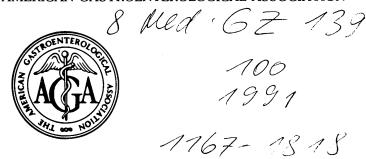
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KEY TO ABBREVIATIONS ar-audiovisual review br-book review c-correspondence cc-clinical conference cr-case report

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KEY TO ABBREVIATIONS

ar-audiovisual review

br-book review

c-correspondence

cc-clinical conference

cr-case report

ctt–clinical trends and topics e–editorial

hs-historical series pa-progress article

ra-review article

ss-selected summary

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Role of High Total Protein in Gallbladder Bile in the Formation of Cholesterol Gallstones

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While it is generally accepted that cholesterol supersaturation of bile is of key importance in the rapid formation of cholesterol crystals, the role of total biliary protein and pH in the pathogenesis of cholesterol gallstones is less well understood. The relation of cholesterol saturation, total protein, and pH was studied in 73 gallbladder bile samples with and 35 gallbladder bile samples without cholesterol crystals. In samples containing crystals, a trend to higher values of cholesterol and to a higher cholesterol saturation index was observed. However, significantly (P = 0.02) higher concentrations of total protein were found in samples with crystals $[0.80 \pm 0.40 \text{ g/dL } (8.0 \pm 4.0 \text{ g/L})]$ than in samples without crystals $[0.63 \pm 0.26 \text{ g/dL} (6.3 \pm 2.6 \text{ g/L})]$. Moreover, of 22 bile samples with total protein concentrations > 10.0 g/L, cholesterol crystals were detected in all but 2. Total lipids, bile acids, phospholipids, and pH values were not significantly different in the two groups of bile samples. It was concluded that high biliary protein concentrations are frequently associated with cholesterol crystals and may, therefore, be a possible risk factor in the pathogenesis of cholesterol gallstones.

In recent years, nonsurgical procedures for the treatment of cholesterol gallstones have gained importance (1–4). These methods are hampered by a high rate of stone recurrence after successful dissolution therapy (5–8). Studies of the factors predisposing to gallstone formation may yield clues about the risk of stone recurrence. In gallbladder bile from patients with cholesterol gallstones, cholesterol crystal formation is accelerated compared with equally supersaturated bile from patients with pigment stones or healthy controls (9,10). This suggests that factors other than cholesterol supersaturation play a role in gallstone

formation. Different bile proteins appear to promote cholesterol nucleation (11–14). Furthermore, it has been postulated that quantitative abnormalities in total biliary protein may favor gallstone formation (15,16).

Recently, Shiffman and Moore (17) have reported a defective acidification of gallbladder bile in gallstone patients. This defect renders bile supersaturated with CaCO₃ and may lead to precipitation of crystals of calcium carbonate, which could act as nidus for cholesterol stones. Changes in pH may also affect the conformation of proteins and by this mechanism change their properties with respect to their nucleating activity.

Therefore, in addition to cholesterol supersaturation, changes in biliary protein concentration and pH may facilitate the development of cholesterol gallstones. These considerations prompted us to compare total protein concentrations and pH in gallbladder bile samples with and without cholesterol crystals.

Materials and Methods

Patients and Collection of Bile

One hundred eight patients, 78 women and 30 men, who underwent elective cholecystectomy for symptomatic gallstone disease were included in the study. Fourteen patients in whom cystic duct obstruction was suspected because of white gallbladder bile and total lipid concentrations $<1~\rm g/dL$ ($<10~\rm g/L$) were excluded from the study. During surgery, bile was aspirated with an 18-gauge needle from the gallbladder. Because of the known stratification of

Abbreviations used in this paper: BSA, bovine serum albumin; TCA, trichloracetic acid.

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human gallbladder bile (18), particular care was taken to collect gallbladder bile completely. Clamping of the gallbladder wall before bile aspiration was avoided to prevent mucosal damage. Stones were removed with the gallbladder, washed with distilled water, dried, and weighed. The cholesterol content of the gallbladder stones was measured chemically after extraction with organic solvents and expressed as percentage of dry weight.

Biliary Microscopy and pH Measurement

After collection, bile samples were mixed thoroughly and one drop of bile was immediately examined by polarized light microscopy for cholesterol crystals. Because small numbers of crystals may be overlooked by the microscopic examination of unspun bile, the sediment of the bile samples was reexamined for cholesterol crystals after ultracentrifugation (100,000 \times g for 2 hours). Parallel to microscopy, pH was measured in fresh anaerobic bile samples with a pH-sensitive electrode that was calibrated at 22°C using hydrogen phosphate and tetraborate buffers at pH 6.86 and pH 9.18, respectively. The bile samples were maintained in closed syringes until analysis to avoid loss of CO, to the atmosphere. Under these conditions, pH values remained constant to within ±0.1 during the 1-hour observation time.

Analysis of Biliary Lipids

For the analysis of bile lipids, duplicate aliquots were stored at -70°C until determination. Cholesterol concentration was determined colorimetrically with the Liebermann-Burchard reaction after extraction of a 1-mL methanolic bile sample with petrol ether (19). The recovery of biliary cholesterol after extraction exceeded 90%, with an interassay variance of <5%. Phospholipids were measured as total biliary phosphate after hydrolysis with sulfuric acid at 150°C using the colorimetric assay described by Fiske and Subbarow (20) (interassay variance, <5%). The contribution of inorganic phosphate to total biliary phosphate is negligible (<5%; unpublished observation); therefore, total phosphate adequately reflects lipid bound phosphate. Total bile salt concentrations were determined by a modified 3-hydroxysteroid dehydrogenase method (21). For this assay, methanolic bile solutions were prepared by diluting 0.1 mL of bile to a volume of 1.0 mL with methanol. The reaction mixture contained 1 mL of 1 mol/L glycine buffer (pH 9.4) containing ethylenediaminetetraacetic acid (EDTA) (5.6 mmol/L) and hydrazine sulfate (0.4 mmol/L), 0.1 mL of NAD solution (5.4 mmol/L), 0.1 mL of hydroxy steroid dehydrogenase solution (0.7 U/mL), and 0.02 mL of methanolic bile solution. The mixture was incubated at 26°C for 1 hour and the extinction measured at 340 nm against appropriate blanks (interassay variance, <5%).

The cholesterol saturation index of each sample was calculated according to the method of Carey (22) by dividing the cholesterol concentration by the maximum cholesterol solubility and corrected for the total lipid content of each individual bile sample.

Analysis of Biliary Protein

Biliary proteins were precipitated from bile by the addition of 7% trichloracetic acid (TCA) (100 µL bile in 2 mL TCA). The mixture was kept at 4°C for at least 12 hours and then centrifuged at $1000 \times g$ for 10 minutes. The pellet was resuspended in 2 mL of 7% TCA and recentrifuged. The precipitated protein was washed twice in 2 mL of cold ether-ethanol 3:1 (vol/vol). After a final centrifugation at $1000 \times g$ for 10 minutes, the organic solvents were decanted and the remaining protein pellet was dissolved in 500 µL of 1N NaOH. This solution was kept at 37°C for 1 hour to ensure complete dissolution of the proteins. Bovine serum albumin (Bio-Rad, Richmond, CA) was used as standard protein. Standards with protein concentrations ranging from 1.0 to 20 g/L were prepared like the native bile samples. For the Lowry procedure (23), 0-, 10-, 20-, 30-, 40-, and 50-L aliquots of the 500-L 1N NaOH/protein solutions were diluted with 1N NaOH to an equal volume of 100 uL. After addition of 1.0 mL of the alkaline reagent, the mixture was incubated for 10 minutes. Two hundred microliters of Folin reagent/H2O (1:1) were added and absorbance was read at 750 nm after an additional 30 minutes of incubation. The protein content of the bile samples was expressed as the mean value of five measurements at different dilutions (1:10-1:50). By this method, an interassay variance of < 5% was determined. The modified Lowry procedure described above was compared with a fluorometric assay for biliary protein determination (24-26). For this purpose five gallbladder bile samples were "spiked" with bovine serum albumin (BSA) and human immunoglobulin G (IgG) in five different concentrations (0.5-7.5 g/L) and the recovery measured using both Lowry and fluorometric assays. For the fluorometric assay the final protein pellet was dissolved in 500 µL borate buffer (0.2 mol/L, pH 8.5) and incubated for 1 hour at 37°C. Ten- and 30-μL aliquots were diluted up to 200 μL with borate buffer (0.2 mol/L, pH 8.5). One minute after addition of 100 μ L of fluorescamine solution (0.03% in acetone) the reaction mixture was diluted with 3.5 mL H₂O and fluorescence was determined at 390 nm on a fluorometer (Fluorescence Spectrophotometer; Perkin-Elmer, Norwalk, CT). The final dilution of native bile was 1:617-1:1850 in the fluorometric assay and 1:10-1:50 in the modified Lowry assay.

Statistical Analysis

All results are expressed as mean \pm SD or median and range. Student's unpaired t test was used for group comparison with the level of statistical significance set at $P \leq 0.05$. Spearman's rank correlation coefficients were calculated using the method of least squares.

Results

Cholesterol Crystals

Gallbladder bile samples obtained from 108 patients during cholecystectomy were divided into two groups according to the presence or absence of microscopic cholesterol crystals. Seventy-three pa-

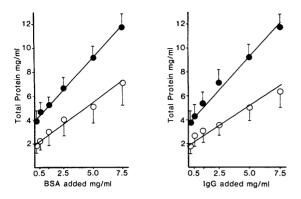


Figure 1. Mean \pm SD total protein concentration in gallbladder biles "spiked" with different concentrations of BSA and IgG as measured by the Lowry (\bullet) or fluorometric (\bigcirc) method (n = 5).

tients, 58 women and 15 men, had multiple cholesterol crystals of typical shape both in their native bile samples and in the sediment after ultracentrifugation. The cholesterol content of the stones in this group of patients varied from 36% to 99% (median, 70.4%). In the remaining 35 patients, 20 women and 15 men, no cholesterol crystals were detected. In this group stone analysis showed a cholesterol content between 0 and 76% (median, 9.6%). In 84 samples of patients with cholesterol stones (>50% cholesterol), crystals were found in 84% (70 patients). In all 18 samples of patients with pigment stones (<10% cholesterol), crystals were absent. In 3 of 6 bile samples from patients with mixed stones (10%–50% cholesterol), cholesterol crystals were found.

Analysis of Protein, Lipids, and pH in Bile

Figure 1 compares total protein concentrations determined in five native bile samples using the Lowry procedure and a fluorometric method as described above. The mean protein concentrations measured were 0.39 and 0.19 g/dL (3.9 and 1.9 g/L), respectively. A linear increase in total protein was obtained with both methods after addition of increasing amounts [0.05–0.75 g/L (0.5–7.5 g/L) bile] of BSA or human IgG. However, the fluorometric assay seemed

to underestimate the amounts of protein added to the bile samples, whereas more complete recovery was achieved with the Lowry method (Figure 1) used in the study.

Significantly higher concentrations of total protein were found in bile samples with crystals $[0.80 \pm 0.40]$ g/dL (8.0 ± 4.0 g/L)] than in samples without crystals $[0.63 \pm 0.26 \text{ g/dL} (6.3 \pm 2.6 \text{ g/L})] (P = 0.02) \text{ (Table 1)}.$ In samples containing cholesterol crystals, a trend to higher mean concentrations of cholesterol and to a higher cholesterol saturation index was observed (Table 1), while total lipid, bile acid, phospholipid concentrations, and pH values were not significantly different in the two groups of bile samples. Figure 2 shows the relationship between individual values of the total lipids and total protein in bile samples with and without crystals. Although a large overlap in bile samples with and without crystals was observed, high protein concentrations were found more frequently in samples containing crystals and only rarely in those without crystals. Indeed, of 22 samples with total protein concentrations > 1.0 g/dL (> 10.0 g/L), 20 contained cholesterol crystals and only two contained no crystals. No significant correlation between total lipids and total protein was found in either of the two groups. However, bile acids showed a highly significant correlation to phospholipid and cholesterol concentrations in samples both with and without cholesterol crystals (data not shown). Biliary pH was not different in the two groups of bile samples (Table 1), and no correlation between individual values of pH and bile acid concentrations was found (Figure 3).

Discussion

Our study demonstrates that total protein concentration is significantly higher in bile with cholesterol crystals than in bile without crystals. This finding is in accordance with earlier observations by Gallinger et al. (15) and Yu and Guan-Zang (16), who found significantly higher protein concentrations in bile samples from patients with cholesterol gallstones than in samples from patients with pigment stones.

Table 1. Concentrations of Total Protein, Bile Acids, Phospholipids, Cholesterol, and Total Lipids, Cholesterol Saturation Index, and pH in 73 Gallbladder Bile Samples With and 35 Samples Without Cholesterol Crystals

	Samples with cholesterol crystals	Samples without cholesterol crystals
Protein; $g/dL(g/L)$	$0.80 \pm 0.40 (8.0 \pm 4.0)$	$0.63 \pm 0.26 (6.3 \pm 2.6)^a$
Bile acids; g/dL ($mmol/L$)	$4.02 \pm 2.21 (82.1 \pm 45.1)$	$4.49 \pm 2.50 (91.5 \pm 50.9)$
Phospholipids; g/dL ($mmol/L$)	$2.33 \pm 1.24 (30.1 \pm 16.0)$	$2.41 \pm 1.65 (31.1 \pm 21.4)$
Cholesterol; g/dL ($mmol/L$)	$0.46 \pm 0.22 (12.0 \pm 5.8)$	$0.39 \pm 0.20 (10.1 \pm 5.3)$
Total lipids; g/dL (g/L)	$6.8 \pm 3.2 (68 \pm 32)$	$7.4 \pm 4.1 (74 \pm 41)$
Cholesterol saturation index	1.6 ± 0.7	1.4 ± 0.8
pH	7.71 ± 0.43	7.60 ± 0.44

 $^{^{}a}P = 0.02$ compared with samples with crystals.

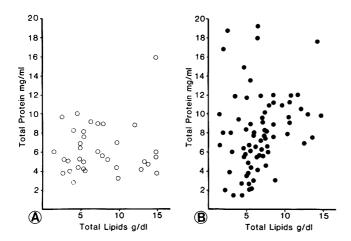


Figure 2. Relation of total protein to total lipid concentration in (A) 35 bile samples without (r = 0.13) and (B) 73 samples with cholesterol crystals (r = 0.19).

Contrary to these findings, Yamazaki et al. (26) did not observe differences in the concentrations of total biliary proteins between patients with cholesterol or pigment gallstones and subjects without gallstones, but a type II error due to the relatively small number of stone-free and pigment bile samples cannot be excluded. In our study, there was a considerable overlap between the total protein concentrations in gallbladder bile with crystals from patients with cholesterol stones and in bile without cholesterol crystals from patients with pigment or mixed stones. In accordance with the observations of Yamazaki et al. (26), patients with cholesterol gallstones could not be separated from those with pigment gallstones on the basis of the total protein concentration of gallbladder bile. However, in 22 bile samples with protein concentrations > 1.0 g/dL (> 10.0 g/L), cholesterol crystals were detected in all but 2 samples. The close association of high biliary protein [>1.0 g/dL (>10.0 g/L)] and cholesterol crystals suggests that high total

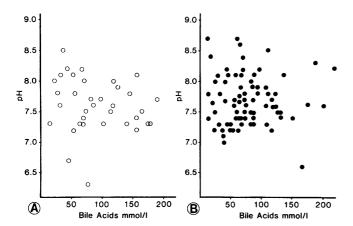


Figure 3. Relation of pH to total bile acid concentration in (A) 35 bile samples without (r = -0.15) and (B) 73 samples with cholesterol crystals (r = -0.02).

protein concentration may be an important factor in the formation of cholesterol crystals. Our findings are supported by a recent study of Strasberg et al. (27) in which high protein concentrations were found to be associated with reduced metastability of bile in an early stage of cholesterol gallstone formation.

The fluorometrically measured protein concentrations in bile in the study of Gallinger et al. (15) were only one third of the protein concentrations measured in our study using the Lowry assay. A similar difference between the two methods was shown in a more recent study by Harvey et al. (24). A carefully performed evaluation of several quantitative techniques of protein determination was performed by Yamazaki et al. (26) in bile samples from subjects with and without gallstones. The authors also observed lower biliary protein concentrations with the fluorometric method than with the Lowry procedure. However, despite large quantitative differences an excellent correlation of the two methods was obtained. The authors concluded that if bile is adequately pretreated (e.g., by dialysis or TCA precipitation and delipidation), use of either method will yield comparable results. We compared both protein determinations in bile using native bile samples that were "spiked" with BSA or human IgG in several physiological concentrations. As shown in Figure 1, the added amounts of BSA and IgG were correctly measured by the Lowry procedure and underestimated by the fluorometric assay. It is conceivable that the absorption caused by biliary pigment explains partially the higher values obtained with the Lowry procedure. On the other hand, because it appears that recovery of added protein is better at lower concentrations than at higher concentrations in the fluorometric assay (Figure 1), it is also conceivable that not enough fluorescamine reagent is being added in the assay at higher protein concentrations and therefore recovery is lower because of insufficient reagent. This would be particularly true if the samples were not sufficiently diluted before the addition of the fluorescamine reagent. In our study the final dilution of the bile samples before the fluorometric readings was similar to the dilutions recommended by Harvey et al. (24).

The data presented in this study allow only speculation about the origin of the elevated protein concentration in crystal-containing bile samples. No significant correlation between total lipid and total biliary protein concentrations was found in our study. Therefore, it is unlikely that the high biliary protein levels are merely caused by concentration of bile. In fact, even in very dilute bile samples with total lipid concentrations as low as 1–5 g/dL (10–50 g/L), high biliary protein concentrations were measured (Figure 2). Elevated protein levels in bile may reflect increased amounts of biliary mucus. A role for biliary

mucus in gallstone formation has been demonstrated in a number of studies (11,12,28–30). Mucus is a complex mixture of glycoproteins, water, electrolytes, lipids, serum proteins, and immunoglobulins with variable protein contents (15%–30%) (31). Harvey et al. (32) described maximal elevations of mucin glycoproteins to 0.62 ± 0.41 g/dL (6.2 ± 4.1 g/L) in four samples from patients with complete obstruction of the cystic duct. Values below 0.1 g/dL (1 g/L) were measured in bile samples from patients with functioning gallbladders. These data suggest that the maximal increase in biliary protein concentrations [> 1.0 g/dL (> 10 g/L)] observed in our study cannot be explained by alterations in biliary glycoproteins alone.

Mucosal inflammation of the gallbladder is common in cholesterol gallstone disease. In the intestinal mucosa the cytotoxic activity of activated inflammatory cells causes an increase in mucosal permeability with a subsequent leakage of plasma proteins into the intestinal lumen (33). Similarly, inflammatory changes of the gallbladder mucosa may allow an increased flux of plasma proteins into the bile. The high biliary protein concentrations observed in our study may therefore reflect mucosal inflammation of the gallbladder wall, and it is conceivable that high protein concentrations in bile are only one aspect of inflammation-induced changes in bile that together favor cholesterol crystal formation.

Biliary pH in our study was not significantly different in bile with and without crystals (Table 1). Our values are higher than those reported by Magnuson et al. (34), who found different mean pH in gallbladder bile samples from patients with cholesterol stones (pH 7.41), in samples from patients with pigment stones (pH 7.36), and in control samples (pH 7.27). Because bile samples were maintained in closed syringes before analysis, a loss of CO₂ to the atmosphere cannot explain the more alkaline pH in our study. The question of whether defective acidification (17) was responsible for our findings could not be tested in this study because a control group without stones was not available for comparison.

In conclusion, our results show that besides cholesterol supersaturation, high total biliary protein concentration may facilitate the formation of cholesterol crystals and the subsequent development of cholesterol stones in bile. Further studies are needed to elucidate the origin of the increased protein concentrations in bile and to define the mechanisms by which protein-rich bile favors cholesterol crystal formation.

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