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ORIGINAL ARTICLE

The physical presence of gallstone modulates *ex vivo* cholesterol crystallization pathways of human bile

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Abstract

Background: Cholesterol crystallization is an essential step toward gallstone formation. Although model bile studies showed that competition occurs between the gallstone surface and the surrounding aqueous phase for cholesterol molecules available for crystallization, this has not been investigated in human bile.
Methods: Fresh gallbladder bile was obtained during laparoscopic cholecystectomy from 13 patients with cholesterol ($n = 10$) or pigment ($n = 3$) stones. Small cholesterol gallstones were collected from another two patients. Both native and ultrafiltered bile with or without added gallstones was analysed by polarized light microscopy for the presence of arc-like and needle-like anhydrous cholesterol crystals and classic cholesterol monohydrate crystals. Weight of the added stones was evaluated before and after 21 days of bile incubation.
Results: In unfiltered bile, the presence of stones was associated with a trend towards less anhydrous cholesterol crystals, but significantly more aggregated cholesterol monohydrate crystals. In ultrafiltered bile, the presence of stones tended to inhibit the formation of arc-like or needle-like crystals and was associated with significantly greater amounts of both plate-like and aggregated cholesterol monohydrate crystals. After 21 days of the incubation, stone weight was decreased in both unfiltered ($-4.5 \pm 1.6\%$, $P = 0.046$) and ultrafiltered bile ($-6.5 \pm 1.5\%$, $P = 0.002$). Bile from pigment-stone patients was clear in the absence of stones, but showed early appearance of plate-like and aggregated cholesterol monohydrate crystals in all samples to which cholesterol gallstones were added.
Conclusions: The physical presence of cholesterol gallstones in both native and filtered bile greatly influences cholesterol crystallization pathways. Whereas cholesterol monohydrate crystals increase, anhydrous cholesterol crystals tend to be inhibited. Detachment of solid cholesterol crystals from the gallstone surface may explain these findings.

Key words: Bile; cholesterol crystals; cholesterol stones; pigment stones; polarizing light microscopy; supersaturated bile

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Introduction

Cholesterol gallstones are solid conglomerates of different sizes, which are made of solid cholesterol crystals, mucin, calcium bilirubinate and proteins, as well as grow mainly in the gallbladder. In industrialized countries, cholesterol gallstones account for about 75% of the stones, black pigment stones for 20% and brown pigment stones for 5% [1–3]. The formation and growth of cholesterol gallstones are deemed as a failure of biliary cholesterol homeostasis and five primary defects play a critical role in the pathogenesis of cholesterol gallstones: (i) *LITH* genes and genetic factors; (ii) hepatic hypersecretion of cholesterol governing supersaturated gallbladder bile; (iii) rapid phase transitions of cholesterol in bile with precipitation of solid cholesterol crystals; (iv) gallbladder stasis harvesting hypersecretion and accumulation of mucin gel in the lumen along with immune-mediated gallbladder inflammation; and (v) intestinal factors involving cholesterol absorption, slow intestinal motility and altered gut microbiota [4, 5]. The sterol molecule is poorly soluble in an aqueous environment, and is solubilized in mixed bile salt micelles in bile. Phosphatidylcholine is the predominant (>95% of total) phospholipid in bile. In case of cholesterol supersaturation, the excess sterol may be solubilized in vesicles that consist mainly of phospholipids or precipitated as solid cholesterol crystals [6]. The studies of Wang and Carey [7] revealed the importance of the relative amounts of bile salts vs phospholipids in the bile system for crystallization behavior (Figure 1). The physical presence of gallstones, however, might affect the cholesterol crystallization process. An *in vitro* model for stone growth and cholesterol crystallization into the surrounding aqueous phase in supersaturated model bile has been studied, suggesting that there is competition between the gallstone surface and the surrounding aqueous phase for cholesterol molecules available for crystallization [8, 9].

An adequate comprehension of mechanisms underlying cholesterol crystallization pathways in bile and, in turn, the formation and growth of cholesterol gallstones are still mandatory to identify effective primary prevention strategies in subjects at risk of developing gallstones [5]. The still available medical therapy by oral litholysis with the hydrophilic tertiary bile acid ursodeoxycholic acid (UDCA) and some recent surgical approaches by interventional radiology-operated cholecystoscopy with stone removal [10–12], endoscopic-laparoscopic cholecystolithotomy [13] and minimally invasive cholecystolithotomy [14] keep the gallbladder *in situ* and open novel perspectives in terms of secondary prevention. The pathochemistry studies of biliary cholesterol crystallization and the effects of the physical presence of stone(s) are therefore of great interest, in this respect.

Thus, this study investigated the effect of the physical presence of cholesterol gallstones on several biliary cholesterol crystallization pathways in different *ex vivo* gallbladder biles from patients harvesting cholesterol or pigment stones.

Patients and methods

Patients

Bile collected from 13 patients (5 males and 8 females) at age of 57 ± 2 years with symptomatic gallstones scheduled for elective laparoscopic cholecystectomy were included in the study. The ratio between solitary and multiple stones was 7:6. Pre-operative ultrasonography had shown stones in the gallbladder with normal liver and bile ducts in all cases. All patients had a thin-walled gallbladder (i.e. less than 3 mm in the fasting state).

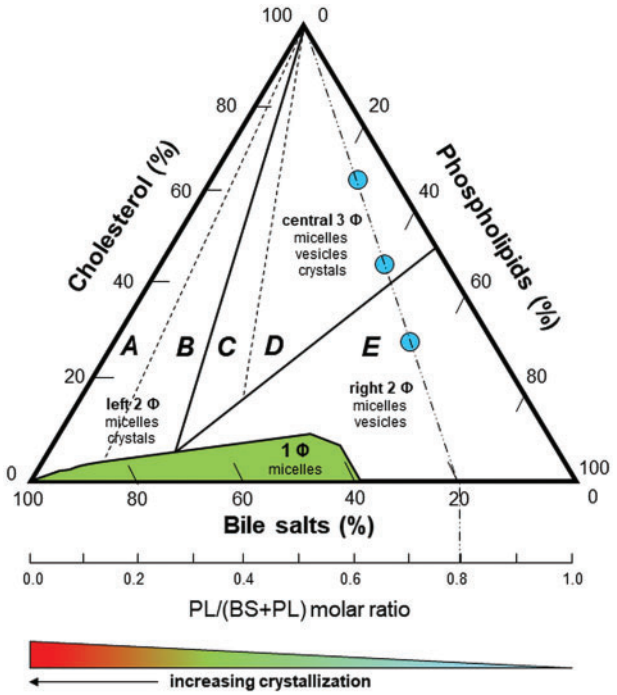

Figure 1. The ternary equilibrium cholesterol-taurocholate-phosphatidylcholine phase diagram. Adapted from Wang and Carey [7]. Components are expressed in mole percent. Depicted are a one-phase (φ) (micellar) zone at the bottom, a left two-phase zone (containing micelles and solid cholesterol crystals), a central three-phase zone (containing micelles, liquid crystals and solid cholesterol crystals) and a right two-phase zone (containing micelles and liquid crystals). At the bottom, phospholipids/(bile salts + phospholipids) molar ratios are also given, which is abbreviated as PL/(BS + PL). Interrupted lines indicate identical PL/(BS + PL) molar ratios, as in the case of the three-model bile systems plotting on the line (in this case ratio of 0.2).

All patients had normal blood count, kidney and liver function tests at entry (data not shown) and were not on medications known to affect biliary lipid composition. Patients with acute cholecystitis, pancreatitis, bile duct or cystic duct obstruction were excluded. Also excluded were patients with diabetes, hemolytic diseases, vagotomy or gastrointestinal surgery, liver cirrhosis or chronic hepatitis. After surgery, an experienced pathologist examined all the removed gallbladders, and the presence of gallbladder cancer was excluded in all patients. The studies were approved by the Human Subjects Committees at the University of Bari Medical School (Bari, Italy), as per the ethical guidelines of the 1975 Declaration of Helsinki. Written informed consent was obtained from all participants before entry.

Chemical analysis of bile

After cholecystectomy, fresh gallbladder bile was aspirated using a sterile syringe, and particular care was taken to avoid the effect of stratification [15]. Histological examination of the removed gallbladders revealed no or at most mild chronic inflammation. Two milliliters of bile were immediately extracted according to the methods of Bligh and Dyer [16] and stored at –20°C until further lipid analysis. The remaining bile was processed for cholesterol crystallization studies (see below). Commercially available kits employing colorimetric assays were used for enzymatic measurement of cholesterol in bile

and gallstones [17] and phospholipids in bile [18]. Bile salt concentrations were determined enzymatically with the 3α-hydroxysteroid dehydrogenase method [19]. The cholesterol saturation index (CSI) was calculated according to Carey's critical tables [20].

Cholesterol crystallization studies

After cholecystectomy, aliquots of fresh native gallbladder bile were immediately examined by polarizing light microscopy for the presence of solid cholesterol crystals, and then were ultrafiltered to obtain crystal-free bile [21, 22]. Samples of native and ultrafiltered bile without and with the added gallstones were subsequently incubated at 37°C, and gently shaken and checked every day for first appearance and growth of solid cholesterol crystals for 21 days [7, 22]. The time interval of 21 days was selected based on previous largely validated experimental protocols [1, 22, 23]. A semi-quantitative score was measured for each crystal form during the 21-day microscopic observation in a standard field of 100× with and without polarizing light [22]. The score consisted of a four-point scale: 0 = no crystals, 1 = 1 crystal, 2 = 2–4 crystals, 3 = 5–9 crystals, 4 = 10 or more crystals. At the end of the observation time (Day 21), indices of crystallization were: maximum score, number of crystals (counted in KOVA Glasstic™ slide, Hycor Biomedical Inc., Garden Grove, CA), size of crystals (min. and max.) and area under the crystallization curve [24].

Gallstone analyses

Cholesterol concentrations of gallstones from 13 patients were determined chemically after crushing stones thoroughly [17]. From two other patients, virtually identical round-shaped, yellowish twin small cholesterol gallstones were collected (0.4–0.8 mm in diameter; weight 15.2–91.0 mg). All twin stones had a cholesterol content >80% and the surrounding native bile was enriched with typical birefringent, plate-like cholesterol monohydrate crystals [22]. These gallstones were washed in demineralized water, dried at 37°C overnight and weighted on an analytical balance with 0.1-mg accuracy. Afterwards, stones were kept in sterile plastic tubes at –20°C in the freezer in a sealed container protected from humidity. Upon collection of the above-mentioned gallbladder bile samples, two equivalent stones were isolated, dried and added to the test tube containing unfiltered or ultrafiltered bile. The weight of the stones before and after preservation in the freezer was found to be basically the same. To avoid a possible effect of different stone weight on biliary cholesterol crystallization, the final volume of bile in the control test tube or to be added to each stone was normalized according to the following formula: added bile (mL) = 10 × stone weight (grams). The weight of the dried stones was measured before and at the end of the experiment (i.e. at Day 21).

Statistics

Results are expressed as mean ± standard error (SE) or as median and range. Differences were evaluated by ANOVA followed by Fisher's LSD multiple comparison test or by Student's t-test for unpaired data, as appropriate. The non-parametric Mann-Withney U test was used to compare medians of crystal observation time. Life-table analysis and Kaplan–Meier curves was used to compare the incidence of cholesterol crystallization in bile [25]. Differences between subgroups were analysed for statistical significance by the log-rank test. Statistical analyses were carried out using the NCSS software package [26]. A two-tailed P-value of less than 0.05 was considered statistically significant.

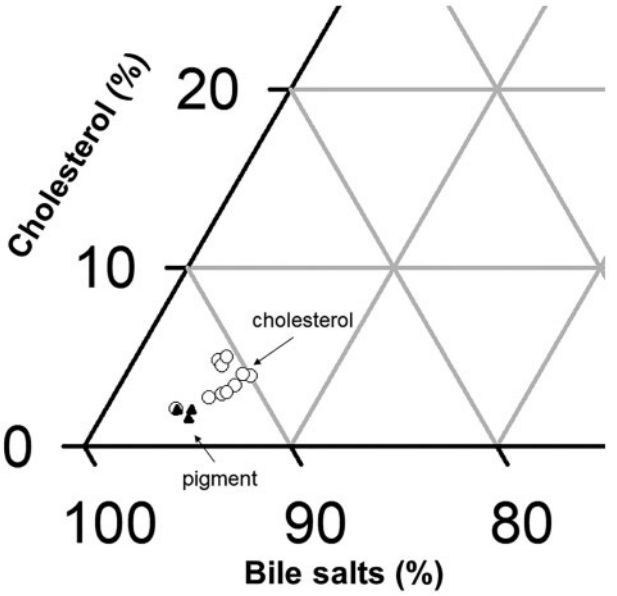

Figure 2. The magnification of the left bottom corner of the ternary equilibrium phase diagram shows the position of the lipid composition of bile of pigment-stone patients ($n = 3$) and cholesterol stone patients ($n = 10$) plot. Distinct positions are visible for bile collected from pigment-stone patients (i.e. meaning a cholesterol saturation index <1 and no propensity to grow solid cholesterol crystals), compared to bile collected from cholesterol stone patients.

Results

Ten patients had cholesterol gallstones with a mean cholesterol concentration of 82% (range 51–100%), whereas the other three patients had pigment stones with a mean cholesterol concentration of 3% (range 0–15%). CSIs of bile were significantly higher in cholesterol stone patients than those in pigment-stone patients (136.2 ± 10.1% vs 84.3 ± 8.6%, $P = 0.01$). The compositions of the bile of 13 patients in terms of percent cholesterol, phospholipids and bile salts were plotted in the phase diagram, which showed distinct positions for cholesterol stone patients (higher percent cholesterol, lower bile acids) and pigment-stone patients (lower percent cholesterol) (Figure 2).

Bile from cholesterol stone patients

Unfiltered bile

Indices of cholesterol crystallization for plate-like and aggregated cholesterol monohydrate crystals are given in Table 1. Several indices of crystallization of both plates and aggregates tended to be greater in bile with than without the added gallstones and, for aggregates, both numbers and sizes on Day 21 were significantly higher in bile with the added stones than without the added stones. The study of the daily semi-quantitative score of cholesterol crystallization showed that both plate-like and aggregated cholesterol crystals appear earlier and at greater score with the added stones (Figure 3A and B). In contrast, arc-like and needle-like crystals and tubules scores were lower in the presence of the added stones and this resulted in a significantly smaller area under the curve (AUC) for arcs-needles (36.2 ± 3.5 vs 55.9 ± 3.0 in bile with and without the

Table 1. Indices of plate-like and aggregated cholesterol crystallization with or without the added cholesterol gallstones in fresh gallbladder bile collected from 10 cholesterol gallstone patients

	Unfiltered biles			Ultrafiltered biles		
	Without stones	With stones	*P*-value	Without stones	With stones	*P*-value
Plate-like crystals						
Bile with solid cholesterol crystals (%)	100	100	NS	100	100	NS
Crystal observation time (days)	0.2 ± 0.1	0.2 ± 0.1	NS	4.9 ± 1.7	1.0 ± 0.1	0.012
Max crystallization score	3.9 ± 0.1	4.0 ± 0.01	NS	3.1 ± 0.4	3.9 ± 0.1	NS
Cumulative crystallization score	79.8 ± 4.4	85.9 ± 1.0	NS	46.6 ± 7.8	76.1 ± 2.4	0.006
Number of crystals/μL at Day 21	1181 ± 383	2218 ± 1052	NS	293 ± 85	1064 ± 528	0.012
Crystal size min (μm)	35.0 ± 6.5	47.5 ± 10.0	NS	35.0 ± 9.6	37.5 ± 8.8	NS
Crystal size max (μm)	173.0 ± 37.2	215.0 ± 16.7	NS	153.0 ± 46.8	180.0 ± 20.0	NS
Aggregated crystals						
Biles with crystals (%)	100	100	NS	70	100	NS
Crystal observation time (days)	1.6 ± 0.9	0.5 ± 0.3	NS	4.5 ± 0.8	2.2 ± 0.5	0.030
Max crystallization score	3.6 ± 0.2	4.0 ± 0.1	NS	2.0 ± 0.5	3.8 ± 0.1	0.012
Cumulative crystallization score	64.5 ± 6.3	74.4 ± 4.0	NS	22.1 ± 6.6	63.1 ± 3.7	0.0002
Number of crystals/μL at Day 21	102.0 ± 34.7	881.0 ± 703.5	0.019	29.0 ± 8.9	149.0 ± 58.7	0.011
Crystal size min (μm)	77.0 ± 20.9	115.0 ± 23.6	0.028	80.0 ± 18.8	101.0 ± 18.6	NS
Crystal size max (μm)	253.0 ± 56.1	350.0 ± 30.7	NS	288.3 ± 59.7	345.0 ± 45.0	NS

Data are expressed as mean ± standard deviation. NS, not significant.

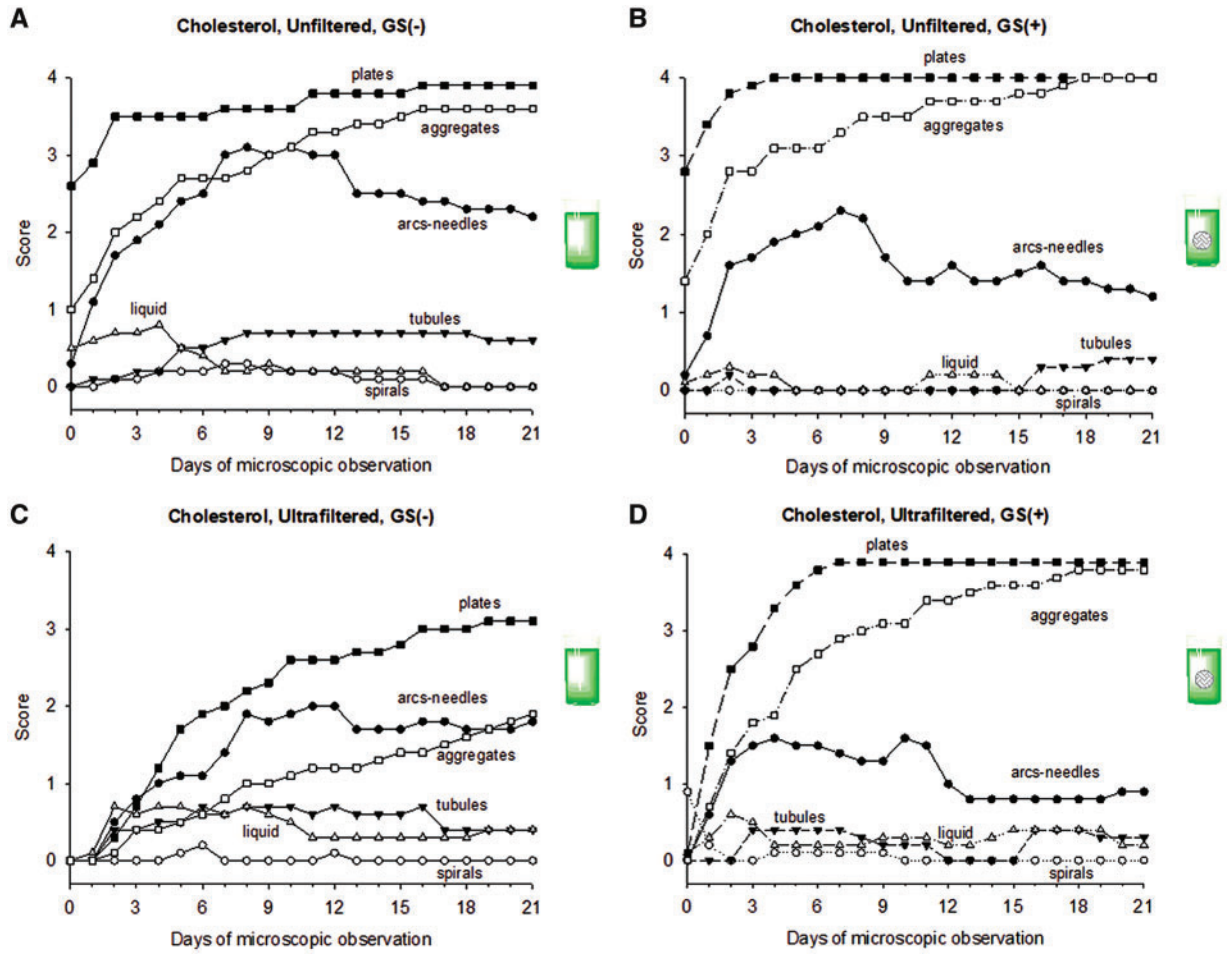

Figure 3. Daily observation of the score of six crystal forms in bile collected from cholesterol stone patients. Unfiltered bile: (**A**) bile without addition of cholesterol gallstones (GS–); (**B**) bile with addition of cholesterol gallstones (GS+). Ultrafiltered bile: (**C**) bile without addition of cholesterol gallstones (GS–); (**D**) bile with addition of cholesterol gallstones (GS+). The semi-quantitative score is plotted against the 21 days of observation by polarizing light microscopy. Invariably, the presence of gallstones in bile greatly modulates the crystallization pathway, enhancing the formation of classical plate-like and aggregated cholesterol monohydrate crystals, while inhibiting the formation of arc-like needle-like anhydrous cholesterol crystals.

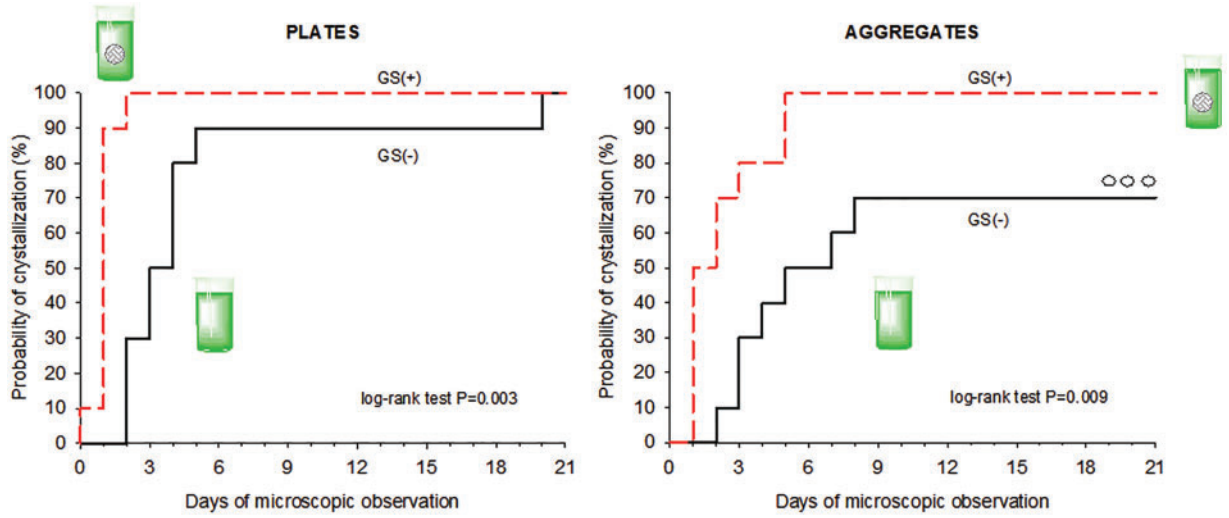

Figure 4. Kaplan–Meier curves of the cumulative percentage of the formation of plate-like (left panel) or aggregated (right panel) cholesterol monohydrate crystals in ultrafiltered bile with (GS+) or without the added cholesterol gallstones (GS–). Open circles indicate bile not growing crystals after 21 days. Cumulative probability of cholesterol crystallization for both plate-like and aggregated cholesterol monohydrate crystals is significantly greater in ultrafiltered bile with addition of gallstones compared to that without addition of gallstones (log-rank test).

added stones, respectively; $P = 0.03$). The addition of gallstones did not influence the appearance of the liquid crystals.

Ultrafiltered bile

Indices of cholesterol crystallization for plate-like and aggregated cholesterol monohydrate crystals are listed in Table 1. When cholesterol gallstones were added, there were significantly shorter crystal observation times, as well as greater cumulative scores and more solid cholesterol crystals after 21 days of incubation. All other indices of crystallization of both plates and aggregates tended to be greater in bile with the added stones compared to that without the added stones. The daily score of cholesterol crystals showed that plates and aggregates appeared earlier and at greater scores when gallstones were present (Figure 3C and D). Again, arc-like and needle-like anhydrous cholesterol crystals showed a trend towards smaller AUC (43.1 ± 9.2 vs 32.9 ± 10.1 in bile without and with the added stones, respectively). The addition of stones did not influence the appearance of liquid crystals.

The probability of crystallization in ultrafiltered bile is shown in Figure 4. For both plate-like and aggregated cholesterol monohydrate crystals, the probability of the observed solid cholesterol crystals was significantly greater in bile with the added gallstones. Median crystal observation times were 1 day and 4 days in bile with and without the added gallstones, respectively (log-rank test $P = 0.0032$). For aggregates, median crystal observation times were 2 and 7 days in bile with and without the added gallstones, respectively (log-rank test $P = 0.009$). There was no difference in liquid crystal formation between bile with or without the added stones. In addition, when indices of crystallization (i.e. max. and cumulative score during 21 days, number of crystals on Day 21 and minimum–maximal size of crystal on Day 21) of arcs-needles, spirals, tubules and liquid crystals were compared, there was no significant difference in ultrafiltered samples with and without the added gallstones, possibly due to the scant number of bile crystallizing with non-plate-like forms (data not shown).

The analysis of several indices of cholesterol crystallization in both native and ultrafiltered bile revealed that plate-like cholesterol crystals and their aggregates occurred in greater amount ($P < 0.04$) in bile from patients with multiple gallstones than those with solitary gallstone. Also, the smallest size of aggregates in ultrafiltered bile without the added stones was larger in bile from patients with multiple gallstones than those with solitary gallstone (125.0 ± 43.3 vs 45.0 ± 7.2 μm, $P = 0.04$). All these differences, however, became smaller in bile with the added stones, due to their intense crystallization propensity, compared with bile without the added stones.

Sequences of cholesterol crystallization

Bile from cholesterol stone patients

Unfiltered native bile without the added gallstones showed early appearance of plate-like cholesterol monohydrate crystals (0–1 day), promptly followed by their aggregates within 1–2 days. Arc-like and needle-like anhydrous cholesterol crystals, by contrast, were virtually absent in 80% of bile, but appeared—mainly persistently—in 90% of cases thereafter. The most frequent pattern was plate-like and aggregated cholesterol monohydrate crystals followed by arc-like and needle-like crystals within 1–6 days. Tubules and/or spirals were rarely found and invariably associated with arcs-needles. In ultrafiltered bile, virtually all the sequences were possible with plate-like cholesterol crystals appearing first. Spirals and/or tubules appeared invariably in those filtered bile that exhibit arcs-needles. As a result, paired studies between native and ultrafiltered bile showed exactly comparable qualitative sequences in only two biles. The addition of gallstones to native bile, in general, shortened the observation time of aggregates and delayed or frankly inhibited the appearance of arc-like and needle-like crystals. Also, a shift towards simpler sequences (i.e. mainly plate-like cholesterol crystals followed by their aggregates, and then by arc-like and needle-like crystals) was noticed. Both spirals and tubules were invariably absent. The addition of gallstones to ultrafiltered bile shifted 80% of the bile towards the two simplest patterns, i.e. plates followed by aggregates and, eventually, arcs-needles.

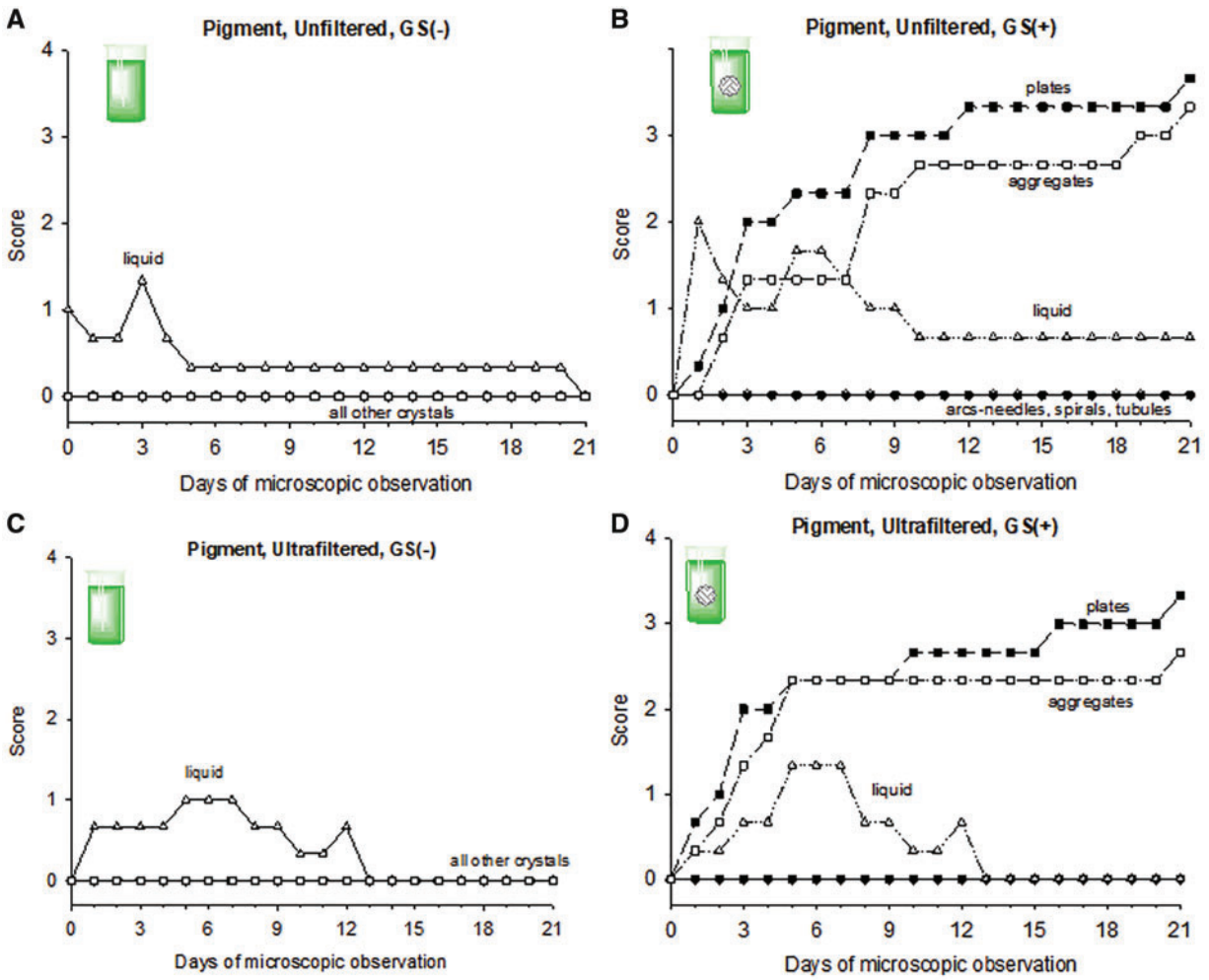

Figure 5. Daily observation of the score of six crystal forms in bile collected from pigment-stone patients. (**A**) Unfiltered bile without cholesterol gallstones (GS–); (**B**) unfiltered bile with addition of cholesterol gallstones (GS+); (**C**) ultrafiltered bile without cholesterol gallstones; (**D**) ultrafiltered bile with addition of cholesterol gallstones. The semi-quantitative score is plotted against the 21 days of observation by polarizing light microscopy. The addition of gallstones was associated with a dramatic increase in the daily crystallization score of plate-like and aggregated cholesterol monohydrate crystals, but not arc-like and needle-like anhydrous cholesterol crystals, in both native and ultrafiltered bile. No significant difference in liquid crystal appearance time is found between bile with or without the addition of cholesterol gallstones.

Bile from pigment-stone patients

In both native and ultrafiltered bile, solid cholesterol crystals were absent throughout the study period. In contrast, all the bile with the added cholesterol gallstones showed cholesterol crystals during the study period. The addition of gallstones was associated with a dramatic increase in daily crystallization scores for plate-like and aggregated cholesterol monohydrate crystals in both native and ultrafiltered bile (Figure 5). However, no arc-like or needle-like anhydrous cholesterol crystals were found. There was no significant difference also in liquid crystal appearance time between bile with or without the added gallstones.

Gallstone weight

In general, a similar decrement in stone weight was found in both native and ultrafiltered bile (1.7 ± 0.9 vs 2.1 ± 0.5 mg, respectively, $P > 0.05$). There was a highly positive correlation between stone weight on Days 0 and 21 in both native and ultrafiltered bile. The percent decrease in stone weight after the 21-day incubation was $4.5 \pm 1.6\%$ in native bile ($P = 0.046$ vs basal) and $6.5 \pm 1.5\%$ in ultrafiltered bile ($P = 0.002$ vs basal) (Figure 6). The decrease in stone weight was associated with morphological changes on the stone surface (Figure 7).

Discussion

The current studies focus on the effect of the physical presence of cholesterol gallstones on crystal dynamics in bile collected from cholesterol and pigment-stone patients. We found that the cholesterol crystallization sequence was shifted to simpler patterns (i.e. plates and aggregates), especially evident in ultrafiltered bile from cholesterol stone patients.

As the precipitation of solid cholesterol crystals from supersaturated bile is a prerequisite for the formation of cholesterol gallstones [1, 21], accurate quantitation of crystallized cholesterol becomes essential when investigating gallstone pathogenesis [22]. Several pieces of evidence point to cholesterol crystallization pathways leading to gallstone formation and growth as a result of complex interplays between systemic and local factors [5], but the direct effects generated by the physical presence of gallstones in the gallbladder lumen are still poorly

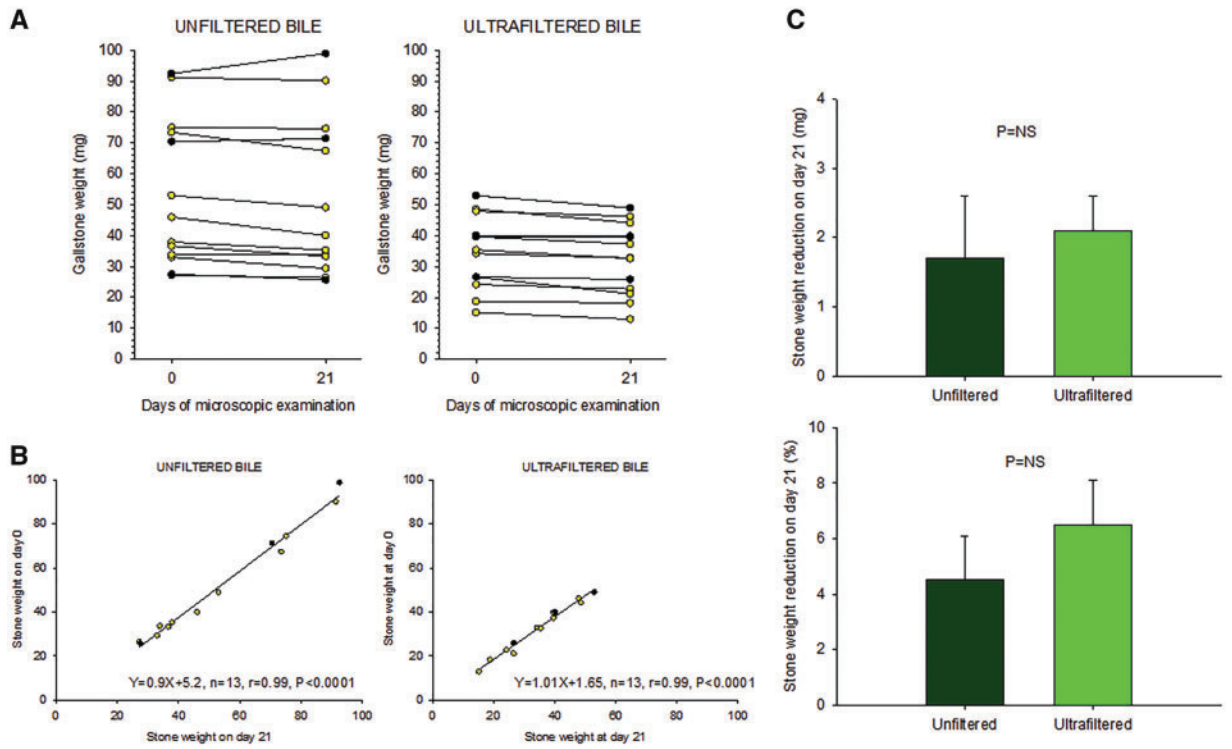

Figure 6. Paired studies assessing the weight of small cholesterol gallstones before (Day 0) and at Day 21 after being added to native and ultrafiltered human bile. Each paired point represents one gallstone. Cholesterol stones are depicted as white dots; pigment stones appear as black dots. (**A**) A significant decrease in absolute stone weight is observed in both native and ultrafiltered bile. (**B**) There is a correlation between stone weight on Day 0 and on Day 21 in both native and ultrafiltered bile. (**C**) Percent and absolute stone weight reduction tends to be grater in ultrafiltered bile than that in native bile.

explored. Besides pathogenic implications, this aspect is of clinical interest in terms of secondary prevention of gallstones. Although surgical gallbladder removal is still the gold-standard therapy in symptomatic gallstone patients (i.e. developing biliary pain or complications [2, 27]), alternative therapeutic options maintaining gallbladder *in situ* are still conceivable using oral litholysis with UDCA in symptomatic patients with small (≤5 mm in diameter), X-ray radiolucent cholesterol gallstones in a functioning gallbladder with a patent cystic duct [27], interventional radiology-operated cholecystoscopy with stone removal in the presence of multiple comorbidities precluding cholecystectomy [10–12], endoscopic-laparoscopic cholecystolithotomy or minimally invasive cholecystolithotomy in children [13, 14].

Outcomes deriving from these gallbladder-preserving therapeutic approaches are still under investigation and available evidence shows a variable gallstone recurrence rate, which seems high after oral dissolution (30–43% of patients within 3–5 years [28, 29]) and low but dependent on coexisting risk factors after surgical stone removal and gallbladder preservation [11, 13, 30]. Besides the persistence of systemic factors increasing the risk of gallstone recurrence [5], some outcomes might partly derive by the surgical removal of gallstones from the gallbladder lumen. Studies on cholesterol crystallization in either model or human bile have never focused on the potential effects of the presence of gallstone per se.

In a preliminary study, Van den Berg *et al.* [9] added human cholesterol gallstones into supersaturated model bile and found that the degree of supersaturation of cholesterol in the model bile enhanced stone growth and such a growth was inversely related to the rate of cholesterol crystallization in the aqueous phase. These findings suggest competition between gallstone surface and the surrounding aqueous phase for cholesterol molecules available for cholesterol crystallization [31].

We found that bile from pigment-stone patients could form solid cholesterol crystals if cholesterol gallstones were added. There also was a significant loss (6–7%) of stone weight during the 21-day incubation period, ruling out that a significant amount of material is adsorbed on the stone surface, as was the case in another study with model bile [32]. All these findings suggest that cholesterol crystals detach progressively from the gallstone surface.

Alternatively, the gallstone per se may enhance the effect of pro-nucleating agents in bile. Excess amounts of mucin and other soluble proteins are often found in the bile of cholesterol stone patients and may play a role in promoting cholesterol crystallization [33–37]. Also, there may be a direct delivery of pro-nucleating agents from the gallstone surface or interior. Substances of different molecular weight may diffuse in and out of cholesterol gallstones [38]. Moreover, virtually all cholesterol gallstones have a matrix of mucus glycoproteins [39] and/or calcium salts [40], which can enhance the crystallization process [41]. Matrix is frequently found in pigment gallstones [42] and might contain pro-nucleating agents also in the bile of pigment-stone patients. However, this effect is not found in bile with pure pigment stones, which do not display any cholesterol crystallization in unsaturated or supersaturated bile (unpublished observations).

Patients with multiple cholesterol gallstones usually display a greater propensity to cholesterol crystallization [22, 43–45]. This was also the case in the present study, although a small number of samples prevented a broader analysis.

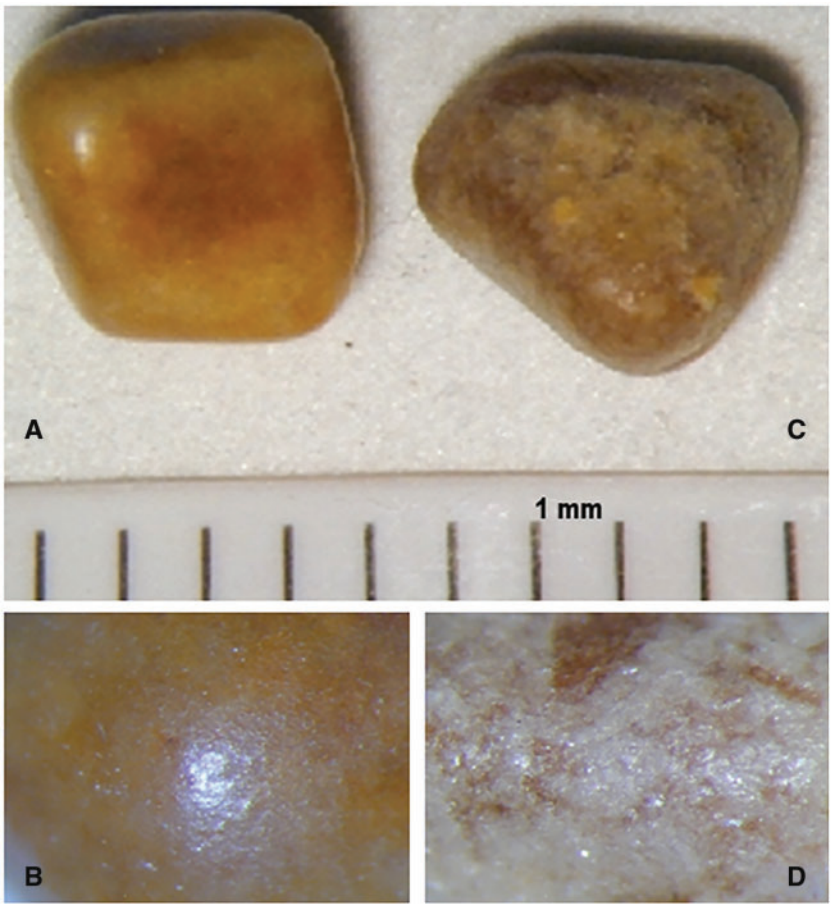

Figure 7. Effect of 21-day incubation of a small cholesterol gallstone in human gallbladder bile. (**A**) A 4-mm pure cholesterol stone from a pool of similar stones of the same patient is taken as control (no incubation in bile); (**B**) the stone surface looks regular and smooth, as confirmed at higher magnifications; (**C**) a similar 'twin' gallstone after 21 days of incubation in ultrafiltered bile; (**D**) at higher magnifications, the crystals are visible on stone surface. As a consequence, stone weight decreased from 47 mg before (Day 0) to 39 mg at Day 21 after bile incubation. Photos were taken with a Leitz Wild Heerbrugg intravital microscope (magnifications 10× and 40×, respectively).

Gallstone addition caused a delayed effect on the formation and growth of arc-like and needle-like anhydrous cholesterol crystals. In a previous study investigating the behavior of cholesterol crystals in human bile, we found that arc-like and needle-like crystals, as well as spiral and tubular crystals, are rarely found in fresh unfiltered bile collected from cholesterol stone patients [22]. This is confirmed by an analysis of cholesterol crystallization pathways predicted in the ternary phase diagram [5, 7, 46]. We showed that the gallstone itself may have an inhibitory effect on the formation of anhydrous cholesterol crystals.

We found a decrease in the weight of cholesterol stones upon bile incubation. While studying gallstone growth in supersaturated model bile, van den Berg *et al.* [32] revealed, for most gallstones, a slight decrease in the dry weight of stones after the maximal value was reached. Nevertheless, our findings are partly at variance with previous reports, in which stone growth *in vitro* was inversely related to biliary crystallization [9, 32]. There are, however, several differences between the experimental conditions in the above-mentioned studies and the present study: we used human bile, rather than model bile, and a stone weight-dependent amount of bile, rather than a fixed amount. Furthermore, methodologies used to assess cholesterol crystallization might also account for different findings.

We previously found that, in supersaturated model bile with or without added cholesterol gallstones, stone growth increased in the case of relatively low amounts of phospholipids in the model bile system, but decreased in the case of relatively high amounts of phospholipids, related to excess vesicular storage capacity [8]. Apparently, in *ex vivo* incubated human bile collected from cholesterol stone patients, as shown in the current study, the latter conditions are present, with a decrease in gallstone weight. Nevertheless, *in vivo* gallbladder stones generally increase in size with time. Events *in vivo* and *ex vivo* could greatly differ due to the presence of a number of additional factors such as the continuous flow of bile from the liver into the gallbladder, the contractile properties of the gallbladder both during the fasting and the postprandial state, the absorptive function of the gallbladder epithelial cells and the possibility that biliary cholesterol saturation changes over time and during the day [47], also in cholesterol stone patients [48]. The present study strengthens the view that the presence of solid cholesterol crystals even in unsaturated bile is highly suggestive of cholesterol gallstone disease, since these cholesterol crystals occurred also in the bile from pigment-stone patients to which a cholesterol gallstone was added.

A limitation of the present study is the apparently small number of enrolled patients and, in particular, of patients with pigment stones. The number of patients is the consequence of

patients' prospective enrollment for elective laparoscopic cholecystectomy when no 'a priori' selection is made. Thus, the expected prevalence of pigment gallstones in the Western world is about 20% of all gallstones, while the remaining gallstones are made of either pure or mixed cholesterol [1, 5, 27, 49, 50]. Despite this limitation, however, differences between bile samples from the two subgroups of patients are statistically sound and results in terms of cholesterol crystallization are evident in bile samples from both cholesterol- and pigment-stone patients. Results from the latter group will be confirmed by further studies.

In conclusion, the present study shows that the physical presence of cholesterol gallstones in both native and filtered bile modifies dynamic cholesterol crystallization processes. Whereas the number of cholesterol monohydrate crystals increases, the formation of arc-like and needle-like anhydrous cholesterol crystals tends to be inhibited. The dramatic increase in classical plate-like and aggregated cholesterol monohydrate crystals in bile could partly depend on the progressive detachment of fragments from the gallstone surface into the surrounding bile. From a clinical point of view, these findings add new elements in terms of pathogenic mechanisms potentially useful in primary and secondary prevention in subjects at risk.

Finally, different features of stone growth *in vivo* or *ex vivo* underscore the relevance of extra-gallbladder factors in the pathogenesis of cholesterol gallstone disease, which could be interpreted as a local expression of the systemic unbalance of cholesterol metabolism.

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