The role of small intestinal bacterial overgrowth, intestinal permeability, endotoxaemia, and tumour necrosis factor α in the pathogenesis of non-alcoholic steatohepatitis

A J Wigg, I C Roberts-Thomson, R B Dymock, P J McCarthy, R H Grose, A G Cummins

Abstract
Background—Small intestinal bacterial overgrowth may contribute to the development of non-alcoholic steatohepatitis, perhaps by increasing intestinal permeability and promoting the absorption of endotoxin or other enteric bacterial products.

Aims—to investigate the prevalence of small intestinal bacterial overgrowth, increased intestinal permeability, elevated endotoxin, and tumour necrosis factor α (TNF-α) levels in patients with non-alcoholic steatohepatitis and in control subjects.

Patients and methods—Twenty-two patients with non-alcoholic steatohepatitis and 23 control subjects were studied. Small intestinal bacterial overgrowth was assessed by a combined 14C-D-xylose and lactulose breath test. Intestinal permeability was assessed by a dual lactulose-rhamnose sugar test. Serum endotoxin levels were determined using the limulus amoebocyte lysate assay and TNF-α levels using an ELISA.

Results—Small intestinal bacterial overgrowth was present in 50% of patients with non-alcoholic steatosis and 22% of control subjects (p=0.048). Mean TNF-α levels in non-alcoholic steatohepatitis patients and control subjects were 14.2 and 7.5 pg/ml, respectively (p=0.001). Intestinal permeability and serum endotoxin levels were similar in the two groups.

Conclusions—Patients with non-alcoholic steatohepatitis have a higher prevalence of small intestinal bacterial overgrowth, as assessed by the 14C-D-xylose-lactulose breath test, and higher TNF-α levels in comparison with control subjects. This is not accompanied by increased intestinal permeability or elevated endotoxin levels.

Keywords: non-alcoholic steatohepatitis; small intestinal bacterial overgrowth; intestinal permeability; endotoxin; tumour necrosis factor α

The pathogenesis of non-alcoholic steatohepatitis (NASH) remains unclear. Several observations have suggested that small intestinal bacterial overgrowth (SIBO) may play a role in NASH. Firstly, NASH was encountered as a common complication of jejunoileal bypass surgery for morbid obesity during the 1980s and could be reversed by treatment with metronidazole. Secondly, several patients with jejunoileal bypass associated NASH required liver transplantation and NASH recurred rapidly following transplantation, particularly in patients who did not have the jejunoileal bypass reversed at the time of transplantation. Thirdly, NASH has been reported in one individual with jejunal diverticulosis and SIBO diagnosed by a 14CO2 bile acid breath test. Finally, various rat models of SIBO have been associated with liver lesions similar to NASH that improved following antibiotics. Despite these observations the prevalence of SIBO has not been investigated in patients with NASH. SIBO could increase intestinal permeability and absorption of endotoxin. Considerable evidence already exists demonstrating that endotoxin can induce steatohepatitis, mediated chiefly via the cytokine tumour necrosis factor α (TNF-α). In alcoholic liver disease, which shares histological similarities to NASH, endotoxin-induced stimulation of Kupffer cells has been proposed as an important initiating event leading to the production of proinflammatory cytokines and oxygen free radicals. Yang and coworkers have suggested that systemic endotoxaemia contributes to TNF-α production and steatohepatitis in genetically obese rats. It seems plausible therefore that gut derived endotoxin, perhaps from SIBO, is important in the pathogenesis of NASH via Kupffer cell stimulation and TNF-α production. To our knowledge however no human study of NASH patients has measured intestinal permeability, endotoxin, or TNF-α levels.

The aim of this study therefore was to determine the prevalence of SIBO in a series of NASH patients and to assess if this is accompanied by increased intestinal permeability or elevated serum levels of endotoxin and TNF-α.

Subjects and methods

Twenty-two patients with NASH were studied. They were consecutive cases diagnosed in our gastroenterology clinic between 1994 and 1997. Three diagnostic criteria were used for...
Small intestinal bacterial overgrowth and NASH

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ated with a significant false positive rate. 11 12
D-xylose and lactulose breath tests on 11 of our

14C-D-xylose and lactulose breath test which has been associ-

14C -D-XYLOSE AND LACTULOSE BREATH TEST

14C-D-xylose tests were positive whereas only four

22 patients drank no alcohol.

significant alcohol consumption, defined as no
greater than 20 g of alcohol per day. Alcohol

histogy was taken by two physicians on two dif-

fatty infiltration on ultrasound or CT scan of the

liver, abnormal liver function tests, and

 conceded essential for the diagnosis. Three patients did

not consent to liver biopsy. These patients had

false positive rate. 11 12

RBD. The minima histological
criteria were the presence of macrovesicular

steatosis and inflammation. Hepatic fibrosis

and Mallory's bodies were not considered

for the diagnosis. Three patients did

not consent to liver biopsy. These patients had

fatty infiltration on ultrasound or CT scan of the

liver, abnormal liver function tests, and

otherwise fulfilled the other inclusion criteria.

The second inclusion criterion was no

significant alcohol consumption, defined as no

greater than 20 g of alcohol per day. Alcohol

history was taken by two physicians on two dif-

ferent occasions and confirmed with family

members where possible. Patients with detect-

able blood alcohol on a fasting morning speci-
mom or an elevated mean corpuscular volume

were excluded. The mean alcohol consump-

tion of NASH patients was 14 g per week; 12 of

the 22 patients drank no alcohol.

The third criterion was the absence of other

relevant liver diseases. All patients were nega-
tive for hepatitis B surface antigen and

antibody to hepatitis C. No patient had anti-

mitochondrial antibody or significantly
elevated titres of antinuclear or antismooth

muscle antibody.

Control subjects were age and sex matched

with NASH patients. All control subjects had

normal liver function tests and no history of

der liver disease. They were made up of volunteers

and ambulatory outpatients with minor gastro-

intestinal complaints.

In addition, a drug history, recording of

height and weight for calculation of body mass

index, and bloods for triglyceride and choles-
terol levels were obtained for all NASH

patients and control subjects. Fasting glucose

levels and immunoglobulins were also assessed

in NASH patients and random glucose levels in

control subjects.

14C-D-XYLOSE AND LACTULOSE BREATH TEST

Twenty two NASH patients and 23 control

subjects were studied by a 14C-D-xylose and

lactulose breath test. This combined breath test

was designed to improve the specificity of the

14C-D-xylose breath test which has been associ-

ated with a significant false positive rate. 11 12

This may be due to rapid colonic transit and
catabolism of unabsorbed 14C-D-xylose, result-
ing in early 14CO2 expiration and the erroneous
diagnosis of SIBO. The combined breath test
has been designed to overcome this problem.

Lactulose, which requires much greater con-

centrations of bacteria to produce an observed

H2/CH4 rise in breath, acts as an internal tran-

sit marker of colonic bacterial catabolism in

individuals both with and without SIBO. To

help validate this test, we performed both 14C-

D-xylose and lactulose breath tests on 11 of our

NASH patients. Nine (82%) of the 14C-D-

xylose tests were positive whereas only four

(36%) of the 14C-D-xylose and lactulose tests

were normal. In a study population in which

the majority had no clear predisposing syn-
dromes for SIBO (four of the 11 had diabetes),

we believe that the lower number of positive
tests in the 14C-D-xylose and lactulose tests

reflects the greater specificity of this test.

We did not use culture of jejunal aspirates to
diagnose SIBO because this test has not been

firmly established as a gold standard. 13 Ethical

and logistical issues also prevented the use of

jejunal culture in this case control study.

To perform the test, subjects ingested 1 µCi

of radiolabelled 14C-D-xylose together with

6.68 g (10 ml) of lactulose made up to a

volume of 100 ml with distilled water. Bacterial

catabolic products of 14C-D-xylose (14CO2) and

lactulose (CH3 and H2) were measured every

30 minutes from ingestion for a minimum of

four hours. Breath tests were interpreted as

positive for SIBO if significant 14CO2 (>70×10–6

DPM) was expired before the colonic H2 and

CH4 rise, or if a double H2 and CH4 peak

occurred. Breath tests were interpreted as

negative if a significant 14CO2 rise was detected

simultaneously with the colonic H2 and CH4

rise. Equivocal tests, where there was no

significant H2 and CH4 rise (>10 PPM), were

recorded as negative. To prevent disturbances

colonic flora, antibiotics, colonoscopy,

barium enema, or other bowel washouts were

avoided for one month prior to testing. Patients

were also required to comply with a low residue

diet the day before the test and not to smoke

within two hours of the test to prevent high

basal levels of H2.

An estimate of oral-caecal or small intestinal

transit time was calculated, where possible, by

observing the time taken from ingestion of lac-

tulose to the appearance of the H2 and CH4

peak, indicating colonic catabolism of lactu-

LACTULOSE-RHAMNOSE INTESTINAL

PERMEABILITY TEST

Eighteen NASH patients and 20 control

subjects completed permeability testing. Sub-

jects fasted overnight and emptied their

bladder before drinking 100 ml of a hypertonic

solution (1500 mosmol) containing 1.0 g

α-1-rhamnose (R-3875, Sigma, St Louis, Mis-

souri, USA), 5.0 g lactulose (Duphalac,

67% w/v syrup, Dulphar BV, Holland), and

22.6 g of glucose as an osmotic filler. Urine

was encouraged to drink water after the first 30

minutes and could eat after three hours. Urine

volume was measured and the concentration of

urinary lactulose and rhamnose determined

using a modified HPLC method as described

by Miki and colleagues. 14 Intestinal permeab-

ility was expressed as the excretion ratio of

urinary lactulose to rhamnose with each

expressed as a percentage of the ingested dose.

ENDOTOXIN ASSAY

Endotoxin was measured in all NASH patients

and control subjects. Sera were diluted 1:10

and heated (70°C) for five minutes to remove

non-specific inhibitors of endotoxin. The assay

was performed using the Bio Whittaker (Bio
Table 1 Clinical and biochemical variables (mean (SD)) for patients with non-alcoholic steatohepatitis (NASH) (n=22) and controls (n=23)

<table>
<thead>
<tr>
<th>Variable (normal range)</th>
<th>NASH patients</th>
<th>Control subjects</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>54 (17)</td>
<td>50 (16)</td>
<td>0.4</td>
</tr>
<tr>
<td>BMI (19–25 kg/m²)</td>
<td>30 (6)</td>
<td>24 (5)</td>
<td>0.002</td>
</tr>
<tr>
<td>Cholesterol (&lt;5.5 mmol/L)</td>
<td>5.7 (1.3)</td>
<td>5.4 (0.9)</td>
<td>0.3</td>
</tr>
<tr>
<td>Triglyceride (0.7–2.1 mmol/l)</td>
<td>2.4 (2.1)</td>
<td>1.6 (1.0)</td>
<td>0.1</td>
</tr>
<tr>
<td>Fasting glucose (3–5.4 mmol/l)</td>
<td>6 (1.9)</td>
<td>6 (1.9)</td>
<td></td>
</tr>
<tr>
<td>Random glucose (3–7.7 mmol/l)</td>
<td>5.3 (1)</td>
<td>5.3 (1)</td>
<td></td>
</tr>
<tr>
<td>AST (0–45 U/l)</td>
<td>124 (159)</td>
<td>19 (5)</td>
<td>0.003</td>
</tr>
<tr>
<td>ALT (0–55 U/l)</td>
<td>153 (161)</td>
<td>18 (9)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>AST:ALT ratio</td>
<td>0.8 (0.4)</td>
<td>1.3 (0.6)</td>
<td>0.008</td>
</tr>
<tr>
<td>GGT (0–55 U/l)</td>
<td>125 (133)</td>
<td>19 (10)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>ALP (30–110 U/l)</td>
<td>118 (96)</td>
<td>70 (23)</td>
<td>0.03</td>
</tr>
<tr>
<td>Bilirubin (1–20 µmol/l)</td>
<td>18 (23)</td>
<td>11 (5)</td>
<td>0.2</td>
</tr>
</tbody>
</table>

AST, aspartate aminotransferase; ALT, alanine aminotransferase; GGT, gamma glutamyltransferase; ALP, alkaline phosphatase.

Whittaker, Inc, Walkersville, USA) QCL-1000 chromogenic limulus amoebocyte lysate test kit, using the manufacturer’s instructions.

**Results**

**CLINICAL AND BIOCHEMICAL CHARACTERISTICS OF NASH PATIENTS AND CONTROL SUBJECTS**

Patient characteristics are shown in table 1. Patient and control groups were well matched for age and sex. The male:female ratio for NASH patients was 8:14 and 7:16 for the control subjects. NASH patients were significantly more obese than control subjects (mean body mass index (BMI) 30 ± 24 kg/m²; p=0.002). In addition, the proportion of obese NASH patients was significantly higher than that of obese control subjects (77% v 27%; p=0.002).

Five (23%) of the NASH group had known type 2 diabetes mellitus. In addition, a further four NASH patients had glucose intolerance diagnosed on the basis of fasting glucose levels of 5.5–7.7 mmol/l. One (4%) control subject had known type 2 diabetes mellitus and no further diabetes or glucose intolerance was discovered following random glucose measurement. The prevalence of type 2 diabetes mellitus was significantly higher in NASH patients (p=0.03).

The prevalence of hyperlipidaemia, defined as serum triglyceride >2.1 mmol/l and/or cholesterol >5.5 mmol/l, was not significantly different between NASH patients and control subjects (59% v 43%; p=0.3).

**FIBROTIC SEVERITY OF NASH PATIENTS**

Twelve of 19 (63%) NASH patients who had liver biopsy demonstrated fibrosis. The mean Scheuer grade for fibrosis was 0.8 (SD 0.8, range 0–2).

**14C-D-XYLOSE AND LACTULOSE BREATH TESTS**

All data for breath tests and small intestinal transit time are presented in tables 2 and 3. Eleven (50%) NASH patients and five (22%) control subjects had positive 14C-D-xylose and lactulose breath tests indicating SIBO (p=0.048, 95% CI for the difference between proportions 3.4–57.9%).

**Table 2** 14C-d-xylose and lactulose breath tests, endotoxin levels, intestinal permeability, tumour necrosis factor a (TNF-a) levels, small intestinal transit time, and diabetes prevalence in patients with non-alcoholic steatohepatitis (NASH)

<table>
<thead>
<tr>
<th>Patient No</th>
<th>14C-d-xylose + lactulose breath test</th>
<th>Endotoxin level (EU/ml)</th>
<th>Intestinal permeability (L/R ratio)</th>
<th>TNF-a (pg/ml)</th>
<th>Small intestinal transit time (minutes)</th>
<th>Diabetes (yes/no/gi)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Positive</td>
<td>1.0</td>
<td>5.8</td>
<td>180</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Negative</td>
<td>1.2</td>
<td>0.13</td>
<td>6.6</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Positive</td>
<td>0.9</td>
<td>0.19</td>
<td>27</td>
<td>gi</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Negative</td>
<td>1.0</td>
<td>0.06</td>
<td>14.3</td>
<td>180</td>
<td>gi</td>
</tr>
<tr>
<td>5</td>
<td>Positive</td>
<td>1.4</td>
<td>0.33</td>
<td>10.8</td>
<td>150</td>
<td>No</td>
</tr>
<tr>
<td>6</td>
<td>Negative</td>
<td>1.2</td>
<td></td>
<td>22.3</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Negative</td>
<td>0.9</td>
<td>0.09</td>
<td>5.4</td>
<td>gi</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Negative</td>
<td>1.0</td>
<td>0.05</td>
<td>14.3</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Positive</td>
<td>0.7</td>
<td>0.5</td>
<td>20</td>
<td>210</td>
<td>No</td>
</tr>
<tr>
<td>10</td>
<td>Positive</td>
<td>0.8</td>
<td>8.9</td>
<td>210</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>Negative</td>
<td>1.2</td>
<td>0.36</td>
<td>18.9</td>
<td>210</td>
<td>gi</td>
</tr>
<tr>
<td>12</td>
<td>Positive</td>
<td>0.9</td>
<td>0.10</td>
<td>7.3</td>
<td>210</td>
<td>gi</td>
</tr>
<tr>
<td>13</td>
<td>Negative</td>
<td>1.5</td>
<td>0.05</td>
<td>20.8</td>
<td>120</td>
<td>No</td>
</tr>
<tr>
<td>14</td>
<td>Positive</td>
<td>3.5</td>
<td>0.13</td>
<td>210</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>Negative</td>
<td>1.6</td>
<td>0.29</td>
<td>4.7</td>
<td>120</td>
<td>No</td>
</tr>
<tr>
<td>16</td>
<td>Positive</td>
<td>1.2</td>
<td>0.49</td>
<td>32.3</td>
<td>180</td>
<td>No</td>
</tr>
<tr>
<td>17</td>
<td>Negative</td>
<td>0.9</td>
<td>13.5</td>
<td>120</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>Negative</td>
<td>1.6</td>
<td>0.9</td>
<td>3.5</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>Negative</td>
<td>1.3</td>
<td>0.05</td>
<td>11.6</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>Positive</td>
<td>1.2</td>
<td>0.2</td>
<td>20.4</td>
<td>240</td>
<td>Yes</td>
</tr>
<tr>
<td>21</td>
<td>Positive</td>
<td>0.9</td>
<td>0.09</td>
<td>17.3</td>
<td>120</td>
<td>No</td>
</tr>
<tr>
<td>22</td>
<td>Positive</td>
<td>1.0</td>
<td>0.25</td>
<td>3.9</td>
<td>210</td>
<td>Yes</td>
</tr>
</tbody>
</table>

gs, glucose intolerance.
Small intestinal bacterial overgrowth and NASH

Six NASH patients (27%) and seven control subjects (30%) had equivocal tests and did not demonstrate a significant H₂ or CH₄ rise. This could be due to these individuals not being colonised by H₂ or CH₄ producing bacteria or having mouth to caecum transit times greater than the observation period of four hours. Small intestinal transit time could not be estimated in these 13 individuals. The mean small intestinal transit time for NASH patients and control subjects was 182 minutes and 180 minutes, respectively (p=0.3). The mean small intestinal transit time for NASH patients with positive breath tests for SIBO was longer than that of control subjects (196 vs 180 minutes; p=0.4).

**LACTULOSE-RHAMNOSE INTESTINAL PERMEABILITY TESTS**

Intestinal permeability data are presented in tables 2 and 3. The mean lactulose/rhamnose permeability ratio (L/R ratio) for NASH patients and control subjects were 0.16 and 0.13, respectively (p=0.37). The mean L/R ratio for NASH patients with SIBO was higher than that of NASH patients without SIBO (0.19 vs 0.10; p=0.13).

**ENDOTOXIN LEVELS**

Endotoxin levels are presented in tables 2 and 3. Mean endotoxin levels for NASH patients and control subjects were 1.2 and 1.3 EU/ml, respectively (p=0.5). Mean endotoxin levels were similar in NASH patients with and without SIBO (1.2 vs 1.2 EU/ml).

**TNF-α ASSAY**

TNF-α levels are shown in tables 2 and 3. NASH patients had significantly higher mean TNF-α levels than control subjects (14.2 vs 7.5 pg/ml, p=0.001, 95% CI for the difference between means 2.8–7.0 pg/ml). NASH patients with SIBO on breath testing did not have a statistically significant higher mean TNF-α levels than NASH patients without SIBO (15.4 ± 12.4 pg/ml, p=0.4). In NASH patients there was no statistically significant correlation between TNF-α values and endotoxin levels (r=−0.3, p=0.5), permeability (r=0.3, p=0.3), or alanine aminotransferase levels (r=−0.3, p=0.08). There was a statistically significant negative correlation between TNF-α values and BMI (r=−0.5, p=0.02).

**Discussion**

One of the major findings of this study was a significantly higher prevalence of SIBO in NASH patients compared with an age and sex matched control group. This is the first controlled study of NASH patients to document this association.

There are several possible explanations for an increased prevalence of SIBO in NASH. Diabetes, an important association of NASH, may predispose to SIBO due to intestinal dysmotility and stasis. However, diabetes or glucose intolerance was present in only a minority (5/11) of NASH patients with SIBO. Nevertheless, if the data are reanalysed and NASH patients with diabetes or glucose intolerance are excluded, there is no longer a significant difference in the prevalence of SIBO between the remaining NASH patients and control subjects (46% vs 22%, p=0.13). It is possible therefore that an association between diabetes and SIBO could explain some of the increased prevalence of SIBO in the NASH group.

We were unable to find other predisposing causes to account for the increased prevalence of SIBO in our NASH patients. We could not detect any significant difference in small intestinal transit time between NASH patients and control subjects to suggest impaired intestinal motility. There was no immunodeficiency among NASH patients that could have predisposed them to SIBO. None had evidence of an isolated IgA or generalised immunoglobulin...
of most bacterial species, would be an important future study.

TNF-α levels were twofold higher in NASH patients than control subjects (p<0.001). This is the first controlled study to demonstrate elevated TNF-α levels in NASH patients. This finding supports the concept that TNF-α plays a significant role in the pathogenesis of NASH as a “second hit” following the development of steatosis. More direct support for endotoxin induced stimulation of TNF-α was lacking from this study as we were unable to demonstrate a statistically significant positive correlation between TNF-α and endotoxin levels.

An association with obesity and elevated TNF-α levels has been described, and the relative obesity of our NASH patients is a possible explanation for the higher TNF-α values of this group. Interestingly however, there is a statistically significant negative correlation between TNF-α values and BMI in NASH patients. It is difficult therefore to attribute elevated TNF-α values to obesity.

In summary, we found an increased prevalence of SIBO among NASH patients compared with control subjects. This was not associated with increased intestinal permeability or elevated endotoxin levels as we had postulated. The significance of our findings remain uncertain. The possible pathogenic role of SIBO in NASH could be further investigated by treating SIBO and assessing any improvement in NASH.

The authors are indebted to D Wigg (Department of Clinical Radiobiology, Royal Adelaide Hospital, statistics), E Southcott (Department of Radiobiology, Royal Adelaide Hospital—statistical analysis), W Johnson, E Southcott, C Kandil (Department of Pathology, Royal Children’s Hospital—intestinal permeability studies), and C Gray (GroPep Pty Ltd—endotoxin assay).

References


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