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RESEARCH ARTICLE

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Oxidative stress in metabolic dysfunction-associated steatotic liver disease (MASLD): How does the animal model resemble human disease?

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Abstract

Despite decades of research, the pathogenesis of metabolic dysfunction-associated steatotic liver disease (MASLD) is still not completely understood. Based on the evidence from preclinical models, one of the factors proposed as a main driver of disease development is oxidative stress. This study aimed to search for the resemblance between the profiles of oxidative stress and antioxidant defense in

Abbreviations: 4-HNE, 4-hydroxy-2-nonenal; ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); ALT, aspartate transaminase; AST, alanine transaminase; BMI, body mass index; CAT, catalase; DT, di-tyrosine; ETC, electron transport chain; GGTP, gamma-glutamyl transpeptidase; GPX, glutathione peroxidase; H&E, hematoxylin and eosin; HDL, high-density lipoprotein; HEL, hexanol-lysine adducts; HOMA-IR, homeostatic model assessment of insulin resistance; LC–MS/MS, liquid chromatography-MS3 spectrometry; LDL, low-density lipoprotein; MASLD, metabolic dysfunction-associated steatotic liver disease; MSRA, methionine sulfoxide reductase A; MSRB2, methionine sulfoxide reductase B2; NAFLD, non-alcoholic fatty liver disease; NAS, NAFLD activity score; OXPHOS, oxidative phosphorylation; PCA, principal component analysis; PRDX, peroxiredoxin; ROS, reactive oxygen species; SD, standard diet; SOD, superoxide dismutase; TAA, total antioxidant activity; TAG, triglycerides; TRX, thioredoxin; TXNRD, thioredoxin reductase; WD, Western diet.

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the animal model of MASLD and the group of MASLD patients. C57BL/6J mice were fed with the Western diet for up to 24 weeks and served as the animal model of MASLD. The antioxidant profile of mice hepatic tissue was determined by liquid chromatography-MS3 spectrometry (LC–MS/MS). The human cohort consisted of 20 patients, who underwent bariatric surgery, and 6 controls. Based on histological analysis, 4 bariatric patients did not have liver steatosis and as such were also classified as controls. Total antioxidant activity was measured in sera and liver biopsy samples. The hepatic levels of antioxidant enzymes and oxidative damage were determined by Western Blot. The levels of antioxidant enzymes were significantly altered in the hepatic tissue of mice with MASLD. In contrast, there were no significant changes in the antioxidant profile of hepatic tissue of MASLD patients, except for the decreased level of carbonylated proteins. Decreased protein carbonylation together with significant correlations between the thioredoxin system and parameters describing metabolic health suggest alterations in the thiol-redox signaling. Altogether, these data show that even though the phenotype of mice closely resembles human MASLD, the animal-to-human translation of cellular and molecular processes such as oxidative stress may be more challenging.

KEYWORDS
antioxidant defense system, mitochondrial respiratory chain, obese patients, oxidative damage, reactive oxygen species

1 | INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD), currently known as metabolic dysfunction-associated steatotic liver disease (MASLD),[1] has become the most common liver disease worldwide. Its prevalence reaches as much as 32.4% of the global population according to the most recent estimations.[2,3] MASLD development is multifactorial and the mechanisms that drive its onset and progression have not yet been fully understood. Among several factors, oxidative stress is regarded as one of the key players in the pathogenesis of MASLD.[4,5] Oxidative stress, manifested as increased levels of markers of oxidative damage (e.g., protein carbonylation, lipid peroxidation), is a consequence of inefficient antioxidant protection from excessive levels of reactive oxygen species (ROS). The involvement of oxidative stress in the pathogenesis of MASLD has been reported in studies involving cellular models of hepatic steatosis as well as animal models of fatty liver.[6,7] Consequently, a variety of studies in patients with MASLD showed correlations between elevated indicators of oxidative stress, diminished antioxidant levels in serum, and the progression of MASLD.[4,8–10]

Animal models used to study the natural history of MASLD or to assess new therapeutic compounds should mimic the phenotype of human disease not only at histopathological but also at cellular and molecular levels, including, for example, the occurrence of oxidative stress.[11] Oxidative stress has been hypothesized to be either a cause or a consequence of mitochondrial dysfunction accompanying MASLD development.[6,12] The association between oxidative stress and mitochondrial dysfunction is based on the essential observation: enhanced mitochondrial activity in the liver, in particular the fatty acid oxidation, is considered a natural adaptation to extensive accumulation of lipids. This leads to an augmented production of superoxide anion ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2).[13,14] As such, a protracted pro-oxidant state could be the main contributor to hepatic mitochondrial dysfunction, e.g., impairments in oxidative phosphorylation (OXPHOS) activity.[12] Our recent study in C57BL/6J mice fed with a Western diet showed that mitochondrial remodeling in early liver steatosis is not accompanied by the presence of mitochondrial oxidative stress.[15] Indeed, neither increased ROS production nor signs of oxidative damage were found in the mitochondria of analyzed mice.[15] In contrast, only mild oxidative stress was manifested as slightly increased antioxidant defense systems but without signs of oxidative damage at the cytosolic level.[15] These observations are in line with results presented by Einer et al., who also did not find signs of oxidative damage or enhanced ROS generation in mitochondria isolated from steatotic livers of mice fed with a Western diet.[16] Such contradictory results have put into question the relevance of mitochondrial oxidative

stress to disease initiation and implied that more evidence linking observations from animal models with those from human studies is needed to better comprehend the natural history of MASLD at cellular level.[17] Therefore, in this study, we determined the levels of markers of oxidative damage, antioxidant enzymes, and total antioxidant activity in liver biopsies from the group of MASLD patients and compared their antioxidant profile with that of the mice model of MASLD.

2 | MATERIALS AND METHODS

2.1 | Ethics

Experiments performed in an animal model were approved by Local Ethical Committees (Resolution No. 200/2016 on December, 11, 2016). All procedures were carried out in accordance with the January, 15, 2015 Act on the Protection of Animals Used for Scientific Purposes in Poland, which follows the Directive 2010/63/EU of the European Parliament. The part of the study involving human subjects was performed in accordance with the ethical guidelines of the 1975 Declaration of Helsinki (latest revision, 2013). The study protocol (KB/237/2018) was approved by the Ethics Committee of the Medical University of Warsaw. Written informed consent was obtained from all participants included in the study.

2.2 | Animal model

The animal model, dietary regimen, and liver histology were previously described in detail by Simoes et al.[15,18] Shortly, four-week-old male C57BL/6J mice (20–30 g) purchased from the Experimental Medicine Centre of the Medical University of Bialystok (Bialystok, Poland) were used. The mice were fed with standard chow (standard diet, SD) or high-fat high-sugar (Western diet, WD) ad libitum for 16 and 24 weeks starting at the age of seven weeks. WD was composed of 35% carbohydrate, 21% protein, and 30% fat (E15126—Ssniff, Soest, Germany), supplemented with 30% (w/v) sucrose in the drinking water. Details regarding diet composition are provided in Table S1 (Supplementary Information—Methods). During these periods, the mice were kept in laboratory cages with a temperature range of 21–23°C and a humidity level of 50%–60%. The cages had 10–15 air exchanges per hour, and the mice had unrestricted access to tap water and food. To perform the necessary procedures, the mice were first subjected to isoflurane inhalation anesthesia and then sacrificed by cervical dislocation. The livers were excised, weighed, and stored (−80°C) until subsequent analysis.

2.3 | Proteomic analysis

The proteomic analysis of mice hepatic tissue was performed as previously described in detail by Simoes et al.[15,18] The liquid chromatography-MS3 spectrometry (LC–MS/MS) analysis was conducted at the Thermo Fisher Center for Multiplexed Proteomics, located in the Department of Cell Biology at Harvard Medical School in Cambridge, MA, USA; the LC-MS3 data collection was performed using an Orbitrap Fusion mass spectrometer from Thermo Fisher Scientific.

2.4 | Patients

Between March 2019 and June 2020, we prospectively enrolled 20 (8 males, 12 females, age range 23–59 years) consecutive nontransplanted adults subjected to bariatric surgery at the Medical University of Warsaw, Warsaw, Poland. Inclusion criteria were: obesity WHO grade 2 or 3 (BMI $\geq 35\,kg/m^2$) and age 18–60 years. Lack of consent to participate in the study, chronic and acute liver diseases other than MASLD, previous bariatric operations or permanent endoscopic bariatric procedures changing the anatomy of the digestive tract, previous major abdominal operations, chemotherapy, alcohol abuse, and pregnancy were regarded as exclusion criteria. Liver samples were obtained intraoperatively. Six individuals without fatty liver, for whom liver resection was performed due to other medical indications, were recruited to the control group. Venous blood samples were collected from all subjects at inclusion.

2.5 | Clinical data

All patients underwent careful clinical examination. Blood samples were drawn from fasted subjects. Liver biopsies were analyzed independently by two pathologists from The Children's Memorial Health Institute, Warsaw, Poland, experienced in liver diseases using Kleiner's histological scoring system for the assessment of MASLD. The results of the analysis were convergent. The available clinical dataset was used to search for possible correlations between blood test values, and changes in the oxidative stress status.

2.6 | Histological evaluation of liver biopsies

All the histological and immunohistochemistry procedures for animal and human samples were performed at

the Department of Pathology of The Children's Memorial Health Institute (Warsaw, Poland). NAFLD activity (NAS) score was evaluated as described by Kleiner et al.[19] Fibrosis was evaluated histologically separately from NAS. The histological and immunohistochemical analysis was performed blindly by two pathologists at The Children's Memorial Health Institute (Warsaw, Poland). A detailed description of the methods used can be found in Supplementary Information—Methods.

2.7 | Western blot analysis

Protein levels were analyzed using sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of human biopsy liver homogenates. Western blotting was performed using the following primary antibodies: SOD1 (cat. number sc101523, Santa Cruz Biotechnology), SOD2 (cat. number sc133134, Santa Cruz Biotechnology), catalase (cat. number sc271803, Santa Cruz Biotechnology), PRX Pathway cocktail (TRX, TXNRD1, PRDX1, cat. number ab184868, Abcam), OXPHOS cocktail (cat. number ab110413, Abcam). The levels of investigated proteins in liver lysates were normalized by the REVERT total protein stain signal (LI-COR Biosciences, NE, USA). A detailed description of the method is provided in Supplementary Information—Methods.

2.8 | Oxidative damage markers in liver biopsy samples

The scale of oxidative damage to proteins (protein carbonylation) in liver biopsies was estimated using OxyBlot™ Protein Oxidation Detection Kit (S7150, Sigma-Aldrich, MO, USA) (see Supplementary Information—Methods). Additionally, the scale of oxidative damage (lipid and protein oxidation markers) in the liver biopsies, that is hexanol-lysine adducts (HEL), 4-hydroxy-2-nonenal (4-HNE), and di-tyrosine (DT), was evaluated on histological slices. For the immunohistochemistry of HEL, 4-HNE, and DT formalin-fixed embedded paraffin samples were used. Before antigen detection, antigen retrieval was performed using a low pH Target Retrieval Solution (DAKO, Glostrup, Denmark) at 99.5°C for 20 min. Then, the following antibodies were used: Anti-HEL antibody (cat. number MHL-021P; Clone 5F12; Japan Institute for the Control of Aging) and Anti-4-HNE monoclonal antibody (cat. number MHN-020P; Japan Institute for the Control of Aging)—lipid oxidation markers and DT antibody (cat. number MDT-020P; Clone 1C3; Japan Institute for the Control of Aging)—protein oxidation marker.

2.9 | Total antioxidant activity (TAA)

Total antioxidant activity was measured in liver biopsies' homogenates and serum samples by the colorimetrical assessment of 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical reduction as described previously by Amorim et al.[20] In short, the stabilized solution of ABTS radical was prepared by mixing phosphate buffer, ABTS, hydrogen peroxide, and horseradish peroxidase, followed by incubation at 4°C for at least 4 h in the darkness. Next, liver biopsies' homogenates or serum samples were mixed with the stabilized ABTS radical, and the absorbance was measured at 730 nm with the aid of a microplate reader (Infinite 200Pro, Tecan, Männedorf, Switzerland) for 15 min. The measurement of TAA in each sample was carried out in three technical replicates.

2.10 | Statistical analysis

All data are presented as means ± standard deviations for the proteomic analysis of mouse hepatic tissue, and as median with interquartile range for patients data. Based on the data distribution, changes in the hepatic proteome of mice fed with the Western diet were analyzed using two-way ANOVA with Sidak's multiple comparisons test. Unpaired two-tailed *t*-test or two-tailed Mann–Whitney test were used, as appropriate, to compare the levels of serum biomarkers, TAA, markers of oxidative damage, antioxidant enzymes, and OXPHOS complexes between the control group and MASLD patients. Clinical characteristics, the parameters describing oxidative stress, antioxidant defense status, and levels of OXPHOS complexes subunits or disease stage (NAS score) were correlated using Spearman's rank correlation test. Statistical analysis was performed using GraphPad Prism 9 (San Diego, CA, USA). R statistical software (version 4.2.2) with R Commander and FactoMineR packages was used to perform the principal component analysis (PCA).[21] The level of statistical significance was set at p-value $< .05$.

3 | RESULTS

3.1 | Histopathological evaluation of the hepatic tissue of mice

Feeding C57BL/6J mice with the Western diet for 16 and 24 weeks induced advanced liver steatosis (grade 3: >66%) with the presence of a few ballooned hepatocytes (grade 2) and mild fibrosis (1c) (Table 1), however, without any signs of liver inflammation (Figure S1). At the same time, the livers of mice fed with the standard (chow) diet

showed no signs of lipid accumulation, hepatocyte ballooning, inflammation, or fibrosis (Figure 1). Mice fed with the Western diet reached a NAS score equal to 5 after both timepoints (Table 1).

3.2 | Oxidative stress and antioxidant profile in the hepatic tissue of MASLD mice

Next, we used the data from the former proteomic analysis[15] on the major antioxidant proteins in the hepatic tissue of mice and analyzed how the profile of antioxidant enzymes changed over time (16 vs. 24 weeks of the Western diet) to investigate a possible adaptation to the obesogenic diet (Figure 2, Table S2). After 16 weeks of the Western diet, the levels of five major antioxidant enzymes were unaffected (catalase—CAT, superoxide dismutase 1—SOD1, peroxiredoxin 1—PRDX1, thioredoxin—TRX, and thioredoxin reductase 1—TXNRD1), the levels of five enzymes were significantly increased (glutathione peroxidase 4—GPX4, superoxide dismutase 3—SOD3, peroxiredoxins 2 and 3—PRDX2, PRDX3, methionine sulfoxide reductase A and B2—MSRA and MSRB2) while the level of one mitochondrial enzyme (superoxide dismutase 2—SOD2) was significantly decreased. After 24 weeks of the Western diet, the levels of SOD1 and TRX remained unaffected while those of CAT, PRDX1, and TXNRD1 slightly increased. Consistently increased levels across both time

TABLE 1 Staging of MASLD in mice.

Mice		Steatosis	Ballooning	Inflammation	Fibrosis	NAS score[a]
16w	SD	Grade 0	Grade 0	Grade 0	Stage 0	0
	WD	Grade 3	Grade 2	Grade 0	Stage 1c	5
24w	SD	Grade 0	Grade 0	Grade 0	Stage 0	0
	WD	Grade 3	Grade 2	Grade 0	Stage 1c	5

Note: Mouse NAFLD activity score (NAS) was calculated based on the Hematoxylin & Eosin (H&E), Masson Trichrome, and immunohistochemistry of CD3, CD45, and CD68 stainings. Fibrosis was evaluated histologically separately from NAS.

Abbreviations: SD, standard diet; WD, Western diet.

[a]NAS value is considered as the sum of the grades obtained for steatosis, ballooning and inflammation. Steatosis, grade 0 ≤ 5%; grade 1 = 5%–33%; grade 2 = 34%–66%; grade 3 ≥ 66%. Ballooning, grade 0 = absent; 1 = a few ballooned hepatocytes; 2 = many ballooned hepatocytes. Inflammation grade (magnification 200×): 0 = absent; 1 = up to two foci per field of view; 2 = two to four foci per field of view; 3 = more than four foci per field of view (lipogranulomas are counted in this category). Fibrosis grade: Stage 0 = absent; Stage 1a = zone 3, perisinusoidal fibrosis; Stage 1b = zone 3, perisinusoidal fibrosis; Stage 1c = only periportal/portal fibrosis; Stage 2 = zone 3, plus portal/periportal fibrosis; Stage 3 = zone 3, plus portal/periportal fibrosis with bridging fibrosis; Stage 4 = cirrhosis.

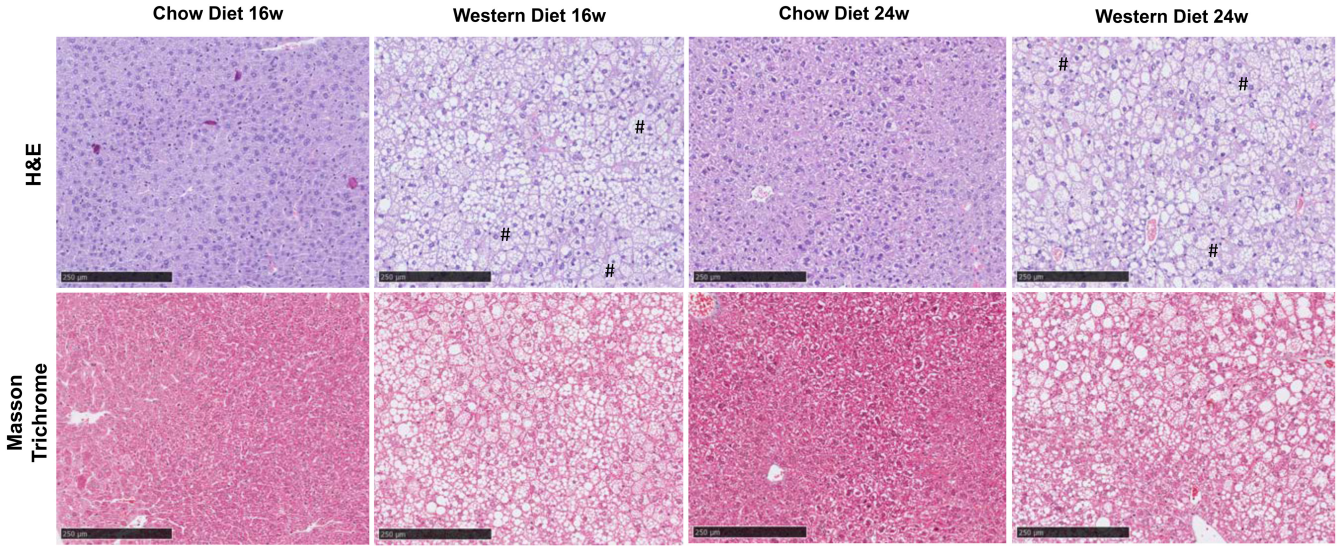


FIGURE 1 Mouse histology. Representative images of paraffin-embedded liver sections with hematoxylin and eosin (H&E) and Masson Trichrome stains. Scale bar, 250 μm; 10× magnification. Ballooned hepatocytes are indicated with #.

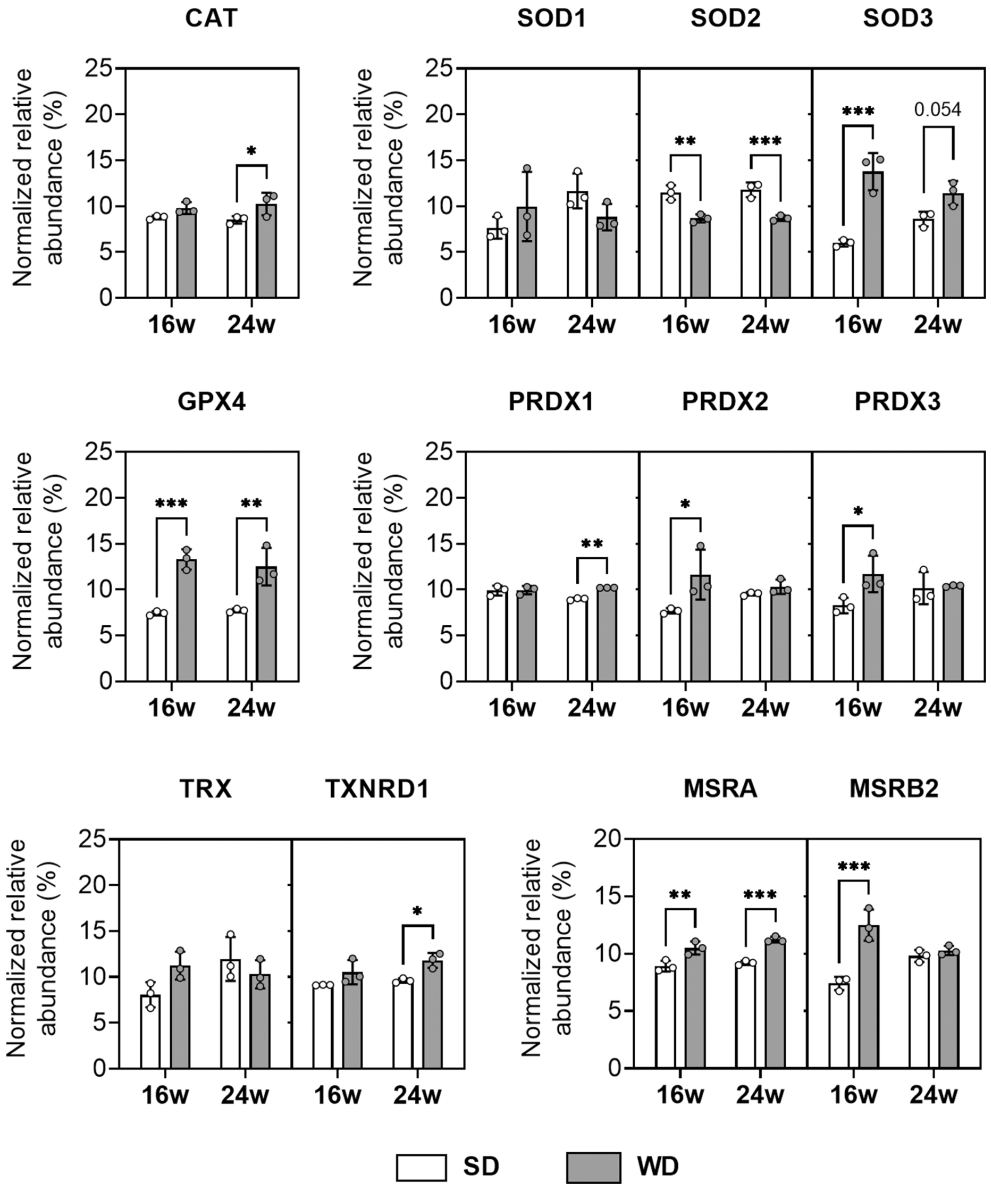


FIGURE 2 Hepatic levels of proteins involved in antioxidant defense in mice fed with standard diet (SD) or Western diet (WD) for 16 or 24 weeks (16w and 24w, respectively). Antioxidant enzymes: catalase (CAT), glutathione peroxidase 4 (GPX4), methionine sulfoxide reductase A (MSRA), methionine sulfoxide reductase B2 (MSRB2), peroxiredoxin 1-3 (PRDX1-3), superoxide dismutase 1-3 (SOD1-3), thioredoxin (TRX), thioredoxin reductase 1 (TXNRD1). Results are expressed as mean ± SD of three biological replicates. Statistically significant changes determined by two-way ANOVA with Sidak's multiple comparisons test are marked as (*)—p-value < .05, (**)—p-value < .01, (***)—p-value < .001.

points were observed for GPX4 and MSRA while the levels of SOD2 remained consistently decreased compared to their hepatic levels in mice fed with the standard diet. After 24 weeks of the Western diet, the levels of some antioxidant proteins (SOD3, PRDX2, PRDX3, MSRB2) were no longer different from the levels observed in the group of mice fed with the standard diet. Altogether, these results show that antioxidant defense systems were significantly altered in the hepatic tissue of mice; however, the magnitude of observed effects may change over time.

3.3 | OXPHOS profile in the hepatic tissue of MASLD mice

To investigate whether any OXPHOS remodeling occurred alongside the observed changes in the hepatic antioxidant profile, we determined the levels of the electron transport chain (ETC) and ATP synthase subunits in the hepatic tissue of mice fed either with the standard chow diet or with the Western diet for 16 and 24 weeks. As shown in Figure 3, 16 weeks of the Western diet significantly decreased the level of only

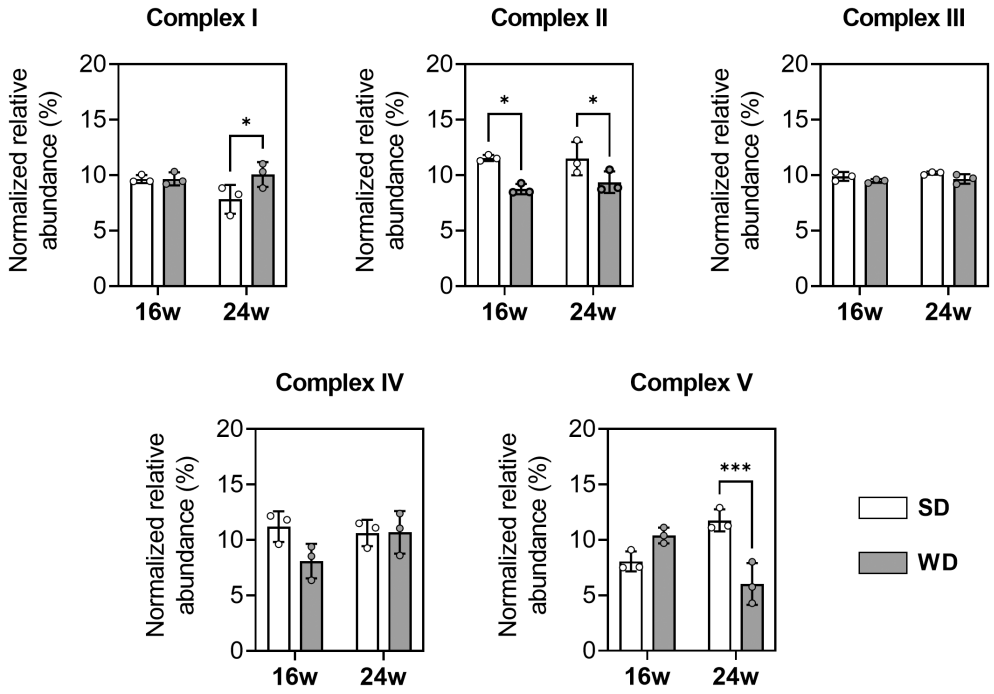


FIGURE 3 Hepatic levels of oxidative phosphorylation (OXPHOS) subunits in mice fed with standard (SD) or Western diet (WD) for 16 or 24 weeks (16w and 24w, respectively). Results are expressed as mean ± SD of three biological replicates. Statistically significant changes determined by two-way ANOVA with Sidak's multiple comparisons test are marked as (*)—p-value < .05, (**)—p-value < .01, (***)—p-value < .001, (****)—p-value < .0001.

one complex—complex II, which remained decreased to the same extent also after the longer feeding period. An even more significant decrease was observed for complex V (subunit e). In contrast, 24 weeks of the Western diet markedly increased the level of complex I. The levels of complexes III and IV remained unaffected regardless of the duration of the Western diet. Altogether, these results point to more significant mitochondrial alterations (at least at the level of OXPHOS subunits) occurring after the longer feeding period resulting in the late stage of liver steatosis. Nevertheless, despite a significant decrease in the level of complex V, mitochondrial OXPHOS remodeling proved to be rather modest.

3.4 | Histological evaluation of liver biopsies of MASLD patients

In the next step, we assessed the status of oxidative stress and antioxidant defense in the cohort of 26 Caucasian individuals. Among 20 patients undergoing bariatric surgery, the presence of MASLD was confirmed in 16 individuals, while 4 did not have liver steatosis (i.e., had fat accumulation below 5% in the liver biopsy). For that reason, these 4 patients were classified to the control group together with 6 other individuals without diagnosed parenchymal liver pathology, for whom the liver was excised due to other medical indications. Histological analysis confirmed the lack of liver steatosis and hepatocyte ballooning in all the subjects ($n = 10$) classified to the control group (Figure S2).

In the MASLD group, 8 patients had mild steatosis and 9 had a few ballooned hepatocytes. Ten patients with MASLD had mild lobular inflammation and 9 had mild liver fibrosis. The detailed results of the histological analysis are shown in Table S3. Histochemical assessment (H&E and Masson trichrome) and immunohistochemical assessment of CD68, CD3, and CD45LCA inflammatory markers of liver biopsies from the control and MASLD groups are presented in Figures S2 and S3, respectively.

3.5 | Clinical characteristics of MASLD patients

The clinical data of the studied individuals are presented in Table 2. The median age within the control and MASLD groups was 45.5 and 41 years, respectively. Gender distribution was equal in the MASLD group; however, 70% of the control group consisted of females. The median body mass index (BMI), fasting glucose, insulin, and homeostatic model assessment of insulin resistance (HOMA-IR) levels were significantly higher in the MASLD group

TABLE 2 Clinical characteristics of the studied cohort ($n = 26$).

Variables	Reference range	Control group $n = 10$	MASLD patients $n = 16$
Clinical characteristics			
Female, *n* (%)	n.a.	7 (70.0)	8 (50.0)
Age (years)	n.a.	45.5 (34.0–70.0)	41.0 (23.0–59.0)
BMI (kg/m^2)	n.a.	30.8 (18.8–46.2)	43.4 (36.1–63.3)
Glucose (mg/dL)	70.0–99.0	91.0 (74.0–111.0)	99.0 (79.0–272.0)
Insulin (μL·U/mL)	2.6–24.9	11.2 (6.2–18.5)	21.4 (10.9–41.1)
HOMA-IR	<2.0	2.5 (1.1–4.4)	4.9 (2.2–19.6)
Total cholesterol (mg/dL)	120.0–190.0	160.0 (111.0–229.0)	175.0 (121.0–247.0)
Triglycerides (mg/dL)	50.0–150.0	97.0 (61.0–182.0)	153.0 (64.0–372.0)
HDL cholesterol (mg/dL)	≥40.0 (male) ≥45.0 (female)	62.0 (35.0–87.0)	41.0 (29.0–59.0)
LDL cholesterol (mg/dL)	<100.0	85.0 (50.0–134.0)	96.0 (62.0–168.0)
AST (U/L)	5.0–40.0	27.5 (17.0–81.0)	33.0 (17.0–107.0)
ALT (U/L)	7.0–56.0	26.5 (17.0–157.0)	45.0 (18.0–127.0)
GGTP (U/L)	7.0–50.0	30.5 (16.0–810.0)	35.0 (17.0–130.0)
Total bilirubin (mg/dL)	0.2–1.2	0.6 (0.4–1.5)	0.8 (0.5–1.9)

Note: Values are expressed as medians (range) unless stated otherwise.

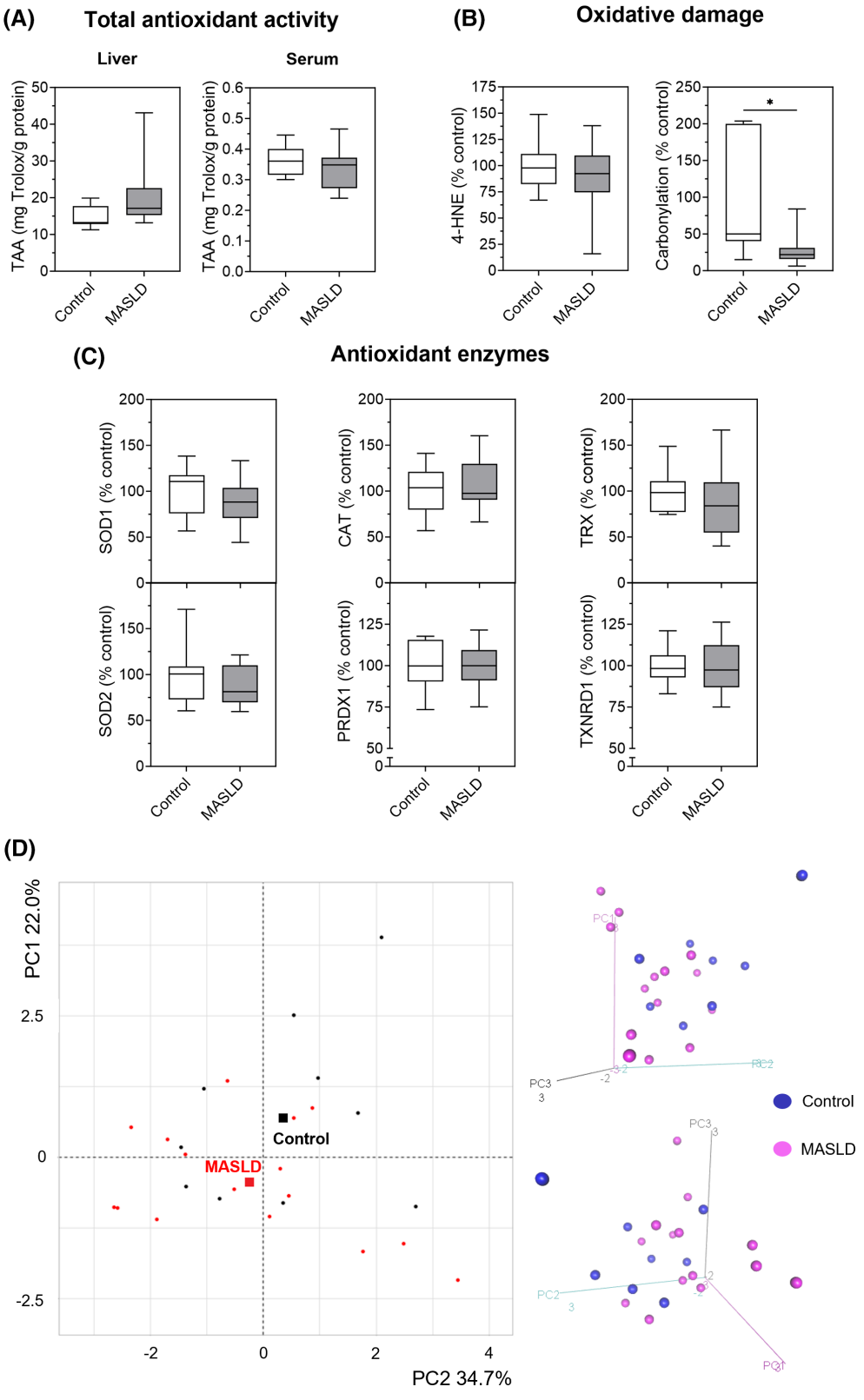
Abbreviations: ALT, alanine transaminase; AST, aspartate transaminase; BMI, body mass index; GGTP, gamma-glutamyl transpeptidase; HDL, high-density lipoprotein; HOMA-IR, homeostatic model assessment of insulin resistance; LDL, low-density lipoprotein; MASLD, metabolic dysfunction-associated steatotic liver disease; n.a., not applicable.

compared to the control group (p-values = .0003, .0162, .0124, and .0027, respectively). All these parameters were strongly correlated with the subjects' health status described by the NAS score (BMI, $r = 0.613$, p-value = .0009; fasting glucose, $r = 0.655$, p-value = .0003; insulin, $r = 0.6129$, p-value = .0019; HOMA-IR, $r = 0.684$, p-value = .0003) (Figure S4). The median levels of total cholesterol, low-density lipoprotein (LDL), and triglycerides (TAG) were higher in the MASLD group; however, only in the case of the last parameter, the difference was statistically significant (p-value = .0251). The median level of high-density lipoprotein (HDL) was significantly higher in the control group compared to MASLD patients (p-value = .0032). The health status of patients was significantly correlated with TAG and HDL levels ($r = 0.687$, p-value = .0001 and $r = -0.558$, p-value = .0038, respectively). None of the liver-related parameters significantly differed between the control and MASLD groups; however, the levels of aspartate transaminase (ALT) and alanine transaminase (AST) were moderately correlated with the NAS score ($r = 0.574$, p-value = .0022 and $r = 0.455$, p-value = .0195, respectively) (Figure S4).

FIGURE 4 Oxidative stress and antioxidant profile in controls and patients with MASLD. (A) Total antioxidant activity measured in liver biopsy homogenates and serum samples of the studied cohort. (B) Hepatic oxidative damage represented by the levels of lipid peroxidation (4-HNE) and protein carbonylation. (C) Hepatic levels of antioxidant enzymes. Protein levels are expressed as a percentage of control. (D) On the left, a two-dimensional principal component analysis (2D PCA) score plot generated from the analysis of parameters describing the hepatic status of antioxidant defense (levels of antioxidant enzymes and markers of oxidative damage). On the right, three-dimensional (3D) visualization of the PCA score plot. Box plots represent the data as medians, interquartile range, and minimal and maximal values. Statistical analysis was performed using an unpaired two-tailed *t*-test or two-tailed Mann–Whitney test (depending on data distribution) with the level of significance set at p-value < .05 marked as (*). 4-HNE, 4-hydroxy-2-nonenal; CAT, catalase; MASLD, metabolic dysfunction-associated steatotic liver disease; PRDX1, peroxiredoxin 1; SOD1, superoxide dismutase 1; SOD2, superoxide dismutase 2; TAA, total antioxidant activity; TRX, thioredoxin; TXNRD1, thioredoxin reductase 1.

3.6 | Oxidative stress and antioxidant profile in patients with MASLD

To verify whether MASLD was accompanied by the aggravating oxidative stress and disrupted antioxidant status, in the first step, we evaluated the TAA in serum and liver samples obtained from the studied cohort. Surprisingly, we found no notable differences in the hepatic and serum TAA values between controls and patients with MASLD (Figure 4A). Next, we evaluated



the levels of protein carbonylation and lipid peroxidation in the liver biopsies to verify whether the manifestation of oxidative stress could be detected at the level of oxidative damage to biomolecules. As presented in Figures 4B and S5, the level of 4-HNE, that is, a marker of lipid peroxidation, did not significantly differ between the studied groups. In contrast to lipid peroxidation, the level of protein carbonylation was significantly lower in the group of MASLD patients (p-value = .0128) than in the control group. Additionally, the scale of oxidative damage (lipid and protein oxidation markers such as HEL, 4-HNE, and DT) was also evaluated in histological liver slices (Figure S6). The immunohistochemical evaluation confirmed the absence of substantial signs of oxidative damage in the liver sections from all the studied subjects.

The presence of oxidative stress is reflected not only by the level of markers of oxidative damage to biomolecules but also by the efficiency of the endogenous antioxidant defense system. Therefore, to have a more complete overview, we evaluated the protein levels of six main antioxidant enzymes. As in the case of TAA, the levels of antioxidant enzymes did not significantly differ between the studied groups (Figures 4C and S5).

Finally, by using PCA, we analyzed whether the oxidative stress and antioxidant profiles (consisting of the level of oxidative damage to proteins and lipids and the levels of six antioxidant enzymes) can differentiate patients with MASLD from controls. In this case, PCA allowed us to transform 8 variables and to visualize graphically (in two- and three-dimensional graphs) the data, with minimal loss of information regarding studied individual parameters. The first two components of the PCA (PC1 and PC2) explained 22.0% and 34.7% of the variance, respectively, within the studied cohort. As shown in Figure 4D, the oxidative stress and antioxidant profile of the controls turned out to slightly differ from that of the MASLD group, which might suggest the presence of subtle alterations in the overall redox status of the liver of patients with MASLD.

3.7 | OXPHOS profile in patients with MASLD

Similarly to the mouse model of MASLD, we measured the levels of representative OXPHOS complexes in the liver biopsies to assess potential alterations in the ETC function. In contrast to the results from mice hepatic tissue (Figure 3), none of the changes in the levels of ETC complexes between the group of MAFLD patients and controls was statistically significant (Figures 5 and S5).

3.8 | Relationship between clinical parameters and antioxidant status of MAFLD patients

In order to identify potential correlations between common clinical parameters and parameters describing antioxidant status in the hepatic tissue of MASLD patients, we performed Spearman's correlation rank test (Figure 6A). We found moderate positive correlations between the levels of two antioxidant enzymes (peroxiredoxin 1, PRDX1; thioredoxin, TRX) and HDL ($r = 0.503$, p-value = .010, and $r = 0.466$, p-value = .019, respectively) as well as between the levels of TXNRD1 and TAG ($r = 0.419$, p-value = .037). Moderate negative associations were detected between the levels of CAT and AST ($r = -0.398$, p-value = .044) or GGTP ($r = -0.448$, p-value = .044), as well as between SOD2 and total bilirubin ($r = -0.414$, p-value = .035). BMI was negatively correlated with the levels of protein carbonylation ($r = -0.506$, p-value = .016) and TRX ($r = -0.554$, p-value = .003). Figure 6B shows the graphical representation of the strongest correlations between parameters evaluated in the controls and MASLD patients.

4 | DISCUSSION

The wealth of evidence describing the role of oxidative stress in the pathogenesis of MASLD is based on *in vitro* and *in vivo* studies, in which decreased levels and/ or activity of antioxidant enzymes as well as increased levels of ROS and/ or markers of oxidative damage have been reported.[6,22] Except for *in vitro* and animal studies, decreased antioxidant status, which suggests the presence of oxidative stress, has been also reported in several human studies involving MASLD patients.[4,8–10] Nevertheless, the cause–effect relationship between oxidative stress and MASLD has not yet been established. As such, the translation of the results from animal models of fatty liver to human subjects turned out to be challenging.[17,23] Therefore, in this work, we aimed to investigate whether the oxidative stress and antioxidant profile determined in the animal model of MASLD is in line with the profile established in the hepatic tissue obtained from the group of patients with MASLD.

Animal models of liver steatosis induced by a Western diet have been shown to exhibit the best resemblance to human MASLD.[24] In our former study, we showed that C57BL/6J mice fed with the Western diet for up to 24 weeks to induce hepatic steatosis (without inflammation) had increased activities of two cytosolic antioxidant enzymes (i.e., SOD1 and glutathione reductase) as well as enhanced total antioxidant capacity of cytosolic (but not mitochondrial) fraction of hepatic tissue.[15,18] Using the available data from the proteomic analysis, in

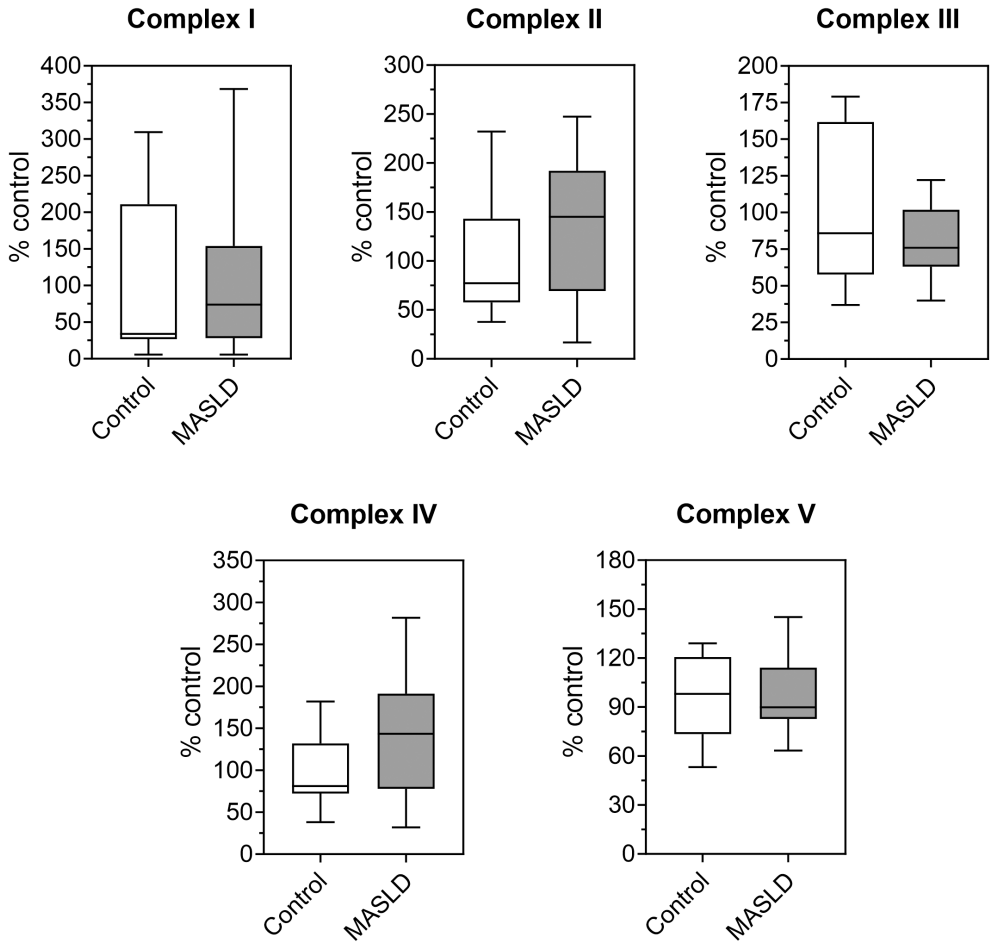


FIGURE 5 Hepatic levels of oxidative phosphorylation (OXPHOS) subunits in controls and patients with MASLD. Protein levels were expressed as a percentage of control. Box plots represent the data as medians, interquartile range, and minimal and maximal values. Statistical analysis was performed using an unpaired two-tailed *t*-test or two-tailed Mann–Whitney test (depending on data distribution) with the level of significance set at p-value < .05.

this study, we further identified the levels of the major antioxidant proteins involved in the maintenance of redox homeostasis and antioxidant defense and monitored how they changed over time by comparison of the results obtained after 16 and 24 weeks of feeding with the Western diet. Protein levels of several major antioxidant enzymes were increased either at both time points (GPX4, MSRA), only after 16 (SOD3, PRDX2-3, MSRB2) or 24 weeks (CAT, PRDX1, TXNRD1) of the Western diet. Consistently increased levels of GPX4, which catalyzes the reduction of lipid hydroperoxides to the corresponding nontoxic lipid alcohols,[25] and MSRA, which is involved in the repair of oxidatively modified proteins,[26] might explain the lack of notable signs of oxidative damage to lipids and proteins as reported previously.[15] The loss of significant differences in the levels of SOD3, PRDX2-3, and MSRB2 between the groups of mice fed with the standard or Western diets suggests that a certain adaptation to a newly established redox homeostasis may occur with time.[27] The level of mitochondrial SOD (SOD2) was the only enzyme with decreased protein level, which further supports the lack of, or even decreased, mitochondrial oxidative stress. Proteomic analysis of mice hepatic tissue revealed minor changes in the levels of OXPHOS subunits, especially after the longer feeding period. Nevertheless, as shown in our previous study,[15] mitochondrial remodeling accompanying the disease progression was not associated with oxidative stress at the mitochondrial level.

In contrast to what was observed in the mice model of MASLD,[15] we did not find any significant changes in both hepatic and serum TAA between the controls and patients with MASLD. Such results have been also reported by a few other human studies.[28,29] In terms of oxidative damage, patients with MASLD had significantly lower levels of protein carbonylation than control subjects. Interestingly, a similar decrease was also observed in our previous animal studies but only at a 16-week time point,[15,18] and recently such an observation has been also reported by Chienwichai and colleagues in

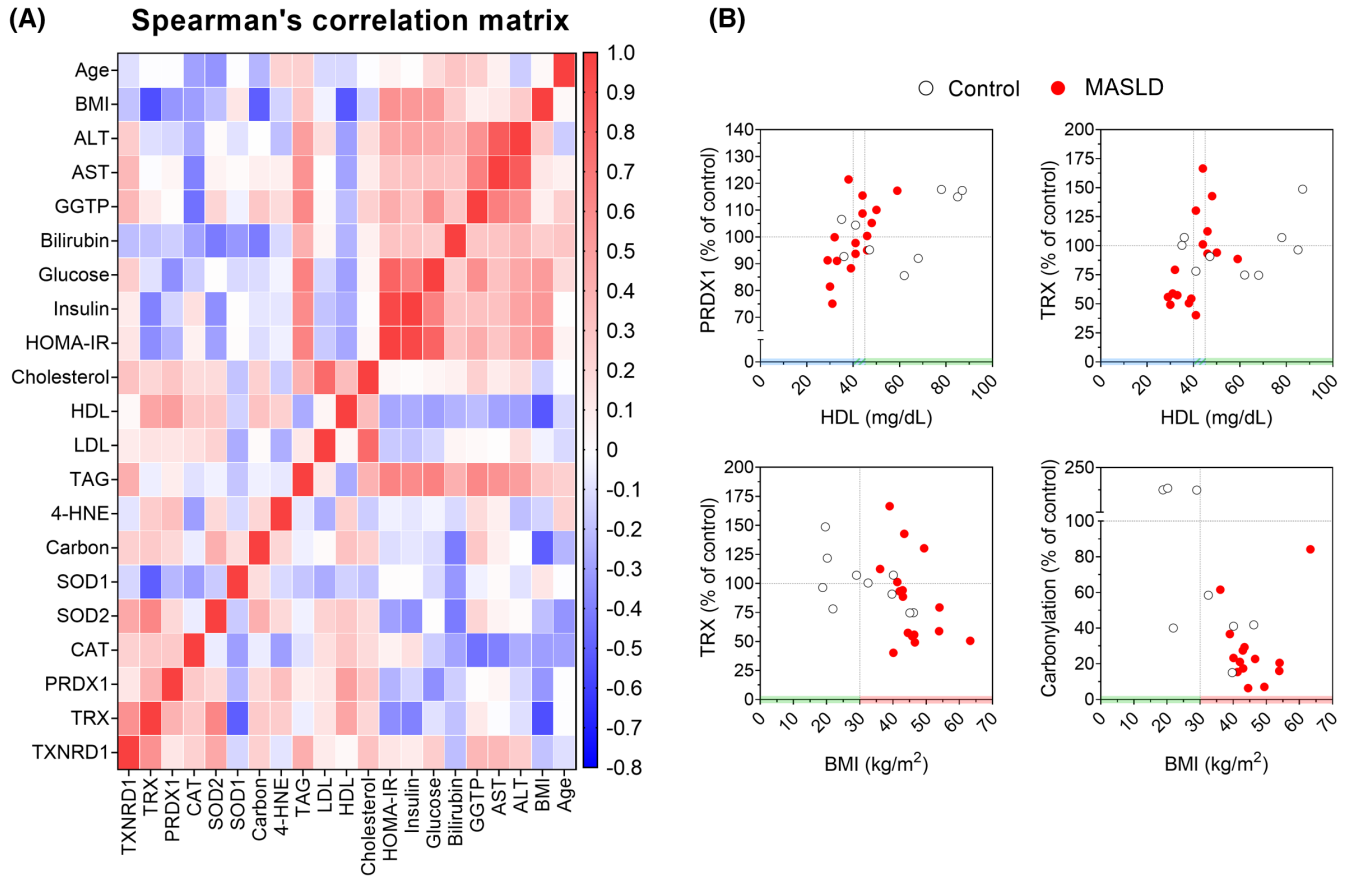


FIGURE 6 Parameters describing antioxidant and oxidative stress status correlate with clinical parameters. (A) Graphical presentation of Spearman's rank correlation coefficients between clinical parameters and parameters describing antioxidant status. (B) Graphical representation of the strongest correlations between parameters evaluated in controls and MASLD patients. The dotted lines indicate the reference range of specific clinical parameters as well as 100% of the control level of the second variable. 4-HNE, 4-hydroxy-2-nonenal; ALT, alanine transaminase; AST, aspartate transaminase; BMI, body mass index; carbon., protein carbonylation; CAT, catalase; GGTP, gamma-glutamyl transpeptidase; HDL, high-density lipoprotein; LDL, low-density lipoprotein; MASLD, metabolic dysfunction-associated steatotic liver disease; PRDX1, peroxiredoxin 1; SOD1, superoxide dismutase 1; SOD2, superoxide dismutase 2; TAG, triglycerides; TRX, thioredoxin; TXNRD1, thioredoxin reductase 1.

an *in vitro* study.[30] In accordance with our results from the animal model,[15] but in contrast to the majority of reports involving MASLD patients[8,28,31,32] the level of 4-HNE, a marker of lipid peroxidation, was not significantly increased in patients with fatty liver as compared to controls. The absence of significant oxidative damage in the liver sections was confirmed for all investigated groups by immunohistochemical evaluation of HEL, 4-HNE, and DT. To determine the antioxidant profile of the hepatic tissue of MASLD patients, we also measured the levels of the most important antioxidant enzymes: SOD1, SOD2, CAT, PRDX1, TRX, and TXNRD1. In contrast to what was observed in the hepatic tissue of mice, no significant changes in the levels of antioxidant enzymes were found in the liver biopsies of MASLD patients. Finally, we demonstrated by PCA that the oxidative stress profile of MASLD patients turned out to be slightly distinct from the control group. As such, the overall oxidative stress status seems to be, at least to some extent, altered in MASLD patients, which is in line with the available evidence from several human studies.[28,33]

Even though the role of mitochondrial ETC is fundamental for cellular metabolism, under pathological conditions, it has been considered one of the major sources of ROS. Excessive ROS generation related to abnormal function or expression level of individual ETC complexes may lead to oxidative stress and together with mitochondrial dysfunction could also contribute to MASLD development and progression.[34] However, in this scenario, we did not observe any significant changes in the levels of hepatic ETC complexes in MASLD patients compared to the controls. This together with our previous data[15] imply nonsignificant role of the ETC disturbances and ETC-derived ROS in the pathogenesis of MASLD. To the best of our knowledge, the evaluation of the hepatic levels of

the ETC complexes in the hepatic tissue of MASLD patients has not been yet performed along with the thorough profiling of oxidative stress and antioxidant status, analogously to what was done previously in our mice model of early MASLD.[15]

Finally, we identified moderate correlations between a few common clinical parameters and parameters describing antioxidant status in the hepatic tissue of MASLD patients. The levels of HDL turned out to be negatively correlated with two interrelated antioxidant enzymes: PRDX1 and TRX. PRDXs are omnipresent antioxidant proteins that catalyze the reduction of H_2O_2 and other organic hydroperoxides to water and alcohol. In the course of such reduction, the peroxidatic cysteine in the catalytic center of PRDX1 becomes oxidized, which can be reversed by TRX in an NADPH-dependent manner.[35] The cooperated activity of both enzymes is also known to be involved in the control of redox signaling.[36] Furthermore, the TRX system is known to decrease protein carbonylation levels, which may serve as one of the mechanisms of thiol-mediated signal transduction.[37,38] Reduced levels of protein carbonylation, as well as significant correlations found between the TRX system (i.e., TRX or TXNRD1), PRDX1, and parameters describing metabolic health (i.e., BMI, the levels of HDL or TAG), may indicate disturbances in thiol-redox signaling accompanying MASLD. Noteworthy, the levels of antioxidant enzymes (PRDX1, TXNRD1) as well as proteins involved in the repair of oxidatively modified proteins (MSRA, MSRB2)[26] were also affected by the Western diet in our animal model of hepatic steatosis. The importance of thiol-redox signaling in MASLD pathogenesis has been recently recognized also by other groups.[39] Therefore, the connection between MASLD and thiol-redox signaling is worthy of further investigation to verify whether there is a cause–effect relationship between these variables.

Our study has several strengths. First of all, we thoroughly evaluated and compared the oxidative stress and antioxidant profiles in the hepatic tissue of the animal model and MASLD patients. Second, along with the assessment of the redox-related parameters, we determined the hepatic levels of OXPHOS complexes, the remodeling of which could be assumed to accompany the presence of oxidative stress-related mitochondrial dysfunction. Our study has also a few limitations that need to be acknowledged. First, liver biopsy may not always be representative of pathology in the whole organ due to an intrinsic heterogeneity in the distribution of the histological characteristics.[40] Second, no significant alterations in the oxidative stress profile of MASLD patients may be attributed to the limited sample size and, as a result, low statistical power; therefore, the discussion and generalization of the results should be done with caution.

5 | CONCLUSIONS

Altogether, these data show that even though the phenotype of mice may closely resemble human MASLD, the translation of cellular and molecular processes such as oxidative stress seems to be more challenging. The relationship between disrupted antioxidant status and MASLD has been reported by several studies, however, in our cohort the manifestation of oxidative stress was not detected. The lack of notable changes in parameters describing the oxidative stress profile of patients with MASLD may be due to the small sample size and consequently low statistical power. Another possibility explaining the lack of differences between the controls and patients may be an adaptation to a dynamically changing redox environment, as suggested by the diminishing changes in the levels of antioxidant proteins that occurred over time in the animal model. Nevertheless, decreased levels of protein carbonylation and significant correlations between the TRX system and some relevant clinical parameters suggest possible alterations in the thiol-redox signaling and its role in the MASLD pathogenesis. These findings may point to new direction in the development of novel therapeutic strategies targeting thiol-redox signaling pathways.

AUTHOR CONTRIBUTIONS

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DISCLOSURES

The authors have read the journal's policy on conflicts of interest and declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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