MINI REVIEW

Pathogenesis of cholesterol and pigment gallstones: An update

Karel Johannes Van Erpecum*

Dept of Gastroenterology and Hepatology, University Medical Center Utrecht, HP F.02.618, PO Box 85500, 3508 GA Utrecht, The Netherlands

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Summary Phase separation of cholesterol crystals from supersaturated bile is still considered the key event in cholesterol gallstone formation. In this review, we will first provide a basal framework of the interactions between the sterol, bile salts and phospholipids in aqueous solutions and then summarize new developments. The hepatocytic apical membrane harbours specific transport proteins for these lipids. Polymorphisms in the gene encoding the cholesterol transporter ABCG5-G8 have been found to increase overall gallstone risk, whereas functional mutations in the gene encoding the phospholipid floppase ABCB4 lead to the rare clinical syndrome of low phospholipid associated cholelithiasis. Expression of bile salt and phospholipid transport proteins is regulated by the bile salt nuclear receptor Farnesoid X receptor (FXR), while the Liver X Receptor (LXR) α regulates ABCG5-G8. Although data from murine experiments suggest a critical role of FXR in gallstone formation, its role in human lithogenesis remains controversial. Variants of the gene encoding UGT1A1 (uridine 5'-diphosphate (UDP)-glucuronosyltransferase 1A1) responsible for bilirubin conjugation were recently associated with risk of gallstones as well as stone bilirubin content, suggesting common factors in cholesterol and pigment gallstone pathogenesis.

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Introduction

In the Western world, prevalence of cholelithiasis has increased considerably in recent years, related to increased prevalence of overweight and/or a higher proportion of elderly subjects in the population. In the Western world, approximately 70% of gallstone carriers exhibit cholesterol gallbladder stones (cholesterol content > 50%), and 30% black pigment gallbladder stones. In East Asia, there is traditionally a high prevalence of brown pigment stones residing in the bile ducts, and causing potentially devastating cholangitis. The current review will first provide a basal framework of the interactions between the sterol, bile salts and phospholipids in aqueous solutions and then summarize new developments since our previous reviews [1,2].

Bile lipids and cholesterol gallstone pathogenesis

Although solubility of cholesterol in aqueous solutions is extremely limited, in gallbladder bile a relatively large
amount (~20 \times 10^{-3} \text{M}) can be kept in solution, due to incorporation of the sterol in mixed micelles, together with bile salts and phospholipids (mainly phosphatidylcholine). The traditional view is that supersaturation and cholesterol crystallization (the earliest step to gallstone formation) occur when either too much cholesterol or not enough solubilizing bile salt and phosphatidylcholine molecules are secreted to allow complete micellar solubilization of cholesterol. Nevertheless, the key role of relative amounts of bile salts versus phospholipids for the process of cholesterol crystallization should be appreciated: Excessive cholesterol may be kept in vesicles (i.e. spherical bilayers of cholesterol and phospholipids) and vesicle formation is promoted by excess phospholipid over bile salt. Vesicles are particular suitable to prevent excess cholesterol from crystallization (vesicular supersaturation above cholesterol/phospholipid ratio of 1, compared to micellar supersaturation above cholesterol/phospholipid ratio of -0.25 depending on conditions). Nevertheless, in case of vesicular cholesterol/phospholipid ratios above 1, cholesterol crystal nucleation will eventually occur. In fact, in human gallstone pathogenesis, cholesterol crystals are thought to nucleate exclusively from supersaturated vesicles rather than from supersaturated micelles. Pivotal information on the process of cholesterol crystal nucleation has been obtained from model bile systems. The equilibrium bile salt-phospholipid-cholesterol ternary phase diagram allows to predict behaviour of mixtures of the three biliary lipids when present in various proportions [3]. As shown in Fig. 1, the phase diagram contains a bottom one-phase zone (only micelles), a left two-phase (micelles and cholesterol crystals-containing) zone, a central three-phase (micelles, vesicles and cholesterol crystals-containing) zone and a right two-phase (micelles and vesicles-containing) zone. Going from baseline to top in the phase diagram the relative percentage cholesterol increases, with progressive cholesterol crystallization. Secondly, a shift from left to right in the phase diagram increases relative amounts of phospholipids compared to bile salts, thus allowing more solubilization of cholesterol in vesicles, lower vesicular cholesterol/phospholipid ratios and less cholesterol crystallization.

Three additional factors strongly affect the ternary equilibrium bile salt-phospholipid-cholesterol ternary phase diagram: bile concentration; bile salt hydrophobicity and phospholipids containing unsaturated acyl chains all promote cholesterol crystallization. Corresponding effects on the ternary equilibrium bile salt-phospholipid-cholesterol ternary phase diagram are in all three cases, an increase of the bottom one-phase (micellar) zone, expansion of the cholesterol crystal-containing zones to the right. The bile sample initially plotting in the right two-phase zone (indicated by the dot) now plots in the central three-phase (cholesterol crystal-containing zone), despite identical relative lipid composition.

(i.e. cholesterol-phospholipid ratio > 1) with cholesterol crystal nucleation. This sequence of events explains why gallstones are generally formed in the gallbladder rather than in the bile ducts. In gallbladder bile of cholesterol gallstone patients, increased amounts of the hydrophobic bile salt deoxycholate are associated with fast crystallization [5]. In selected patients with cholesterol gallstones, treatment with the hydrophilic bile salt ursodeoxycholate may desaturate bile and dissolve the stones. In \textit{in vitro} studies with model biles, phospholipid class and phospholipid acyl chain composition exert profound effects

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**Figure 1** Equilibrium bile salt-phospholipid-cholesterol phase diagram. The components are expressed in mol percent. Depicted are a one-phase (micellar) zone at the bottom, a left two-phase zone (containing micelles and crystals), a central three-phase zone (containing micelles, vesicles and crystals) and a right two-phase zone (containing micelles and vesicles). Phospholipid/(bile salt + phospholipid) ratios are given at the bottom axis, and going from left to right indicates increased relative amounts of phospholipids compared to bile salts, with increased vesicular cholesterol solubilization. In the phase diagram, relative amounts of phospholipids compared to bile salts are expressed as phospholipid/(bile salt + phospholipid) ratios since this allows easy (linear) plotting along the bottom axis of the phase diagram. Any line connecting the bottom axis with the top of the triangle (100% cholesterol) represents identical phospholipid/(bile salt + phospholipid) ratio, with increased relative amounts of cholesterol when moving from bottom to top. For example, in the figure, all biles plotting on the coarse interrupted line exhibit identical phospholipid/(bile salt + phospholipid) ratio of 0.8. In the Figure, fine dotted lines indicate zones in case of diluted bile, hydrophilic bile salts or saturated phospholipid acyl chains. Continuous lines indicate zones in case of concentrated bile, hydrophobic bile salts or unsaturated phospholipid acyl chains. Under the latter circumstances, cholesterol crystallization is promoted by an expansion of the crystal-containing zones to the right. The bile sample initially plotting in the right two-phase zone (indicated by the dot) now plots in the central three-phase (cholesterol crystal-containing zone), despite identical relative lipid composition.

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on cholesterol crystallization. Human biliary phospholipid composition is tightly regulated, and almost exclusively composed of phosphatidylcholine with unsaturated acyl chains, thus contributing to human vulnerability for gallstone formation [6]. Although modification of biliary phospholipids towards a more saturated acyl chain composition would be in theory attractive, dietary modifications to accomplish this have not been successful so far. For an extensive review on biliary lipids we refer to references [1,2].

The ternary bile salt-phospholipid-cholesterol phase diagram is based on equilibrium conditions. Nevertheless, time to reach equilibrium is essential for human gallstone formation, since bile is stored in the gallbladder only for a limited period of time. A shift from left to right in the phase diagram is associated with an increase in time to reach equilibrium (from a few hours to more than 30 days, depending on experimental conditions). Numerous biliary proteins have previously been suggested to shorten or lengthen time to equilibrium and cholesterol crystallization in gallbladder bile. Various pronucleating proteins are present in much higher concentrations in the fast nucleating bile of patients with multiple cholesterol stones. Immunoglobulins M and G [7,8], haptoglobin [9], α1-acid glycoprotein [10,11], aminopeptidase-N [12], α1-antichymotrypsin and mucin [13] are regarded as pro-nucleating proteins and apolipoprotein A-I [14] and IgA [15] have been postulated to exert anti-nucleating activity. Nevertheless, in more recent years, a growing number of publications have marshalled experimental evidence arguing against a role of most of these biliary proteins in human cholesterol gallstone formation [16,17]. In line with these publications, it was recently reported that bile composition and risk of gallstones were not altered in apolipoprotein A-I "knockout" mice [18]. Mucin remains one of the few candidate proteins with a potential role in human gallstone formation.

The gallbladder and cholesterol gallstone pathogenesis

Impaired postprandial gallbladder emptying, often present in cholesterol gallstone patients, may prolong residence of bile in the gallbladder, thus allowing more time for nucleation of cholesterol crystals from supersaturated bile and their growth/aggregation into macroscopic stones. Significant absorption of cholesterol appears to occur from supersaturated bile in the gallbladder [19]. Excess cholesterol is then incorporated within the sarcolemmal plasma membrane of the gallbladder smooth muscle cell, with decreased membrane fluidity, impaired contractility and impaired relaxation as a result. Recent in vitro data indicate that reduced contraction is caused by a lower cholcystokinin binding to CCK-1 receptors due to caveolar sequestration of the receptors [20,21]. Gallbladder wall inflammation may also be critical in gallstone formation The gallbladder wall is exposed to detergent bile salts, unesterified cholesterol and bacteria, which all could induce inflammation [22,23]. The murine gallstone model which was employed by the group of Carey in Boston so extensively to study gallstone pathogenesis, proved to be dependent on gallbladder infection with helicobacter species [24]. Subsequent studies utilizing immunocompetent Helicobacter spp.-infected and -uninfected BALB/c and congenic immunodeficient Rag2(−/−) mice confirmed the critical role of helicobacter in murine lithogenesis and pointed to the relevance of T-cell proinflammatory response [25]. As a result, hypomotility and increased mucin secretion may occur [22,23]. Although various helicobacter species have been detected in human gallbladders and bile, and antibodies to helicobacter hepaticus were found at increased frequency in gallstone patients [26], the role of helicobacter in human gallstone pathogenesis remains to be defined. Also the extensive literature available on murine Lith genes is now difficult to interpret without knowledge on status of Helicobacter infection of the various strains applied. Although impaired motility is generally secondary to biliary cholesterol supersaturation, it may still facilitate the process of gallstone formation. Gallbladder motility is often impaired in high-risk situations for gallstone formation such as pregnancy, obesity, diabetes mellitus, gastric surgery, treatment with the somatostatin analogue octreotide, very low calorie dieting and total parenteral nutrition.

Enterohepatic circulation of bile salts, intestinal cholesterol absorption, lipid transport proteins and cholesterol gallstone formation

In a polygenetic disorder as cholesterol gallstone disease, several underlying mechanisms may be involved in pathogenesis and their relevance may differ between different populations. Increased bile salt and cholesterol synthesis have been reported in Chilean patients [27]. The defect was supposed to be secondary to increased intestinal loss of bile salts, and preceded gallstone formation. Interestingly, decreased expression of ileal bile salt transport proteins apical sodium-dependent bile acid transporter (ASBT), cytosolic ileal lipid binding protein (ILBP) and basolateral organic solute transporter (OST)α and β were recently described in female non-obese patients, as a possible explanation of these findings [28,29]. In line with these findings, a variant of the SLC10A2 gene encoding the apical sodium-dependent bile acid transporter was recently identified as a risk factor for gallstone disease in a large group of German gallstone patients [30].

It has also been reported, that a high cholesterol diet increases biliary cholesterol secretion and decreases bile salt synthesis and bile salt pool in cholesterol gallstone subjects but not in controls [31]. These findings point to the importance of intestinal cholesterol absorption in gallstone pathogenesis. Interestingly, increased expression of the intestinal cholesterol uptake protein protein NPC1L1 (Niemann Pick C1 Like protein 1) and ACAT2 (Acylic Coenzyme A-cholesterol acyl transferase: enzyme involved in cholesterol esterification) were recently reported in jejenum of Chinese gallstone patients [32]. These findings suggest that increased intestinal uptake and esterification of the sterol could be important in gallstone pathogenesis. Also, inhibiting cholesterol absorption with etezimibe prevents gallstone formation in the mouse model and decreases biliary cholesterol saturation in gallstone patients with slower crystallization as a result [33].
Figure 2  Nascent bile formation at the hepatocytic canalicular membrane, biliary cholesterol solubilization and gallstone formation. ABCG5-G8 transports cholesterol into bile, and is regulated by nuclear receptor LXR. ABCB11 and ABCB4 transport bile salts and phosphatidylcholine into bile, and are regulated by nuclear receptor FXR. Bile cholesterol is solubilized in mixed micelles or kept in cholesterol-phospholipid vesicles. Cholesterol crystallization and gallstones occur in most patients because of excess biliary cholesterol secretion and subsequent crystallization from supersaturated vesicles (continuous lines). In patients with LPAC (low phospholipid associated cholelithiasis) there are insufficient amounts of phospholipids in bile due to loss of function mutations in the ABCB4 gene. In patients with BRIC (benign recurrent intrahepatic cholestasis) type 2, there are insufficient amounts of bile salts in bile due to loss of function mutations in the ABCB11 gene. In patients with LPAC and BRIC type 2, there is an increased risk of gallstone formation. It is unknown, whether cholesterol crystallization in LPAC and BRIC type 2 (fine resp. coarse interrupted lines) occurs from supersaturated vesicles or supersaturated micelles.

The process of nascent bile formation is maintained by an elaborate network of ATP-binding cassette (ABC) transporters in the hepatocyte canalicular membrane which enable biliary secretion of cholesterol, bile salts and phospholipids (Fig. 2). The ABCG5/G8 genes encode protein half-transporters which heterodimerize to form the functional transporter localized in the canalicular membrane of hepatocytes and facilitating cholesterol secretion into bile [34]. Recently, variants of ABCG5/8 were linked to gallstone disease; ABCGB D19H in Caucasians and ABCG5 Q604E in Chinese populations [35,36]. Similar findings were recently reported in a Swedish twin study [37]. ABCB5-B8 may also provide a mechanistic link between gallstones and the metabolic syndrome. Mice solely with hepatic insulin resistance, created by liver-specific disruption of the insulin receptor (LIRKO mice) were found to be markedly predisposed toward cholesterol gallstone formation due to at least two distinct mechanisms. Disinhibition of the forkhead transcription factor FoxO1, increased expression of the biliary cholesterol transporters Abcg5 and Abcg8, resulting in an increase in biliary cholesterol secretion. Hepatic insulin resistance also decreased expression of the bile salt synthetic enzymes, particularly Cyp7b1, and produced partial resistance to the farnesoid X receptor (see below), leading to a lithogenic bile profile [38].

The bile salt export pump (BSEP: current nomenclature ABCB11) pumps bile salts over the membrane into bile (Fig. 2). In patients with benign recurrent cholestasis type 2 (BRIC 2) due to mutations in the ABCB11 gene there is a strongly increased frequency of associated gallstones, supposedly due to insufficient amounts of biliary bile salts [39]. The human Multi Drug Resistance 3 (MDR3) P-glycoprotein (current nomenclature ABCB4) functions as a "floppase", translocating phosphatidyicholine molecules from the inner to the outer leaflet of the canalicular membrane thus enabling their secretion into bile (Fig. 2). 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. A subgroup of these patients exhibits a severe phenotype with large uni- or multifocal spindle-shaped dilations of the intrahepatic bile ducts without any bile duct stenosis, and filled of gallstones [40]. The underlying pathogenetic mechanism of this so-called "low phospholipid associated cholelithiasis" is thought to be relative biliary phospholipid deficiency due to a missense mutation in the MDR3 gene [41]. Nevertheless, recent data from sib pairs with gallstones and control do not support a link between ABCB4 and ABCB11 polymorphisms and gallstone formation in the large majority of patients [42]. Also, there appears to be no increased frequency of mutations in ABCB4 and ABCB11 genes in pediatric gallstone patients with gallstone disease and a positive family history of gallstones [43].

Nuclear receptors and gallstone formation

Farnesoid X receptor (FXR: NR1H4) is a member of the nuclear receptor superfamily and functions as a bile salt
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Pathogenesis of pigment gallstones

Brown pigment stones

Brown pigment stones are formed in the bile ducts [49]. They are primarily composed of calcium salts of unconjugated bilirubin and varying amounts of cholesterol and protein. Brown pigment stones are associated with chronic bacterial or parasitic infection of the bile ducts. Bacteria may produce β-glucoronidase, phospholipase A and bile acid hydrolase leading to increased amounts of unconjugated bilirubin, palmitic and stearic acids, and unconjugated bile acids, which can complex with calcium, resulting in stone formation. Parasites in the bile ducts may stimulate stone formation by the calcified overcoat of the parasitid egg, which may serve as a nidus and enhance precipitation of calcium bilirubinate.

Black pigment stones

In the Western world, approximately 30% of gallstone carriers exhibit black pigment gallbladder stones (<20% cholesterol content). Black pigment stones are formed in sterile bile in the gallbladder and are primarily composed of calcium bilirubinate. Normally, the large majority of bilirubin — the breakdown product of hemoglobin — is conjugated in the liver to bilirubin monoglucuronide and subsequently to water-soluble bilirubin diglucuronide. Unconjugated bilirubin is poorly soluble in water. In case of hemolysis, biliary excretion of bilirubin may increase ten-fold with increased risk of calcium bilirubinate precipitation. This phenomenon explains the high prevalence of black pigment stones in chronic hemolytic disorders such as sickle cell anemia, hereditary spherocytosis and Gilbert syndrome. Concomitant presence of Gilbert syndrome is associated with increased gallstone prevalence in sickle cell disease [50]. Enterohepatic cycling of bilirubin may contribute to high frequency of pigment stones in patients with ileal Crohn disease (especially in case of ileal resection) and cystic fibrosis [51]. The proposed mechanism is that increased amounts of bile salts reach the caecum and solubilize unconjugated bilirubin thus allowing their reabsorption with subsequent hyperbilirubinobilia [52—54]. Prevalence of Gilbert syndrome is increased in patients with cystic fibrosis and gallstones, which may contribute to pigment stone formation [55]. Interestingly, a recent report on a large cohort of (both cholesterol and pigment) gallstone patients found that variants of the UTG1A1 gene (encoding for uridine 5′-diphosphate (UDP)-glucuronosyltransferase 1A1 responsible for bilirubin conjugation) and the SLO1B1 gene (encoding for a basolateral hepatocytic membrane transporter for various exogenous and endogenous compounds including bilirubin and its glucuronides) determined not only serum bilirubin levels, but also stone bilirubin content and risk of gallstone formation [56]. Of note, the combination of UTG1A1 and ABCG5/8 variants as major genetic risk factors explained 11% of gallstone risk in males of this German cohort. Importantly, the increased gallstone risk applied to both pigment and cholesterol stones, consistent with the classic pathophysiological model of bilirubin serving as a nucleation core for gallstone formation in general. These findings could provide a potential link between pathogenesis of pigment and cholesterol gallstones.

Conflict of interest statement

The author has no relevant financial disclosures.

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