Oxidant stress is a significant feature of primary biliary cirrhosis

Ali Aboutwerata, Philip W. Pemberton, Alexander Smith, Peter C. Burrows, Raymond F.T. McMahon, Sanjiv K. Jain, Thomas W. Warnes*

The Liver Unit, Department of Gastroenterology, Manchester Royal Infirmary, Oxford Road, Manchester M13 9WL, UK
Department of Pathological Sciences, University of Manchester, Manchester, UK

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Abstract

Primary biliary cirrhosis (PBC) is a chronic cholestatic disorder characterised by an immunological, and often granulomatous, attack on bile ducts leading to fibrosis, cirrhosis, liver failure and death. Animal and human studies suggest that oxidant stress plays a key role in progression of other liver diseases, but no comprehensive investigation has been performed previously in PBC. A wide range of lipid peroxidation and antioxidant markers were measured in the blood and urine of 41 patients with histologically confirmed PBC. Lipid peroxidation markers were significantly elevated [plasma and urinary 8-isoprostane, \( P < 0.001 \); plasma malondialdehyde (MDA), \( P = 0.007 \)] compared to age- and sex-matched controls. The most striking antioxidant depletion occurred with plasma total glutathione where levels were significantly reduced (30% of controls). Total serum antioxidant levels were decreased \( (P = 0.013) \) and serum selenium and vitamin A were also lower (both \( P < 0.001 \)); vitamins C and E were normal. Most patients had early disease biochemically and were Child-Pugh grade A. Urinary 8-isoprostane correlated positively with Ludwig stage and markers of hepatic injury and cholestasis. This study clearly demonstrates that oxidant stress, as reflected in a comprehensive spectrum of lipid peroxidation and antioxidant markers, is a significant feature of early-stage PBC.

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Keywords: Primary biliary cirrhosis; Oxidant stress; Lipid peroxidation; Antioxidant

1. Introduction

Primary biliary cirrhosis (PBC), predominantly affecting middle-aged women, is a chronic cholestatic disorder which has a prevalence of 20–150 cases per 100,000 population in the Western World. The disease is characterised by an immunological attack on medium size (interlobular) bile ducts; this produces cholestasis with retention of copper and toxic primary bile acids, followed by the development of fibrosis. The natural history of the disease and its histological evolution have been well documented [1] and whilst a number of drugs, including ursodeoxycholic acid [2], colchicine [3–6] and corticosteroids [7], may modify the course of the disease, none is curative. PBC therefore remains a major indication for liver transplantation.

The pathophysiological mechanisms underlying progression from the initial autoimmune attack to the development of fibrosis and, ultimately, cirrhosis and liver failure in PBC are poorly understood, but recent work indicates that generation of reactive oxygen species (ROS) such as the superoxide anion and the hydroxyl radical may play a role. Oxidant stress has been demonstrated in animal models of acute and chronic liver disease and, in particular, in a rat model of cholestasis, induced by bile duct ligation [8–10]. The authors suggest that ROS were generated by inflammatory processes and possibly by endotoxin or bile acids; copper retention in cholestasis may also play a part as demonstrated in a rat model of copper accumulation [11]. Oxidant stress has also been observed in a variety of human liver disorders, including chronic viral hepatitis [12,13].
alcoholic liver disease [14], Wilson’s disease and haemachromatosis [15]. In PBC, one histological study in liver biopsies from five patients demonstrated the presence of malondialdehyde (MDA) and 4-hydroxynonenal (HNE) adducts, major metabolites of lipid peroxidation [16]. In a more recent and larger study, HNE protein adducts have been detected in hepatocytes preferentially located around the portal tracts as well as in the cytoplasm of damaged, but also intact, biliary cells [17]. Elevated levels of Mn-superoxide dismutase have been found, and immunostaining of liver biopsies has shown increased expression of this protein in damaged epithelial cells of interlobular bile ducts, bile ductules and degenerated hepatocytes. This finding suggests that free radicals, including the superoxide anion, may be involved in the pathogenesis of the disease [18]. Micronutrient studies of individual vitamins and selenium in liver disease have shown some deficiencies in small numbers of PBC patients but these individual factors were examined in a dietary context rather than as a panel of markers of oxidant stress [19–21].

Oxidant stress arises when there is an imbalance between radical-generating and radical-scavenging activity; it may therefore cause an increase in the formation of oxidation products and a decrease or depletion of endogenous antioxidant protection mechanisms. Oxidative damage of lipids leads to the generation of MDA and 8-isoprostanate. MDA is the end product of peroxidative decomposition of polyenic fatty acids and, in the experimental animal, the level in tissues [10] is indicative of the extent of lipid peroxidation. Whilst lipid peroxidation is traditionally assessed by measurement of MDA, a more sensitive measure can be provided by determination of 8-isoprostanate, a product of arachidonic acid peroxidation [22]. Individual antioxidant components include vitamins A, C and E, and selenium, an essential co-factor of glutathione peroxidase. Glutathione is a major component of the antioxidant system and synthesis in, and efflux from, the liver is the main source of plasma glutathione [23]. However, previous work has suggested that glutathione metabolism is disturbed only in late stage, decompensated hepatic disorders [24]. In the present study, in addition to the above markers, we have also measured total antioxidant capacity, which reflects the effective sum of all cooperative chain-breaking antioxidants.

To date, no comprehensive investigation of oxidant stress in PBC has been published. The present study is designed to determine, first, whether oxidant stress occurs in this disease by measuring, in blood and urine, a wide spectrum of markers reflecting both pro- and antioxidant facets of the process. Second, we wish to determine whether this process occurs in early stage PBC and may therefore play a pathophysiological role in the early progression of this disease or, alternatively, is confined to late stage disease when it presumably reflects major, but nonspecific, disturbances in hepatic metabolism. Finally, we wish to elucidate the mechanisms underlying this pathophysiology by assessing the relationship between oxidant stress and markers of fibrosis and cholestasis. For the former we have measured serum type III procollagen peptide (PIIINP), which we have previously shown to accurately reflect hepatic fibrogenesis and prognosis in PBC, and for the latter, serum bilirubin, which is of major prognostic value in this disease as well as serum bile acids, the most sensitive indicator of cholestasis, in addition to assessment of copper binding protein by orcein, a surrogate histological marker of cholestasis.

2. Materials and methods

2.1. Study groups

The study was approved by the Central Manchester Health Authority Research Ethics Committee and all patients gave written informed consent. All subjects were nonsmokers, took no vitamin supplements and had a normal nutritional status. All PBC patients (n = 41) fulfilled conventional diagnostic criteria, with a raised serum alkaline phosphatase (ALP), a positive anti-mitochondrial antibody test and a liver biopsy diagnostic of, or compatible with this diagnosis [4]. Pruritis was present in 34% of patients whilst the other 66% had few, if any, symptoms directly referable to the disease. No patient had received ursodeoxycholic acid, colchicine or corticosteroids for at least 3 months. As the PBC patients were all Caucasian and predominantly older females, care was taken to ensure that patient and control groups were age-, sex- and race-matched (control group n = 34, 7 females, age = 53.1 ± 11.9 years, range 37–79 years; PBC group n = 41, 4 females, age = 57.9 ± 9.0 years, range 37–72 years). Disease severity was assessed biochemically (serum bilirubin [25]), and by Child-Pugh grade [26].

2.2. Liver biopsy

A subset of PBC patients (n = 20) had been biopsied within 9 months of entry to this study (mean time = 18 months). Needle liver biopsies were fixed in formalin and stained with haematoxylin/eosin, orcein and Gordon and Sweats’ reticulin. All biopsies were assessed blindly by a single histopathologist for Ludwig stage [27] and for the presence of orcein staining (for the detection of copper binding protein, a surrogate marker for histological cholestasis) as well as for the presence and number of granuloma in each biopsy.

2.3. Specimen processing

Blood and urine samples were collected after overnight fasting. For measurement of MDA and 8-isoprostanate, blood was collected into ice-cold tubes containing EDTA (1 mg/ml) and GSH (1 mg/ml) and, after centrifugation (800 × g, 10 min), the plasma was protected from oxidation during storage by addition of the chain-breaking antioxidant buty-
lated hydroxytoluene (BHT) (200 μg/ml). Urine was protected by addition of indomethacin (0.001% w/v) to prevent in vitro formation of prostanooids due to any leucocyte contamination [28]. Serum (1 ml) was added to 1-ml ice-cold 10% (w/v) trichloroacetic acid and, after centrifugation (3300 × g, 20 min), the deproteinated sample was used for vitamin C determination. Whole blood for glutathione assay was kept on ice and analysed on the morning of collection. All other samples were stored at −70 °C prior to assay.

2.4. Analytical procedures

2.4.1. 8-Isoprostane

Prostagland or urine (0.5 ml) was mixed with 10-μl [3H]-prostaglandin F2α (NEN, Boston, MA; ca. 200 000 dpm; added to estimate recovery), 3-μl BHT (10% w/v in methanol) and 2-ml methanol. After standing on ice for 5 min, precipitated protein was removed by centrifugation (1200 × g, 10 min) and the pH of the supernatant adjusted to below 4.0 by addition of 0.3 M sodium phosphate and 1 M HCl. Solid phase extraction [28] was carried out on C18 BondElut (500 mg; Varian, Harbor City, CA). Extracts were reconstituted in 1-ml 0.1 M phosphate buffer pH 7.4 containing 1% BSA and 0.02% (w/v) BHT. After taking 0.25 ml for [3H] counting, 50-μl aliquots were assayed for 8-isoprostane using an enzyme-linked immunossay kit (Cayman Chemicals, Ann Arbor, MI). Urinary creatinine was estimated using an alkaline picrate solution [29].

2.4.2. MDA

This was determined in serum by the 2-thiobarbituric acid reactive substances (TBARS) method. Precipitation of serum lipoproteins using a phosphotungstic acid–sulfuric acid system was performed to ensure removal of interfering water-soluble substances [30]. 2-Thiobarbituric acid (TBA) was added to the precipitate and, after boiling for 10 min, the red pigment generated was extracted with butan-1-ol and measured fluorimetrically. 1,1,3,3-Tetraethoxypropane (Sigma-Aldrich Company Ltd., Poole, Dorset, UK) was used as a standard. As the TBARS method proved relatively insensitive for MDA measurement in acid-precipitated serum, an improved and more reproducible fluorimetric method [31] was also employed using 1,3-diethy1-2-thiobarbituric acid (DETBA) to measure MDA in whole serum.

2.4.3. Total antioxidant capacity

This was measured in whole and protein-free serum by an enhanced chemiluminescent technique [32]. A stable light output was generated by mixing 200-μl luminol-based reagent (Supersignal CL-HRP Substrate System, Pierce & Warriner, Chester, UK) with 2.3-ml water and 25-μl antimouse IgG horse radish peroxidase-linked whole antibody from sheep (1:250; Sigma-Aldrich). Addition of 50-μl whole serum (diluted 1:14 with distilled water), protein-free serum (1:5) or Trolox standard (50 μM; Sigma-Aldrich) resulted in depression of the signal for a period of time which is proportional to the antioxidant concentration. Results were expressed as trolox equivalents, assuming a stoichiometric factor of 2. Serum was deproteinated by centrifugation through an Amicon Centrifree™ Micropartition System (Millipore Corporation, Bedford, MA).

2.4.4. Selenium

A simple single-tube fluorimetric assay was employed [33]. Serum or selenium standard (100 μl) (500 μg/l; Merck, Lutterworth, Leics) was digested with 100 μl perchloric acid (60% v/v) + 0.4 ml nitric acid (69% v/v) for 90 min at 190 °C to free selenium from organic materials. The Se(VI) produced was then reduced to Se(IV) by incubation with 0.5-ml hydrochloric acid (37% v/v) at 150 °C for a further 30 min. Following incubation with 0.5-ml 2,3-diaminonaphthalene-HCl (Sigma-Aldrich; 0.1% w/v) and 2-ml EDTA (2.5 mM) at 60 °C for 30 min, the resulting fluorophore was extracted into 1.3-ml cyclohexane and its fluorescence measured at λex = 373 nm, λem = 515 nm.

2.4.5. Vitamin A

Methanol (1.4 ml) + water (0.28 ml) was added to 100-μl serum or all-trans-retinol acetate standard (Sigma-Aldrich; 15 μM in cyclohexane) followed by 1.4-ml cyclohexane [34]. After centrifugation (1200 × g, 10 min) to separate phases, fluorescence was measured at λex = 323 nm, λem = 470 nm in the upper layer.

2.4.6. Vitamin C

2,4-Dinitrophenylhydrazine (0.1 ml) (3% w/v; Sigma-Aldrich)/thiourea (0.4% w/v)/copper sulfate (0.05% w/v) in 4.5 M sulfuric acid was incubated at 37 °C for 3 h with 0.5-ml deproteinated serum or ascorbic acid standard (20 mg/l in 5% (w/v) TCA) [35]. After addition of 0.75-ml ice-cold sulfuric acid (65% v/v) and incubation for a further 30 min at RT, absorbance was measured at λ = 500 nm.

2.4.7. Vitamin E

Pyrogallol (0.7 ml) (Sigma-Aldrich; 2% w/v in methanol) and 105-μl saturated potassium hydroxide were incubated at 70 °C for 30 min with 0.35-ml serum or α-tocopherol standard (Sigma-Aldrich; 50 mg/l in methanol) [36]. Water (0.35 ml) and hexane (1.4 ml) were added and, after centrifugation (1200 × g, 10 min) to separate phases, the fluorescence was measured at λex = 287 nm, λem = 321 nm in the upper layer.

2.4.8. Glutathione

Three distinct measurements were performed: (1) Total glutathione, the sum of reduced (GSH) and oxidised glutathione (GSSG) in fresh whole blood. Ten microliters was haemolysed in 3.99-ml ice-cold phosphate-EDTA buffer, pH 7.5. (2) Total glutathione (GSH + GSSG) in plasma. Fresh whole blood (0.6 ml) was added to 0.6 ml 5,5’-dithio-bis(2-nitrobenzoic acid) (DTNB) (10 mM in ice-cold phosphate-EDTA buffer, pH 7.5), and centrifuged (1200 × g, 6
min) immediately. By adding the reagent directly, DTNB reacts rapidly with free sulfhydryl groups and prevents oxidation of GSH during sample preparation. (3) GSSG in plasma. Fresh whole blood (0.6 ml) was added to 0.6 ml 10 mM N-ethyl-maleimide (NEM) in phosphate-EDTA buffer pH 6.5, and centrifuged as before. NEM chemically traps GSH and prevents autooxidation to GSSG. NEM was removed by solid phase extraction prior to assay.

The reduction of DTNB by NADPH was catalysed by GSH or GSSG and glutathione reductase and the resulting absorbance measured at λ = 412 nm [37].

2.4.9. PIIINP

This serum marker of hepatic fibrogenic activity was measured using the Type III procollagen intact PIIINP radioimmunoassay (Orion Diagnostica, Espoo, Finland).

2.4.10. Other serum markers

Aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), γ-glutamyl transferase (γGT), total protein, albumin, bilirubin, urate, cholesterol, high density lipoprotein (HDL), triglycerides and prothrombin index were determined by standard automated techniques. Total serum bile acid concentration, the most sensitive indicator of cholestasis, was measured spectrophotometrically using the Enzabile test kit (Bio-Stat Diagnostic Systems, Stockport, UK).

2.5. Statistics

Since much of the data were not normally distributed, all values were expressed as medians and nonparametric statistics were employed. Differences between groups were assessed using the Mann–Whitney U-test. The significance of correlations between parameters was assessed using the Spearman rank test. For all analyses, a P-value of < 0.05 was considered to be statistically significant.

3. Results

The great majority of patients were shown to have early stage disease (Child-Pugh grade A); only two patients were grade B (both with a score of 9 points). The concentrations, in PBC and control groups, of all biochemical markers used in this study are shown in Table 1.

8-Isoprostane was significantly elevated in both urine and plasma. Although MDA by TBARS was raised in the PBC patients, this increase was not significant. In contrast, when MDA was measured by the more sensitive 1,3-diethyl-2-thio-barbituric acid reactive substances (DETBARS) method, PBC patients showed a significant elevation. Urinary 8-isoprostane and MDA (by DETBARS) were the two lipid peroxidation marker assays of choice (Fig. 1a and b).

Antioxidant status was compromised in PBC, with several important components of the antioxidant defence mechanism being significantly decreased. The total antioxidant capacity in whole serum was significantly reduced in PBC patients (Fig. 1c), but in protein-free serum was normal, suggesting that protein-bound components make an important contribution. Total glutathione (GSH + GSSG) in whole blood, a measure of intracellular antioxidant reserve, was not significantly different from the control group. The most striking antioxidant depletion occurred with total glutathione in plasma, where levels were significantly reduced to about 30% of control values (Fig. 1d). Plasma GSSG, expressed as a percentage of total plasma glutathione, was normal in the PBC group. Individual antioxidants were also shown to be affected; serum levels of selenium and vitamin A were significantly diminished (Fig. 1e and f). In contrast, serum levels of vitamins C and E and urate were normal. When vitamin E was expressed as a ratio compared to lipid (vitamin E/cholesterol, vitamin E/HDL and vitamin E/TG; results not shown), the ratios were also normal.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Oxidant stress in control and PBC groups: markers of lipid peroxidation, antioxidant status, hepatic fibrogenesis, hepatic function and other biochemical parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td>Assay</td>
<td>Control</td>
</tr>
<tr>
<td>8-Isoprostane (u)</td>
<td>0.33</td>
</tr>
<tr>
<td>8-Isoprostane (p)</td>
<td>15.5</td>
</tr>
<tr>
<td>MDA (TBARS)</td>
<td>2.05</td>
</tr>
<tr>
<td>MDA (DETBARS)</td>
<td>2.49</td>
</tr>
<tr>
<td>Total antioxidant (ws)</td>
<td>0.95</td>
</tr>
<tr>
<td>Total antioxidant (pfs)</td>
<td>0.45</td>
</tr>
<tr>
<td>Total glutathione (wb)</td>
<td>1.42</td>
</tr>
<tr>
<td>Total glutathione (p)</td>
<td>2.65</td>
</tr>
<tr>
<td>GSSG/(GSH + GSSG)</td>
<td>9.6</td>
</tr>
<tr>
<td>Selenium</td>
<td>99.8</td>
</tr>
<tr>
<td>Vitamin A</td>
<td>3.61</td>
</tr>
<tr>
<td>Vitamin C</td>
<td>17.9</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>14.4</td>
</tr>
<tr>
<td>PIIINP</td>
<td>2.80</td>
</tr>
<tr>
<td>AST</td>
<td>21</td>
</tr>
<tr>
<td>ALT</td>
<td>17</td>
</tr>
<tr>
<td>ALP</td>
<td>170</td>
</tr>
<tr>
<td>γGT</td>
<td>16</td>
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<tr>
<td>Total protein</td>
<td>78</td>
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<tr>
<td>Albumin</td>
<td>46</td>
</tr>
<tr>
<td>Bilirubin</td>
<td>10</td>
</tr>
<tr>
<td>Urate</td>
<td>0.26</td>
</tr>
<tr>
<td>Cholesterol</td>
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</tr>
<tr>
<td>HDL</td>
<td>1.58</td>
</tr>
<tr>
<td>TG</td>
<td>1.35</td>
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<tr>
<td>Prothrombin index</td>
<td>0.89</td>
</tr>
<tr>
<td>Total bile acids</td>
<td>3</td>
</tr>
</tbody>
</table>

Values shown as medians. P values derived by the Mann–Whitney test. ns = not significant. Units were expressed as follows: urinary 8-isoprostane in ng/g creatinine; plasma 8-isoprostane in ng/l; MDA (TBARS and DETBARS), total glutathione (p), vitamin A, total bile acids and bilirubin in μmol/l; total glutathione (wb), urate, cholesterol, HDL and TG in mmol/l; total antioxidant (ws and pfs) in μmol/l trolox equivalents; GSSG/ (GSH + GSSG) × 100 as %; selenium and PIIINP in μg/l; vitamins C and E in mg/l; total protein and albumin in g/l; AST, ALT, ALP and γGT in U/l. ur = urine, p = plasma, ws = whole serum, pfs = protein-free serum, wb = whole blood.
Serum PIIINP was significantly elevated in PBC patients, confirming that, as we have previously demonstrated, hepatic fibrogenesis is a prominent feature even in the early stages of this disease [38]. All conventional serum enzyme markers of hepatic injury (AST, ALT, ALP and γGT) were also significantly raised in the PBC group. Albumin was reduced significantly in the PBC group whilst, in contrast, total protein was significantly increased, reflecting increased serum globulin levels characteristically found in this putative autoimmune disorder. Although serum bilirubin was significantly higher in the PBC group than in the controls, almost all values were within the normal range and the great majority of patients had early disease; only four patients had serum bilirubin levels >34 μmol/l, the level above which patients in general enter an accelerated phase of disease progression [25]. The prothrombin index was normal and all clotting times were within 2 s of normal. Median levels of cholesterol, HDL and triglycerides were no different compared to controls. In contrast, serum total bile acids were significantly elevated in PBC patients, with a median level threefold higher than the normal group.

In the patient group, the most sensitive indicator of lipid peroxidation, urinary 8-isoprostane, correlated positively with markers of hepatocellular injury (AST: $r = 0.57$, $P < 0.001$; ALT: $r = 0.39$, $P = 0.013$), and cholestasis (ALP: $r = 0.41$, $P = 0.008$; bilirubin: $r = 0.38$, $P = 0.018$; total bile acids $r = 0.47$, $P = 0.0024$) and negatively with vitamin A ($r = -0.40$, $P = 0.011$). MDA, measured by the DETBARS assay, correlated similarly with markers of hepatocellular injury and cholestasis. The two methods used to measure MDA correlated significantly ($r = 0.46$, $P = 0.003$), but the poor reproducibility of the TBARS method resulted in a lower than expected $r$ value. Serum selenium concentrations showed a highly significant, positive correlation with albumin ($r = 0.56$, $P < 0.001$). The important contribution of the chain-breaking antioxidant urate to the total antioxidant capacity was reflected in excellent correlations between the two parameters in both whole ($r = 0.90$, $P < 0.001$) and protein-free serum ($r = 0.89$, $P < 0.001$).

In the patient group in whom recent liver histology was available, Ludwig stage (Fig. 2) correlated positively with urinary 8-isoprostane ($r = 0.45$, $P = 0.045$), with serum
4. Discussion

Oxidant stress is now a recognised feature of chronic liver disease and, in PBC, a number of mechanisms can be envisaged by which excess free radical activity can contribute to both initial bile duct damage and later progression to cirrhosis and death. Inflammation is a common and early feature of PBC and invading macrophages will generate ROS, such as superoxide and hydrogen peroxide, leading to lipid peroxidation [39]. Cholestasis, also a major feature of PBC, leads to accumulation of bile acids and hepatic copper, both of which can lead to generation of free radicals [15,40]. Hepatic decompensation in late stage disease [24] can itself lead to further generation of free radicals.

The present study complements recent publications [16,17] which have demonstrated, immunohistochemically, the presence of enhanced lipid peroxidation in the bile ducts and hepatocytes of PBC patients. Although, as we have previously discussed [13], the assessment of oxidant stress in blood and urine has limitations, our work supports these immunohistochemical findings. We have assessed a broad spectrum of markers reflecting different facets of the disease process and oxidative and antioxidant activity. This approach may give an insight concerning the importance of the various mechanisms proposed.

Oxidative damage to lipids has traditionally been assessed using the TBARS assay, which detects MDA, a peroxidation product of polyunsaturated fatty acids. However, we have previously demonstrated in hepatitis C [13] that urinary 8-isoprostane, a free radical mediated product of arachidonic acid peroxidation, is more sensitive. This has been confirmed in the present study, where plasma and urinary 8-isoprostane levels were significantly elevated, being two- to threefold higher in the PBC group than in controls. Although MDA, measured by the DETBARS method, was also significantly increased, median levels were only 20% higher than the control group. Elevated levels of 8-isoprostanes have been demonstrated in the plasma and urine of smokers, who are known to be under oxidant stress [41] and in the bile [42] and urine [43] following carbon tetrachloride-induced liver injury in the rat.

The antioxidant defence system includes a wide range of enzymatic and nonenzymatic components [44] including some serum markers of prognostic value in PBC (e.g. albumin and bilirubin). The total antioxidant assay employed provides a global assessment of the body’s antioxidant reserve, in both the aqueous and lipid phases. It reflects a complex array of factors, such as antioxidant turnover rates in the tissues, recycling of ascorbic acid and tocopherol, and production of bilirubin. Urate, probably the most important

**Fig. 2. Correlation of urinary 8-isoprostane, PIIINP and total bile acids with Ludwig stage in PBC patients.** 

PIIINP ($r = 0.61, P = 0.004$) and with total bile acids ($r = 0.57, P = 0.009$). Granuloma score correlated negatively with urinary 8-isoprostane ($r = -0.45, P = 0.048$) and positively with selenium ($r = 0.45, P = 0.048$). Orcein staining correlated positively with urinary 8-isoprostane ($r = 0.59, P = 0.006$), PIIINP ($r = 0.50, P = 0.026$) and total bile acids ($r = 0.60, P = 0.005$) and negatively with vitamin A ($r = -0.49, P = 0.026$).
single factor in determining total antioxidant capacity [45], binds iron and copper and scavenges hydroxyl and peroxo radicals. Our results show that total antioxidant capacity in whole serum is reduced in PBC despite the fact that urate levels are unchanged. The total antioxidant capacity in protein-free serum was also unchanged. Therefore, the observed fall in whole serum total antioxidant capacity probably results from a decrease in protein-bound factors such as –SH groups on proteins, vitamin E residing in the lipoprotein moiety and bilirubin located on albumin. However, in the PBC group, whilst albumin was reduced, vitamin E was unchanged and bilirubin and total protein were increased.

Selenium is an essential co-factor of glutathione peroxidase, the enzyme responsible for catalyzing the reduction of hydroperoxides in the presence of glutathione. In both alcoholic and nonalcoholic liver disease, selenium levels are reduced in the liver and serum, but not in urine [19]. Burk et al. [46] showed that plasma selenium in a variety of liver diseases declined in proportion to the severity of the cirrhotic condition as indicated by the Child’s grade. However, as plasma glutathione peroxidase actually increased, patients with cirrhosis do not appear to have functional selenium deficiency. Selenium levels in our PBC group were slightly but significantly reduced, consistent with these earlier observations, and reflecting the early stage of the disease.

As well as being a major component of the aerobic antioxidant system, glutathione plays a central role in such diverse biological processes as the detoxification of xenobiotics, maintenance of protein thiol levels and amino acid transport across membranes [23]. Plasma glutathione is known to reflect intracellular glutathione concentration, and is an index of the balance between glutathione efflux from the liver and uptake by the kidney [47]. In our PBC group, there was a striking reduction in plasma total glutathione (GSH + GSSG), suggesting that intracellular reserves were severely compromised. Plasma oxidized glutathione (GSSG) arises from both hepatic and extrahepatic sites, and provides a sensitive index of whole-body oxidant stress. However, as no increase in the plasma GSSG/(GSH + GSSG) ratio was observed, it must be assumed that the glutathione redox cycle is working efficiently in our PBC group and that GSSG is being recycled to GSH by NADPH/glutathione reductase. These results are in agreement with observations using a rat, bile-duct ligation model [10], where plasma total glutathione was significantly reduced, but the GSSG/(GSH + GSSG) ratio was found to be unchanged. In our experience, high GSSG/(GSH + GSSG) ratios only occur in cirrhotic patients with severe disruption of hepatic metabolism (unpublished results). A recent publication [48] found that a marked reduction in the expression of glutathione-S-transferase (reflecting reduced intracellular glutathione) was linked to increased perinuclear expression of HNE in the damaged bile ducts of PBC patients.

A predictable consequence of cholestasis is malabsorption of fat-soluble factors (vitamins A, D, E and K) and other free radical scavengers such as carotenoids. Retinol, α-tocopherol and total carotenoids have been shown to be reduced in PBC, but plasma levels were not affected by the histological stage in one study [49]. Kaplan et al. [20] also found vitamin A commonly decreased in PBC but, in contrast to our study, decreased levels correlated with both the histological stage and clinical severity of disease. Vitamin A was also significantly reduced in our PBC group but no correlation was found with Ludwig stage. Malabsorption is unlikely to explain the low vitamin A levels found in our patients who were mainly Child’s grade A and had biochemically early stage disease. It could be anticipated that a loss of vitamin A storage capacity might arise when hepatic stellate cells (HSCs) undergo transformation into myofibroblasts. However, Nyberg et al. [21] observed that the number and size of HSCs and the charging intensity of the retinol binding protein were actually higher in PBC patients than in controls, suggesting that low serum vitamin A in PBC is not necessarily a consequence of vitamin A deficiency, but may instead reflect defective mobilisation of vitamin A from the liver. Vitamin E was found by Jeffrey et al. [50] to be decreased in 44% of patients with PBC, those with severe cholestasis being most likely to be affected. Our PBC patients showed no evidence of significant vitamin E deficiency; this is in accordance with the findings of Kaplan et al. [20] who found only 1/52 patients were affected. He concluded that deficiencies of fat-soluble vitamins are most likely to be present in jaundiced patients with long standing severe cholestasis. Water-soluble vitamin C levels were unaffected in our PBC group, and patients therefore retain their capacity for regeneration of vitamin E by the vitamin C pathway.

Bile acids, which accumulate in the diseased liver and plasma in PBC, are pro-oxidants causing direct tissue damage mediated by ROS [40], or indirectly through activation of tissue resident macrophages [39]. Results from the rat bile-duct ligation model [8–10] indicate that bile acid-induced lipid peroxidation may be enhanced in hepatocytes in PBC patients presenting with chronic cholestasis. In our PBC group, serum total bile acids were significantly elevated and, not surprisingly, correlated positively with Ludwig stage. However, we have also shown for the first time a significant correlation between serum bile acid levels and the concentration of urinary 8-isoprostane.

Copper-mediated lipid peroxidation, as proposed for Wilson’s disease [15], may also contribute to hepatocellular injury during cholestasis in PBC where copper is known to accumulate in the liver. In our study, orcein-staining of copper-associated protein correlated positively with lipid peroxidation (urinary 8-isoprostane) as well as with hepatic fibrogenesis (PIIINP) and serum bile acids but negatively with vitamin A.

Muriel and Suarez [51], in a rat model of acute liver damage induced by biliary obstruction, demonstrated that hepatocyte necrosis (as evidenced by serum markers of tissue damage) preceded lipid peroxidation. This suggests that the latter is a consequence, rather than the cause, of
liver injury. The moderate elevation of ALT seen in our PBC group indicates that, although present, necrosis is not a major feature in these patients with early stage disease.

Only very limited information is available on the treatment of PBC with antioxidants. One study showed that administration over a period of 3 months of large oral doses of vitamin E to PBC patients with very low serum concentrations failed to restore levels to normal in the great majority of cases [50]. In contrast, antioxidant therapy using a combination treatment of Bio-Antox and Bio-Quinone Q10 over 3 months has been shown to reduce symptoms of fatigue and chronic pruritus in PBC patients [52] but no markers of oxidant stress were studied. Given the very low intrahepatic levels of glutathione in our patient group, there may be a case for combining antioxidant therapy with other markers of oxidant stress [8]. However, in the same rat model, Baron and Muriel [9] found that although antioxidant treatment inhibited lipid peroxidation and oxidation of glutathione, it had no effect on conventional biochemical markers of liver injury and on liver histology. The authors concluded that oxidant stress does not play a causative role in this model of cholestatic liver disease, but is a consequence of bile-salt induced solubilisation of plasma membranes and cell death.

Therefore, although it is still unclear whether oxidant stress is involved in the initiation of the disease process in PBC, it is almost certainly an important factor in disease progression. Hepatic stellate cells play a key role in the development of fibrosis, being a major source of collagen and also of other extracellular matrix proteins in the injured liver. It has previously been shown that stimulation of lipid peroxidation or treatment with HNE increases procollagen mRNA expression in human HSCs [53,54] leading to fibrogenesis and eventually to cirrhosis. Therefore, oxidant stress in PBC provides a likely mechanism for disease progression following the initial immunological insult and leading to the subsequent development of fibrosis and cirrhosis.

In conclusion, our study shows that oxidant stress, assessed for the first time in a large group of PBC patients using a wide range of markers of both oxidant and antioxidant processes in blood and urine, is not just a feature of late stage decompensated liver disease but is a significant feature of early PBC. Trials of antioxidant therapy, adequately monitored, are therefore now indicated to determine whether early treatment can prevent or slow progression to cirrhosis and liver failure.

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References