statistical significance level was defined as $p < 0.05$ for all analyses.

Results

The study group consisted of 57 (47.5%) male and 63 (52.5%) female subjects, with a mean age of 46.1 ± 10.1 years. There were 30 (25%) subjects in the NASH group, 40 (33.3%) subjects in the simple steatosis group and 50 (41.7%) subjects in the control group. All study groups were found to be similar in terms of age and gender distribution.

Table 1: Clinical, demographical and laboratory data of the groups.

	Control group (n = 50)	Simple steatosis (n = 40)	NASH (n = 30)	p-Value
Age, years	45.2 ± 9.3	45.3 ± 10.1	48.6 ± 11.2	0.308
Male (n, %)/female (n, %)	24 (48)/26 (52)	19 (47.5)/21 (52.5)	14 (46.7)/16 (53.3)	0.993
BMI, kg/m^2	24.0 ± 3.5	28.9 ± 4.3	28.6 ± 6.0	<0.001[a,b]
Waist to hip ratio, %	85.7 ± 8.9	89.7 ± 5.7	94.4 ± 9.8	<0.001[a,b,c]
SBP, mmHg	104.8 ± 9.8	127.6 ± 11.3	124.3 ± 15.7	<0.001[a,b]
DBP, mmHg	69.2 ± 7.5	76.6 ± 8.0	81.3 ± 10.0	<0.001[a,b,c]
Glucose, mg/dL	92.3 ± 10.2	99.7 ± 11.2	138.0 ± 58.3	<0.001[a,b,c]
Urea, mg/dL	27.1 ± 7.6	26.5 ± 6.8	28.2 ± 9.5	0.695
Creatinine, mg/dL	0.75 ± 0.13	0.75 ± 0.14	0.73 ± 0.20	0.803
ALT, U/L	18.5 ± 10.0	25.7 ± 15.4	109.6 ± 117.0	<0.001[a,b,c]
AST, U/L	21.1 ± 8.5	20.2 ± 5.6	63.2 ± 41.5	<0.001[b,c]
GGT, U/L	19.4 ± 10.6	38.8 ± 42.5	63.9 ± 48.6	<0.001[a,b,c]
ALP, U/L	64.1 ± 13.3	78.4 ± 32.2	97.5 ± 82.1	0.005[a,b]
T.Bil, mg/dL	0.75 ± 0.38	0.69 ± 0.38	0.76 ± 0.38	0.505
D.Bil, mg/dL	0.23 ± 0.12	0.23 ± 0.10	0.28 ± 0.17	0.28
Total protein, g/dL	7.2 ± 0.7	7.3 ± 0.3	7.0 ± 0.8	0.506
Albumin, g/dL	4.4 ± 0.3	4.4 ± 0.3	4.1 ± 0.6	0.12
Triglyceride, mg/dL	84 (64–129)	179 (129–220)	142 (98–218)	0.001[a,b]
Total cholesterol, mg/dL	183 ± 42	214 ± 53	198 ± 62	0.104
LDL-C, mg/dL	112 ± 41	130 ± 45	118 ± 60	0.429
HDL-C, mg/dL	46.6 ± 11.9	45.5 ± 10.9	43.6 ± 13.0	0.645
Hemoglobin, g/dL	14.4 ± 1.6	14.8 ± 1.2	14.0 ± 2.10	0.132
Hct, %	42.2 ± 4.1	42.9 ± 6.1	41.1 ± 5.0	0.099
Leukocyte, mm^3	6.9 ± 1.4	7.50 ± 1.7	7.7 ± 2.0	0.125
Thrombocyte, mm^3	253 ± 56	263 ± 51	269 ± 99	0.573
Native thiol, μmol/L	451 ± 36	416 ± 41	378 ± 62	<0.001[a,b,c]
Total thiol, μmol/L	480 ± 37	447 ± 38	415 ± 64	<0.001[a,b,c]
Disulfide, μmol/L	14.9 ± 3.6	15.5 ± 4.8	18.5 ± 6.3	0.005[b,c]
Disulfide/native thiol, %	3.32 ± 0.84	3.81 ± 1.45	5.00 ± 2.17	<0.001[b,c]
Disulfide/total thiol, %	3.10 ± 0.74	3.50 ± 1.21	4.49 ± 1.64	<0.001[b,c]
Native thiol/total thiol, %	93.8 ± 1.5	93.0 ± 2.4	91.0 ± 3.3	<0.001[b,c]

NASH, non-alcoholic steatohepatitis; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, γ-glutamyltransferase; ALP, alkaline phosphatase; T.Bil, total bilirubin; D.Bil, direct bilirubin; LDL-C, low density lipoprotein; HDL, high density lipoprotein; Hct, hematocrit. [a,b,c]Denote for the results of binary comparisons between the study groups. [a]The difference between the control group and the simple steatosis group is statistically significant. [b]The difference between the control group and the NASH group is statistically significant. [c]The difference between the simple steatosis group and the NASH group is statistically significant.

Demographical characteristics, anthropometric measurements and laboratory findings of the NASH, simple steatosis and control groups are summarized in Table 1.

In the NASH group, 19 (63.4%) patients had diabetes mellitus, 10 (33.3%) patients had arterial hypertension, eight (26.7%) patients had hyperlipidemia and five (16.6%) patients had atherosclerotic coronary artery disease. Metabolic syndrome was present in 21 (70%) patients. Three patients (10.0%) in the NASH group were on insulin treatment, 16 patients (53.3%) were using oral antidiabetic drugs, 13 patients (43.3%) were using oral antihypertensive drugs (10 patients: angiotensing converting enzyme inhibitor, five patients: β-blockers, five patients: calcium channel blockers). Eight (26.6%) patients were on statin treatment for hyperlipidemia. In the simple steatosis group, five (12.5%) patients had diabetes mellitus, two (5.0%) patients had arterial hypertension, six (15.0%) patients had hyperlipidemia and one (2.5%) patient had atherosclerotic coronary artery disease. Metabolic syndrome was present in 16 (43.2%) patients. Five patients (12.5%) were using oral antidiabetic drugs, two patients (43.3%) were using oral antihypertensive drugs (one patient: angiotensing converting enzyme inhibitor, one patient: calcium channel blockers) and two (5%) patients were using statins.

The mean serum total thiol concentrations in the NASH, simple steatosis and control groups were 415 ± 64 μmol/L, 447 ± 38 μmol/L and 480 ± 37 μmol/L, respectively, and the difference between the groups was statistically significant ($p < 0.001$). Intergroup comparisons showed that both the simple steatosis ($p < 0.001$) and NASH groups ($p < 0.001$) had statistically lower mean serum total thiol concentrations than the healthy controls. The mean serum total thiol concentration was also significantly lower in the NASH group compared to the simple steatosis group ($p = 0.044$).

The mean serum native thiol concentrations in the NASH, simple steatosis and control groups were 378 ± 62 μmol/L, 416 ± 41 μmol/L and 451 ± 36 μmol/L, respectively, and the difference between the groups was statistically significant ($p < 0.001$). Intergroup comparisons showed that both the simple steatosis ($p < 0.001$) and NASH groups ($p < 0.001$) had statistically lower mean serum native thiol concentrations than the healthy controls and the difference between the simple steatosis and NASH groups was also statistically significant ($p = 0.013$).

The Mean serum disulfide concentrations in the NASH, simple steatosis and control groups were 18.5 ± 6.3 μmol/L, 15.5 ± 4.8 μmol/L and 14.9 ± 3.6 μmol/L, respectively, and the difference between the groups was statistically significant ($p = 0.005$). Intergroup comparisons showed that the difference between the simple steatosis and control groups was not statistically significant ($p = 0.76$), on the other hand, the NASH group had significantly higher mean serum disulfide concentration than both the simple steatosis group ($p = 0.03$) and healthy controls ($p = 0.006$).

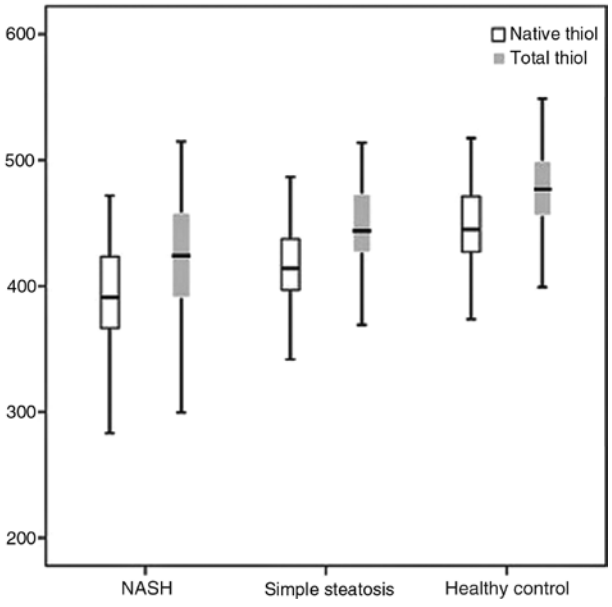

Figure 1: The mean serum native thiol, total thiol and disulfide concentrations in the NASH, simple steatosis and control groups.

Figure 1 summarizes the mean serum native thiol, total thiol and disulfide concentrations in the NASH, simple steatosis and control groups.

The mean native thiol/total thiol ratio was 91.0 ± 3.3%, 93.0 ± 2.4% and 93.8 ± 1.5% in the NASH, simple steatosis and control groups, respectively, and statistical analyses showed that the NASH group had a significantly lower native thiol/total thiol ratio than both the simple steatosis ($p = 0.002$) and control groups ($p < 0.001$) whereas the difference between the simple steatosis and control groups was not statistically significant ($p = 0.14$).

The mean disulfide/total thiol ratio was 4.49 ± 1.64%, 3.50 ± 1.21% and 3.10 ± 0.74% in the NASH, simple steatosis and control groups, respectively, and statistical analyses showed that the NASH group had a significantly higher disulfide/total thiol ratio than both the simple steatosis ($p = 0.002$) and control groups ($p < 0.001$) whereas the difference between the simple steatosis and control groups was not statistically significant ($p = 0.13$).

The mean disulfide/native thiol ratio was 5.00 ± 2.17%, 3.81 ± 1.45% and 3.32 ± 0.84% in the NASH, simple steatosis and control groups, respectively, and statistical analyses showed that the NASH group had a significantly higher disulfide/total thiol ratio than both the simple steatosis ($p = 0.002$) and control groups ($p < 0.001$) whereas

Table 2: Correlation analyses.

	Native thiol, μmol/L		Total thiol, μmol/L		SH/total thiol, %		Disulfide, μmol/L	
	p-Value	P	p-Value	P	p-Value	P	p-Value	P
BMI, kg/m^2	0.001	−0.318	0.002	−0.297	0.022	−0.218	0.120	0.149
Waist to hip ratio	0.004	−0.275	0.014	−0.235	0.001	−0.311	0.013	0.238
Glucose, mg/dL	<0.0001	−0.433	<0.0001	−0.414	0.003	−0.281	0.122	0.146
Creatinine, mg/dL	0.0408	0.077	0.323	0.092	0.556	−0.053	0.452	0.070
AST, U/L	<0.0001	−0.444	<0.0001	−0.412	<0.0001	−0.374	0.019	0.219
ALT, U/L	<0.0001	−0.331	0.002	−0.284	<0.0001	−0.388	0.002	0.288
GGT, U/L	0.013	−0.269	0.024	−0.246	0.023	−0.248	0.209	0.138
Albumin, g/dL	<0.0001	0.543	<0.0001	0.551	0.024	0.238	0.726	−0.037
TG, mg/dL	0.045	−0.217	0.189	−0.143	<0.0001	−0.412	<0.0001	0.410

BMI, body mass index; AST, aspartate aminotransferase; ALT, alanine aminotransferase; GGT, γ-glutamyltransferase; TG, triglyceride; P, correlation coefficient.

the difference between the simple steatosis and control groups was not statistically significant (p = 0.13).

Correlation analyses showed that the serum native thiol and total thiol concentrations were negatively correlated with the serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), GGT, triglyceride levels, BMI and waist to hip ratio and positively correlated with the serum albumin concentration in patients with NAFLD. On the other hand, the serum disulfide concentration was found to be positively correlated with the serum AST, ALT, triglyceride levels and waist to hip ratio in NAFLD patients. Correlation analyses are summarized in Table 2.

The control group was excluded from the analysis and ROC curves were obtained to test the ability of the serum native thiol, total thiol and disulfide concentrations and the native thiol/total thiol, disulfide/total thiol and disulfide/native thiol ratios to differentiate NASH patients from patients with simple steatosis. Best results were obtained

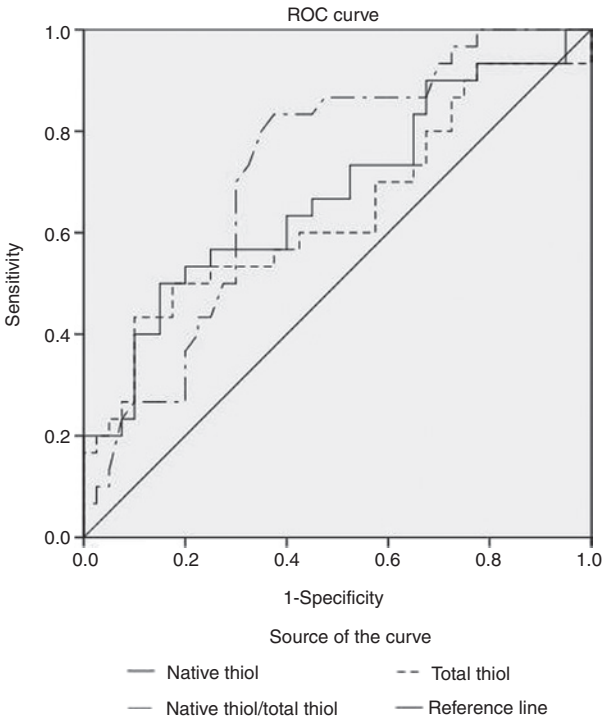

Figure 2: ROC curves for the serum native thiol and total thiol concentrations and the native thiol/total thiol ratio to differentiate NASH patients from patients with simple steatosis.

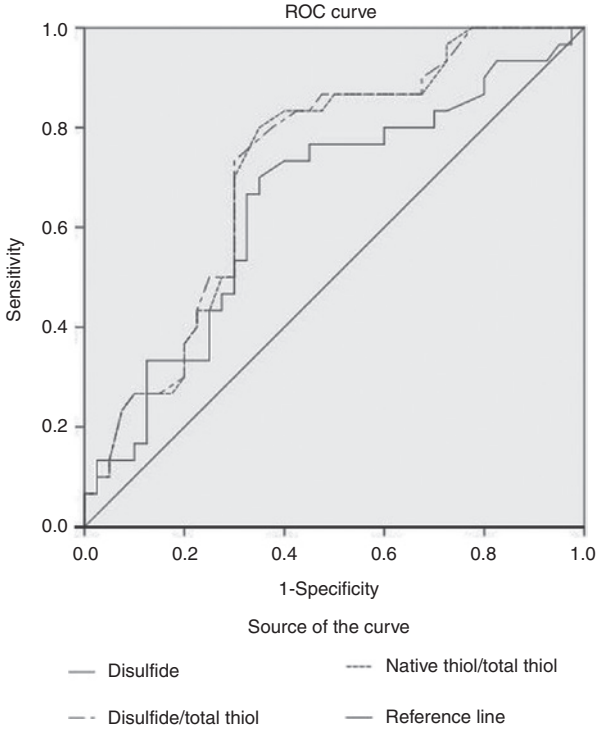

Figure 3: ROC curves for the serum disulfide concentration, the disulfide/total thiol and disulfide/native thiol ratios to differentiate NASH patients from patients with simple steatosis.

from the native thiol/total thiol, disulfide/total thiol and disulfide/native thiol ratios. For the native thiol/total thiol ratio, AUC was 0.720 [95% confidence interval (CI): 0.60–0.84] and the specified cut-off value of 92.95 yielded 83.3% sensitivity and 62.5% specificity. For the disulfide/total thiol ratio, AUC was 0.722 (95% CI: 0.602–0.841) and the specified cut-off value of 3.45 yielded 83.3% sensitivity and 57.5% specificity. Lastly for the disulfide/native thiol ratio, AUC was 0.720 (95% CI: 0.599–0.840) and the specified cut-off value of 3.75 yielded 83.3% sensitivity and 60.0% specificity.

ROC curves are summarized in Figures 2 and 3.

Discussion

The liver is the main organ for metabolism and detoxification of many toxic substances. These detoxification reactions result in continuous generation of pro-oxidants, such as reactive oxygen and nitrogen species. In health, these pro-oxidants are balanced with antioxidants at a similar rate to avoid oxidative injury within the liver. Maintenance of this oxidative homeostasis is of vital importance for the liver. Among these antioxidants, glutathione, which is the most abundant intracellular thiol, is of particular importance [16]. A disturbance in the balance between pro-oxidants and antioxidants is known as oxidative stress and it has been shown to contribute to the pathogenesis of several chronic liver diseases including NAFLD and NASH. The NAFLD pathogenesis is believed to be complex and multifactorial. Currently, the "two hit" hypothesis is widely accepted to explain the pathogenesis of NAFLD and NASH [17]. According to this hypothesis the first insult is insulin resistance which promotes peripheral lipolysis and causes increased serum concentrations of free fatty acids. Excessive delivery of free fatty acids to the liver results in lipid accumulation within the hepatocytes. Another consequence of insulin resistance is hyperglycemia and increased hepatic insulin dependent lipogenesis from glucose, which further augments hepatic steatosis. The mechanisms underlying progression from simple steatosis to steatohepatitis are multifactorial and complex and this is the point where the "second hit" gains importance. The "second hit" comes from lipid peroxidation, mitochondrial dysfunction and inflammation, which result in hepatocyte damage and development of liver fibrosis. Oxidative stress which can mediate inflammation and cytotoxicity through different mechanisms is particularly believed to be important in this step. Indeed several studies in the literature point out the role of oxidative stress in the pathogenesis of NAFLD and NASH [18–21]. Oxidative stress may contribute to the pathogenesis of NAFL and NASH through several mechanisms. First, reactive oxygen and nitrogen species may contribute to the "first hit" in the NAFLD pathogenesis, that is insulin resistance, through induction of signaling pathways that may trigger insulin resistance [22]. Second, reactive oxygen and nitrogen species have been shown to activate or inhibit several signaling pathways that can modulate cellular lipid metabolism [23]. Third, oxidative stress causes damage to hepatocyte mitochondria and triggers mitochondrial dysfunction which is considered to be one of the key factors in the NASH pathogenesis. The mitochondrial dysfunction causes further increase in reactive oxygen species and a vicious cycle is initiated. There is also evidence in the literature that oxidative stress is related to endoplasmic reticulum stress within the cells and endoplasmic reticulum stress together with mitocondrial dysfunction triggers inflammation via activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κβ) [24]. The inflammation in turn further increases oxidative stress through production of several oxidative mediators from the hepatic Kuppfer cells and neutrophils, initiating another vicious cycle. On the other hand, oxidative stress may simultaneously promote hepatic fibrogenesis by activating hepatic stellate cells and also by causing upregulation of expression of genes involved in fibrogenesis, such as pro-collagen type I, monocyte chemoattractant protein 1 (MCP-1) and tissue inhibitor of metalloproteinase-1 (TIMP1), possibly via activation of transcription factors [25].

The results of the current study showed that thiol-disulfide homeostasis is disturbed in NAFLD patients. Serum native and total thiol concentrations were found to be lower in the simple steatosis and NASH groups than in healthy controls. Serum native and total thiol concentrations were also significantly lower in NASH patients compared to patients with simple steatosis. On the other hand, the mean serum disulfide concentration was found to be higher in the NASH group than in both the simple steatosis group and healthy controls. The net result of these findings can be summarized as shifting of dynamic thiol-disulfide balance toward disulfide formation which indicates the presence of oxidative stress in NAFLD patients. To our knowledge, this is the first study in the literature investigating dynamic thiol-disulfide homeostasis in NAFLD patients using the Erel's method [10]. Although the contribution of oxidative stress to the NAFLD and NASH pathogenesis is well documented, the number of studies investigating serum markers of systemic oxidative stress in these patients is limited. In a study conducted by Başkol et al. [26], the serum total thiol concentration was found to be lower in the NASH patients than in healthy controls. They have also reported that plasma

advanced oxidation protein products, total oxidant status and oxidative stress index were significantly higher in NASH patients compared to healthy controls. In another study, the serum concentration of protein carbonyls, another marker of systemic oxidative stress, have been shown to increase in pediatric NASH patients [21]. Also in a study conducted by Leach et al. [27], a lower glutathione peroxidase activity and lower serum levels of total and reduced glutathione have been reported in NASH patients compared to healthy controls. All these findings indicate that, concentrations of different serum markers of oxidative stress are increased in patients with NAFLD and NASH confirming the possible role of oxidative stress. In this aspect, the results of our study can be considered to be consistent with past researches.

There are several studies investigating various biomarkers of necroinflammation and fibrosis in NAFLD patients to differentiate NASH from simple steatosis but currently there is no optimal serum marker. Cytokeratin-18 (CK-18), leptin, adiponectin, C-reactive protein, interleukin-6, interleukin-8 and tumor necrosis factor α were investigated in different studies to differentiate NASH patients [28]. Among these, CK-18 is a promising biomarker. CK-18 is a protein found in the liver and the serum level of CK-18 has been shown to increase in NASH patients [29, 30]. In the current study, the calculated sensitivities and specificities for different dynamic thiol-disulfide homeostasis parameters as markers to differentiate NASH patients from patients with simple steatosis were moderate; therefore, using these parameters alone does not seem to be suitable as serum markers to diagnose NASH patients. But, they may be combined with other clinical and laboratory parameters in the management of NAFLD patients. Our findings indicate that changes in the serum native thiol/total thiol ratio, disulfide/total thiol ratio and disulfide/native thiol ratio were prominent in NASH patients. Therefore rather than individually measuring the serum native thiol, total thiol or disulfide concentrations, determination of all parameters of dynamic thiol-disulfide homeostasis using the Erel's method may be more appropriate in patients with NAFLD.

In conclusion, thiol-disulfide homeostasis is disturbed and shifted towards disulfide side in patients with NAFLD and NASH which supports the role of oxidative stress in the pathogenesis of fatty liver disease. Further studies are needed to clarify the pathophysiological mechanisms underlying this association. Better understanding of these mechanisms would facilitate development of new therapeutic strategies in the era of NAFLD and NASH.

Author contributions: All the authors have accepted responsibility for the entire content of this submitted manuscript and approved submission.

Research funding: None declared.
Employment or leadership: None declared.
Honorarium: None declared.
Competing interests: The funding organization(s) played no role in the study design; in the collection, analysis, and interpretation of data; in the writing of the report; or in the decision to submit the report for publication.

References

1. Younossi ZM, Stepanova M, Afendy M, Fang Y, Younossi Y, Mir H, et al. Changes in the prevalence of the most common causes of chronic liver diseases in the United States from 1988 to 2008. Clin Gastroenterol Hepatol 2011;9:524–30.e1; quiz e60.
2. Vernon G, Baranova A, Younossi ZM. Systematic review: the epidemiology and natural history of non-alcoholic fatty liver disease and non-alcoholic steatohepatitis in adults. Aliment Pharmacol Ther 2011;34:274–85.
3. Sanyal AJ, Campbell-Sargent C, Mirshahi F, Rizzo WB, Contos MJ, Sterling RK, et al. Nonalcoholic steatohepatitis: association of insulin resistance and mitochondrial abnormalities. Gastroenterology 2001;120:1183–92.
4. Oliveira CP, Stefano JT. Genetic polymorphisms and oxidative stress in non-alcoholic steatohepatitis (NASH): a mini review. Clin Res Hepatol Gastroenterol 2015;39(Suppl 1):S35–40.
5. Abdou RM, Zhu L, Baker RD, Baker SS. Gut microbiota of nonalcoholic fatty liver disease. Dig Dis Sci 2016;61:1268–81.
6. Diesen DL, Kuo PC. Nitric oxide and redox regulation in the liver: part II. Redox biology in pathologic hepatocytes and implications for intervention. J Surg Res 2011;167:96–112.
7. Turell L, Radi R, Alvarez B. The thiol pool in human plasma: the central contribution of albumin to redox processes. Free Radic Biol Med 2013;65:244–53.
8. Winterbourn CC, Hampton MB. Thiol chemistry and specificity in redox signaling. Free Radic Biol Med 2008;45:549–61.
9. Circu ML, Aw TY. Reactive oxygen species, cellular redox systems, and apoptosis. Free Radic Biol Med 2010;48:749–62.
10. Erel O, Neselioglu S. A novel and automated assay for thiol/disulphide homeostasis. Clin Biochem 2014;47:326–32.
11. Ates I, Ozkayar N, Inan B, Yilmaz FM, Topcuoglu C, Neselioglu S, et al. Dynamic thiol/disulphide homeostasis in patients with newly diagnosed primary hypertension. J Am Soc Hypertens 2016;10:159–66.
12. Yuksel M, Ates I, Kaplan M, Alışık M, Erel Ö, Saygılı F, et al. The dynamic thiol/disulphide homeostasis in inflammatory bowel disease and its relation with disease activity and pathogenesis. Int J Colorectal Dis 2016;31:1229–31.
13. Ates I, Kaplan M, Yuksel M, Mese D, Alisik M, Erel Ö, et al. Determination of thiol/disulphide homeostasis in type 1 diabetes mellitus and the factors associated with thiol oxidation. Endocrine 2016;51:47–51.
14. Kundi H, Ates I, Kiziltunc E, Cetin M, Cicekcioglu H, Neselioglu S, et al. A novel oxidative stress marker in acute myocardial infarction; thiol/disulphide homeostasis. Am J Emerg Med 2015;33:1567–71.

15. Topuz M, Kaplan M, Akkus O, Sen O, Yunsel HD, Allahverdiyev S, et al. The prognostic importance of thiol/disulfide homeostasis in patients with acute pulmonary thromboembolism. Am J Emerg Med 2016;34:2315–9.
16. Chen Y, Dong H, Thompson DC, Shertzer HG, Nebert DW, Vasiliou V. Glutathione defense mechanism in liver injury: insights from animal models. Food Chem Toxicol 2013;60:38–44.
17. Day CP, James OF. Steatohepatitis: a tale of two "hits"? Gastroenterology 1998;114:842–5.
18. Köroğlu E, Canbakan B, Atay K, Hatemi İ, Tuncer M, Dobrucalı A. Role of oxidative stress and insulin resistance in disease severity of non-alcoholic fatty liver disease. Turk J Gastroenterol 2016;27:361–6.
19. Petrosillo G, Portincasa P, Grattagliano I, Casanova G, Matera M, Ruggiero FM, et al. Mitochondrial dysfunction in rat with nonalcoholic fatty liver Involvement of complex I, reactive oxygen species and cardiolipin. Biochim Biophys Acta 2007;1767:1260–7.
20. Yesilova Z, Yaman H, Oktenli C, Ozcan A, Uygun A, Cakir E, et al. Systemic markers of lipid peroxidation and antioxidants in patients with nonalcoholic fatty liver disease. Am J Gastroenterol 2005;100:850–5.
21. Nobili V, Parola M, Alisi A, Marra F, Piemonte F, Mombello C, et al. Oxidative stress parameters in paediatric non-alcoholic fatty liver disease. Int J Mol Med 2010;26:471–6.
22. Houstis N, Rosen ED, Lander ES. Reactive oxygen species have a causal role in multiple forms of insulin resistance. Nature 2006;440:944–8.
23. Serviddio G, Bellanti F, Vendemiale G. Free radical biology for medicine: learning from nonalcoholic fatty liver disease. Free Radic Biol Med 2013;65:952–68.
24. Ashraf NU, Sheikh TA. Endoplasmic reticulum stress and oxidative stress in the pathogenesis of non-alcoholic fatty liver disease. Free Radic Res 2015;49:1405–18.
25. Mormone E, George J, Nieto N. Molecular pathogenesis of hepatic fibrosis and current therapeutic approaches. Chem Biol Interact 2011;193:225–31.
26. Başkol M, Dolbun Seçkin K, Başkol G. Advanced oxidation protein products, total thiol levels and total oxidant/antioxidant status in patients with NASH. Turk J Gastroenterol 2014;25(Suppl 1):32–7.
27. Leach NV, Dronca E, Vesa SC, Sampelean DP, Craciun EC, Lupsor M, et al. Serum homocysteine levels, oxidative stress and cardiovascular risk in non-alcoholic steatohepatitis. Eur J Intern Med 2014;25:762–7.
28. Jayakumar S, Harrison SA, Loomba R. Noninvasive markers of fibrosis and inflammation in nonalcoholic fatty liver disease. Curr Hepatol Rep 2016;15:86–95.
29. Feldstein AE, Wieckowska A, Lopez AR, Liu YC, Zein NN, McCullough AJ. Cytokeratin-18 fragment levels as noninvasive biomarkers for nonalcoholic steatohepatitis: a multicenter validation study. Hepatology 2009;50:1072–8.
30. Feldstein AE, Alkhouri N, De Vito R, Alisi A, Lopez R, Nobili V. Serum cytokeratin-18 fragment levels are useful biomarkers for nonalcoholic steatohepatitis in children. Am J Gastroenterol 2013;108:1526–31.