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Primary biliary cirrhosis is associated with oxidative stress and endothelial dysfunction but not increased cardiovascular risk

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Aim: Primary biliary cirrhosis (PBC) is a chronic cholestatic disease which is associated with hypercholesterolaemia. Further, cholestatic diseases are associated with deficiencies of anti-oxidant vitamins. Despite these associations PBC is not associated with an increase in cardiovascular mortality. The aim of this study is to assess if primary biliary cirrhosis is associated with oxidative stress, endothelial dysfunction and alteration of vascular compliance which is a surrogate marker for cardiovascular risk.

Methods: Fifty-one PBC patients and 34 control subjects were studied. Lipid soluble vitamins A, and E in addition to ascorbate and carotenoids were measured to assess anti-oxidant status. C-reactive protein, hydroperoxides and adhesion molecules sICAM-1/sVCAM-1 were assessed as serological measures of endothelial function. Finally, measures of vascular compliance were assessed by applanation tonometer.

Results: CRP, sICAM and sVCAM were all significantly higher in PBC patients (469.14 vs 207.13, \( P < 0.001 \); 768.12 vs 308.03, \( P < 0.001 \); 708.40 vs 461.31, \( P < 0.001 \)) whilst anti-oxidant vitamin levels were lower in PBC patients, with ascorbate, vitamin E and vitamin A all significantly lower in PBC patients (39.91 vs 72.68, \( P < 0.001 \); 2.63 vs 3.14, \( P = 0.02 \); 1.08 vs 1.81, \( P < 0.001 \)). Despite these findings PBC patients have a lower pulse wave velocity than control subjects (8.22 m/s vs 8.78 m/s, \( P = 0.022 \)).

Conclusion: PBC patients appear to have reduced vascular risk as assessed by pulse wave velocity but concurrently have evidence of endothelial dysfunction, inflammation and anti-oxidant deficiency.

Key words: cardiovascular risk, endothelial dysfunction, hyperlipidaemia, oxidative stress, primary biliary cirrhosis, pulse wave velocity

INTRODUCTION

PRIMARY BILIARY CIRRHOSIS (PBC) is a chronic cholestatic disease which is associated with hypercholesterolaemia.1 Despite this association, only three studies have explored any link between PBC and cardiovascular risk, and in general these have failed to demonstrate an increased risk in cardiovascular mortality.2–4 A small number of studies however have demonstrated elevated biochemical markers of endothelial damage suggesting vascular dysfunction is present in liver disease.5–7 Blann et al. demonstrated that serum from patients suffering from PBC (and other cholestatic liver diseases) was cytotoxic to cultured endothelial cells.5 This cytoxicity correlated with levels of von Willebrand factor (vWF; an endothelial cell product) and bilirubin. Further evidence suggesting endothelial dysfunction in PBC came from Marasini et al. who used digital blood flow to demonstrate the existence of structural changes in peripheral blood vessels in PBC and elevation of levels of vWF and tissue plasminogen activator (tPA) indicating endothelial cell damage.7 An association between PBC and elevated serum levels of adhesion molecules would also point to endothelial dysfunction.6

There is also evidence of oxidative stress in cholestatic liver diseases including PBC. Indeed, there is some evidence that generation of reactive oxygen species such as superoxide and hydroxyl radical due to ongoing inflammation may play a role in the pathophysiological mechanisms underlying progression of the liver disease.
in PBC from normal to fibrosis and subsequently cirrhosis. This theory is supported by animal models of cholestasis. Products of lipid peroxidation, a key feature of oxidative stress, have been identified in both hepatocytes located around portal tracts and within biliary epithelial cells of PBC patients. Further, increased levels of superoxide dismutase have been demonstrated in injured biliary epithelial cells and hepatocytes suggesting superoxide may have a role to play in PBC pathogenesis.

PBC patients are also predisposed to oxidative stress through depletion of lipid soluble antioxidants. Prevalence rates of vitamins A, D, E and K deficiency are variable and seem to be related to both laboratory techniques and severity of disease, with most studies being published before Ursodeoxycholic acid was recommended as treatment for PBC.

Aboutwerat et al. recently assessed anti-oxidant status in 41 PBC patients and compared these with age- and sex- matched controls. They found significant depletion of glutathione, selenium and Vitamin A in addition to elevation of urinary 8-isoprostanes in the PBC group. Vitamin C and E (including vitamin E levels expressed as a ratio to total cholesterol) levels were normal in the PBC group.

The initiating event in the pathogenesis of the liver damage in PBC is uncertain and may be an immunological reaction, viral infection or toxic substance. Abnormal hepatic vascular dysfunction, excessive lipid peroxidation, or anti-oxidant deficiency might contribute to ongoing hepatic damage by leading to alterations in blood flow, causing localized ischemia, hyperperfusion or inadequate anti-oxidant defence. Further, abnormal systemic vascular function as a consequence of these same processes may lead to an increased risk of atherosclerosis.

The aim of this study is to assess if primary biliary cirrhosis is associated with oxidative stress, endothelial dysfunction and alteration of vascular compliance.

**METHODS**

**Subjects**

PBC PATIENTS WERE recruited from the Liver Clinic at the Royal Victoria Hospital, Belfast. PBC was defined by the presence of (i) Positive antimitochondrial antibodies to a titre of >1:40 on two occasions along with (ii) Elevation of alkaline phosphatase above the reference range or (iii) Compatible or diagnostic liver histology.

Fifty-one patients aged between 20 and 75 years were included and 36 control subjects were recruited from hospital and laboratory staff and members of the public. Written informed consent was obtained from all subjects. Ethical approval was obtained from the regional ethics committee for Northern Ireland, at Queens University, Belfast. Subjects with known hypertension (blood pressure >160/90 mmHg), diabetes mellitus, a history of cardiovascular disease and those taking lipid lowering agents or hormonal preparations were excluded from the study. Subjects attended for studies in the morning following an overnight fast. Each individual’s height and weight were recorded to allow calculation of body mass index. Blood pressure (semi-automated device; Omron 705-CP) was measured in duplicate following a 15 minute rest in the supine position and the average reading recorded.

**Estimation of vascular compliance**

The methodologies of pulse wave analysis and pulse wave velocity have been described in detail. After the patient had rested in the supine position in a temperature controlled room for a minimum of 15 minutes, radial pulse wave analysis was recorded with a Millar tonometer and the Sphygmocor system model SCOR-Px, incorporating pulse wave velocity system Model SCOR-Vx (SPC-301; Millar instruments and Atcor medical, Sydney, Australia). Data were continuously recorded on a computer using commercially available software (Sphygmocor Blood pressure Analysis System BPAS-1 version 7.0; Atcor medical). For pulse wave analysis, triplicate measurements were made with the patient in the supine position from the radial artery of the dominant arm and the average reading calculated. The software automatically processed the radial artery waveform data and using a generalised transfer function generated measures of vascular compliance including augmentation index, time to reflectance, Buckberg’s subendocardial viability ratio and ejection duration percentage (Fig. 1).

To obtain pulse wave velocity (PWV) the procedure was similar to PWA with the exception that the analysis was gated to the cardiac cycle by attaching electrocardiograph leads to the patient. Readings were taken from the dominant radial artery (distal site) and then ipsilateral carotid artery (proximal site) following measurement of distance from sternal manubrium to both the distal and proximal sites using a tape measure. Carotid-radial PWV was measured rather than carotid-femoral due to ease of reproducibility and acceptability to patients.
Peripheral venous blood sampling

Each subject had venous blood collected for measurement of markers of liver function, endothelial function and anti-oxidant status (see below). Peripheral venous blood samples were collected after subjects were sitting quietly for five minutes following arterial compliance assessment described above. A tourniquet was used to improve venous access, but was kept on for the minimum possible time and for a maximum of two minutes.

Laboratory analysis

Fasting plasma glucose, serum lipid profile and liver function tests were assayed by routine methods. Plasma soluble intercellular adhesion molecule-1 (sICAM-1) and soluble vascular cell adhesion molecule-1 (sVCAM-1) were determined using commercially available monoclonal ELISA kits (Diaclone; Immunodiagnostics Systems, Boldon) with all measurements performed manufacturer recommendations.

Serum concentrations of lipid soluble antioxidants were measured according to the method of Craft et al.21 This high performance liquid chromatography assay using diode array detection was used to assess retinol, γ-tocopherol, α-tocopherol, lutein, zeaxanthin, β-cryptoxanthin, α-carotene, β-carotene and lycopene. The detection limits for retinol and the tocopherols were 0.05 μmol/l and 0.005 μmol/l for carotenoids.

Ascorbic acid concentrations were determined as described by Vuillemier & Keck.22 The procedure is based on the enzymatic oxidation of ascorbic acid and subsequent quinoxaline formation to generate a fluorescent derivative measured on the Cobas Faro centrifugal analyser.

High sensitivity C-reactive protein (hsCRP) was assessed by latex-enhanced immunoturbidimetric assay (Randox Pharmaceuticals, Curmlin) using an ILab 600 biochemical analyzer and ILab 600 computer software (Instrumentation Laboratories, Warrington).

Ferrous ion oxidation xylene orange-1 (FOX-1) was used to determine hydroperoxides (HPO) in the aqueous phase of serum. Hydroperoxides oxidise ferrous ions to ferric ions in dilute acids and the resultant ferric ions can be determined using ferric-sensitive dyes as an indirect measure of hydroperoxide concentration.

Statistical analysis

All data were recorded in Microsoft Excel (Redmond, WA) and formed the basis for final statistical analysis. Analyses were performed using SPSS (version 15.0; SPSS, Chicago, IL). Parameters were tested for normal distribution using both q-q plots and histograms. Log transformations were performed on those parameters not normally distributed. Differences between groups were assessed using student’s t-test and expressed as arithmetic mean difference with standard deviation (sd), P-values and 95% confidence intervals. For log transformed data results were expressed as geometric means (GM) with inter-quartile range (IQR), ratio of GM’s with 95% confidence intervals (95% CI) and P-values also described. To ensure no significant interaction existed between age, gender and group, results were also adjusted for both age and sex using univariate analysis.

RESULTS

Demographic

Fifteen PBC (49 females) and 36 control subjects (27 female) were recruited to the study. Demographic characteristics for participants are shown in Table 1, with no significant difference between the
groups for age, body mass index, systolic or diastolic blood pressure and smoking status.

Twelve of 51 (29.4%) PBC patients were receiving treatment with Ursodeoxycholic acid at the time of study.

Liver enzymes, immunoglobulins and lipid profile results for PBC patients are shown in Table 2. These parameters were not assessed in controls and therefore no comparisons could be drawn.

### Arterial compliance

The results of pulse wave analysis and pulse wave velocity studies are presented in Table 3. In addition to direct comparison, adjustment for age and gender was undertaken as the groups were not matched and previous studies have demonstrated differences in augmentation index, pulse wave velocity and other markers of arterial compliance with gender and age. Arterial compliance data was normally distributed and therefore analysed with student’s t-test.

PBC patients had a significantly lower PWV. When adjusted for age, gender and blood pressure the mean difference remained significant. Augmentation index at heart rate 75 (AIx adjusted to a standardized heart rate of 75 beats per minute by the sphygmocor software) was initially significantly different between groups but following adjustment for age and gender significance was lost. Time to reflectance was also significantly lower before age and gender adjustment but like AIx75, significance was lost following adjustment for age and gender. There was no significant difference in Buckberg’s subendocardial viability ratio or ejection duration percentage.

### Endothelial dysfunction

PBC patients had evidence of endothelial dysfunction as measured by serological markers in this study. Results are presented in Table 4. sICAM-1, sVCAM-1 and hsCRP levels were all significantly higher in PBC patients compared with controls. These differences remained significant after adjustment for age and gender.

Given the evidence for bilirubin having anti-inflammatory effects and protecting against endothelial dysfunction, correlation between log-bilirubin and the aforementioned serological markers was calculated.
Table 3 Results of student’s t-test analysis of arterial compliance analysis comparing primary biliary cirrhosis (PBC) subjects with controls

<table>
<thead>
<tr>
<th></th>
<th>PBC (n = 51)</th>
<th>Control (n = 36)</th>
<th>Mean difference</th>
<th>95% Confidence Intervals</th>
<th>P</th>
<th>ADJUSTED FOR AGE AND GENDER</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Mean difference</td>
</tr>
<tr>
<td>Aix75 (%)</td>
<td>31.24 (8.92)</td>
<td>25.71 (11.11)</td>
<td>5.53</td>
<td>1.25–9.81</td>
<td>0.012</td>
<td>2.57</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>78.82 (14.47)</td>
<td>77.36 (9.77)</td>
<td>1.46</td>
<td>−4.08–7.01</td>
<td>0.601</td>
<td>0.60</td>
</tr>
<tr>
<td>ED (%)</td>
<td>37.08 (3.31)</td>
<td>36.10 (3.77)</td>
<td>0.98</td>
<td>−0.53–2.50</td>
<td>0.201</td>
<td>0.23</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>132.41 (21.71)</td>
<td>135.72 (16.06)</td>
<td>−3.31</td>
<td>−11.78–5.16</td>
<td>0.439</td>
<td>−5.18</td>
</tr>
<tr>
<td>SEVR (%)</td>
<td>142.15 (23.33)</td>
<td>146.50 (26.15)</td>
<td>−4.35</td>
<td>−14.97–6.27</td>
<td>0.418</td>
<td>1.26</td>
</tr>
<tr>
<td>PWV (m/s)</td>
<td>8.22 (1.11)</td>
<td>8.78 (1.08)</td>
<td>−0.56</td>
<td>−1.03–0.08</td>
<td>0.022</td>
<td>−0.67</td>
</tr>
<tr>
<td>HR (bpm)</td>
<td>68.05 (10.34)</td>
<td>65.72 (8.68)</td>
<td>2.33</td>
<td>−1.86–6.53</td>
<td>0.272</td>
<td>1.59</td>
</tr>
<tr>
<td>TR (msec)</td>
<td>134.52 (14.11)</td>
<td>141.27 (15.36)</td>
<td>−6.75</td>
<td>−13.09–0.42</td>
<td>0.037</td>
<td>−3.23</td>
</tr>
</tbody>
</table>

ADJUSTED FOR AGE/ GENDER/SBP/DBP

<table>
<thead>
<tr>
<th></th>
<th>PBC (n = 51)</th>
<th>Control (n = 36)</th>
<th>Mean difference</th>
<th>95% Confidence Intervals</th>
<th>P</th>
<th>ADJUSTED FOR AGE/ GENDER/SBP/DBP</th>
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<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>Mean difference</td>
</tr>
<tr>
<td>PWV (m/s)</td>
<td>8.22 (1.11)</td>
<td>8.78 (1.08)</td>
<td>7406</td>
<td>−1.03–0.08</td>
<td>0.022</td>
<td>−0.614</td>
</tr>
</tbody>
</table>

Aix75, augmentation index adjusted to heart rate 75; DBP, diastolic blood pressure; ED%, ejection duration; SBP, systolic blood pressure; SEVR, subendocardial viability ratio; PWV, pulse wave velocity; HR, heart rate; TR, time to reflectance.

Table 4 Results of student’s t-test analysis of markers of endothelial dysfunction following log transformation comparing primary biliary cirrhosis (PBC) subjects with controls

<table>
<thead>
<tr>
<th></th>
<th>Geometric mean</th>
<th>Ratio of GM</th>
<th>95% CI</th>
<th>Inter-quartile range</th>
<th>P</th>
<th>Adjusted for age and sex</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PBC</td>
<td>control</td>
<td></td>
<td>IQR PBC</td>
<td>IQR Control</td>
<td>Ratio of GM</td>
</tr>
<tr>
<td>HPO (μmol/l)</td>
<td>0.45</td>
<td>0.47</td>
<td>0.96</td>
<td>0.82–1.12</td>
<td>0.36–0.61</td>
<td>0.39–0.54</td>
</tr>
<tr>
<td>HSCRP (μg/dl)</td>
<td>469.14</td>
<td>207.13</td>
<td>2.26</td>
<td>1.52–3.37</td>
<td>259.28–887.11</td>
<td>101.31–305.04</td>
</tr>
<tr>
<td>sICAM1 (ng/ml)</td>
<td>768.12</td>
<td>308.03</td>
<td>2.49</td>
<td>2.02–3.08</td>
<td>509–1423</td>
<td>255.5–402.50</td>
</tr>
<tr>
<td>sVCAM1 (ng/ml)</td>
<td>708.40</td>
<td>461.31</td>
<td>1.54</td>
<td>1.22–1.93</td>
<td>478–1059</td>
<td>385.25–600.50</td>
</tr>
</tbody>
</table>

HPO, hydroperoxides; HSCRP, high sensitivity C-reactive protein; sICAM-1, soluble intercellular adhesion molecule-1; sVCAM-1, soluble vascular adhesion molecule-1.
using Spearman’s rank. There was no significant correlation between bilirubin and hsCRP but both sICAM and sVCAM were positively correlated. Further, sICAM correlated significantly with all liver enzymes and sVCAM with AST suggesting these elevations are due to disease activity. (Table 5)

There was no difference in hydroperoxide (HPO) levels between the groups with or without adjustment for age and gender.

**Anti-oxidant status**

Consistent with previous studies, PBC patients were found to have diminished levels of fat soluble anti-oxidants. (Table 6)

Retinol levels were significantly lower in PBC patients and following adjustment for age and gender this difference was maintained. As expected g-tocopherol was significantly lower in PBC patients but a-tocopherol was only minimally lower with no statistical difference. Vitamin C was significantly lower in PBC patients, although the mean level did not fall within the deficient range (<30 μmol/L). Again, this result was not affected by adjustment for age and gender. Analysis of carotenoids revealed no significant association between PBC and levels. Lutein and b-carotene levels were in fact marginally higher in PBC patients compared to controls, although this was not significant. The remainder of the carotenoids (lycopene, b-cryptoxanthin and a-carotene), were lower, but not significantly, in PBC patients.

**DISCUSSION**

**OVERALL ANALYSIS OF arterial compliance suggests that pulse wave velocity in PBC patients is reduced which corresponds to a reduced risk of cardiovascular events. This would be consistent with epidemiological studies which have assessed cardiovascular risk in PBC.**

Somewhat paradoxically, the results of
this study demonstrate a pattern of increased endothelial dysfunction and reduced serum anti-oxidant levels in patients with primary biliary cirrhosis.

The pulse wave velocity measured by applanation tonometry is regarded as the most reproducible parameter, mostly due to the lack of a generalized transfer factor required to generate a result. It would normally be anticipated that the Augmentation index and PWV results would follow similar trends (e.g. if PWV rises so would AIx). In this study we found PWV to be lower in PBC patients than controls. PWV predicts cardiovascular events, and the lower level detected in PBC patients is consistent with previous reports of lack of an increase in cardiovascular risk in these patients. Indeed, a lower PWV indicates a lower risk of cardiovascular events and our results are consistent with the conclusion that not only do PBC patients have no increased cardiovascular risk but they actually have diminished risk.

In light of the gender difference between the disease group and controls it is difficult to interpret differences detected prior to age and gender adjustment. Both time to reflectance (TR) and augmentation index at heart rate 75 (AIx75) were significantly different between groups but lost significance following correction for age and gender. This is not surprising as previous studies have shown indices of pulse wave analysis differ significantly by gender. AIx is known to be higher in females, by a margin of up to 10% while PWV has not been universally shown to vary between gender. The higher AIx seen in our study may be consistent with the gender difference between groups.

It is worth noting that PWV and AIx would be expected to show agreement; if PWV is low, AIx would also be expected to be low. In our study AIx was higher, albeit not significantly, in the PBC patients who had a low PWV. There are, however, other studies that have demonstrated poor agreement between PWV and other pulse wave analysis parameters including AIx. These studies have concluded AIx is not a reliable marker of arterial compliance.

Our study also showed serological evidence of endothelial dysfunction associated with primary biliary cirrhosis. sICAM, sVCAM and hsCRP were all significantly higher in the PBC group. This confirms previous reports of elevation of adhesion molecules in PBC. hsCRP is a marker of both inflammation and cardiovascular risk and it may be expected that PBC patients would have increased cardiovascular risk given the levels detected in this study. It is worth noting that both sICAM and sVCAM correlated significantly with bilirubin levels in PBC patients (Figs 2 and 3). sICAM also correlated significantly at the 0.01 level with all liver enzymes. A number of studies have described how elevated bilirubin levels may protect against endothelial dysfunction, inflammation and even cardiovascular disease. Obviously elevations of bilirubin in PBC are consistent with disease progression, while a previous report suggested sICAM levels may predict disease stage in PBC with a high sensitivity and specificity. The raised sICAM, sVCAM and hsCRP levels seen in PBC may therefore be due to disease activity and may not confer the same degree of cardiovascular risk in this disease due to protective factors offered by bilirubin. No significant correlation existed however between bilirubin and markers of vascular compliance.
Deficiency of vitamin A has been well documented in association with PBC while there are also a number of reports of vitamin E deficiency. This study has not only confirmed previous reports of lower levels of Vitamin A in PBC patients but also demonstrates lower concentrations of g-tocopherol (vitamin E), although a-tocopherol was similar to the control group. Unfortunately we did not measure serum lipid profiles in both PBC and control subjects and are therefore unable to cite vitamin E/lipid ratios. This study establishes a previously unreported link between PBC and relative vitamin C deficiency.

Authors of the previous reports of fat soluble deficiency in PBC have explained their findings on a purely theoretical physiological basis. That is impaired micellar formation in PBC patients prevents absorption of fat soluble vitamins. While this study was not designed to assess the mechanism of lower levels of these vitamins it is perceivable that consumption, rather than malabsorption, of anti-oxidant vitamins is contributing to the lower levels detected in serum. If that were the case we would perhaps have expected more evidence of lipid peroxidation which was not observed by analysis of hydroperoxides. Vitamin A levels were comparatively much lower than the other anti-oxidants in the PBC group. Although consumption may account for this, another explanation may be impaired hepatic synthesis of retinol binding protein or pre-albumin which would result in relative lower measurable levels compared to the other fat soluble vitamins. These proteins were not measured in this study.

There are few studies assessing the effect of anti-oxidant supplementation in PBC on disease progression. In one small study of 24 individuals over a three month period there were improvements in symptoms but not liver biochemistry.

In summary, PBC patients appear to have reduced vascular risk as assessed by pulse wave velocity but concurrently have evidence of endothelial dysfunction, inflammation and anti-oxidant deficiency. It seems likely that these biochemical abnormalities are related to disease activity. Whilst it is not clear why PBC patients have a reduced PWV this finding is consistent with previous findings of no significant increased cardiovascular risk. Despite the lack of correlation between bilirubin and pulse wave velocity in this study it is possible that bilirubin is conferring cardiovascular protection. The pathophysiological relevance of endothelial dysfunction and vascular compliance in PBC remains unclear. Given the well established link between PBC and hyperlipidaemia and between raised lipids and endothelial dysfunction it would be of interest to explore the effect of lipid lowering agents and anti-oxidants on endothelial function, vascular compliance and anti-oxidant status in this disease.

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