Leading article

Cholesterol crystallisation in bile

Cholesterol gallstones occur when at least three simultaneous defects are present: (i) cholesterol supersaturation of bile with formation of cholesterol-rich unstable vesicles; (ii) accelerated crystallisation of cholesterol in bile owing to a defect of crystallisation inhibiting or an abundance of crystallisation promoting factors; and (iii) prolonged bile stasis due to decreased gall bladder motility.[1]

Precipitation of solid cholesterol crystals from supersaturated bile has an essential role in cholesterol gallstone formation.[2 3] The number of days it takes before microscopic plate-like cholesterol monohydrate crystals are observed in human bile has been referred to as the crystal nucleation time.[2] This term, however, indicates early aggregation of cholesterol molecules from supersaturated bile into submicroscopic nuclei.[4] This crucial step is followed by precipitation, growth and agglomeration of cholesterol crystals, which then become visible at light microscopy. In this respect, the term crystal detection time seems more appropriate than crystal nucleation time.[5] Recent data indicate that solid cholesterol can also precipitate as crystal forms other than plate-like structures,[6–8] suggesting that crystallisation in bile is a rather complex phenomenon.

The study of pathways leading to cholesterol crystallisation in bile has clinical relevance as the appearance of biliary crystals reliably distinguishes between patients with and without cholesterol gallstones.[2 9] Crystallisation of cholesterol is faster in patients with multiple than in those with solitary stones,[8 10–12] a finding which might be linked to the increased risk of gallstone recurrence after non-surgical treatment in patients with more than one stone.[13–16] Moreover, a better understanding of the physical/chemical events occurring during crystallisation in bile would provide a useful method for the identification of factors delaying or preventing precipitation of cholesterol crystals and, therefore, gallstone formation in humans.

This article will discuss current views on the formation of cholesterol crystals in bile.

Cholesterol secretion in bile

The elucidation of the exact events leading to lipid secretion in bile is still being investigated. In an elegant ultrastructural study, Crawford et al[17] have proved that biliary phospholipid molecules are secreted by hepatocytes into the bile canalicular lumina as unilamellar vesicles 63–67 nm in diameter. This process occurs rapidly and seems to be facilitated by the detergent action of luminal bile salts at the level of the exoplasmic part of the canalicular membrane. Cholesterol is also secreted into hepatic bile and is taken up by biliary lipid vesicles in which the cholesterol:phospholipid ratio is normally 0.34–0.38. In lithogenic bile, however, this ratio is larger.[18–21] As mixed micelle formation requires the solubilisation of more phospholipid than cholesterol, the cholesterol:phospholipid ratio in the remaining vesicles progressively increases to >1 (about 2:1).[22] These cholesterol-rich vesicles tend to aggregate or to fuse into multilamellar vesicles of increasing size. As emphasised by Cohen et al,[23] cholesterol solubilisation and transport in bile is explained by the classic paradigm of the simple micelle-mixed micelle-unilamellar vesicle, with multilamellar unstable vesicles heralding the initiation of the lithogenic process. Classic triclinic cholesterol crystals may precipitate from multilamellar vesicles, as observed by video enhanced time lapse microscopy.[24] The process of mixed micelle formation starts in the biliary tree but is strongly promoted in the gall bladder[11] as the gall bladder absorbs electrolytes actively and water passively. The concentration of biliary lipids then increases and high bile salt concentrations promote mixed micelle formation with growth of cholesterol-rich vesicles. Thus, vesicular cholesterol is important for cholesterol crystal formation.[25] Recent studies in cholesterol fed prairie dogs, however, have suggested that supersaturated mixed micelles may also have a role in cholesterol crystal precipitation.[26]

Biliary proteins and cholesterol crystallisation

Bile from patients with cholesterol stones cannot be discriminated from those of control patients simply on the basis of lipid composition.[2 27 28] Thus, other factors, such as biliary proteins, have been suggested to play a part in the pathogenesis of cholesterol crystallisation and stone formation.[29] Although several proteins with cholesterol crystallisation inhibiting as well as promoting properties have been isolated, the active factor has not been identified as yet. Apolipoprotein A-1 and A-2[30] and a 120 kDa glycoprotein isolated from normal human gall bladder bile[31] inhibit cholesterol crystallisation.

Mucin and non-mucin glycoproteins might have a role as promoting agents of cholesterol crystallisation. In fact, gall bladder mucin promotes crystallisation of cholesterol[18 32 33] and mucin hypersecretion precedes cholesterol crystal precipitation and stone formation both in animals[34 35] and humans (such as in obese people with weight reduction and gallstone formation).[36–38] Mucin provides a microenvironment and might act as a nidus for cholesterol crystal aggregation. In this way sludge is formed, a viscous gel of mucin and entrapped cholesterol crystals or calcium bilirubinate precipitates.[39 40] Sludge may disappear spontaneously[41] but progressive cholesterol crystal precipitation and subsequent aggregation into macroscopic gallstones may also occur.[33] Groen et al[12] isolated from T-tube bile of patients with cholesterol gallstones a concanavalin-A binding non-mucin glycoprotein (molecular weight 130 kDa) that stimulates cholesterol crystallisation in model bile. As it is found in hepatic bile, this factor is likely to be of hepatic origin. Other non-mucin factors have crystallisation promoting effects in vitro: a pronase resistant low density lipoprotein particle (±90% lipid),[42] aminopeptidase N (a 130 kDa biliary canalicular glycoprotein),[43 44] phospholipase C,[45] immunoglobulins of the IgM, IgA and IgG classes,[46–48] fibronectin,[49] a biliary anionic polypeptide fraction (APF),[50] acute phase proteins, such as a 42 kDa α_1-acid glycoprotein,[51–54] α_1-antichymotrypsin,[55] and haptoglobin.[56] Although the liver secretes promoting and inhibiting factors simultaneously, the ultimate pathophysiological importance of these proteins in human bile remains controversial.[51] There is also

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evidence for the existence of pronase resistant pronucleating proteins as protein hydrolysis does not influence rapid[42 57] crystallisation in human bile.[55 58]

Cholesterol crystallisation in bile

Precipitation of crystalline cholesterol in bile is likely to occur when the cholesterol concentration exeeds the micellar solubility limit and fusion or aggregation of cholesterol-rich vesicles occurs.[24 59] The "classic" crystallisation pathway was believed to result in the appearance of typical monohydrate cholesterol plates in both human[2 60] and model bile.[60 61] Although early studies showed that hypovitaminotic C guinea-pigs fed a high cholesterol diet developed randomly arranged, needle shaped (possibly anhydrous cholesterol) crystals in the gall bladder[62] and that needle shaped crystals existed both in model and human bile,[63–65] the possibility that crystal forms other than plate-like structures could develop in bile was only recently tackled. In an important study Konikoff *et al*[7] described filamentous crystals that appeared within two to four hours of dilution of model bile and within one to three days of ex vivo incubation of human gall bladder bile (preliminary report: in six of eight patients with cholesterol gallstones but in none of those with pigmented stones). Over a few days filaments evolved through needle, helical, and tubular structures to end as typical plate-like cholesterol monohydrate crystals. These findings were confirmed in a subsequent study from a taurocholate-rich dilute bile salt-rich model bile.[6] In this system cholesterol low density (1.030 g/ml) filamentous crystals reached a maximal concentration at 24 hours and disappeared gradually after 156 hours of incubation, whereas high density (1.045 g/ml) plate-like crystals increased reciprocally. The authors used several analytical procedures which pointed to the mainly anhydrous nature of early cholesterol filaments,[6] with subsequent hydration as water becomes incorporated within the crystals. In an initial report[66] two non-perturbing techniques (that is, cryotransmission electron microscopy and video enhanced light microscopy) were used for direct imaging of cholesterol crystallisation. This study confirmed the dynamic evolution of lipid microstructure which is similar in model and human bile. According to a recent hypothesis,[5] the two putative pathways of cholesterol nucleation in bile might involve (i) precipitation of classic plate-like cholesterol crystals from multilamellar vesicles[24] and (ii) nucleation of anhydrous cholesterol occurring internally as a liquid core within unilamellar vesicles (that is, "internal nucleation").[5 57]

Studies on the different habits of cholesterol crystals have been extended in model bile.[4 67] Our group has studied this in model[68 69] and human bile.[8 70 71] We believe that the complete process of cholesterol crystallisation in bile is still poorly understood and that the pathophysiological importance of the pathway through cholesterol microcrystalline intermediates needs to be carefully investigated. In this respect, a major contribution comes from the recent work of Wang and Carey[4] who used complementary physical–chemical techniques, and have now carefully defined five different crystallisation pathways which are based on the main forms of liquid and solid cholesterol crystals in pathophysiologically relevant model bile. As shown by the analysis of crystallisation sequences as functions of time and increasing lecithin content within the phase diagram for the mixed bile salt-egg yolk lecithin-cholesterol system, at equilibrium five regions were identified above the one-phase micellar zone and named *A* to *E* (moving from left to right across the phase diagram). Crystal observation times for plate-like crystals were progressively retarded between pathways *A* and *D*, whereas plates never appeared in pathway *E*. Studies from the same group

suggest that the events in model biles correlate with crystallisation pathways in humans[72] and in inbred mice on a lithogenic diet.[35] Accordingly, it is anticipated that factors in bile such as total lipid concentration, phospholipids and bile salt hydrophobicity can modulate crystal growth by shifting the relative composition of bile to different regions of the phase diagram, as discussed in the following paragraphs.

The finding that cholesterol crystallisation is longer in dilute supersaturated gall bladder[11] and model[73] bile would be explained by a shift delution of the crystallisation pathway towards the *E* region (liquid crystals) of the phase diagram. The opposite would apply to concentrated bile.[4]

Phospholipid molecular species and class can influence cholesterol crystallisation in model and human bile.[74–76] Saturated lecithins delay whereas polyunsaturated phospolipids accelerate the crystal observation time in model bile.[7 74] Although the surface layer of phosphatidylcholine, which covers the surface of cholesterol filamentous crystals, might interfere directly with crystal growth,[7] a valid alternative explanation is that modulation of phospholipid species can shift phase boundaries towards pathways where the plate-like crystal observation time is either shorter (a leftward shift) or longer (a rightward shift) within the phase diagram.[4] Possible strategies for cholesterol crystallisation and gallstone prevention might theoretically imply increased biliary secretion of phospholipids[75] as dietary factors can influence phospholipid content.[76] Further studies are warranted on this topic.

Several studies have focused on the relation between cholesterol crystal shape in model bile and the hydrophobic–hydrophilic balance of bile salts, and have concluded that crystallisation of several forms is increased in the presence of more hydrophobic bile salts.[4 21 67–69] At least seven different crystal forms have been identified, including helical, thread-like and ribbon-like structures, the growth of which decreased as bile salt hydrophobicity decreased (that is, deoxycholate > chenodeoxycholate > cholate > taurocholate > ursodeoxycholic acid (UDCA)).[68 77] Van Erpecum *et al*[69] found that the most striking crystallisation of all cholesterol crystal forms, including large plate aggregates, was seen in the very hydrophobic system in taurodeoxycholate-rich model bile and that bile salt hydrophobicity influenced both crystal shape and extent of crystallisation. Similar studies with an accurate description of cholesterol crystal shapes have been carried out by Juste *et al*[67] and by Wang and Carey[4] in supersaturated model biles with a number of bile salts at different hydrophobicities. It is possible that hydrophobic bile salts which exhibit lower critical micellar concentrations induce rapid formation of mixed micelles with increased formation of cholesterol-rich vesicles and rapid crystal precipitation.[21] A better explanation is provided by the study of the equilibrium phase diagram[4]: crystallisation pathways shift with decreasing bile salt hydrophobicity and the right two-phase area (that is, region *E*, where only liquid cholesterol crystals occur) becomes progressively larger. This mechanism would explain why crystallisation of all forms is greatly inhibited or prevented by very hydrophilic bile salt species such as UDCA, tauroursodeoxycholate, taurohyodeoxycholate, etc.[4 67–69]

Recently, we studied cholesterol crystal forms in both ultrafiltered (isotropic) bile and native bile of patients with cholesterol or pigment gallstones.[8] Daily polarising microscopy was used to quantify crystal observation time and to construct crystal-time growing curves of the different forms of solid crystals such as arcs, needles, spirals, tubules, plates, and their aggregates over three weeks. Figure 1 shows most of these crystal forms. Pigment stone biles never crystallised; cholesterol gallstone bile, in
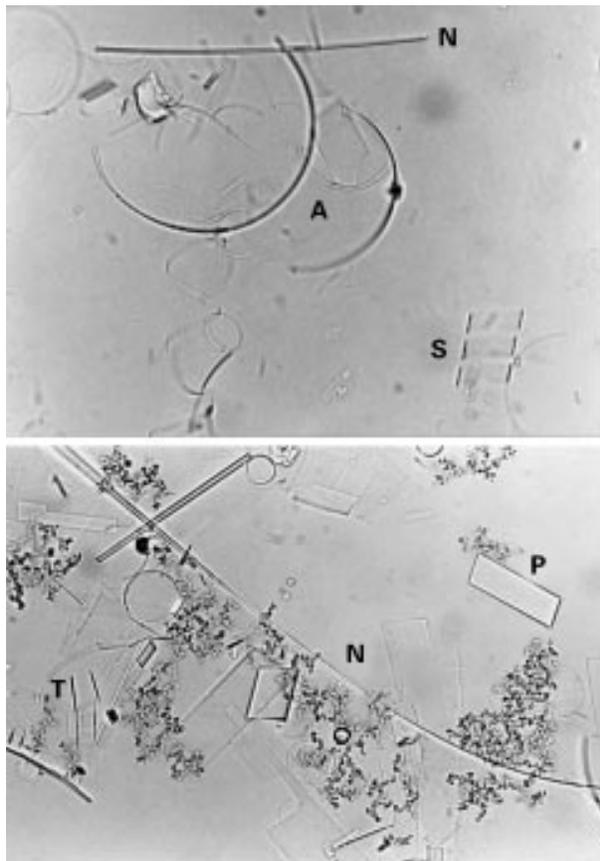
Figure 1: Light microscopy of ultrafiltered bile from a patient with cholesterol gallstones showing classic plate-like monohydrate cholesterol crystals (P) and other cholesterol crystal shapes such as needles (N), arcs (A), spirals (S), and tubules (T). Original magnification ×100.

contrast, had a rich assortment of crystals forms. Apparently, arcs and needles act as substrates for the development of progressively thicker forms giving rise to young and mature (stable) plates. This evolution suggests that slow hydration of filamentous anhydrous crystals occurs with time.[6] It has been suggested that arcs/needles are transient forms.[6 7] Indeed, arcs and needles are seen infrequently in freshly sampled bile of patients with cholesterol stones.[8] However, we also found that in vitro these filamentous forms of cholesterol increased progressively to a maximum and persisted throughout the observation time.[8] Whether this behaviour is relevant in vivo during gallstone formation is still unclear. Both tubules and spirals were transient forms usually seen within the first week of observation and with a tendency to decrease and/or to convert into mature plates. The presence of tubules or spirals was associated with a high concentration of the hydrophobic deoxycholate and rapid growth of other crystal forms. As anticipated by a number of studies,[10–12] all crystal forms had a high rate/extent of crystallisation in patients with multiple cholesterol stones, compared with those with solitary stones. Overall, the two most important factors independently associated with fast crystallisation of plates were the number of gallstones and the presence of tubules/spirals in ultrafiltered bile.

We found that treatment with UDCA (which results in bile enriched with glycine and taurine conjugates of UDCA) led to some interesting effects on crystallisation of solid cholesterol. UDCA, in fact, totally prevented the precipitation of all crystal forms in isotropic bile of patients with gallstones treated with UDCA[8] and was associated—in native bile—with the appearance of rather thickened atypical cholesterol crystals with multilobate

edges which, in some cases, tended to decrease in size and even disappeared after prolonged observation.[8 71] This has been described previously in in vitro dissolution studies in UDCA containing systems.[78] As anticipated[4] from in vitro studies the effect of UDCA on crystallisation might be due to the expansion of the liquid crystal region of the equilibrium phase diagram by reducing bile salt hydrophobicity. We also found recently[71] that treatment with UDCA leads to a decrease in the concentrations of various crystallisation promoting proteins (IgG, IgA, haptoglobin, and α_1-acid glycoprotein, aminopeptidase N, and total proteins) and reduces the crystallisation promoting activity of concanavalin A eluate in patients with cholesterol gallstones. This was confirmed by two independent techniques including a nephelometric assay[79] and microscopy.[8]

Conclusions

Rapid crystallisation of cholesterol in bile is as a key factor in cholesterol gallstone formation. The original observation that solid cholesterol precipitates only as characteristic plates of monohydrate cholesterol has been recently revised as filamentous, helical and tubular forms of non-hydrated cholesterol can grow together with plates and aggregates of plates in both isotropic and native bile. Several factors such as biliary proteins and lipids might act as potential cofactors of cholesterol crystallisation. The most active crystallisation of cholesterol in bile is found in patients with multiple cholesterol gallstones. Gall bladder bile enriched with the more hydrophilic bile salts, such as glycine and taurine conjugates of UDCA, inhibits cholesterol crystallisation and decreases the concentrations of crystallisation promoting biliary proteins. Exciting studies are awaited to clarify whether prevention of cholesterol gallstone formation in at-risk subjects can be effectively achieved by manipulating biliary factors which have pronucleating or antinucleating effects.

P PORTINCASA
K J VAN ERPECUM
G P VANBERGE-HENEGOUWEN
Department of Gastroenterology, University Hospital Utrecht, The Netherlands

Correspondence to: Professor G P vanBerge-Henegouwen, Department of Gastroenterology, University Hospital Utrecht, PO Box 85500, Utrecht, The Netherlands.

1 Carey MC. Formation of cholesterol gallstones: the new paradigm. In: Paumgartner G, Stiehl A, Gerok W, eds. *Trends in bile acid research.* Dordrecht: Kluwer Academic Publishers, 1989: 259–81.
2 Holan KR, Holzbach RT, Hermann RE, Cooperman AM, Claffey WJ. Nucleation time: a key factor in the pathogenesis of cholesterol gallstone disease. *Gastroenterology* 1979; **77:** 611–7.
3 Sedaghat A, Grundy SM. Cholesterol crystals and the formation of cholesterol stones. *N Engl J Med* 1980; **302:** 1274–7.
4 Wang QH, Carey MC. Complete mapping of crystallization pathways during cholesterol precipitation from model bile: influence of physical-chemical variables of pathophysiologic relevance and identification of a stable liquid crystalline state in cold, dilute and hydrophilic bile salt-containing system. *J Lipid Res* 1996; **37:** 606–30.
5 Carey MC. Formation and growth of cholesterol gallstones: the new synthesis. In: Fromm H, Leushner U, eds. *Bile acids—Cholestasis—Gallstones. Advances in basic and clinical bile acid research.* Dordrecht: Kluwer Academic Publishers, 1996: 147–75.
6 Konikoff FM, Carey MC. Cholesterol crystallization from a dilute bile salt-rich model bile. *J Crystal Growth* 1994; **144:** 79–86.
7 Konikoff FM, Chung DS, Donovan JM, Small DM, Carey MC. Filamentous, helical and tubular microstructures during cholesterol crystallization from bile. Evidence that biliary cholesterol does not nucleate classic monohydrate plates. *J Clin Invest* 1992; **90:** 1156–61.
8 Portincasa P, van Erpecum KJ, Jansen A, Renooij W, Gadellaa M, vanBerge-Henegouwen GP. Behavior of various cholesterol crystals in bile from gallstone patients. *Hepatology* 1996; **23:** 738–48.
9 van Erpecum KJ, vanBerge-Henegouwen GP, Stoelwinder B, Stolk MFJ, Eggink WF, Govaert WHA. Cholesterol and pigment gallstone disease: comparison of the reliability of three bile tests for differentiation between the two stone types. *Scand J Gastroenterol* 1988; **23:** 948–54.
10 Jüngst D, Lang T, von Ritter C, Pratschke E, Paumgartner G. Cholesterol nucleation time in gallbladder bile of patients with solitary or multiple cholesterol gallstones. *Hepatology* 1992; **15:** 804–8.
11 van Erpecum KJ, vanBerge-Henegouwen GP, Stoelwinder B, Schmidt YMG, Willekens FLH. Bile concentration is a key factor for nucleation of cholesterol crystals and cholesterol saturation index in gallbladder bile of gallstone patients. *Hepatology* 1990; **11:** 1–6.
12 Groen AK, Noordam C, Drapers JAG, Egbers P, Jansen PLM, Tytgat GNJ. Isolation of a potent cholesterol nucleation-promoting activity from human

gallbladder bile of patients with solitary or multiple cholesterol gallstones. *Hepatology* 1990; **11:** 525–33.

13 Villanova N, Bazzoli F, Taroni F, Frabboni R, Mazzella G, Festi D, *et al*. Gallstone recurrence after successful oral bile acid treatment: a 12 year follow-up study and evaluation of long term postdissolution treatment. *Gastroenterology* 1989; **97:** 726–31.

14 Petroni ML, Jazrawi RP, Lanzini A, Facchinetti D, Heaton KW, Northfield TC. Characteristics of recurrent gallstones following non-surgical treatment: implication for retreatment. *Eur J Gastroenterol Hepatol* 1991; **3:** 473–8.

15 Pelletier G, Capdeville R, Mosnier H, Raymond JM, Delmont J, Moreaux J, *et al*. Low early gallstone recurrence after successful extracorporeal lithotripsy in patients with solitary stones. *J Hepatol* 1992; **16:** 102–5.

16 Pelletier G, Raymond JM, Capdeville R, Mosnier H, Caroli-Bosc FX. Gallstone recurrence after successful lithotripsy. *J Hepatol* 1995; **23:** 420–3.

17 Crawford JM, Möckel GM, Crawford AR, Hagen SJ, Hatch VC, Barnes S, *et al*. Imaging biliary lipid secretion in the rat: ultrastructural evidence for vesiculation of the hepatocyte canalicular membrane. *J Lipid Res* 1995; **36:** 2147–63.

18 LaMont JT, Carey MC. Cholesterol gallstone formation. 2. Pathobiology and pathomechanics. *Prog Liver Dis* 1992; **10:** 165–91.

19 Schubert R, Beyer K, Wolburg H, Schmidt K. Structural changes in membranes of large unilamellar vesicles after binding of sodium cholate. *Biochemistry* 1986; **25:** 5263–9.

20 Schubert R, Schmidt K. Structural changes in vesicle membranes and mixed micelles of various lipid compositions after binding of different bile salts. *Biochemistry* 1988; **27:** 8787–94.

21 van de Heijning BJM, Stolk MFJ, van Erpecum KJ, Renooij W, Groen AK, vanBerge-Henegouwen GP. Bile salt-induced cholesterol crystal formation from model bile vesicles: a time-course study. *J Lipid Res* 1994; **35:** 1002–11.

22 Donovan JM, Carey MC. Separation and quantitation of cholesterol "carriers" in bile. *Hepatology* 1990; **12:** 94S–105S.

23 Cohen DE, Kaler EW, Carey MC. Cholesterol carriers in human bile: are "lamellae" involved? *Hepatology* 1993; **18:** 1522–31.

24 Halpern Z, Dudley MA, Kibe A, Lynn MP, Breuer AC, Holzbach RT. Rapid vesicle formation and aggregation in abnormal human biles: A time-lapse video-enhanced contrast microscopy study. *Gastroenterology* 1986; **90:** 875–85.

25 Harvey PRC, Sömjen GJ, Gilat T, Gallinger S, Strasberg SM. Vesicular cholesterol in bile. Relationship to protein concentration and nucleation time. *Biochim Biophys Acta* 1988; **958:** 10–18.

26 Ahrendt SA, Fox-Talbot MK, Kaufman HS, Lillemoe KD, Pitt HA. Cholesterol nucleates rapidly from mixed micelles in the prairie dog. *Biochim Biophys Acta* 1994; **1211:** 7–13.

27 Holzbach RT, Marsh M, Olszewski M, Holan K. Cholesterol solubility in bile. Evidence that supersaturated bile is frequent in healthy man. *J Clin Invest* 1973; **52:** 1467–79.

28 Carey MC, Small DM. The physical chemistry of cholesterol solubility in bile. Relation to gallstone formation and dissolution in man. *J Clin Invest* 1978; **61:** 998–1026.

29 Burnstein MJ, Ilson RG, Petrunka CN, Taylor RD, Strasberg SM. Evidence for a potent nucleating factor in the gall-bladder bile of patients with cholesterol gallstones. *Gastroenterology* 1983; **85:** 801–7.

30 Kibe A, Holzbach RT. Inhibition of cholesterol crystal formation by apolipoproteins in supersaturated model bile. *Science* 1984; **255:** 514–6.

31 Ohya T, Schwarzendrube J, Busch N, Gresky S, Chandler K, Takabayashi A, *et al*. Isolation of a human biliary glycoprotein inhibitor of cholesterol crystallization. *Gastroenterology* 1993; **104:** 527–38.

32 Lee SM, LaMont JT, Carey MC. Role of gallbladder mucus hypersecretion in the evolution of cholesterol gallstones. Studies in the prairie dog. *J Clin Invest* 1981; **67:** 1712–23.

33 Carey MC, Calahane MJ. Whither biliary sludge? *Gastroenterology* 1988; **95:** 508–23.

34 Pemsingh RS, MacPherson BR, Scott GW. Mucus hypersecretion in the gallbladder epithelium of ground squirrels fed a lithogenic diet for the induction of cholesterol gallstones. *Hepatology* 1987; **7:** 1267–71.

35 Wang DQH, Paigen B, Carey MC. Phenotipic characterization of *Lith* genes determining susceptibility to cholesterol (Ch) gallstone formation in inbred mice [abstract]. *Hepatology* 1995; **22:** 289A.

36 Shiffman ML, Shamburek RD, Schwartz CC, Sugerman HJ, Kellum JM, Moore EW. Gallbladder mucin, arachidonic acid, and bile lipids in patients who develop gallstones during weight reduction. *Gastroenterology* 1993; **105:** 1200–8.

37 Shiffman ML, Sugerman HJ, Kellum JM, Moore EW. Changes in gallbladder bile composition following gallstone formation and weight reduction. *Gastroenterology* 1992; **103:** 214–21.

38 Marks JW, Bonorris GG, Albers G, Schoenfield LJ. The sequence of biliary events preceding the formation of gallstones in humans. *Gastroenterology* 1992; **103:** 566–70.

39 Lee SP, Nicholls JF. Nature and composition of biliary sludge. *Gastroenterology* 1986; **90:** 677–86.

40 Allen B, Bernhoff N, Blankaert N. Sludge is calcium-bilirubinate associated with bile stasis. *Am J Surg* 1981; **141:** 51–6.

41 Maringhini A, Ciambra M, Baccelliere P, Raimondo M, Orlando A, Tine F, *et al*. Biliary sludge and gallstones in pregnancy: incidence, risk factors, and natural history. *Ann Intern Med* 1993; **119:** 116–20.

42 de Bruijn MAC, Mok KS, Nibbering CP, Out T, van Marle J, Stellaard F, *et al*. Characterization of the cholesterol crystallization-promoting low-density particle isolated from human bile. *Gastroenterology* 1996; **110:** 1936–44.

43 Offner GD, Gong D, Afdhal NH. Identification of a 130-kilodalton human biliary concanavalin A binding protein as aminopeptidase N. *Gastroenterology* 1994; **106:** 755–62.

44 Nuñez L, Amigo L, Mingrone G, Rigotti A, Puglielli A, Raddatz A, *et al*. Biliary aminopeptidase-N and the cholesterol crystallisation defect in cholelithiasis. *Gut* 1995; **37:** 422–6.

45 Pattinson NR, Willis KE. Effect of phospholipase C on cholesterol solubilization in model bile: A concanavalin A-binding nucleation-promoting factor from human gallbladder bile. *Gastroenterology* 1991; **101:** 1339–44.

46 Upadhya GA, Harvey PR, Strasberg SM. Effect of human biliary immunoglobulins on the nucleation of cholesterol. *J Biol Chem* 1993; **268:** 5193–200.

47 Harvey PRC, Upadhya GA, Strasberg SM. Immunoglobulins as nucleating proteins in the gallbladder bile of patients with cholesterol gallstones. *J Biol Chem* 1991; **266:** 13996–4003.

48 Lipsett PA, Hilddreth J, Kaufman HS, Lillemoe KD, Pitt HA. Human gallstones contain pronucleating nonmucin glycoproteins that are immunoglobulins. *Ann Surg* 1994; **219:** 25–33.

49 Chijiiwa K, Koga A, Yamasaki T, Shimada K, Noshiro H, Nakayama F. Fibronectin: A possible factor promoting cholesterol monohydrate crystallization in bile. *Biochim Biophys Acta* 1991; **1086:** 44–8.

50 Ostrow JD. APF/CBP, an anionic polypeptide in bile and gallstones that may regulate calcium salt and cholesterol precipitation from bile. *Hepatology* 1992; **16:** 1493–6.

51 Abei M, Schwarzendrube J, Nuutinen H, Broughan TA, Kawczak P, Williams C, *et al*. Cholesterol crystallization-promoters in human bile: comparative potencies of immunoglobulins, alpha-1-acid glycoprotein, phospholipase C, and aminopeptidase N1. *J Lipid Res* 1993; **34:** 1141–8.

52 Abei M, Kawczak P, Nuutinen H, Langnas A, Svanvik J, Holzbach RT. Isolation and characterization of a cholesterol crystallization promoter from human bile. *Gastroenterology* 1993; **104:** 539–48.

53 Afdhal NH, Offner GD, Murray FE, Troxler RF, Smith BF. Isolation and characterization of peptides from the protein core of bovine gallbladder mucin. *Gastroenterology* 1990; **98:** 1633–41.

54 Nuutinen H, Abei M, Schwarzendrube J, Ginanni Corradini S, Walsh RM, Kawczak P, *et al*. Biliary α1-acid glycoprotein concentration in gallstone-free controls and in patients with multiple or solitary cholesterol gallstones. *Dig Dis Sci* 1995; **40:** 1786–91.

55 Zijlstra AI, Offner GD, Afdhal NH, van Overveld M, Tytgat GNJ, Groen AK. The pronase resistance of cholesterol-nucleating glycoproteins in human bile. *Gastroenterology* 1996; **110:** 1926–35.

56 Yamashita G, Ginanni Corradini S, Secknus R, Takabayashi A, Williams C, Hays L, *et al*. Biliary haptoglobin, a potent promoter of cholesterol crystallization at physiological concentrations. *Gastroenterology* 1995; **36:** 1325–33.

57 Apstein MD, Carey MC. Pathogenesis of cholesterol gallstones: a parsimonious hypothesis. *Eur J Clin Invest* 1996; **26:** 343–52.

58 Pattinson NR, Willis KE. Nucleation of cholesterol crystals from native bile and the effect of protein hydrolysis. *J Lipid Res* 1991; **32:** 215–21.

59 Kibe A, Dudley MA, Halpern Z, Lynn MP, Breuer AC, Holzbach RT. Factors affecting cholesterol monohydrate crystal nucleation time in model systems of supersaturated bile. *J Lipid Res* 1985; **26:** 1102–11.

60 Holzbach RT, Kibe A, Thiel E, Howell JH, Marsh M, Hermann RE. Biliary proteins: unique inhibitors of cholesterol crystal nucleation in human gallbladder bile. *J Clin Invest* 1984; **73:** 35–45.

61 Whiting MJ, Watts JM. Cholesterol crystal formation and growth in model bile solutions. *J Lipid Res* 1983; **24:** 861–68.

62 Jenkins SA. Biliary lipids, bile acids and gallstone formation in hypovitaminotic C guinea-pigs. *Br J Nutr* 1978; **40:** 317–22.

63 Lichtenberg D, Ragimova S, Bor A, Almog S, Vinkler C, Peled Y, Halpern Z. Stability of mixed micellar bile models supersaturated with cholesterol. *Biophys J* 1988; **54:** 1013–25.

64 Toor EW, Evans DF, Cussler EL. Cholesterol monohydrate growth in model bile solutions. *Proc Natl Acad Sci USA* 1978; **75:** 6230–4.

65 Lichtenberg D, Ragimova S, Bor A, Almog S, Vinkler C, Peled Y, *et al*. Stability of mixed micellar systems made by solubilizing phosphatidylcholine-cholesterol vesicles by bile salts. *Hepatology* 1990; **12:** 149S–54S.

66 Konikoff FM, Kaplun A, Rubin M, Vilan A, Laufer H, Eitan A, *et al*. Direct visualization of supra-molecular lipid aggregates during cholesterol crystallization in bile [abstract]. *Gastroenterology* 1995; **108:** A1102.

67 Juste C, Catala I, Henry R, Chabanet C, Gueugneau AM, Béguet F, *et al*. Influence of bile salt molecular species on cholesterol crystallization from supersaturated model biles. *Biochim Biophys Acta* 1995; **1254:** 88–97.

68 Stolk MFJ, van de Heijning BJM, van Erpecum KJ, van de Broek AMWC, Renooij W, vanBerge-Henegouwen GP. The effect of bile salt hydrophobicity on nucleation of several types of cholesterol crystals from model bile vesicles. *J Hepatol* 1994; **20:** 802–10.

69 van Erpecum KJ, Portincasa P, Gadellaa M, van de Heijning BJM, Renooij W, vanBerge-Henegouwen GP. Effects of bile salt hydrophobicity on nucleation behaviour of cholesterol crystals in model bile. *Eur J Clin Invest* 1996; **26:** 602–8.

70 van Erpecum KJ, Portincasa P, vanBerge-Henegouwen GP, Groen AK. Pronucleating proteins in gallbladder bile: relation to stone type, number, speed of cholesterol crystallization and effect of ursodeoxycholic acid (UDCA) [abstract]. *Gastroenterology* 1996; **110:** A1351.

71 van Erpecum KJ, Portincasa P, Eckhardt E, Go PMNYH, vanBerge-Henegouwen GP, Groen AK. Ursodeoxycholic acid reduces protein levels and their nucleation-promoting activity in gallbladder bile. *Gastroenterology* 1996; **110:** 1225–37.

72 Wang QH, Carey MC. Cholesterol (CH) crystal (C) nucleation paths in human gallbladder bile are identical to model bile: increased secondary bile salts (BS) and CH saturation index (CSI) are partly responsible for accelerating CH nucleation [abstract]. *Gastroenterology* 1995; **108:** A1196.

73 van Erpecum KJ, Stolk MFJ, van den Broek AMWC, Renooij W, van de Heijning BJM, vanBerge-Henegouwen GP. Bile concentration promotes nucleation of cholesterol monohydrate crystals by increasing the cholesterol concentration in the vesicles. *Eur J Clin Invest* 1993; **23:** 283–8.

74 Konikoff FM, Cohen DE, Carey MC. Phospholipid molecular species influence crystal habits and transition sequences of metastable intermediates during cholesterol crystallization from bile salt-rich model bile. *J Lipid Res* 1994; **35:** 60–70.

75 Jüngst D, Lang T, Huber P, Lange V, Paumgartner G. Effect of phospholipids and bile acids on cholesterol nucleation time and vesicular/micellar cholesterol in gallbladder bile of patients with cholesterol stones. *J Lipid Res* 1993; **34:** 1457–64.

76 Nervi F, Covarrubias C, Bravo P, Velasco N, Ulloa N, Cruz F, *et al*. Influence of legume intake on biliary lipids and cholesterol saturation in young Chilean men. *Gastroenterology* 1989; **96:** 825–30.

77 Armstrong MJ, Carey MC. The hydrophobic-hydrophilic balance of bile salts. Inverse correlation between reverse-phase high performance liquid chromatographic mobilities and micellar cholesterol solubilizing capacities. *J Lipid Res* 1982; **23:** 70–80.

78 Salvioli G, Igimi H, Carey MC. Cholesterol gallstone dissolution in bile. Dissolution kinetics of crystalline cholesterol monohydrate by conjugated chenodeoxycholate-lecithin and conjugated ursodeoxycholate-lecithin mixtures: dissimilar phase equilibria and dissolution mechanisms. *J Lipid Res* 1983; **24:** 701–20.

79 Busch N, Tokumo H, Holzbach RT. A sensitive method for determination of cholesterol growth using model solutions of supersaturated bile. *J Lipid Res* 1990; **31:** 1903–9.