Biliary lipids, water and cholesterol gallstones

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Cholesterol supersaturation, hydrophobic bile salts, pronucleating proteins and impaired gall-bladder motility may contribute to gallstone pathogenesis. We here show that both gallstone-susceptible C57L and gallstone-resistant AKR male inbred mice exhibit supersaturated gall-bladder biles during early lithogenesis, whereas bile-salt composition becomes hydrophobic only in susceptible C57L mice. In vitro, cholesterol crystallization occurs depending on relative amounts of lipids; excess cholesterol may exceed solubilizing capacity of mixed bile salt–phospholipid micelles, whereas excess bile salts compared with phospholipids leads to deficient cholesterol-storage capacity in vesicles. In vivo, bile lipid contents are mainly determined at the level of the hepatocyte canalicular membrane, where specific transport proteins enable lipid secretion [ABCG5/G8 (ATP-binding cassette transporter G5/G8) for cholesterol, MDR3 (multi-drug resistant 3) for phospholipid, BSEP (bile salt export pump)]. These transport proteins are regulated by farnesoid X and liver X nuclear receptors. After nascent bile formation, modulation of bile water contents in biliary tract and gall-bladder exerts critical effects on cholesterol crystallization. During progressive bile concentration (particularly in the fasting gall-bladder), cholesterol and, preferentially, phospholipid transfer occurs from cholesterol-unsaturated vesicles to emerging mixed micelles. The remaining unstable cholesterol-enriched vesicles may nucleate crystals. Various aquaporins have recently been discovered throughout the biliary tract, with potential relevance for gallstone formation.

Introduction
The incidence of gallstones is relatively high in the Western world, with approx. 10% of the whole population being affected. In the U.S.A., 1 million new cases of gallstones are detected each year. Most gallstone carriers do not need any therapy, since they are asymptomatic. Nevertheless, gallstone disease remains a significant health and economic burden. Most patients have cholesterol gallstones. Nucleation of cholesterol crystals is considered the essential initial step in cholesterol gallstone formation. Also, in normal subjects there is significant gall-bladder emptying (approx. 70% after meal ingestion, and approx. 30% in the fasting state). Impaired gall-bladder emptying could contribute to gallstone formation by providing time for crystallization of cholesterol from supersaturated gall-bladder bile and their subsequent aggregation into macroscopic stones (van Erpecum and van Berge-Henegouwen, 1999). Many proteins are present in bile, e.g. IgG, IgA, IgM, haptoglobin, α-1 acid glycoprotein, aminopeptidase N and mucin. Although some of these proteins may promote cholesterol crystallization in models in vitro, their role in human gallstone formation in vivo has become increasingly controversial, with the possible exception of mucin (Miquel et al., 1998; van Erpecum et al., 2001). Rather, biliary cholesterol supersaturation and hydrophobic bile salts appear to be the critical factors for cholesterol crystal nucleation. In this review, we will therefore focus on biliary lipids (including regulation of their secretion by recently discovered specific transport proteins in the hepatocyte canalicular membrane and hepatocytic nuclear receptors) in relation to cholesterol crystallization. Bile water is another important determinant of supersaturation and cholesterol crystallization. Indeed,

| Biliary cholesterol supersaturation: This indicates that more cholesterol is present in bile than can be solubilized in bile salt–phospholipid micelles. The excess cholesterol can be kept in vesicles (spherical cholesterol–phospholipid bilayers) or nucleate as cholesterol crystals. |

Key words: bile, bile salt, cholesterol, mouse, nuclear receptor, phospholipid.
Abbreviations used: ABC transporter, ATP-binding cassette transporter; BSEP, bile salt export pump; MDR3, multidrug resistant 3; FXR, farnesoid X receptor; LXR, liver X receptor; SHP, short heterodimer partner.

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www.biolcell.org | Volume 97 (11) | Pages 815–822 815
various aquaporins have recently been detected in liver, bile ducts and gall-bladder that could affect biliary water content and consequently, cholesterol crystallization (Ferri et al., 2003; Masyuk et al., 2002; Portincasa et al., 2003). We therefore also discuss effects of water on cholesterol crystallization and gallstone formation.

Biliary lipids and gallstone formation

Although solubility of cholesterol in aqueous solutions is extremely limited, in gall-bladder bile a relatively large amount (\(\sim 20 \text{ mM}\)) of the sterol can be kept in solution. This significant increase in solubility is explained by incorporation of cholesterol in mixed micelles, together with bile salts and phospholipids (mainly phosphatidylcholine). Supersaturation occurs when either too much cholesterol or not enough solubilizing bile salt and phosphatidylcholine molecules are secreted to allow complete micellar solubilization of all cholesterol. Excessive cholesterol may be kept in vesicles (i.e. spherical bilayers of cholesterol and phospholipids, without bile salts), provided that enough phospholipid is available. When relatively low amounts of phospholipids are present, cholesterol crystal formation occurs in supersaturated bile, which is the beginning of gallstone formation. Primary bile salts are synthesized from cholesterol in the liver (i.e. cholate and chenodeoxycholate). Secondary bile salts (mainly deoxycholate) are formed from primary bile salts in the intestine by bacterial transformation. Cholesterol crystallization is promoted by hydrophobic bile salts (chenodeoxycholate, deoxycholate) and by phospholipids with unsaturated acyl chains. In human bile, there is considerable variation in hydrophobicity of bile salt composition (especially amounts of deoxycholate), but phosphatidycholine acyl chain composition is tightly regulated (mainly C_{16:0} acyl chains on SN-1 position, C_{18:2} > C_{18:1} > C_{20:4} acyl chains on SN-2 position). Therefore biliary bile salt, rather than phospholipid, composition may affect human gallstone formation with potential therapeutic implications. Indeed, human gallstone patients exhibit a more hydrophobic biliary bile salt composition than subjects without gallstones (Hussaini et al., 1995; Shoda et al., 1995). Nevertheless, one has to bear in mind that in patients with gallstones, stones are already present when bile is collected, with the potential for secondary effects; changes in bile composition could be the consequence rather than the cause of gallstone formation. This disadvantage does not apply to the inbred mouse model for gallstone formation. Due to the presence of Lith genes, male C57L inbred mice are highly susceptible, but male AKR inbred mice highly resistant, to cholesterol gallstone formation when fed a lithogenic diet (15% fat, 1% cholesterol, 0.5% cholic acid) (Khanuja et al., 1995; Wang et al., 1997). In the mouse model, potentially relevant factors can therefore easily be explored during the earliest time points of gallstone formation. In Figures 1 and 2, we show biliary cholesterol saturation and bile salt composition in gall-bladder bile of gallstone-susceptible C57L and gallstone-resistant AKR male mice at several time points on the lithogenic diet. At baseline, both strains demonstrate cholesterol unsaturated gall-bladder bile (i.e. cholesterol saturation

Figure 1 | Cholesterol saturation index values of gall-bladder biles of gallstone-susceptible male C57L
inbred mice and gallstone-resistant male AKR mice on a lithogenic diet (15% fat, 1% cholesterol, 0.5% cholic acid)
In both strains bile is unsaturated at baseline, and becomes supersaturated from day 7 on the lithogenic diet. Cholecystectomy was performed and fasting gall-bladder bile was aspirated completely at 09:00 hours (pooled biles of 15 mice of each strain at each time point to obtain sufficient quantities) (Wang et al., 1999). Biles were lipid extracted (Bligh and Dyer, 1959) after short centrifugation (5 min at 3000 \(g\)) to spin down cholesterol crystals. Cholesterol (Fromm et al., 1980) and phospholipid (Allain et al., 1974) concentrations were measured enzymatically in the extracted biles, and total bile salt concentrations in whole bile samples by the 3\(\alpha\)-hydroxysteroid dehydrogenase method (Turley and Dietschy, 1978). Cholesterol saturation index was calculated according to Carey (1978). ■, Gallstone-susceptible male C57L mice; ●, gallstone-resistant male AKR mice.
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Figure 2 | Biliary bile-salt compositions as a function of time while on a lithogenic diet (15% fat, 1% cholesterol, 0.5% cholic acid) in gallstone-susceptible C57L male mice (A) and gallstone-resistant AKR male mice (B)

Hydrophilic tauro-β-muricholate and taurocholate are the predominant bile salts when mice are fed laboratory chow. Because the lithogenic diet contains 0.5% cholic acid, taurocholate replaces tauro-β-muricholate over the first few days in both strains. In gallstone-susceptible C57L mice, percentages of the hydrophobic bile salts, taurochenodeoxycholate and taurodeoxycholate rise markedly after starting the lithogenic diet. In contrast, in gallstone-resistant AKR mice, percentages of taurochenodeoxycholate and taurodeoxycholate remain low throughout the study period. As a result, the bile-salt hydrophobicity index \((\text{Heuman, 1989})\), displayed on the right-hand \(y\)-axes\] becomes markedly hydrophobic for C57L mice, but remains moderately hydrophilic for AKR mice. Conjugated bile salt species were analysed by HPLC (Rossi et al., 1987) using a C-18 Waters Bondapack 10 µm column. Methanol/phosphate buffer was used as eluent (pH 5.2, flow rate of 1 ml/min). □, taurocholate; ○, tauro-β-muricholate; ●, taurochenodeoxycholate; ▲, taurodeoxycholate; ●, tauroursodeoxycholate; □, bile-salt hydrophobicity index.

Cumulative bile salt hydrophobicity index: This indicates hydrophobicity of a bile salt mixture, on the basis of their retention times by HPLC. Hydrophobic bile salt composition promotes cholesterol crystallization.

From day 7 on the lithogenic diet, gall-bladder bile becomes cholesterol supersaturated, without much difference between both strains (Figure 1). Bile salt composition is similar at baseline in gallstone-susceptible C57L and gallstone-resistant AKR male mice (displaying mainly hydrophilic tauro-β-muricholate and taurocholate, Figure 2). When fed on a lithogenic diet, taurocholate becomes the major bile salt in both mouse strains. Hydrophobic bile salts rise sharply in gallstone-susceptible C57L mice (taurochenodeoxycholate to 28% and taurodeoxycholate to 10%), but remain low in gallstone-resistant AKR mice. As a consequence, cumulative bile salt hydrophobicity index (Heuman, 1989) rises in gallstone-susceptible C57L mice from very hydrophilic to moderately hydrophobic values (from −0.35 at baseline to +0.16 at the end of the study; right-hand \(y\)-axis in Figure 2A). In contrast, in gallstone-resistant AKR mice, bile salt composition remains relatively hydrophilic (cumulative bile salt hydrophobicity index −0.35 at baseline and −0.14 at the end of the study; right-hand \(y\)-axis in Figure 2B). Therefore, although bile cholesterol supersaturation is an essential prerequisite for murine gallstone formation, additional factors also need to be present, such as hydrophobic bile salt pool. In line with these factors, many human subjects exhibit gall-bladder bile cholesterol supersaturation without developing gallstones. These subjects may be protected against gallstone formation by a relatively hydrophilic bile salt composition. Also, treatment with the hydrophilic bile salt ursodeoxycholate leads to a hydrophilic bile salt pool composition. In selected patients, cholesterol gallstones may be dissolved and gallstone formation prevented with ursodeoxycholate therapy.

In vivo, biliary lipid composition is determined to a large extent at the level of the hepatocyte canalicular membrane. The process of nascent bile formation is maintained by an elaborate network of ABC (ATP-binding cassette) transporters in the hepatocyte canalicular membrane that regulate biliary secretion of bile salts, phospholipids and cholesterol (Figure 3). BSEP (bile salt export pump; present nomenclature, ABCB11) pumps bile salts across the...
Figure 3 Various transport proteins in hepatocyte canalicular membrane mediate lipid secretion into bile

The MDR3 glycoprotein ‘flips’ phospholipids over the membrane, BSEP mediates bile-salt secretion and ABCG5/G8 enables cholesterol secretion. FXR regulates the BSEP and MDR3 phospholipid flippase, and LXR regulates ABCG5/G8 cholesterol transport protein (see the text for details). Excessive biliary cholesterol secretion or, alternatively, relative excess of (especially hydrophobic) bile salt compared with phospholipid secretion could result in cholesterol crystallization from cholesterol-enriched vesicles or micelles (see the text for details).

membrane into bile (Gerloff et al., 1998). The human MDR3 (multi-drug resistant 3) P-glycoprotein (present nomenclature, ABCB4) functions as a ‘flippase’, translocating phosphatidylcholine molecules from the inner to the outer leaflet of the canalicular membrane, thus enabling their secretion into bile (Smit et al., 1993). Recently, a subset of gallstone patients has been identified with intrahepatic and bile duct stones at young age (<40 years) and high risk of recurrent biliary symptoms after cholecystectomy. The underlying pathogenetic mechanism of this so-called ‘low-phospholipid-associated cholelithiasis’ is thought to be a relative biliary phospholipid deficiency due to a missense mutation in the MDR3 gene (Rosmorduc et al., 2003). Other ABC transporters, namely ABCG5/G8 have been found to be directly involved in biliary cholesterol secretion (Yu et al., 2002). Certain polymorphisms in the ABCG5/G8 gene encoding the hepatocytic canalicular membrane cholesterol-transport protein may be associated with increased risk of gallstone formation. The underlying mechanism could be primary enhanced biliary cholesterol secretion. ABCG5/G8 is also present in the small intestinal cell and transports excess absorbed cholesterol (as well as other sterols) from the enteroocyte back to the intestinal lumen (Berge et al., 2000). Therefore, ABCG5/G8 polymorphisms associated with increased gallstone risk could also primarily enhance intestinal cholesterol absorption. In turn, the lipid-transport proteins in the hepatocytic canalicular membrane are regulated by nuclear receptors. The farnesoid X receptor (FXR, NR1H4) is a member of the nuclear receptor superfamily (Mangelsdorf et al., 1995) and functions as a bile-salt receptor that regulates transcription of numerous genes involved in maintaining cholesterol and bile-salt homeostasis [reviewed in Lu et al., 2001]. In the liver, the activation of FXR by endogenous bile salts induces the expression of the short heterodimer partner (SHP; NR0B2), which is an unusual member of the nuclear receptor family in that it lacks the typical zinc-finger DNA-binding domain. SHP inhibits the transcription of the gene encoding cholesterol 7α-hydroxylase, the rate-limiting enzyme in the major synthetic pathway of bile salts (Lu et al., 2000). The FXR/SHP signalling pathway is therefore an important molecular basis for the feedback repression of bile-salt synthesis. The primary bile salts, chenodeoxycholic and cholic acid, are the highest affinity endogenous ligands characterized for FXR in the enterohepatic system (Makishima et al., 1999).

FXR has also been shown to regulate expression of BSEP and the MDR3 phospholipid flippase in the hepatocyte canalicular membrane. Another subfamily of nuclear receptors, liver X receptor (LXR) regulates expression of the ABCG5/G8 cholesterol-transport protein. These nuclear receptors are therefore expected to be important factors in gallstone formation, as already shown in the inbred mouse model, at least for FXR (Wittenburg et al., 2003). There is convincing evidence for a genetic gallstone predisposition in certain families or populations (Carey and Paigen, 2002; Galman et al., 2004). As mentioned above, an excess of biliary secretion of cholesterol compared with solubilizing bile salts and phospholipids or, alternatively, an excess secretion of bile salt compared with

Low-phospholipid-associated cholelithiasis: This is cholesterol crystallization and gallstone formation due to a deficiency in solubilizing phospholipid. It occurs in bile ducts, rather than in the gall-bladder.
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Figure 4 | Model of cholesterol crystallization
Since nascent cholesterol–phospholipid vesicles in the hepatocyte canalicular lumen are relatively cholesterol-poor (cholesterol/phospholipid ratio far below 1), they are stable and cholesterol crystallization does not occur. During bile concentration in the bile ducts and gall-bladder, mixed cholesterol–phospholipid–bile salt micelles are increasingly formed, because (especially hydrophobic) bile-salt concentrations now exceed critical micellar concentration required for micelle formation. Although less vesicles remain, they are now cholesterol supersaturated (i.e. cholesterol/phospholipid ratio > 1) and may nucleate cholesterol crystals (see the text for details).

Biliary water and gallstone formation
Water is a major component of bile, and modulation of the water content can have major effects on cholesterol crystallization. Significant net water absorption occurs during bile transfer through the bile ducts and during prolonged storage in the gall-bladder. As a result, bile water content decreases from 97 weight percentage in the bile ducts to 90 weight percentage in the gall-bladder. This 3- to 4-fold concentration of bile enhances cholesterol crystallization and gallstone formation considerably (van Erpecum et al., 1990). The underlying mechanism is thought to be as follows. After cholesterol and phospholipid molecules have been made available at the outer leaflet of the hepatocyte canalicular membrane by ABCG5/G8 and MDR3, detergent bile-salt monomers (secreted into the canalicular lumen by BSEP) induce formation of nascent cholesterol–phospholipid vesicles (as shown in Figure 4). These nascent vesicles are stable, because they have a relatively low cholesterol content (the cholesterol/phospholipid ratio is far below 1), and cholesterol crystallization does not occur. Bile salts are mainly present as monomers under these circumstances, since bile salt concentration is initially quite low (in all probability below the critical micellar concentration). During bile concentration in the bileducts and gall-bladder, mixed phospholipids (leading to impaired storage capacity for cholesterol in vesicles), may promote cholesterol crystallization (see Figure 3). Genetic polymorphisms of genes encoding FXR and/or LXR could therefore disturb the delicate balance between cholesterol, bile salts and phospholipids in human bile, resulting in cholesterol crystallization and gallstones.

Bile concentration: This leads to increased lipid concentrations in the fasting gall-bladder and promotes cholesterol crystallization. Aquaporins in the gall-bladder wall may be involved by promoting water transport.
cholesterol–phospholipid–bile-salt micelles are increasingly formed, because the bile-salt concentrations now exceed the critical micellar concentrations required for micelle formation. Since hydrophobic bile salts exhibit much lower critical micellar concentrations than hydrophilic bile salts, the micellization process is markedly enhanced by a hydrophobic bile-salt pool. As a result, cholesterol and phospholipid transfer occurs from vesicles into these mixed micelles. However, since solubilizing capacity of micelles for phospholipids is much higher than for cholesterol, phospholipid transfer occurs preferentially. Thus, although less vesicles remain, they are now cholesterol supersaturated (i.e. cholesterol/phospholipid ratio > 1) and may nucleate cholesterol crystals. This sequence explains why gallstones are normally formed in the gall-bladder, where highest bile concentration exists. Impaired gall-bladder emptying in the fasting state could promote cholesterol crystallization and gallstone formation by allowing time for progressive bile concentration. Although research on gall-bladder emptying has generally focused on the post-prandial period, the fasting state (i.e. at night) would seem to be the most vulnerable period for gallstone formation. During this period, biliary cholesterol saturation is highest due to relatively low bile-salt secretion and relatively high cholesterol secretion. There is also a progressive concentration of gall-bladder bile during this period, which is partially counteracted by periodic interdigestive gall-bladder contractions (30% gall-bladder emptying every 1–2 h) in association with phase III of the migrating motor complex of the intestine and release of the hormone motilin. We found that gallstone patients have less frequent cycles of the intestinal migrating motor complex, an absence of interdigestive gall-bladder emptying and an altered pattern of motilin release compared with controls (Stolk et al., 2001). A similarly prolonged intestinal migrating motor complex cycle has been found in the guinea-pig model of gallstone formation (Xu et al., 1997). All these findings suggest that impaired gall-bladder emptying in the fasting state promotes cholesterol crystallization and gallstone formation by allowing time for progressive bile concentration. Indeed, in the prairie dog model, increased fasting gall-bladder bile concentration occurs during the earliest stages of gallstone formation (before stones have formed), and reducing the concentrating ability of the gall-bladder mucosa with the drug amiloride prevents gallstone formation (Roslyn et al., 1987; Strichartz et al., 1989). Also, in humans with gallstones, highly concentrated gall-bladder bile is associated with fast cholesterol crystallization (van Erpecum et al., 1990). In contrast with the fasting state, gall-bladder water secretion seems to predominate after meal ingestion, possibly influenced by gastrointestinal hormones such as secretin and vasoactive intestinal polypeptide (Igimi et al., 1992; Sweeting, 1993; Peters et al., 1997). These hormones act on the cystic fibrosis transmembrane conductance regulator in epithelial cells (Peters et al., 1997). Increases in intracellular cAMP levels subsequently inhibit the $Cl^-/HCO_3^{-}$ and $Na^+/	ext{H}^+$ exchangers at the apical membrane, with the result that net NaCl entry is inhibited (Masyuk et al., 2002).

Aquaporins (AQP0–AQP10) are a family of transmembrane channels mediating the movement of water through the lipid bilayer in kidney, reproductive and nervous systems, airway, intestine, pancreas and digestive tract (Masyuk et al., 2002). Interestingly, various aquaporins have also been detected in the liver, bile ducts and gall-bladder epithelial cells (Nielsen et al., 1993; Hasegawa et al., 1994; Masyuk et al., 2002; Portincasa et al., 2003; van Erpecum et al., 2004). These aquaporins are discussed extensively elsewhere (Calamita et al., 2005). Although the extensive movement of water across cellular membranes throughout the biliary tract would suggest some involvement of these aquaporins in gallstone formation, their actual role in lithogenesis and potential therapeutic implications remains to be explored.

Acknowledgments

I thank David Q.-H. Wang (Gastroenterology Division, Beth Israel Deaconess Medical Center, Harvard...
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Medical School and Harvard Digestive Diseases Center, Boston, U.S.A.) for all help and assistance.

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Received 2 August 2004; accepted 18 November 2004
Published on the Internet 24 October 2005, doi:10.1042/BC20040088