Biliary lipid composition in cholesterol microlithiasis

M Fracchia, S Pellegrino, P Secreto, L Gallo, G Masoero, A Pera, G Galatola

Abstract
Background—Little information is available on the pathogenesis of cholesterol microlithiasis, and it is not clear if biliary lipid composition in these patients is similar to changes seen in cholesterol gall stone patients.

Aims—to measure biliary lipid composition in patients with cholesterol microlithiasis.

Patients—Eleven patients with cholesterol microlithiasis, 20 cholesterol gall stone patients, and 17 healthy controls.

Methods—Duodenal bile was collected in the fasting state during ceruletid infusion. Biliary cholesterol, phospholipids, and total bile acids were analysed by enzymatic assays, and conjugated bile acids by high pressure liquid chromatography.

Results—Patients with microlithiasis had a cholesterol saturation index significantly higher than controls (mean value 1.30 (95% confidence interval 1.05–1.54) v 0.90 (0.72–1.08)) but similar to gall stone patients (1.51 (1.40–1.63)). This was due to a significant decrease in per cent phospholipid (10.0% (7.1–12.8)) compared with controls (21.4% (18.1–24.6)) and gall stone patients (24.9% (20.5–29.3)). Per cent cholesterol was similar in patients with microlithiasis and controls (5.3% (4.5–6.1) and 5.6% (4.3–6.8), respectively) but was significantly increased in gall stone patients (10.9% (9.3–12.4)). Bile acid composition in patients with microlithiasis was similar to controls whereas in gall stone patients deoxycholic acid was significantly increased: 27.3% (24.8–29.7) v 19.0% (15.7–22.2) in controls and 20.6% (14.9–26.2) in patients with microlithiasis.

Conclusion—Patients with cholesterol microlithiasis have biliary cholesterol supersaturation, similarly to cholesterol gall stone patients. Whereas in the latter this is due to increased per cent cholesterol, in patients with microlithiasis this is caused by phospholipid deficiency, with normal per cent cholesterol and normal biliary bile acid composition.

Keywords: cholesterol microlithiasis; biliary sludge; biliary lipid composition; bile acids; phospholipid; deoxycholic acid

Microlithiasis can be defined as a suspension of precipitates of cholesterol monohydrate crystals or calcium bilirubinate granules in bile.1 Its presence can be suggested by transabdominal ultrasonography as hyperechoic non-shadowing mobile images, the so-called “biliary sludge”,2 but its definitive demonstration is based on finding biliary crystals on microscopic examination of duodenal bile.3

The clinical significance of microlithiasis has been emphasised as it has been considered to have a pathogenic role in acute idiopathic pancreatitis.4 5 However, the natural history of this condition is not completely understood. Based on ultrasonographic follow up studies, sludge may disappear and never recur but usually tends to reappear.6 It may evolve into gall stone disease only in a minority of patients.7 Formation of microlithiasis has been associated with mucus hypersecretion in the gall bladder to an even higher level than in gall stone patients.7

Surprisingly, little information is available on biliary lipid composition of bile containing cholesterol crystals. According to Lee and Nicholls, both hepatic and gall bladder bile of patients with sludge is not different from that found in gall stone patients and healthy subjects, in terms of cholesterol saturation index (CSI), and cholesterol, phospholipid, and total bile acid concentrations.8 Sharma et al have found that patients with cholesterol microlithiasis have a pattern of nucleation time and gall bladder emptying intermediate between healthy subjects and cholesterol gall stone patients, whereas CSI and duodenal bile concentrations of the three main lipids were similar to gall stone patients.8 No data are available on biliary bile acid composition. This is relevant, as bile enriched with deoxycholic acid has been associated with a greater cholesterol secretion and a higher CSI, and is a risk factor for cholesterol gall stone formation.9

Hence our aim was to study the composition of biliary lipids in patients with cholesterol microlithiasis, comparing the results with a group of healthy control subjects and cholesterol gall stone patients.

Subjects and methods
SUBJECTS
We studied three groups of patients. The first group comprised 11 patients with biliary cholesterol microlithiasis, seven men and four women, mean age 47 years (range 33–71). These patients were consecutively enrolled from those referred to our centre with a clinical indication for gall bladder bile sampling because of recurrent episodes of acute idiopathic pancreatitis. Patients were selected for

Abbreviations used in this paper: CSI, cholesterol saturation index; HPLC, high performance liquid chromatography.
this study if five or more cholesterol monohydrate crystals with or without calcium bilirubinate granules were found at microscopic examination of bile rich duodenal fluid. Bile sampling was performed 4–6 weeks after the last episode of acute pancreatitis, in the absence of ultrasonographic evidence of gall stone disease.

The second group consisted of 20 cholesterol gall stone patients, 10 men and 10 women, mean age 53 years (range 34–77). These patients had a functioning gall bladder and radiolucent gall stones at oral cholecystography. The third group consisted of 17 healthy control subjects, 10 men and seven women, mean age 41 years (range 21–62).

At the time of bile collection, all subjects enrolled in the study had a normal body mass index: 23.2 (22.6–23.8) for patients with cholesterol microlithiasis, 22.6 (22.0–23.1) for patients with cholesterol gall stone disease, and 23.1 (22.5–23.8) for healthy controls (NS for all comparisons). All subjects had normal routine blood chemistry, including liver and pancreatic function tests and serum lipid profiles; none was receiving drugs of any kind in the four weeks before the study. Informed verbal consent for obtaining bile samples was given by all subjects. The protocol was approved by the local ethics committee.

**EXPERIMENTAL PROCEDURE**

Duodenal bile was collected from all subjects in the morning after overnight fasting. A nasoduodenal tube was positioned into the third portion of the duodenum under fluoroscopic guidance. Bile rich duodenal fluid was collected during intravenous infusion of 50 ng/kg ceruletide (Takus; Farmitalia, Milan, Italy). A bile aliquot of bile was immediately centrifuged at 3500 rpm for 15 minutes and the sediment examined on a glass slide under a polarising microscope. Cholesterol monohydrate crystals were identified on the basis of their classical rhombohedral shape and by birefringence under cross polarisation. Samples were considered positive for cholesterol micro lithiasis when five or more crystals per slide were found.

An aliquot of bile was processed for chemical analysis. Concentrations of cholesterol, phospholipid, and total bile acids were measured enzymatically. All bile samples satisfied the requirement of a total lipid concentration greater than 5 g/dl, thus rendering possible a reliable calculation of CSI. CSI was calculated using the polynomial equation of Thomas and Hofmann based on cholesterol solubility lines described by Hegardt and Dam. Analysis of conjugated bile acids was carried out by high performance liquid chromatography (HPLC) using a previously described technique recently modified by our group. Analytical grade reagents and deionised distilled water were used. Conjugated bile acids and phenol (internal standard) were purchased from Sigma (St Louis, Missouri, USA). Aqueous KH2PO4 2.0 g/l, (A), H3PO4 85% 2.0 ml/l (B) solutions, and gradient grade acetonitrile (C) (Merck, Darmstadt, Germany) were used as the mobile phase. Bile (200 µl) was diluted to 4.0 ml with 100% ethanol, brought to boiling point for 5–10 minutes, and left overnight at room temperature in the dark. The mixture was spiked with 100 µl ethanolic phenol solution (0.5 mg/ml), shaken well, and filtered through a 0.22 µm Millipore filter; 4.0 ml of an ethanolic solution containing 1.0 mg/ml of each bile acid, spiked with 100 µl of internal standard was used as the standard. A 20 µl aliquot of sample or standard was injected onto the chromatograph.

A Merck Hitachi (Merck, Darmstadt, Germany) liquid chromatograph (L-6200A Intelligent Pump) equipped with a UV-VIS variable wavelength detector (L-4250 UV-VIS detector), automatic injection auto sampler (AS-2000A Autosampler), and a column oven (L-5025 Column Thermostat) were used. An octadecylsilyl LiChroCART 250×4 mm HPLC Cartridge Superspher 100 RP-18 4 µm column (Merck) was used throughout with a LiChroCART 4×4 mm HPLC Cartridge LiChrospher 100 RP-18 5 µm (Merck) guard column. Elution was performed at a flow rate of 1.0 ml/min at 25°C. A multistep linear gradient, starting at time 0 with an A:B:C eluent composition of 41:31:28 (v/v/v), was imposed over 90 minutes. The initial composition, maintained for 10 minutes, was brought to 38:29:33 within five minutes; this was maintained for the following 10 minutes. The composition was then linearly brought to 2:2:96 during the following 45 minutes and then to the original conditions within two minutes. The column was re-equilibrated for 18 minutes before the next injection. The acetonitrile gradient effectively served to elute more retained contaminants, which otherwise could appear during subsequent chromatograms. Optimum chromatographic performance was obtained by accurate preparation of the mobile phase. The column was periodically cleaned by flushing with pure acetonitrile. Peak height was measured using a Merck Hitachi D-2500 Chromato-Integrator. Detection was performed at 200 nm, the detector output was set at 0.002 absorbance units at full scale, and the integrator input was 128–256 mV at full scale. Reproducibility, assessed by repeated assays of bile samples, and accuracy, evaluated by adding increasing concentrations of standards to bile acid specimens, were always greater than 98%.

**STATISTICAL ANALYSIS**

Results are expressed as mean (95% confidence interval). Significant differences between groups were assessed using the Student’s t test for unpaired data. Linear regression analysis was used for assessing the existence of a significant correlation. Values of p<0.05 were considered significant.

**Results**

In addition to patients with cholesterol microlithiasis, for whom it was the entry criterion, cholesterol monohydrate crystals were found on microscopic examination of bile in all patients with gall stone disease; no crystals...
were found in the bile of healthy subjects. Among the 11 patients with cholesterol micro-
lithiasis, one also had biliary bilirubinurate gran-
ules.
Total lipid concentration was 6.36 (5.45–
7.27) g/dl in patients with microolithiasis, 6.06
(5.63–6.49) g/dl in cholesterol gall stone
patients, and 6.16 (5.74–6.57) in healthy sub-
jects (NS for all comparisons).
CSI was significantly increased in patients
with cholesterol microolithiasis (1.30 (1.05–
1.54)) compared with controls (0.90 (0.72–
1.08); p<0.02) and gall stone patients (1.51
(1.40–1.63); p<0.0001 v controls). Figure 1
shows individual data for biliary lipid composi-
tion in the three groups. Per cent biliary
cholesterol was similar in microolithiasis pa-
tients and controls (5.3 (4.5–6.1)% and 5.6
(4.3–6.8)% respectively) whereas it was sig-
ificantly increased in gall stone patients (10.9
(9.3–12.4)%; p<0.0001), and CSI in patients with microlithiasis and gall stone patients,
respectively (NS for all comparisons). There
was a significant increase in deoxycholic acid in gall
stone patients: 27.3 (24.8–29.7)% compared with controls (7.3 (69.3–
76.8)%; p<0.0002) and gall stone patients
(64.7 (58.6–70.7)%; p<0.0002) in whom per
cent bile acid was also significantly lower than
in controls (p<0.05).

As a consequence of this biliary lipid
composition pattern, the cholesterol/
phospholipid ratio was increased in both
microolithiasis (0.69 (0.44–0.93)) and gall stone
patients (0.49 (0.42–0.55)) compared with
healthy controls (0.28 (0.20–0.36); p<0.002
and p<0.001, respectively). The cholesterol/
bile acid ratio was increased in gall stone
patients (0.18 (0.15–0.22)) compared with
patients with microolithiasis (0.06 (0.05–
0.07); p<0.0001) and controls (0.08 (0.06–0.10;
p<0.0001); the bile acid/phospholipid ratio
was increased in patients with microolithiasis
(11.2 (7.3–15.1)) compared with controls (3.9
(3.1–4.8); p<0.001) and gall stone patients
(4.2 (2.1–6.2); p<0.0001).

CSI was significantly associated with per
cent biliary cholesterol in the three groups:
r=0.74 in patients with microolithiasis (p<0.01),
r=0.80 in controls (p<0.0001), and r=0.76 in
gall stone patients (p<0.0001). CSI was
inversely associated with per cent phospholipid
in microolithiasis patients (r=-0.65 (p<0.05))
whereas no association was found in the two
other groups. No correlation was found
between CSI and per cent bile acid in the three
groups.

Results for per cent bile acid composition
were as follows: cholic acid was 35.5 (30.2–
40.8)%, 35.1 (30.8–39.4)%, and 30.1 (25.4–
34.8)% in patients with microolithiasis, con-
trols, and gall stone patients, respectively (NS
for all comparisons); chenodeoxycholic acid
was 40.1 (35.7–44.5)% 40.4 (36.5–44.2)% and
41.5 (37.9–45.2)% respectively (NS for all
comparisons); deoxycholic acid was 1.8
(1.0–2.6)%, 3.4 (2.0–4.8)% and 2.1 (1.5–
2.6)% respectively (NS for all comparisons); and
lithocholic acid was 2.1 (1.1–3.1)% 2.2
(0.8–3.6)% and 1.0 (0.8–1.1)% respectively
(NS for all comparisons). There was a significant
increase in deoxycholic acid in gall
stone patients: 27.3 (24.8–29.7)% v 19.0
(15.7–22.2)% in controls (p<0.001) and v
20.6 (14.9–26.2)% in patients with microolithi-
asis (p<0.02). A deoxycholic/cholic acid ratio
>1 was found more frequently in gall stone
patients (eight of 20 patients) than in controls
(one of of 17) and patients with microolithiasis
(one of 11).

Discussion
In this study we have shown that patients with
cholesterol microolithiasis, similar to cholesterol
gall stone disease, CSI was increased as
a result of an increase in molar per cent biliary
cholesterol, as is widely accepted in the
literature. An increase in CSI may be
caused by an increase in per cent biliary
cholesterol or a reduction in per cent bile acid and/or phospholipid. In our patients with
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Figure 1 Individual data for per cent molar concentration of biliary lipids in healthy control subjects, in patients with cholesterol microolithiasis, and in cholesterol gall stone patients. Arrows indicate statistical differences between groups. (A) % molar cholesterol; (B) % molar phospholipids; and (C) % molar bile acids.

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Biliary lipids in microlithiasis

Sharma et al have recently shown abnormal gall bladder motility patterns in patients with cholesterol microlithiasis, intermediate between those observed in healthy subjects and cholesterol gall stone patients. Cholesterol content in the gall bladder wall affects gall bladder motility, and the cholesterol content of gall bladder muscles has been found to be increased in cholesterol gall stone patients. It is possible to hypothesise that lipid absorption by the gall bladder mucosa in patients with microlithiasis has a different pattern to that in cholesterol gall stone patients, but no information is available on this subject.

The characteristic increase in cholesterol/bile acid ratio of gall stone patients was not present in our patients with microlithiasis, in whom there was an increase in the cholesterol/phospholipids ratio, further suggesting differences in bile composition between these two groups of patients.

Bile acid composition in microlithiasis patients was similar to that found in healthy subjects, lacking the characteristic increase in per cent biliary deoxycholic acid of cholesterol gall stone patients that we confirmed in our group of gall stone patients. Increased deoxycholic acid has a pathogenic role in the formation of cholesterol gall stones by increasing hepatic secretion of cholesterol in bile. This finding may help explain the normal per cent cholesterol in our patients with microlithiasis.

Our results suggest that cholesterol microlithiasis forms in bile supersaturated with cholesterol via a different mechanism to that of cholesterol gall stone patients. The natural history of microlithiasis is not completely defined. Only a minority of patients with ultrasonographic demonstration of sludge develop gall stones, and whether microlithiasis evolves to gall stone disease is still a matter of controversy. The longer nucleation time of bile and better gall bladder emptying, together with the multiple differences in lipid composition of bile in patients with microlithiasis compared with cholesterol gall stone patients suggests that cholesterol microlithiasis may be a different disease from cholesterol gall stone disease. Our data provide further support for this hypothesis but cannot clarify whether microlithiasis is an early stage of cholesterol gall stone formation.

Further studies, both on the natural history and on the pathogenesis of microlithiasis, are needed to address this question.

In conclusion, we have shown that patients with cholesterol microlithiasis have an increased CSI similarly to cholesterol gall stone patients. Bile supersaturation of patients with microlithiasis was however due to a decrease in per cent biliary phospholipid and not to the characteristic increase in per cent biliary cholesterol of gall stone patients. Microlithiasis patients do not have enrichment of their bile acid pool with deoxycholic acid, as is the case in cholesterol gall stone patients.
