Unconjugated secondary bile acids in the serum of patients with colorectal adenomas

E Bayerdörffer, G A Mannes, T Ochsenkühn, P Dirschedl, B Wiebecke, G Paulmgartner

Abstract
A positive association between deoxycholic acid (DCA) in the serum and colorectal adenomas, the precursors of colorectal cancer has recently been found, which supported the hypothesis of a pathogenic role of DCA in colonic carcinogenesis. This approach was based on the hypothesis that DCA formed in the colon is absorbed into the portal venous blood and exhibits a constant spillover to the systemic circulation. To further substantiate this hypothesis this study investigated whether in the serum of adenoma patients DCA was higher in the unconjugated fraction, which originates directly from the colon. DCA was found to be 2.8-fold higher in the unconjugated fraction of patients with colorectal adenomas than in controls (0.89 v 0.32 μmol/l, p<0.0025), 1.9-fold in the total DCA fraction (1.89 v 0.95 μmol/l, p<0.0001), and 1.4-fold in the conjugated fraction (0.67 v 0.47 μmol/l, p<0.05). It was further found that the bacterial isomerisation product 3β-DCA was twofold higher in the unconjugated fraction of adenoma patients than in controls (0.08 v 0.04 μmol/l, p=0.27), 1.8-fold in the total iso-DCA fraction (0.11 v 0.06 μmol/l, p<0.05), and 1.5-fold in the conjugated iso-DCA fraction (0.03 v 0.02 μmol/l, p=0.68). The data suggest that the positive association between the serum DCA concentration and colorectal adenoma as described previously results from the DCA fraction that is absorbed from the colon. This further supports a pathogenic role of DCA in the carcinogenesis of colorectal cancer.

Keywords: bile acids, conjugated/unconjugated bile acid fraction, iso-bile acids, deoxycholic acid, colorectal adenomas.

An increase of secondary bile acids in the colon has been suggested to play a part in the pathogenesis of colorectal neoplasia. Secondary bile acids could be a link between Western diets containing a high proportion of fat and the increased frequency of colorectal cancer in Western countries. The amount of dietary fat influences the amount of bile acids secreted into the intestine, and the composition of gut bacteria and faecal pH, factors, which both influence the degradation of intestinal bile acids. Gut bacteria determine the amount of secondary bile acids, so the proportion of unconjugated bile acids, and the proportion of iso-bile acids, which are absorbed into the portal venous blood.

There are indications that the pattern of colonic bile acids influences that of the serum bile acids, because of a constant spillover of bile acids from the enterohepatic into the systemic circulation and the correlation between the bile acid pattern in serum and bile. It may be assumed that the percentage of deoxycholic acid (DCA) in the serum reflects biliary DCA content and the comparative size of the DCA pool. DCA concentrations in the serum should reflect the amount of conjugated and unconjugated DCA absorbed from the ileum and colon, but the source of unconjugated DCA is primarily the colon. This led us to hypothesise that an increased amount of absorbed secondary bile acids in patients with colorectal adenomas should be reflected by increased concentrations of secondary bile acids in the peripheral venous blood.

In a recent investigation we have found significantly increased serum concentrations and increased percentages of secondary bile acids in patients with colorectal adenomas compared with patients without these precursors of colorectal cancer. We could also show that measurement of secondary bile acids in the serum of patients with colorectal adenomas may be a reliable and reproducible parameter.

Therefore, the aim of this study was to find out if the increase in secondary bile acids in the serum of patients with colorectal adenomas is more pronounced in the unconjugated fraction, which originates directly from the colon, than in the conjugated fraction. An increase in secondary bile acids in the unconjugated fraction would further support the hypothesis of secondary bile acids playing a part in colonic carcinogenesis.

Methods

PATIENT SELECTION
We analysed a group of 10 men with colorectal adenomas and 10 age matched controls for their venous bile acid pattern in the conjugated and the unconjugated fraction, and in the unseparated serum fraction. Only men were chosen for this investigation because they had shown a significant positive association between the presence of colorectal adenoma and serum DCA.

Control patients were age matched to eliminate the main confounding risk factor for the development of colorectal adenomas. Control patients were recruited from a group of patients who had undergone colonoscopy.
with no evidence of adenomas or a history of adenomas. Pairs of patients with and without adenomas did not differ in age by more than one year. Where more than one patient without colonic polyps was available to act as a control, one was selected by use of random number tables. Adenoma patients with previous cholecystectomy were matched with a control patient who had also undergone cholecystectomy.

Patients with previous or current malignant disease, chronic inflammatory bowel disease, familial polyposis, or partial colectomy were excluded from the study. We also excluded from analysis patients adhering to a cholesterol lowering diet or those receiving drugs, and vegetarians. Patients with acute or chronic liver disease were excluded. Patients with malignant disease were excluded because of the influence of colon cancer and also other malignancies on cholesterol metabolism even in a pre-clinical stage. Patients with antibiotic treatment during the last four weeks before blood sampling were also not considered for the study.

Personal and medical histories were obtained, and height and weight were recorded for all patients, and patients were asked whether they adhered to any special diet that could influence the lipid and cholesterol metabolism. Indications for colonoscopy were as follows: occult or overt blood in the stool (but not acute, severe intestinal bleeding); abdominal pain of unknown origin, diarrhoea, constipation, suspected neoplasia, and follow up of patients after resection for colorectal cancer (but not in the immediate postoperative period), follow up after polypectomy, or chronic inflammatory bowel disease.

Colonoscopy was performed in our endoscopy unit with Olympus CF-10I, and CF-201 fibreoptic colonoscopes. After macroscopic evaluation, polyps and tumours were removed or biopsied endoscopically or surgically. Adenomas were classified histologically as tubular, tubulo villous, or villous, in accordance with the criteria of the World Health Organisation. Epithelial dysplasia of removed adenomas was graded mild, moderate or severe.

Blood samples were collected after a 12 hour fast either before starting preparation for colonoscopy between 0800 and 0900, or at least four weeks after colonoscopy.

**Bile acid determination**

Two ml of serum were extracted on Bond Elut C18 cartridges. The bile acids were eluted with 6 ml 75% methanol. The eluate was cleaned with three volumes of n-hexane after acidification to pH 3-5. Solvents were removed from bile acids under nitrogen and they were resolved in one volume hydrogen chloride and nine volumes aceton and solvolysed for three hours at 25°C. Thereafter bile acids were deconjugated enzymatically at pH 5-9 for 16 hours. The deconjugated bile acids were passed through a Lipidex-1000 column and eluted with 10 ml 75% methanol according to Setchell et al.20 Bile acids were then methylated and trimethylsilylated. Solvents from the methylster trimethylsilyl-ether derivates were removed under nitrogen and they were dissolved in 25 µl isocitane, and 1-2 µl were analysed by capillary gas chromatography. A Varian 3700 gas chromatograph and a 25 m×0.32 mm fused silica capillary OV-1701 column (CP-Sil-19-CB, Chrompack, Middleburg, Netherlands) were used for bile acid separation. Samples were introduced by cold on column injection and temperature programming. Quantification of bile acids was carried out using hydoxycholic acid as internal standard.

**SEPARATION OF CONJUGATED AND UNCONJUGATED BILE ACIDS**

Conjugated and unconjugated bile acid fractions were prepared from the same serum sample as the total bile acids. The unconjugated fraction of bile acids was eluted with 6 ml 0·1 mol/l acetic acid in 72% ethanol pH 4-0. The conjugated fraction was eluted with 6 ml 0·15 mol/l acetic acid in 72% ethanol pH 6·4 after cleaning both fractions as described above, the unconjugated fraction was methylated and trimethylsilylated, whereas the conjugated fraction was solvolysed and enzymatically deconjugated as described elsewhere.

**QUALITY CONTROL IN BILE ACID DETERMINATION**

Bile acids were prepared from serum samples in groups of 10 samples. To ensure a constant absolute and relative recovery of the individual bile acid a reference serum was analysed together with each batch of serum samples. Table I shows the coefficients of variation of the individual bile acids in the reference serum.

**STATISTICAL ANALYSIS**

Patients with serum bile acid concentrations above 10 µmol/l were excluded because of suspected liver disease. Reasons for excluding patients were stipulated in the protocol before the study began (see patient evaluation).

**TABLE I Coefficients of variance of total, conjugated, and unconjugated bile acids**

<table>
<thead>
<tr>
<th>Bile acid</th>
<th>Cholic acid</th>
<th>Deoxycholic Acid</th>
<th>Chenedoxycholic Acid</th>
<th>Lithocholic acid</th>
<th>Ursodeoxycholic acid</th>
<th>Iso-deoxycholic acid</th>
<th>Iso-chenodeoxycholic acid</th>
<th>Iso-litocholic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total bile acids</td>
<td>1.0</td>
<td>1.1</td>
<td>1.5</td>
<td>3.3</td>
<td>2.1</td>
<td>5.8</td>
<td>4.3</td>
<td>12.5</td>
</tr>
<tr>
<td>Conjugated bile acids</td>
<td>3.6</td>
<td>2.6</td>
<td>2.1</td>
<td>3.9</td>
<td>1.7</td>
<td>16.1</td>
<td>24.1</td>
<td>31.8</td>
</tr>
<tr>
<td>Unconjugated bile acids</td>
<td>2.4</td>
<td>2.3</td>
<td>2.0</td>
<td>3.7</td>
<td>2.9</td>
<td>35.8</td>
<td>4.8</td>
<td>24.4</td>
</tr>
</tbody>
</table>

Values are calculated from all bile acid preparations and expressed in percentages.
TABLE II Demographic data of patients with colorectal adenoma and controls

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Patients with adenoma (n=10)</th>
<th>Matched controls (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y) (mean (SD))</td>
<td>58.7 (12.9)</td>
<td>57.8 (12.4)</td>
</tr>
<tr>
<td>Cholecystectomy</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Gall stones</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Indications for colonoscopy</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Abdominal pain</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>Occult faecal blood</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Suspected neoplasia</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Irregular bowel movements</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Preventive check</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Constipation</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

Patients with and without colorectal adenoma were compared with respect to lithocholic acid, deoxycholic acid, cholic acid, chenodeoxycholic acid, and Ursodeoxycholic acid. We further analysed the iso-bile acids iso-lithocholic acid, iso-DCA, and iso-chenodeoxycholic acid.

In addition to the absolute values of the individual bile acids we also compared their relative proportion in patients with and without adenoma. Student's t test for unmatched samples was used to examine continuous variables.

Results
Table II shows the demographic data and the indications for colonoscopy for the patients with adenoma and controls. All adenomas were histologically graded as mild or moderate dysplasia. Severe dysplasia of adenomas was not diagnosed in patients of this study.

UNCONJUGATED AND CONJUGATED BILE ACID FRACTION
The mean (SD) recovery of bile acids in the unconjugated and conjugated bile acid fractions were 85.6 (11.9)% and 80.2 (9.7)% respectively (Table III), which explains that the total individual bile acids exceed the sum of the conjugated and unconjugated fractions by an average of about 20% (Table III).

Figure 1 shows the total, conjugated, and unconjugated bile acids absolute values, and Fig 2 shows the relative proportions, respectively. The absolute values were 1.6-fold higher for the unconjugated bile acids (p<0.05) and 1.3-fold higher for conjugated bile acids (p<0.05) in patients with adenomas than in controls (Fig 1). Analysing the relative proportions of unconjugated and conjugated bile acids we found no significant difference between patients with adenomas and controls, but a trend for a higher proportion of unconjugated bile acids in patients with adenoma (Fig 2).

Table III shows the values of all individual bile acids cholic acid, chenodeoxycholic acid, iso-chenodeoxycholic acid, DCA, iso-DCA, lithocholic acid, iso-lithocholic acid, and Ursodeoxycholic acid in the unconjugated and conjugated fractions for patients with adenomas and controls. The unconjugated fractions in patients with adenomas were higher for cholic acid, iso-chenodeoxycholic acid, DCA, iso-DCA, and lithocholic acid (59.5%, 81.8%, 58.1%, 78.8%, and 52.8% respectively, Table III). The conjugated fractions in patients with adenomas were higher for chenodeoxycholic acid, iso-lithocholic acid, and Ursodeoxycholic acid (64.6%, 60%, and 51.5% respectively, Table III).

Figure 3 shows the absolute values of 3α-DCA in the unconjugated and conjugated fraction. Unconjugated 3α-DCA was 2.8-fold higher (p<0.0025) in patients with adenoma than in controls. In the conjugated fraction the difference between adenoma patients and controls for the 3α-DCA serum concentrations was less pronounced than in the unconjugated fraction (Fig 3).

The relative proportions of unconjugated 3α-DCA was 1.4-fold higher in patients with adenomas than in controls (p<0.05) and 1.4-fold lower in the conjugated fraction of patients with adenomas than in controls (Fig 4).

Table III shows the values of all individual bile acids cholic acid, chenodeoxycholic acid, iso-chenodeoxycholic acid, DCA, iso-DCA, lithocholic acid, iso-lithocholic acid, and Ursodeoxycholic acid in the unconjugated and conjugated fractions for patients with colorectal adenoma and in controls. The absolute values of total 3α-bile acids were 1.4-fold higher (p<0.01) and those

Table III Individual bile acids in the conjugated and deconjugated serum fraction of patients with colorectal adenoma and in controls

<table>
<thead>
<tr>
<th>Bile acid</th>
<th>Total bile acids</th>
<th>Conjugated bile acids</th>
<th>Deconjugated bile acids</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Adenoma</td>
<td>Controls</td>
<td>Adenoma</td>
</tr>
<tr>
<td>CA</td>
<td>0.95 (0.66)</td>
<td>0.76 (0.37)</td>
<td>0.32 (0.23)</td>
</tr>
<tr>
<td>CDCA</td>
<td>1.96 (0.96)</td>
<td>1.34 (0.65)</td>
<td>0.93 (0.72)</td>
</tr>
<tr>
<td>iso-CDCa</td>
<td>0.12 (0.051)</td>
<td>0.11 (0.035)</td>
<td>0.03 (0.011)</td>
</tr>
<tr>
<td>DCA</td>
<td>1.99 (0.49)</td>
<td>0.95 (0.31)</td>
<td>0.67 (0.41)</td>
</tr>
<tr>
<td>iso-DCA</td>
<td>0.11 (0.038)</td>
<td>0.06 (0.006)*</td>
<td>0.03 (0.008)</td>
</tr>
<tr>
<td>LCA</td>
<td>0.49 (0.11)</td>
<td>0.42 (0.12)</td>
<td>0.19 (0.03)</td>
</tr>
<tr>
<td>iso-LCA</td>
<td>0.13 (0.086)</td>
<td>0.08 (0.051)</td>
<td>0.06 (0.05)</td>
</tr>
<tr>
<td>UDCA</td>
<td>0.40 (0.12)</td>
<td>0.40 (0.22)</td>
<td>0.17 (0.04)</td>
</tr>
</tbody>
</table>

Values are mean (SD) in umol/L. The values in the conjugated and deconjugated fractions are adapted to a mean recovery of 85.6% in unconjugated fractions and 80.2% in conjugated fractions. Statistical differences were calculated using the Student t test. *p<0.05, **p<0.0025, ***p<0.001. Other differences between men with adenomas and controls were not significant.

CA = cholic acid, CDCA = chenodeoxycholic acid, LCA = lithocholic acid, UDCA = Ursodeoxycholic acid.
Unconjugated secondary bile acids in the serum of patients with colorectal adenomas

We have found a significant increase of DCA in the fraction of unconjugated serum bile acids in patients with colorectal adenomas compared with controls. In a previous study we have seen a significantly higher DCA serum concentration in the combined analysis of bile acids derived from the unconjugated and the conjugated fraction in patients with colorectal adenomas. In this study we found an even larger difference between the DCA concentrations of adenoma patients and controls in the unconjugated fraction. As this fraction directly originates from the colon it suggests that our previous finding of an increased serum DCA concentration in the peripheral blood of patients with colorectal adenomas is caused by DCA absorption from the colon.

Deconjugation of Bile Acids

At least 98% of the bile acids are delivered into the intestine as conjugates. In the colon, and to some extent already in the terminal ileum, about 70 to 90% of these bile acids are deconjugated by bacterial enzymes. About 60–90% of the total bile acids and 60% of unconjugated DCA in the portal venous blood are extracted by hepatocytes in the liver to be reconjugated. Bile acids that pass the liver without being taken up by hepatocytes appear in the peripheral blood. We therefore hypothesised that an increased amount of secondary bile acids absorbed in the colon of patients with colorectal adenomas should be reflected by an increase in their concentrations in peripheral venous blood, and that the positive association between the DCA serum concentration and colorectal adenomas should be more pronounced in the unconjugated bile acid fraction originating directly from the colon. We found 47% of total bile acids unconjugated, which is similar to the data of Makino but higher than that found by Setchell. The concentration of total unconjugated bile acids, however, was similar to that reported by Setchell et al. The concentrations of conjugated bile acids in healthy subjects in this study were in the range of earlier investigations for cholic acid and chenodeoxycholic acid. Conjugated DCA and chenodeoxycholic acid in this study was higher than that reported by Linnet et al. This finding, however, fits the epidemiological background, but it does not mutually exclude speculations about another hypothesis such as a change in permeability of the colon to individual bile acids in patients with adenomatous polyps.

Formation of Iso-Bile Acids

Epimerisation of bile acids to 3β-hydroxy derivatives (iso-bile acids) is generally assumed to be caused by bacterial enzymes. Iso-bile acids are absorbed in the colon, appear in the portal venous blood, and in the systemic circulation.

Colonic Carcinogenesis

Secondary bile acids have long been suspected to be promoters (co-carcinogens) of human colorectal neoplasia as experimental studies have shown that secondary bile acids, especially
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METHODOLOGICAL APPROACH
We investigated the relation between secondary bile acids in serum and colorectal adenomas, which has not been reported so far. There is much evidence to suggest that most cases of colorectal cancer arise from adenomatous polyps,\(^5\)\(^6\) a process referred to as the adenomadyplasia-carcinoma sequence.\(^3\) An investigation of the precursor lesion may elucidate risk factors in the pathogenesis of colorectal cancer uninfluenced by possible metabolic consequences of an established cancer.\(^15\)\(^16\) The analysis of matched pairs of patients eliminated the influence of both sex and age – the strongest risk factors for colorectal adenoma.\(^53\)

In conclusion, the data of this study show that DCA and iso-DCA in the unconjugated fraction of total serum bile acids are higher in patients with colorectal adenomas than in controls. These data substantiate our previous finding that increased DCA in the serum of patients with colorectal adenomas is derived from the colon. The data further confirm the hypothesis of a pathogenic role of DCA in the development of colorectal cancer.

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Unconjugated secondary bile acids in the serum of patients with colorectal adenomas


