

Oxidative stress and antioxidant status in patients with autoimmune liver diseases

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Objective: To estimate oxidative stress and antioxidant components during different stages of autoimmune liver diseases and assess their possible implication on disease progression.

Methods: We determined several markers of oxidative injury (isoprostane, aldehydes, protein carbonyls, 3-nitrotyrosine, and myeloperoxidase) and antioxidant components (glutathione, glutathione peroxidase, glutathione reductase, superoxide dismutase, and catalase) in whole blood, serum, and urine in 49 patients with autoimmune cholestatic liver diseases (AC) and 36 patients with autoimmune hepatitis (AIH) and healthy subjects matched for sex and age.

Results: Both AC and AIH patients had increased levels of all lipid and protein oxidative injury products and significantly decreased whole blood glutathione levels compared to controls. AIH patients had significantly higher levels of aldehydes and glutathione peroxidase activity and significantly lower protein carbonyl levels compared to AC patients. Protein carbonyl and isoprostane levels increased and glutathione levels decreased gradually with progression from mild fibrosis to severe fibrosis and cirrhosis in both AC and AIH patients. In addition, both cirrhotic AC and AIH patients had significantly higher protein carbonyls compared to non-cirrhotics.

Discussion: We provide novel findings in support of a major contribution of oxidant/antioxidant imbalance in the progression of liver injury in AC and AIH.

Keywords: Autoimmune hepatitis, Primary biliary cirrhosis, Lipid peroxidation, Glutathione, Antioxidant enzymes, Protein carbonyls

Introduction

The pathophysiological mechanisms underlying progression from the initial autoimmune attack to the development of fibrosis and ultimately to cirrhosis and liver failure in patients with autoimmune liver diseases, namely, autoimmune hepatitis (AIH), primary biliary cirrhosis (PBC), and primary sclerosing cholangitis (PSC) are poorly understood.^{1–3} Several reports have suggested the involvement of oxidative injury in animal models of acute and chronic liver disease and particularly in rat model of cholestasis induced by bile duct ligation,^{4,5} as well as in a variety of human liver disorders.^{5–7} Data on the presence of components of oxidative stress and antioxidant defence and their potential contribution to progression of AIH and PBC are scarce and limited to evaluation of individual markers in serum or in liver.^{8,9}

The aim of the present study was to determine several components of oxidative injury and antioxidant defence in patients with different stages of AIH and cholestatic liver diseases in order to assess their potential implication in disease progression. We determined lipid peroxidation products like *trans*-4-hydroxy-2-nonenal (4-HNE), malondialdehyde (MDA), and 8-isoprostane (8-iso), protein modification products such as 3-nitrotyrosine (3-NT) or protein carbonyls (PC) in parallel with enzymatic and non-enzymatic antioxidant activities and subsequently correlated their activity with clinical and laboratory parameters associated with disease severity, as well as administration and response to treatment.

Materials and methods

Subjects

Eighty-five patients with autoimmune liver diseases (14 male, 71 female) followed at the Department of Medicine, Medical School, Thessaly University,

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Table 1 Age, gender, BMI, dietary and lifestyle habits of patients and controls

	AC controls	AC patients	AIH controls	AIH patients
Gender (male/female)	(8/42)	(6/43)	(10/31)	(8/28)
Age (years)	54 ± 15	57 ± 14	49 ± 17	50 ± 15
BMI (kg/(h) ²)	26 ± 4	26 ± 4	26 ± 3	26 ± 6
Smokers	33%	16%	18%	16%
Smoking (pack-years)	29 ± 24	29 ± 27	26 ± 50	27 ± 59
Alcohol consumption (1–7 drinks/week)	36%	24%	15%	16%
Meat consumption >4 times/week	35%	20%	30%	26%
Fruit consumption >7 times/week	55%	52%	65%	60%
Vegetables consumption >7 times/week	80%	53%	75%	80%

Values shown as percentages % or as mean ± SD.
BMI, body mass index.

were studied prospectively. Patients were divided into two groups: Group 1 consisted of 49 patients with autoimmune cholestatic liver diseases (AC; 41 PBC and 8 PSC).^{2,3} According to Ludwig's classification 2 patients had stage 1, 26 stage 2, 2 stage 3, and 6 stage 4 PBC.¹⁰ All PSC patients ($n = 8$) had stage I fibrosis and for this reason they were analysed together with stage I PBC patients (stage I AC group). Nine PBC patients were not receiving any treatment, while the remaining (32 PBC, 8 PSC) were under ursodeoxycholic acid (UDCA; 15 mg/kg/day for PBC and 20 mg/kg/day for PSC). Response to treatment was defined as normalization of liver enzymes.²

Group 2 (AIH group) consisted of 36 AIH patients.¹¹ All but three patients (because of the presence of burn-out cirrhosis) were under immunosuppression with prednisolone (median dose: 5 mg/day, range: 0–10 mg/day) and/or mycophenolate mofetil (MMF; median dose: 1 g/day, range: 0–2 g/day) as part of a single-centre uncontrolled trial.¹² Response to treatment was defined according to our report¹² and the American Association for the Study of Liver Diseases criteria.¹³ According to Ishak scoring system,¹⁴ 14 patients had minimal/mild, 6 had moderate, and 10 had severe fibrosis (cirrhosis). Other causes of cholestasis or hepatitis, Wilson's disease, and haemochromatosis were excluded appropriately. Mean follow-up of AC and AIH patients was 59 ± 26 and 68 ± 75 months, respectively.

Voluntary blood donors, age- and sex-matched, for each group of patients were also studied. All were seronegative for hepatitis C virus, hepatitis B virus, and human immunodeficiency virus with no previous history of hepatitis and/or chronic alcohol consumption. None of the patients and controls had received antioxidant supplementation at least 2 months preceding their inclusion in the study. Demographic characteristics and personal habits of patients and controls are presented in Table 1. All patients and controls consented to participate in the study. The study was approved by the Ethical Committee of the University of Thessaly, Medical School.

Specimen processing

Plasma, serum, and urine were collected after overnight fasting. Plasma and urine were protected from oxidation during storage by addition of the antioxidant butylated hydroxytoluene (200 µg/ml). For myeloperoxidase (MPO) measurement, leucocytes were isolated from the blood according to Cooray *et al.*¹⁵

Antioxidants

Copper, zinc superoxide dismutase (CuZn-SOD), catalase (CAT), glutathione peroxidase (GSH-Px), and GSH-reductase activities were measured in the erythrocyte lysates on a UV-VIS Recording Spectrophotometer (UV-2100S, Shimadzu, Japan). Erythrocytes were lysed with cold distilled water (1:4).

Measurement of CuZn-SOD activity was performed using Ransod reagents (Ransod Laboratories, London, UK) according to McCord and Fridovich.¹⁶ GSH-reductase activity was determined according to Goldberg and Spooner,¹⁷ GSH-Px according to Paglia and Valentine,¹⁸ and CAT determination according to Aeby¹⁹ whereas determination of reduced glutathione (GSH) and oxidized glutathione (GSSG) was carried out according to Tietze.²⁰

Oxidative injury markers

15-F_{2t}-isoprostane in urine and serum 3-NT levels were determined using commercially available enzyme-linked immunosorbent assay kit ((Oxford Biomedical Research, Oxford, UK), (HyCult Biotechnology, Uden, The Netherlands)). PC in serum were determined according to Levine *et al.*²¹ while MDA and 4-HNE according to Gérard-Monnier *et al.*²² Leucocyte MPO activity was measured according to the method of Marquez and Dunford.²³

Statistical analysis

The results were expressed as mean ± standard deviation (SD) or median (range) as appropriate. Data were analysed by unpaired *t*-test, Mann-Whitney *U* test, analysis of variance, and the Fisher's *post hoc* least-significant difference as the *post hoc* test corrected for multiple comparisons, as well as the non-

Table 2 Markers of lipid peroxidation, protein oxidation, activities of oxidant enzymes and antioxidant defence in controls and AC group in relation to disease stage and treatment response

Parameters	Controls (n = 50)	AC patients (n = 49)	Cirrhotics with AC (n = 6)	Non-cirrhotics with AC (n = 43)	Naïve patients with AC (n = 9)	Treated patients with AC (n = 40)	Non-responders to treatment with AC (n = 9) (3 patients with cirrhosis)	Responders to treatment with AC (n = 31) (3 patients with cirrhosis)
8-Iso ng/mg creatinine (U)	1 (0.01–2.7)	1.8*** (0.2–3.7)	2.6*** (1.7–3.2)	1.8**** (0.2–3.7)	2.5** (2.2–3.4)	1.8**** (0.2–3.7)	2.1*** (0.6–3.2)	1.8**** (0.2–3.7)
Aldehydes μ M (P)	0.3 (0.05–4.7)	1.2*** (0.1–3.8)	0.4 (0.1–1)	1.3*** (0.1–3.7)	2.1* (0.3–3.8)	0.9** (0.1–3.8)	0.3 (0.1–2.1)	1.7**** (0.2–3.8)
3-NT nM (P)	11 (1.5–20)	14** (1.3–70)	22 (1.8–50)	14 (1.3–70)	6.9 (2.5–20)	14 (1.3–70)	14 (1.9–40)	13 (1.3–70)
PC mg/mg protein (S)	1.6 (0.1–10)	4.1**** (0.01–21)	8.1**** (5.9–21)	3.7****/+ (0.02–19)	5.4 (0.02–4.6)	4*** (0.02–21)	3.6**** (0.02–21)	4.2*** (0.14–15)
MPO U/10 ⁶ cells (L)	12 (1–142)	19 (1.3–511)	13 (2–39)	20 (1.3–511)	7 (1.3–65)	25** (1.3–511)	10 (1.3–511)	26 (2–245)
GSSG (GSH + GSSG) (WB)	5.1 (0.1–36)	14*** (0.2–92)	27** (1.5–75)	12 (0.2–92)	12 (0.2–71)	10** (0.2–92)	13 (0.2–92)	14 (0.4–71)
GSH μ M (WB)	1101 (276–5409)	475**** (4–2743)	209**** (89–659)	495**** (4–2743)	258** (18–2743)	500**** (18–1872)	426**** (4–1400)	494** (18–1872)
Catalase kU/gHb (RC)	142 (50–449)	148 (11–504)	144 (79–265)	153 (11–504)	144 (28–430)	130 (11–504)	124** (14–294)	162 (11–504)
SOD kU/gHb (RC)	43 (8–68)	51*** (14–78)	52 (25–60)	50** (14–78)	46 (24–78)	51** (14–60)	52** (29–78)	50** (14–60)
GSH-Px U/gHb (RC)	3.6 (0.3–25)	4.9 (0.1–63)	7.8 (0.8–16)	3.4 (0.1–63)	3 (0.8–63)	3.5 (0.1–63)	3.4 (0.4–25)	3.7 (0.1–63)
Glutathione reductase U/gHb (RC)	1.6 (0.08–10)	2.6 (0.1–36)	2.9 (0.5–16)	2.6 (0.1–36)	3.3 (0.83–36)	2.6 (0.1–37)	2.7 (0.22–37)	2.6 (0.1–35)
AST U/l (S)	20 (12–44)	23 (12–100)	37* (19–100)	22 (12–48)	18 (15–30)	23 (12–100)	25 (15–100)	21 (12–39)
ALT U/l (S)	20 (11–57)	26** (6–86)	44 (12–58)	25** (6–86)	18 (6–31)	26** (6–86)	31** (12–86)	21 (6–50)
ALP U/l (S)	61 (32–96)	81**** (28–279)	130* (41–191)	79**** (28–279)	67 (41–120)	120**** (28–279)	127**** (46–279)	73 (28–117)
γ -GT U/l (S)	14 (5–44)	28**** (10–213)	71* (11–196)	27**** (10–213)	25 (12–75)	30**** (10–213)	52**** (13–213)	21 (10–37)
Bilirubin mg/dl (S)	0.5 (0.1–1)	0.6*** (0.23–1.7)	1.1** (0.53–1.7)	0.6** (0.23–1.6)	0.45 (0.26–1.6)	0.6**** (0.23–1.7)	0.7**** (0.23–1.7)	0.6 (0.29–1.1)
UDCA (duration of intake in months)		29 (1–168)	50 (12–168)	29 (1–91)		29 (1–168)	25 (2–91)	38 (1–168)

Values shown as medians with range. *P* values (<0.05) derived by the Mann–Whitney *U* test (*vs. control group, +vs. cirrhotic group).

*0.1 > *P* > 0.05, **/+*P* ≤ 0.05, ****P* ≤ 0.01, *****P* ≤ 0.001. GSSG/(GSH + GSSG) was expressed as %.

U, urine; P, plasma; S, serum; WB, whole blood; L, leucocytes; RC, red blood cells; UDCA, ursodeoxycholic acid.

parametric test Kruskal–Wallis, where applicable. A two sided $P < 0.05$ was considered as statistically significant.

Results

Oxidative stress markers in patients with AC, AIH, and their respective controls are shown in Tables 2 and 3. AIH patients had significantly higher levels of aldehydes (MDA + 4-HNE) ($P = 0.02$) and GSH-Px activity ($P = 0.02$) and significantly lower PC levels ($P = 0.001$) compared to AC patients.

Oxidative stress in the AC group

Patients with AC had significantly increased levels of 8-iso ($P < 0.0001$), aldehydes ($P = 0.005$), 3-NT ($P = 0.05$), PC ($P = 0.0009$), GSSG/total GSH ratio ($P = 0.002$), and superoxide dismutase (SOD) activity ($P = 0.01$) compared to controls while whole blood GSH was significantly reduced ($P \leq 0.0001$) (Table 2).

Oxidative stress in relation to cirrhosis in AC patients

8-Iso and PC levels were significantly increased in both cirrhotic ($n = 6$, $P = 0.01$ and $P < 0.0001$, respectively) and non-cirrhotic patients ($n = 43$, $P < 0.001$ and $P < 0.0001$, respectively) compared to controls (Table 2). GSSG/total GSH ratio was also significantly higher in cirrhotics ($P = 0.05$), while there was no difference between non-cirrhotics and controls (Table 2). In contrast, GSH levels were significantly decreased both in patients with cirrhosis and non-cirrhosis compared to controls ($P = 0.001$ and $P = 0.01$, respectively; Table 2).

AC patients had a gradual increase of 3-NT, PC, 8-iso, aldehydes, and the GSSG/total GSH ratio from minimal–mild fibrosis (stages 1–2, $n = 36$) to moderate–severe fibrosis (stages 3–4, $n = 8$) (Fig. 1A–E). 8-Iso and PC levels were significantly increased in all stages compared to controls (Fig. 1B and C).

In contrast, AC patients showed a gradual decrease of GSH from stage 1–2 to stage 3–4 compared to controls ($P = 0.002$ and $P = 0.004$, respectively; Fig. 1F). Apart from PC levels ($P = 0.03$), no other statistical significant difference was observed between cirrhotic and non-cirrhotic AC patients (Table 2).

Oxidative stress in AC patients according to response to treatment

There were no significant differences regarding oxidative stress markers and antioxidant activities between treatment-naïve patients and those receiving UDCA at the time of investigation, as well as between AC patients with response to UDCA treatment ($n = 31$), compared to those not responding to UDCA ($n = 9$), although treatment-naïve and treated patients, as well as responders and non-responders,

had statistically significant differences compared to controls (Table 2).

Oxidative stress in the AIH group

Patients with AIH had significantly increased levels of 8-iso ($P = 0.009$), aldehydes ($P \leq 0.0001$), 3-NT ($P = 0.004$), GSSG/total GSH ratio ($P = 0.03$), and GSH-Px activity ($P = 0.02$) compared to controls while whole blood GSH was significantly reduced ($P = 0.01$) (Table 3).

Oxidative stress in relation to cirrhosis in AIH patients

Aldehyde levels were significantly increased in both cirrhotic ($n = 10$, $P < 0.0001$) and non-cirrhotic patients ($n = 26$, $P < 0.0001$) compared to controls, whereas 8-iso and PC levels were significantly elevated only in cirrhotics compared to controls ($P = 0.05$ and $P = 0.002$) (Table 3). In addition, PC levels were significantly higher in cirrhotics compared to non-cirrhotic patients ($P = 0.01$, Table 3). Analysing cirrhotic patients according to the presence of biochemical activity, only patients with biochemical activity showed significant elevation of 8-iso compared to controls (2.8 ng/mg creatinine (1.5–5.8) vs. 1 ng/mg creatinine (0.02–3), $P = 0.03$). GSH levels were significantly reduced in cirrhotic compared to non-cirrhotic patients ($P = 0.0003$) but were abnormal in both cirrhotic and non-cirrhotic patients ($P < 0.0001$ and $P = 0.01$ respectively; Table 3).

AIH patients had a gradual increase of aldehyde and PC levels from mild to severe fibrosis (Fig. 2A and B). Actually, aldehyde levels were significantly elevated in patients with moderate (0.8 μM (0.2–6.6)) and severe fibrosis (3 μM (0.2–7.8)) compared to controls ($P = 0.03$ and $P < 0.0001$, respectively; Fig. 2A), whereas PC levels were significantly higher only between AIH patients with severe fibrosis and controls (3.8 mg/mg protein (0.6–15) vs. 1.8 mg/mg protein (0.1–9.9); $P = 0.002$) (Fig. 2B). Furthermore, GSH-Px and CAT activities were significantly increased in non-cirrhotic patients with AIH compared to controls ($P = 0.02$ and $P = 0.04$, respectively; Table 3).

Oxidative stress in AIH patients in relation to response to treatment

AIH patients not responding to immunosuppression ($n = 10$) had significantly increased aldehyde ($P = 0.006$), PC levels ($P = 0.05$), GSSG/total GSH ratio ($P = 0.05$), CAT ($P = 0.008$), and GSH-Px activities ($P = 0.05$) and lower GSH levels ($P = 0.0006$) compared to controls (Table 3). AIH patients responding to immunosuppressive treatment ($n = 23$) had significantly higher levels of aldehyde ($P \leq 0.001$), GSH-Px ($P = 0.003$), and GSSG/total GSH ratio ($P \leq 0.05$) and decreased GSH levels ($P \leq 0.05$) compared to controls (Table 3). Differences in oxidative stress

Table 3 Markers of lipid peroxidation, protein oxidation, activities of oxidant enzymes and antioxidant defence in controls and AIH group in relation to disease stage and treatment response

Parameters	Controls (n = 41)	AIH patients (n = 36)	Cirrhosis (n = 10)	No cirrhosis (n = 26)	Non-responders to treatment (n = 10) (4 patients with cirrhosis)	Responders to treatment (n = 23) (6 patients with cirrhosis)
8-Iso ng/mg creatinine (U)	1 (0.02–3)	1.3*** (0.01–8.5)	2.6** (0.4–8.5)	0.9 (0.01–5)	0.9 (0.01–8.5)	1.2 (0.4–4.2)
Aldehydes μ M (P)	0.3 (0.05–4.7)	1.7**** (0.2–7.9)	3**** (0.18–7.8)	1.7**** (0.17–6.6)	1.7*** (0.2–7.9)	0.8**** (0.2–7)
3-NT nM (P)	9 (1.5–20)	12*** (1.6–100)	12 (2.3–48)	15 (1.64–100)	6.5 (2.1–100)	12 (1.6–48)
PC mg/mg protein (S)	1.8 (0.1–9.9)	1.8* (0.1–14)	3.8**** (0.6–15)	1.5 ⁺ (0.15–6.8)	6.7** (0.14–14)	1.9 (0.5–6.3)
MPO U/10 ⁶ cells (I)	12 (1–141)	10.5 (1.2–463)	11.5 (1.5–87)	10.5 (1.2–462)	9.7 (1.2–463)	12 (1.5–160)
GSSG/(GSH + GSSG) (WB)	4.3 (0.2–15)	9.5** (0.1–93)	8.6 (1.4–85)	12.4 (0.1–93)	6.7** (0.3–93)	7.9** (0.1–85)
GSH μ M (WB)	1135 (293–5409)	512*** (51–5541)	293**** (51–5541)	511****/++ (88–2977)	519**** (88–5541)	800** (51–1033)
Catalase kU/gHb (RC)	146 (40–449)	173 (5–772)	147 (5–320)	215** (22–772)	236*** (5–772)	148 (17–409)
SOD kU/gHb (RC)	44 (8–68)	48 (13–59)	37 (16–59)	47 (13–59)	24 (13–59)	45 (13–59)
GSH-Px U/gHb (RC)	3.6 (0.3–25)	8.6** (0.6–36)	7 (2.2–32)	8.8** (0.6–35)	8.9** (1.6–36)	8.9*** (0.6–33)
Glutathione reductase U/gHb (RC)	2.4 (0.1–10)	2.1 (0.08–36)	2.2 (0.08–36)	2.3 (0.08–8.9)	2.6 (0.1–36)	2.3 (0.2–8)
AST U/I (S)	20 (12–30)	25*** (10–117)	29* (10–65)	25** (10–117)	52*** (18–117)	23 (10–49)
ALT U/I (S)	18 (9–29)	25*** (10–160)	23** (10–81)	26*** (12–160)	56 **** (20–160)	20 (10–43)
ALP U/I (S)	61 (32–91)	61 (26–320)*	64 (49–136)*	60 (26–320)*	106*** (30–300)	61 (26–116)
γ -GT U/I (S)	13 (5.2–27)	22.5**** (8–277)	21** (8–170)	23**** (8–277)	35**** (12–240)	15 (8–69)
Bilirubin mg/dl (S)	0.5 (0.1–1)	0.7*** (0.3–1.96)	1*** (0.3–1.96)	0.6** (0.2–1.32)	0.68**** (0.3–1.96)	0.5 (0.3–1)
Prednisolone (duration of intake in months)		30 (1–114)	54 (11–101)	13 (1–114)	16 (1–58)	36 (3–114)
MMF (duration of intake in months)		26 (1–94)	41 (11–84)	16 (1–94)	16 (1–60)	30 (3–94)

Values shown as medians with range. *P* values (<0.05) derived by the Mann–Whitney *U* test (*vs. control group, ⁺vs. cirrhotic group).

*0.1 > *P* > 0.05, ***P* ≤ 0.05, ***/⁺*P* ≤ 0.01, ****/⁺⁺*P* ≤ 0.001. GSSG/(GSH + GSSG) was expressed as %.

U, urine; P, plasma; S, serum; WB, whole blood; L, leucocytes; RC, red blood cells; MMF, mycophenolate mofetil.

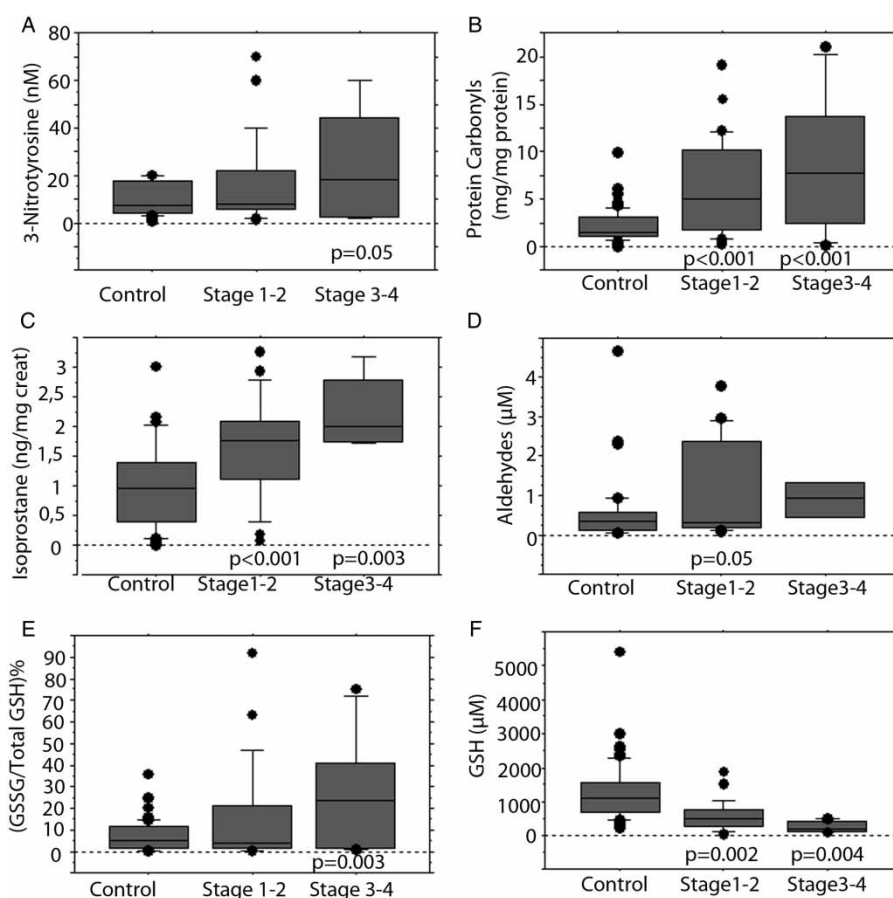


Figure 1 Markers of oxidative stress in controls and AC patients according to Ludwig's stage. Levels of 3-NT (A), PC (B), isoprostane (C), aldehydes (D), oxidized/total GSH ratio (E), and reduced glutathione (F). The results are expressed as median, interquartile range, and range. P values refer to the comparison between each stage to controls (Mann-Whitney U test).

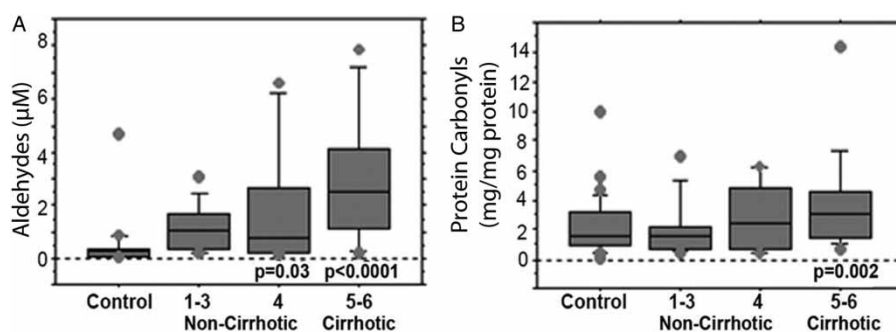


Figure 2 Products of oxidative stress injury products in controls and AIH patients according to the stage of fibrosis. Products of lipid (A) and protein (B) oxidation. The results are expressed as median, interquartile range, and range. P values refer to the comparison between each stage to controls (Mann-Whitney U test).

products and markers of antioxidant defence were evident between responders and non-responder AIH patients, though did not reach statistical significance (Table 3).

Discussion

Three major findings emerge from the present study. First, oxidative stress is a significant feature of autoimmune liver diseases and is evident from early stages of both diseases. Second, disturbances in antioxidant defence are involved in the progress of both

diseases contributing probably to progression towards cirrhosis. Third, immunosuppression can ameliorate protein oxidation, by reducing PC levels.

To our knowledge, this is the first study to evaluate lipid peroxidation products, protein oxidation markers, and MPO concurrent with enzymatic and non-enzymatic antioxidant activities in patients with autoimmune liver diseases. Previous work in patients with autoimmune liver diseases has dealt with the determination of individual parameters of oxidative stress, including GSH status, lipid peroxidation

products, or intrahepatic 3-NT accumulation.^{8,24–26} PC levels are reported for the first time in the literature.

Our results demonstrate a marked increase of lipid peroxidation (8-iso and aldehydes) and protein oxidation (3-NT and PC) products in AIH and AC patients compared to healthy. In our AC patients, we observed a significant augmentation of 8-iso with progression of fibrosis, as assessed by Ludwig's score. Our data complement previous work^{26,27} and demonstrate increased lipid peroxidation to be an early event in the disease progress and probably not just a consequence of the disease. This hypothesis is further supported by Paradis *et al.*,²⁷ who provided immunohistochemical evidence of 4-HNE adducts in normal bile ducts with no detectable features of cell death. Reactive oxygen species (ROS) generation by inflammatory cells, accumulation of cytotoxic bile acids and intrahepatic copper are some of the mechanisms that contribute to lipid peroxidation induction in PBC.²⁸

Similarly, PC levels increased with progression of liver disease. PC was the only marker of oxidative stress that differed significantly between cirrhotic and non-cirrhotic AC patients, being higher in the former group. The use of PC is regarded as more advantageous oxidative stress marker compared to lipid peroxidation products, taking into account that they are more stable compared to other oxidative stress biomarkers and are an index of severe oxidative protein damage.²⁹

Moreover, similar to others²⁴ we observed a significant decrease in total GSH levels in AC patients compared to controls. Reduced intracellular glutathione levels were linked to increased perinuclear expression of 4-HNE in the damaged bile ducts of PBC patients.³⁰ Of note, we showed a gradual decrease of total GSH levels and a gradual increase of 8-iso levels with deterioration of fibrosis stage in AC, suggesting that reduced intrahepatic glutathione reserves might contribute to increased lipid peroxidation which stimulates collagen synthesis.³¹ GSSG/total GSH ratio was also significantly increased only in cirrhotics with AC compared to controls, indicating a defective glutathione redox state.

Of interest, among enzymatic antioxidants, only SOD was increased in AC patients and particularly in early disease stage compared to controls. This may indicate overexpression of enzymatic activity at early stages to counteract for the increased burden of ROS production. Decreased or unaltered levels of antioxidants at advanced stages of disease could be explained by consumption of antioxidant elements necessary for neutralizing the oxidative damage. Changes in enzymatic antioxidants develop *in situ* and this could probably explain the fact that no significant changes were noted in other antioxidant enzymes.

So far, UDCA is the only approved treatment for PBC.² UDCA has been shown to have a hepatoprotective effect mainly via up-regulation of γ -glutamyl-cysteine-synthetase expression, which leads to increased rate of GSH synthesis.³² In our study, UDCA treatment was associated with a relative increase of GSH levels compared to non-treated patients, even though the difference was not significant. UDCA failed to improve any parameter of oxidative stress, as demonstrated also by others.³³

Data on parameters of oxidative stress in AIH are scarce. We have found increased levels of lipid peroxidation products and PC in AIH patients compared to controls. Similar to AC patients, there was a significant correlation between both lipid peroxidation products and PC levels with fibrosis stage, suggesting that increased levels of oxidant stress may contribute to perpetuation of liver injury in AIH. These data are in agreement with Pemberton *et al.*⁸ reporting increased levels of lipid peroxidation that correlated significantly with the necroinflammatory activity and fibrosis score. In a previous study, Sanz-Cameno *et al.*²⁵ revealed enhanced NT accumulation in AIH patients and provided evidence of significant correlation with severity of liver damage. Even though 3-NT was increased in all AIH and AC patients, we could not find significant changes in the blood between different stages of liver disease probably due to small number of patients in each stage of liver injury. Similar to patients with AC, PC was the only marker that was significantly elevated in cirrhotics compared to non-cirrhotic AIH patients. This association is of immense importance since published data have pointed towards a link between oxidative and nitrosative stress and induction/exaggeration of autoimmunity, as covalent binding of lipid peroxidation products with proteins can lead to formation of neoantigens.^{34,35} In parallel, AIH patients with cirrhosis had significantly lower GSH levels compared to non-cirrhotics, suggesting a major contribution of depletion of antioxidant reserves in disease progression.

Of note, comparison between AC and AIH groups with chronic hepatitis B patients revealed significantly higher NT levels in autoimmune liver disease groups (Supplementary Material 1). Whether changes in individual parameters of oxidative stress are disease specific needs further investigation in larger studies and it is not within the aim of the present study.

Systemic use of corticosteroids alone or in conjunction with azathioprine or MMF is considered the mainstay of treatment for patients with AIH.^{12,13} The anti-inflammatory action of corticosteroids depends partially on interference with ROS formation.³⁶ We have demonstrated lower PC and CAT levels, higher median GSH levels in AIH patients responding to immunosuppressive treatment

compared to non-responders. Similar data have been reported in patients with infiltrative Grave's ophthalmopathy.³⁷ Our data suggest that corticosteroids were more effective than UDCA in ameliorating protein oxidation products.

Our data suggest that increased levels of oxidant stress in AC and AIH patients might correspond to liver inflammatory activity and fibrosis in both diseases. In this context, though speculative at present, our results may be of value in order to design future antioxidant therapies that can be used as complementary treatments, mainly for non-responder patients to immunosuppressive regimens. In addition, studies in larger cohort of patients may help to identify individual parameters of oxidative stress or antioxidant protection mechanisms that could serve as markers of treatment response but this needs external validation.

Acknowledgements

We thank Kostas Galanis for assistance in blood collection.

Disclaimer statements

Contributors GND and ANM had the original idea for the study, designed the study protocol and wrote the paper. ETK in collaboration with ANM did the whole laboratory work and along with EIR collected all the demographic and clinical data and performed the statistical analysis and wrote the first draft of the manuscript. GKK did the interpretation of the histological data of the patients. EIR and GND treated the patients and performed the follow-up assessment while contributed to the final version of the paper. GKK and GND wrote the final version of the paper. All authors have seen and approved the final draft of the paper.

Funding Parts of this work has been supported by the Hellenic Ministry of Education and Religious Affairs (Code: Archimidis) and the Research Committee of the University of Thessaly (Code No: 2466).

Conflicts of interest None.

Ethics approval The study was approved by the Ethical Committee of the University of Thessaly, Medical School.

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