

ORIGINAL ARTICLE

Unconjugated bilirubin in human bile: the nucleating factor in cholesterol cholelithiasis?

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7 March 2003**Aims:** To investigate the concentrations of bilirubin, bilirubin conjugates, phospholipid, and cholesterol in the gall bladder bile obtained at surgery from patients with and without cholesterol gallstones.**Methods:** Gall bladder bile was collected during surgery, by puncture, from 20 patients with gallstones undergoing routine cholecystectomy and from eight patients with normal liver blood tests. Concentrations of bilirubin, bilirubin conjugates, phospholipid, and cholesterol were measured using standard procedures.**Results:** The proportion of total bilirubin that was unconjugated was significantly higher in the bile from patients with stones than in bile from control patients, whether or not the bile from either group was saturated with cholesterol or not. Indeed, the mean concentration of cholesterol was significantly higher in control bile samples.**Conclusion:** The presence of stones was more closely related to the proportion of unconjugated bilirubin than to the degree of saturation of bile with cholesterol. Bilirubin and its metabolites probably play an important part in the formation of cholesterol gallstones.

Cholesterol is sparingly soluble in water and its transport in an aqueous medium such as bile is dependent on its aggregation with other biliary lipids. Consequently, studies of the pathogenesis of cholesterol gallstones have for many years concentrated on the relative stabilities of the different modes of cholesterol transport in bile. More recently, attention has been focused on the process of cholesterol crystal formation and, in particular, glycoproteins have been identified in human bile that may promote¹ or inhibit² cholesterol crystal precipitation. However, stones are rarely pure cholesterol; indeed, the "Western" cholesterol gallstone is often only 70% cholesterol by weight and almost invariably contains a pigmented nucleus, often being pigmented throughout.^{3–4} Like cholesterol, bilirubin is also insoluble in water and its transport in human bile is bile acid dependent.⁵ However, there have been few studies of bilirubin metabolites in bile from patients with cholesterol gallstones, even though it has been proposed that unconjugated bilirubin may play an important complementary role in the initiation of cholesterol gallstone formation.⁶

"There have been few studies of bilirubin metabolites in bile from patients with cholesterol gallstones"

Therefore, we compared the concentrations of bilirubin metabolites and biliary lipids in the bile of patients with and without cholesterol gallstones.

PATIENTS AND METHODS

Gall bladder bile was collected during surgery, by puncture, from 20 patients with gallstones undergoing routine cholecystectomy. Oesophagogastroduodenoscopy had been carried out in 14 of the 20 patients and was normal. All preoperative liver blood tests, including serum bilirubin, enzymes, and albumin were normal. Operative cholangiography revealed no bile duct stones.

Gall bladder bile was also obtained from eight patients with normal liver blood tests, six of whom were undergoing surgery for duodenal ulcer, one for adhesions, and one an exploratory laparotomy for unexplained abdominal pain in whom no

abnormality was found. In each case the gall bladder was inspected and palpated and, being normal, the specimen of bile was aspirated before the surgical procedure.

The median age of the 20 patients with stones was 47 years (range, 21–73) and 12 were women. The median age in the eight patients without stones was 43 years (range, 21–66) and four were women. No patient was taking drugs. All stones were cholesterol in type (> 70%), as analysed by routine laboratory procedures.⁶

The concentrations of bilirubin and its conjugates in bile were determined using the base catalysed transesterification procedure of Blanckaert.⁷ Bilirubin standards were prepared in bile acid/lecithin solutions and in chloroform, to cover the concentration range of 0–200 µmol/litre. Recovery was monitored by the addition of 0.1 µCi ¹⁴C-bilirubin (specific activity 15.9 mCi/nmol; Amersham-Buchler, Germany) to 0.2 ml bile, and incubating for 15 minutes at 34°C before extraction; the mean recovery from two bile samples on five consecutive occasions was 97% (SD, 3%) and 96% (SD, 2%), respectively. The recovery from thin layer plates of bilirubin standards prepared in chloroform was indistinguishable from that of bilirubin standards prepared in the bile acid/lecithin solutions, and did not vary over the range of 2–30 µmol applications: mean, 69%; SD, 5% at 2 µmol; mean, 71%; SD, 2% at 30 µmol (n = 5).

Biliary bile acids, phospholipids, and cholesterol were measured as described previously,⁸ and the cholesterol saturation index (CSI) of each bile sample was calculated⁹ as described. The significance of the differences between the results was estimated with the Mann Whitney non-parametric method using "Minitab" software version 2.0.

All patients gave written permission for the bile to be obtained during surgery, and the procedure was approved by the St Thomas's Hospital ethics committee.

RESULTS

One quarter of the 20 patients with predominantly cholesterol gallstones had bile unsaturated with cholesterol (CSI < 1.0), whereas half of the stone free bile samples were unsaturated; consequently, there was no significant difference with respect

Table 1 Lipid concentrations in gall bladder bile from patients with or without gallstones

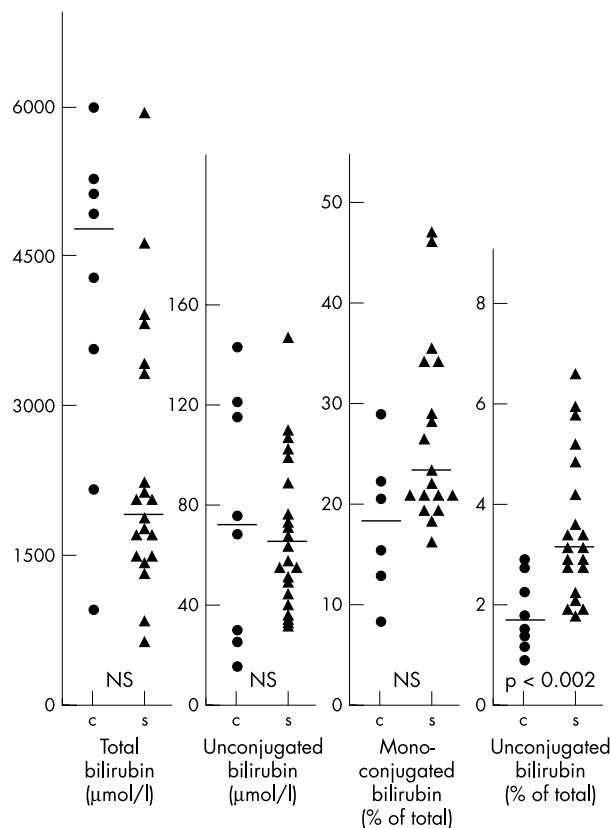
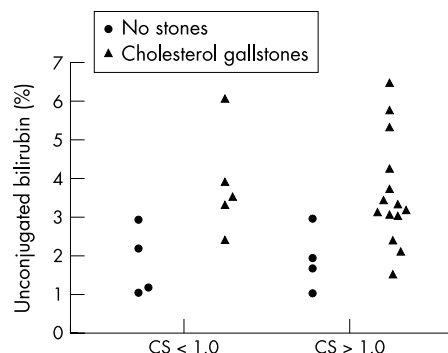
	No stones (n=8)	With stones (n=20)
Cholesterol (mmol/l)	14.03 (5.1)	12.05 (4.9)*
Bile acids (mmol/l)	119.7 (38.2)	91.3 (27.5)
Phospholipids (mmol/l)	54.2 (20.6)	36.3 (15.5)*
Total lipids (g/l)	114.8 (38)	80.8 (26)*
Cholesterol saturation index	0.99 (0.28)	1.30 (0.48)

Values are means (SD).
* $p < 0.02$, stones v no stones.

to the CSI (table 1). Indeed, the mean biliary cholesterol, phospholipid, and total lipid concentrations in the stone group were significantly lower than those in the control group ($p < 0.02$; table 1).

The major bands of bilirubin detected in all samples were bilirubin IX C-8 monomethylester (Rf 0.5), bilirubin IX C-12 monomethylester (Rf 0.45), and bilirubin dimethylester (Rf 0.25). Minor bands were bilirubin III and XIII monomethylesters (Rf 0.54 and Rf 0.01, respectively).

The biliary total and unconjugated bilirubin concentrations were not significantly different between the two groups (fig 1). However, the unconjugated fraction expressed as a proportion of the total bilirubin present was significantly higher in the cholesterol gallstone group (cholesterol gallstone group: mean, 3.41%; median, 3.04%; SD, 1.4%; $n = 20$; controls: mean, 1.74%; median 1.51%; SD, 0.8%; $n = 8$; $p < 0.002$). The mean monoconjugated bilirubin fraction was also higher in the stone group, although the difference was not significant (cholesterol gallstone group: mean, 26.56%; median 22.5%; SD, 9.3%; $n = 18$; controls: mean, 17.67%; median, 18.0%; SD, 6.9%; $n = 6$; $p = 0.062$).

**Figure 1** Biliary bilirubin concentrations in patients with (triangle) and without (circle) predominantly cholesterol gallstones. Medians are shown.**Figure 2** Unconjugated bilirubin (% total) in cholesterol saturated (cholesterol saturation index [CSI] > 1.0) and unsaturated (CSI < 1.0) bile samples from patients with and without gallstones.

Therefore the presence of stones correlated more with the proportion of unconjugated bilirubin than with the CSI. Indeed, in the 15 patients with cholesterol gallstones and bile saturated with cholesterol, the mean percentage of bilirubin present in the unconjugated fraction (mean, 3.33%; median, 2.84%; SD, 1.5%; $n = 15$) was higher than that in the four control subjects also with cholesterol saturated bile but without gallstones (mean, 1.63%; median, 1.51%; SD, 0.8%; $n = 4$; $p < 0.02$), and also higher, but not significantly different, from that in the other four control subjects whose bile was not saturated with cholesterol (mean, 1.85%; median, 1.72%; SD, 0.8%; $n = 4$; $0.05 > p < 0.10$).

In the five patients with cholesterol gallstones but bile unsaturated with cholesterol, the percentage of bilirubin present in the unconjugated fraction (mean, 3.66%; median, 3.25%; SD, 1.4%; $n = 5$) was significantly higher than that seen in the eight subjects without gallstones, whether their bile was saturated with cholesterol (mean, 1.63%; median, 1.51%; SD, 0.8%; $n = 4$; $p < 0.04$) or not (mean, 1.85%; median, 1.72%; SD, 0.8%; $n = 4$; $p < 0.04$; fig 2).

DISCUSSION

Our results confirm that the CSI and lipids in the gall bladder bile of patients with gallstones are not consistently higher than in controls, although the bile of patients with cholesterol gallstones frequently contains a greatly increased proportion of unconjugated (and also probably of monoconjugated) bilirubin. Such observations are in accordance with the hypothesis that deconjugation of bilirubin glucuronides in bile may be an early event in the formation of cholesterol gallstones with a pigmented nidus,¹⁰ rather than a primary abnormality of lipid metabolism being the cause.

The major bilirubin metabolite in human bile is its diglucuronide, which, together with the monoglucuronide, accounts for more than 98% of total biliary bilirubin.^{11,12} Deconjugation may be catalysed by glucuronidases of bacterial or mucosal origin,¹² and bilirubin monoglucuronide may also undergo non-enzymatic hydrolysis to form unconjugated bilirubin.¹³ Even in normal bile, the low concentration of unconjugated bilirubin still exceeds its aqueous solubility 100–1000-fold,^{14,15} so that bile has frequently been described as being supersaturated with calcium salts of bilirubin.^{5,16}

Increased proportions of unconjugated bilirubin in gallbladder bile may be a consequence of impaired gallbladder motility.^{17,18} Prolonged storage of bile in the gallbladder, in addition to increasing the length of exposure of conjugated bilirubin to glucuronidases, may also decrease the biliary pH,¹⁹ although this has not been measured in humans. Endogenous β glucuronidase activity can be detected at pH 7.5,²⁰ but its optimum pH is 4.5–5.0,²¹ so that a lower pH would favour the formation of unconjugated bilirubin. Even in sterile gallstones, bacterial DNA can usually be detected, suggesting

Take home messages

- The presence of stones was more closely related to the proportion of unconjugated bilirubin than to the degree of saturation of bile with cholesterol
- Bilirubin and its metabolites probably play an important part in the formation of cholesterol gallstones

that bacterial glucuronidases have been active.²² Unconjugated bilirubin has been identified as a component of biliary sludge, which is believed to precede gallstones,^{23,24} particularly pigmented stones.^{25,26}

“The proportions of conjugated to unconjugated and/or monoconjugated bilirubin may be important in the nucleation of cholesterol”

The concept that unconjugated bilirubin plays at least as an important role in the formation of cholesterol gallstones as does cholesterol saturation is not new,^{4,6,27} but has received little attention. Our finding of an increased proportion of bilirubin in its unconjugated moiety in the bile of patients with cholesterol gallstones rather than an increase in the total bilirubin concentration has previously been observed mainly in bile containing pigmented stones,^{25,28} but we found that unconjugated bilirubin is a feature of both cholesterol saturated and unsaturated bile from patients with cholesterol gallstones, although this conclusion is based on small numbers. Hence, there was a better correlation between the proportion of unconjugated bilirubin and the presence of stones than the classic cholesterol saturation index, raising the possibility that the proportions of conjugated to unconjugated and/or monoconjugated bilirubin may be important in the nucleation of cholesterol, or that it is simply a marker of glucuronidase activity related to poor gall bladder motility. Many studies have emphasised the roles of impaired motility of the gall bladder^{29–32} and of the intestine³³ in the pathogenesis of cholesterol gallstone disease, and impaired gall bladder motility would favour the formation of unconjugated bilirubin. There is no agreement on the relative importance of these factors,³³ but our results add to the hypothesis that unconjugated bilirubin plays an important role in cholesterol precipitation (M K Dutt. MD Thesis, University of London, 1983).

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REFERENCES

- 1 **Abel M**, Kawczak P, Nuutinen H, *et al*. Isolation and characterisation of a cholesterol crystallization promoter from human bile. *Gastroenterology* 1993;**104**:539–48.
- 2 **Ohya T**, Schwarzendrube J, Busch N, *et al*. Isolation of a human biliary glycoprotein inhibitor of cholesterol crystallization. *Gastroenterology* 1993;**104**:527–38.
- 3 **Been JM**, Bills PM, Lewis D. Microstructure of gallstones. *Gastroenterology* 1979;**76**:548–55.
- 4 **Sutor DJ**, Wooley SE. The organic matrix of gallstones. *Gut* 1974;**15**:487–91.
- 5 **Ostrow JD**. Unconjugated bilirubin and cholesterol gallstone formation. *Hepatology* 1990;**12**:219S–26S.
- 6 **Duvaldestin P**, Mahu JL, Metreau JM, *et al*. Possible role of a defect in hepatic bilirubin glucuronidation in the initiation of cholesterol gallstones. *Gut* 1980;**21**:650–5.
- 7 **Blanckaert N**. Analysis of bilirubin and bilirubin mono- and diconjugates. Determination of their relative amounts in biological fluids. *Biochem J* 1980;**185**:115–28.
- 8 **Oureshi MY**, Murphy GM, Dowling RH. The enzymatic determination of total phospholipids in bile and bile-rich duodenal aspirates. *Clin Chim Acta* 1980;**105**:407–10.
- 9 **Thomas PJ**, Hofmann AF. A simple calculation of the lithogenic index of bile: expressing biliary lipid composition on rectangular coordinates. *Gastroenterology* 1972;**65**:698–700.
- 10 **Smith BF**, LaMont JT. The central issue of cholesterol gallstones. *Hepatology* 1986;**6**:529–631.
- 11 **Boonyapisit ST**, Trotman BW, Ostrow JD, *et al*. Measurement of conjugated and unconjugated bilirubin in bile. A new thin-layer chromatographic method. *J Lab Clin Med* 1976;**88**:857–63.
- 12 **Favery J**, Blanckaert N, Leroy P, *et al*. Analysis of bilirubins in biological fluids by extraction and thin-layer chromatography of the intact tetrapyrroles: application to bile of patients with Gilbert's syndrome, hemolysis, or cholelithiasis. *Hepatology* 1983;**3**:177–83.
- 13 **Spivak W**, Di Venuto D, Yuvey W. Non-enzymatic hydrolysis of bilirubin mono- and di-glucuronide to unconjugated bilirubin in model and native bile systems. *Biochem J* 1987;**22**:323–9.
- 14 **Brodersen R**. Bilirubin solubility and interaction with albumin and phospholipid. *J Biol Chem* 1979;**254**:2364–9.
- 15 **Ostrow JD**, Celic L, Mukerjee P. Molecular and micellar associations in the pH-dependent stable and metastable dissolution of unconjugated bilirubin by bile salts. *J Lipid Res* 1989;**29**:335–48.
- 16 **Ostrow JD**, Celic L. Apparent solubility products (K^{11} sp) of the calcium salts of unconjugated bilirubin (B) indicate supersaturation of human gallbladder bile with Ca (HB)₂ but not CaB [abstract]. *Hepatology* 1987;**7**:1111.
- 17 **Howard PJ**, Murphy GM, Dowling RH. Gall bladder emptying patterns in response to a normal meal in healthy subjects and subjects with gall stones: ultrasound study. *Gut* 1991;**32**:1406–11.
- 18 **Jazrawi RP**, Pazzi P, Petroni ML, *et al*. Postprandial gallbladder motor function: refilling and turnover of bile in health and in cholelithiasis. *Gastroenterology* 1995;**109**:582–91.
- 19 **Gleeson D**, Hood KA, Murphy GM, *et al*. Calcium and carbonate ion concentration in gallbladder and hepatic bile. *Gastroenterology* 1992;**102**:1707–16.
- 20 **Shinya F**, Toshima T, Takahashi W, *et al*. Effects of pH on the deconjugation of conjugated bilirubin in human bile. *Tohoku J Exp Med* 1985;**147**:281–93.
- 21 **Maki T**. Pathogenesis of calcium bilirubinate gallstones: role of E coli, β -glucuronidase, and coagulation by inorganic ions, polyelectrolytes and agitation. *Ann Surg* 1966;**164**:90–100.
- 22 **Swidsinski A**, Khilkin M, Pahlig H, *et al*. Time dependent changes in the concentration and type of bacterial sequences found in cholesterol gallstones. *Hepatology* 1998;**27**:662–5.
- 23 **Allen B**, Bernhoft R, Blanckaert N, *et al*. Sludge is calcium bilirubinate associated with bile stasis. *Am J Surg* 1981;**141**:51–6.
- 24 **Lee SP**, Nicholls JF. Nature and composition of biliary sludge. *Gastroenterology* 1986;**90**:677–86.
- 25 **Boonyapist ST**, Trotman BW, Ostrow JD. Unconjugated bilirubin, and the hydrolysis of conjugated bilirubin, in gallbladder bile of patients with cholelithiasis. *Gastroenterology* 1978;**74**:70–4.
- 26 **Magnuson TH**, Lillemo KD, Peoples GE, *et al*. Oral calcium promotes pigment gallstone formation. *J Surg Res* 1989;**46**:286–91.
- 27 **Wang DQ**, Carey MC. Characterization of crystallization pathways during cholesterol precipitation from human gallbladder bile: identical pathways to corresponding model biles with three predominating sequences. *J Lipid Res* 1996;**37**:2539–49.
- 28 **Masuda H**, Nakayama F. Composition of bile pigment in gallstones and bile and their etiological significance. *J Lab Clin Med* 1979;**93**:353–60.
- 29 **Pauletzki J**, Althaus R, Holl J, *et al*. Gallbladder emptying and gallstone formation: a prospective study on gallstone recurrence. *Gastroenterology* 1996;**111**:765–71.
- 30 **Duane WC**. Something in the way she moves: gallbladder motility and gallstones. *Gastroenterology* 1996;**111**:823–5.
- 31 **Gustafsson U**, Sahlin S, Einarsson C. Biliary lipid composition in patients with cholesterol and pigment gallstones and gallstone-free subjects: deoxycholic acid does not contribute to formation of cholesterol gallstones. *Eur J Clin Invest* 2000;**30**:1099–106.
- 32 **Portincasa P**, Ciauala AD, Vendemiale G, *et al*. Gallbladder motility and cholesterol crystallisation in bile from patients with pigment and cholesterol gallstone. *Eur J Clin Invest* 2000;**30**:317–24.
- 33 **Dowling RH**. Review: pathogenesis of gallstones. *Aliment Pharmacol Ther* 2000;**14**(suppl 2):39–47.



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