HHS Public Access

Author manuscript

Liver Res. Author manuscript; available in PMC 2021 August 05.

Published in final edited form as:

Liver Res. 2017 June ; 1(1): 42–53. doi:10.1016/j.livres.2017.05.005.

New insights into the role of *Lith* genes in the formation of cholesterol-supersaturated bile

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Abstract

Cholesterol gallstone formation represents a failure of biliary cholesterol homeostasis in which the physical-chemical balance of cholesterol solubility in bile is disturbed. Lithogenic bile is mainly caused by persistent hepatic hypersecretion of biliary cholesterol and sustained cholesterol-supersaturated bile is an essential prerequisite for the precipitation of solid cholesterol monohydrate crystals and the formation of cholesterol gallstones. The metabolic determinants of the supply of hepatic cholesterol molecules that are recruited for biliary secretion are dependent upon the input-output balance of cholesterol and its catabolism in the liver. The sources of cholesterol for hepatic secretion into bile have been extensively investigated; however, to what extent each cholesterol source contributes to hepatic secretion is still unclear both under normal physiological conditions and in the lithogenic state. Although it has been long known that biliary lithogenicity is initiated by hepatic cholesterol hypersecretion, the genetic mechanisms that cause supersaturated bile have not been defined yet. Identification of the *Lith* genes that determine hepatic cholesterol hypersecretion should provide novel insights into the primary genetic and pathophysiological defects for gallstone formation. In this review article, we focus mainly on the pathogenesis of the formation of supersaturated bile and gallstones from the viewpoint of genetics and pathophysiology. A better understanding of the molecular genetics and pathophysiology of the formation of cholesterol-supersaturated bile will undoubtedly facilitate the development of novel, effective, and noninvasive therapies for patients with gallstones, which would reduce the

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Conflict of interest

There is no conflict of interest to disclose for all authors.

morbidity, mortality, and costs of health care associated with gallstones, a very prevalent liver disease worldwide.

Keywords

Bile flow; Bile acid; Biliary secretion; *Lith* gene; Micelle; Vesicle

1. Introduction

Gallstone disease is not only a very prevalent liver disease worldwide, but also a very old human disorder, going back thousands of years, as it has been found in ancient mummies in Egypt and China. Although gallstone disease was not recognized by ancient Chinese, abdominal pain as a result of hepatobiliary diseases and gastric malfunction, jaundice caused by liver diseases, and epigastric colic owing most likely to gallstones or biliary ascariasis were often treated with bear's bile.[1] The earliest medical record for these therapeutic interventions was found in Treatise on Properties of Drugs (c. 643 CE or earlier) written by an ancient Chinese doctor Zhen Quan (c. 540 to 643 CE).[1] Modern chemical analysis of the bile of Asian black bears (*Ursus thibetanus* or *Selenarctos thibetanus*) and brown bears (*Ursus arctos*) found that ursodeoxycholic acid (UDCA) is the major composition of the bile acid pool in these animals. Notably, UDCA, a hydrophilic bile acid, is now first-line pharmacological therapy in a subgroup of symptomatic patients with small, radiolucent cholesterol-enriched gallstones.[2] Long-term administration of UDCA promotes the dissolution of cholesterol gallstones, especially in patients with small (≤5 mm in diameter), cholesterol-rich and uncalcified stones (radiolucent on plain X-ray film) in a functioning gallbladder with preserved kinetics and a patent cystic duct.[3–5] However, the therapeutic effect of UDCA is not always achieved in clinical practice because of a high recurrence rate of gallstones.[5] Although laparoscopic cholecystectomy is nowadays the first choice of treatment options for gallstone disease, it is invasive and can cause surgical complications regarding morbidity and mortality, and not all patients with symptomatic gallstones are candidates for surgery.[6]

To reduce the morbidity, mortality and costs of health care associated with gallstones, it is imperative to elucidate the pathogenesis of gallstone disease. This will promote the development of a novel, effective, and noninvasive therapy for patients with gallstones. Since the first gallstone gene, *Lith1* was identified by quantitative locus trait (QTL) mapping methods in inbred strains of mice in 1995,[7] a mouse gallstone gene map that contains 25 *Lith* genes has been established through genetic analysis of cholesterol gallstone formation in different strains of inbred mice fed a lithogenic diet for 8 weeks.[8] This greatly promotes the discovery of human *Lith* genes because of homologues between human and mouse chromosomes. Such a successful study is the confirmation of *ABCG5/G8* as a human *Lith* gene based on mouse studies. The *Abcg5/g8* was first identified as the mouse *Lith9* by the QTL mapping methods,[9–11] and subsequently, two major gallstone-associated variants in *ABCG5/G8* (*ABCG5*-R50C and *ABCG8*-D19H) were found not only in German and Chilean populations, but also in Chinese and Indian populations.[12–19] Therefore, based on

the mouse gallstone (*Lith*) gene map, more human *Lith* genes will be identified and their pathogenic mechanisms will be elucidated in the near future.

2. History of cholesterol and bile acid research

Bile is a yellow, brownish, or olive-green liquid that is composed primarily of water, organic solutes (such as lipids), inorganic salts, and some proteins. In bile, cholesterol, phospholipids, and bile acids are three major lipids, and bile pigments are minor lipids. Chemical studies of bile and gallstones for more than 200 years led to the discovery of cholesterol and bile acids, two major organic molecules in bile. The “cholesterol” was first identified in gallstones in the mid-18th century, and subsequently, this material was isolated from gallstones by some researchers. Accumulated evidence showed during the second half of the 18th century that the major component of gallstones was a white crystalline substance that is soluble in alcohol and ether, but not in water. It was not until 1816 that the compound “cholesterine” was named by chemist Michel Chevreul.[20] After cholesterine was found to be an alcohol by Berthelot in 1859,[21] a new name “cholesterol” was largely used in French and English scientific literature. The term cholesterol originated from the ancient Greek chole- (bile) and stereos (solid) followed by the chemical suffix -ol for an alcohol. Although cholesterol was recognized as a distinct chemical compound in the early 19th century, its chemical structure has not been known for many decades. In 1888, Reinitzer[22] identified that the empirical formula of cholesterol was $C_{27}H_{46}O$, indicating that cholesterol was not a straight-chain compound with a double bond, since it did not have enough hydrogen atoms to bind to all the carbon valency of four. However, he saw it was consistent with a structure containing four rings with two shared carbon atoms at each ring junction (four fused rings). Subsequently, some substances isolated from fungi and green plants were found to be cholesterol-like crystalline compounds. In 1889, Tanret[23] isolated a substance from rye seeds infected with ergot, which closely resembled cholesterol. This compound was named ergostérine (now called ergosterol). Furthermore, the empirical formulae of cholic acid ($C_{24}H_{40}O_5$), which was found by Strecker in 1848,[24] and of deoxycholic acid ($C_{24}H_{40}O_4$), which was found by Mylius in 1886,[25] displayed a highly similar ratio (1.67) of hydrogen to carbon atoms compared with that (1.70) in cholesterol. Because both bile acids and cholesterol are present in bile, it was reasonable to hypothesize that the structural features of these two compounds could be similar. In 1919, Windaus and his colleagues[26] found that the carbon skeleton of bile acids was the same as that of the cholesterol molecule, for the most part. This discovery greatly promoted the study of the chemical structure of cholesterol because the presence of the hydroxyl group in ring C of cholic and deoxycholic acids enabled Windaus and other researchers to further investigate the steroid ring system through the bile acid approach.

In 1928, the Nobel Committee for Chemistry announced that the Nobel Prize in Chemistry 1927 was awarded to Heinrich Wieland “for his investigations of the constitution of the bile acids and related substances,” as well as that the Nobel Prize in Chemistry 1928 was given to Adolf Windaus “for the services rendered through his research into the constitution of the sterols and their connection with the vitamins.” Thus, on December 10, 1928, two Nobel Prizes in Chemistry were awarded to Wieland and Windaus, respectively. In his Nobel lecture,[27] Wieland first described a brief history of how three bile acids (including cholic,

deoxycholic, and lithocholic acids) were discovered and then, summarized his chemical experiments of bile acids. Based on his experimental results, he proposed a possible chemical structure of bile acid. In his Nobel lecture,[28] Windaus presented his discovery that the chemical precursor of vitamin D was a member of the sterol group and also showed how sunlight broke one of the chemical bonds in the parent molecule, converting it into the active vitamin. This finding clearly explained why exposure to sunlight could prevent rickets, a disease caused by vitamin D deficiency in humans. In addition, Windaus proposed a possible chemical structure of cholesterol. He spent some 30 years studying the chemical structure of cholesterol, which was part of his study of the complex alcohols, known as sterols. He found that sterols were closely related to bile acids by transforming cholesterol into cholanic acid. Unfortunately, the steroid nucleus of bile acid and cholesterol shown in their Nobel lectures was incorrect.[29] However, this did not significantly influence their excellent findings and conclusions for which their prizes were awarded.

It must be noted that modern physical techniques for structural analysis of steroids were not available to these early talented scientists that time. It was a challenging task for these early scientists to precisely identify the chemical structures of cholesterol and bile acids. However, the development of new physical techniques led to the discoveries of the correct chemical structures of these steroids. Desmond Bernal used X-ray diffraction methods to study vitamin D, cholesterol, and ergosterol, and reported the chemical structures of these compounds in *Nature* in 1932.[30] Subsequently, two research groups, led by Rosenheim and King in the UK and Wieland and Dane in Germany, further investigated the chemical structure of bile acids.[31] Each group independently proposed the structure of cyclopentanoperhydrophenanthrene for the steroid nucleus of bile acids. These structures were confirmation by both X-ray diffraction and chenodeoxycholic acid synthesis.[32] Obviously, the X-ray diffraction methods played a critical role in the determination of the correct chemical structures of these lipids in bile, which was proposed in 1932 and has been used ever since. The determination of the sterol ring structure promoted identification of the chemical structures of many other biologically important sterols. For example, Adolf Butenandt identified the structures of the male and female sex hormones even from 25 mg of the male hormone sample. Fig. 1 shows, from left to right, the molecular structures, the standard chemical formulae, the perspective formulae, and the space-filling models of cholesterol and cholic acid, respectively.

Of special note, although Edward A. Doisy at Saint Louis University won the Nobel Prize in Physiology or Medicine 1943 for his outstanding work on the discovery of the chemical nature of vitamin K, his other excellent work was the identification of α-, β-, and ω-muricholic acids, three isoforms of the 3,6,7-trihydroxy bile acids in rat bile.[33] Subsequently, William Elliott synthesized these bile acids and investigated their chemical and chromatographic properties.[34–38] These muricholic acids are the major bile acids in mice and rats, and these findings elucidated differences in bile acid composition between rodents and humans.

3. Physical chemistry of cholesterol

Cholesterol is an essential component of mammalian cell membranes and is widely distributed in unesterified and esterified forms. In its unesterified form, the chemical structure of the cholesterol molecule includes the cholestene nucleus with a double bond at the C-5 and C-6 positions and a hydroxyl group on the third carbon. Furthermore, the angular methyl groups at C-10 and C-13, the hydrogen atom at C-8 and the side-chain at C-17 are in β configuration. The hydrogen atoms at C-9 and C-14 are in α configuration. The solubility of cholesterol is very low in water, approximately 4.7 mmol at 25 °C. Furthermore, when one fatty acid attaches to the cholesterol molecule at the C-3 position, its residue increases the hydrophobicity of cholesterol.

In the plasma, approximately one third of cholesterol is in the unesterified form and the remaining two thirds exist as cholesteryl esters. The actual cholesterol concentration in plasma of a healthy individual is usually between 120 and 200 mg/dL. Such a high concentration of cholesterol can be present in the blood because plasma lipoproteins, mainly high-density lipoprotein (HDL), low-density lipoprotein (LDL), and very-low-density lipoprotein (VLDL), carry large amounts of cholesterol, regardless of whether the cholesterol molecule is in a nonesterified or an esterified form.[39–45]

Notably, approximately 95% of the cholesterol molecule in bile is in the unesterified form and <5% of the sterols are cholesterol precursors and dietary sterols.[46–48] In contrast, the concentrations of cholesteryl esters are negligible in human bile. Moreover, cholesterol is abundant in human bile, with normal concentrations being approximately 390 mg/dL in the gallbladder. Bile acids, which are metabolites of cholesterol, can form simple and mixed micelles in bile, which can aid in solubilizing cholesterol in bile.[49–54] Furthermore, the vesicles that are composed primarily of phospholipids also greatly promote the solubility of cholesterol in bile.[55–61]

4. Five primary defects leading to cholesterol gallstone formation

As shown in Fig. 2, compelling evidence from clinical studies and animal experiments has clearly demonstrated that interactions of five primary defects play a critical role in the pathogenesis of cholesterol gallstone disease.[8] These defects include (i) genetic factors and *Lith* genes; (ii) hepatic hypersecretion of biliary cholesterol leading to supersaturated bile; (iii) rapid phase transitions of cholesterol in bile; (iv) impaired gallbladder motility accompanied with hypersecretion of mucins and accumulation of mucin gel in the gallbladder lumen, as well as immune-mediated gallbladder inflammation; and (v) increased amounts of cholesterol of intestinal origin owing to high efficiency of cholesterol absorption and/or slow intestinal motility, which aids “hydrophobe” absorption and augments “secondary” bile acid synthesis by the anaerobic intestinal microflora.[8,46,62] By numerous human and animal studies, hepatic cholesterol hypersecretion is recognized to be the primary pathophysiologic defect, leading to the formation of cholesterol-supersaturated bile and solid cholesterol crystals, as well as their aggregation and growth into cholesterol gallstones. These abnormalities are caused by multiple *Lith* genes, with insulin resistance as part of the metabolic syndrome working with cholelithogenic environmental factors to

induce the phenotype.[46,63,64] Rapid growth and agglomeration of solid plate-like cholesterol monohydrate crystals into microlithiasis and eventually gallstones is a consequence of persistent hepatic hypersecretion of biliary cholesterol together with both gallbladder mucin hypersecretion and incomplete evacuation by the gallbladder owing to its impaired motility dependent on defective smooth muscle response to neuro-hormonal stimuli.[65] Over the past decades, new progress has been made in the genetic analysis of *Lith* genes and the pathophysiology of gallstone disease. Many excellent review articles on these topics have been extensively published, and interested readers can further read these papers.[8,47,65–72]

5. The sources of cholesterol secreted into bile

Bile formation is an osmotic process and solutes are actively transported into the canaliculus by primary active lipid transporters: ABCG5/G8 for biliary cholesterol secretion, ABCB4 for biliary phospholipid secretion, and ABCB11 for biliary bile acid secretion.[46,47] The most important solutes driving bile formation are bile acids. Three important physiological functions of bile formation are[73]: (i) it is a major route for the elimination of cholesterol from the body, either as unesterified cholesterol or as bile acids, the end productions of cholesterol degradation; (ii) it ensures the secretion of bile acids, which are crucial for lipid emulsification in the small intestinal tract and subsequent lipid absorption by the enterocytes; and (iii) it represents an important pathway for the removal of drugs, toxins, and waste productions from the body.

As shown in Fig. 3, the metabolic determinants of the supply of hepatic cholesterol molecules that are recruited for biliary secretion are dependent upon the input-output balance of cholesterol and its catabolism in the liver. Input is dependent on the amount of both unesterified and esterified cholesterol taken up by the liver from plasma lipoproteins (LDL > HDL > chylomicron remnants) plus hepatic *de novo* biosynthesis. Output is dependent upon the amount of cholesterol disposed within the liver after its conversion to cholesteryl esters (to form new VLDL plus ester storage) minus the amount of cholesterol converted to the primary bile acids, such as cholic acid and chenodeoxycholic acid. Overall, the liver can systematically regulate the total amount of cholesterol within it, and any excess cholesterol can be handled efficiently.

When no dietary cholesterol is consumed, bile contains newly synthesized cholesterol from the liver as well as preformed cholesterol, which reach the liver via several different ways. Under the circumstances, it is estimated that ~85% of total biliary cholesterol is derived from the pools of preformed cholesterol within the liver and less than 15% of the cholesterol in bile comes from hepatic *de novo* biosynthesis. The sources of preformed cholesterol are derived from hepatic uptake of plasma lipoproteins, such as HDL, LDL, and VLDL through their respective receptors on the basolateral membrane of hepatocytes.[74] Consistent with its predominant physiological function in reverse cholesterol transport, HDL transfers cholesterol from the extrahepatic tissues to the liver for biliary secretion, which is the major lipoprotein source of cholesterol that is targeted for hepatic secretion into bile. Acetyl-CoA is often used as a substrate for the hepatic *de novo* biosynthesis of cholesterol, which is regulated mainly by 3-hydroxy-3-methylglutaryl-coenzyme A reductase, the rate-limiting enzyme in this cholesterol synthesis pathway in the liver.[75] This enzyme is up- or down-

regulated depending on the overall cholesterol balance in the liver. Increasing its enzymatic activity could enhance hepatic secretion of biliary cholesterol.[76,77] However, its inhibition by statins reduces hepatic cholesterol secretion by less than 10%.

Most, but not all, studies showed that the use of oral contraceptive steroids and conjugated estrogens in premenopausal women significantly increases the incidence of cholesterol gallstones.[78] The administration of estrogen to postmenopausal women and estrogen therapy to men with prostatic carcinoma display similar lithogenic effects, leading to hepatic cholesterol hypersecretion and biliary lithogenicity.[79–83] Animal studies found that hepatic estrogen receptor α (ERα) activated by estrogen interferes with the negative feedback regulation of cholesterol biosynthesis by stimulating sterol-regulatory element binding protein-2 (SREBP-2), which activates the SREBP-2 responsive genes for the cholesterol biosynthetic pathway.[84] Thus, under conditions of high levels of estrogen, mice continue to synthesize cholesterol in the face of its excess availability from the high-cholesterol diet, suggesting that there is a loss in the negative feedback regulation of cholesterol biosynthesis that results in excess secretion of newly synthesized cholesterol and supersaturation of bile.[84] These abnormalities lead to a predisposition to cholesterol gallstone formation. These findings highlight the importance of estrogen in the pathogenesis of gallstones because more newly synthesized cholesterol determined by the estrogen-ERα-SREBP-2 pathway is secreted into bile, leading to biliary cholesterol hypersecretion and the formation of supersaturated bile.[47]

Under conditions of high cholesterol consumption, an appreciable fraction of cholesterol in bile is derived from the diet through the chylomicron pathway to the liver. Dietary cholesterol reaches the liver through the intestinal lymphatic routes as of chylomicrons, and subsequently, chylomicron remnants after chylomicrons are hydrolyzed by plasma lipoprotein lipase and hepatic lipase.[85,86] Under the circumstances, newly synthesized cholesterol in the liver is reduced, which consists of only approximately 5% of biliary total cholesterol.

The small intestine is a unique organ providing dietary and re-absorbed biliary cholesterol to the body.[85] Clinical studies and epidemiological investigations have found that cholesterol cholelithiasis is prevalent in cultures consuming a “Western” diet that consists of high total calories, cholesterol, saturated fatty acids, refined carbohydrates, proteins, and salt, as well as low fiber. In addition, its incidence in North and South America, as well as in European countries, is significantly higher than that in Asian and African populations.[87] Several clinical studies have found an association between the increased incidence of cholesterol gallstones in China and a “westernization” of the traditional Chinese diet. Cholesterol cholelithiasis once was rare in Japan, but the incidence is now increased markedly mostly because of over the past half a century with the adoption of Western-type dietary habits. Because biliary cholesterol hypersecretion is an important prerequisite for cholesterol gallstone formation, biliary cholesterol secretion and saturation could be significantly reduced by inhibiting cholesterol absorption and hepatic uptake of chylomicron remnants.[88] More importantly, there is a significant and positive correlation between the efficiency of intestinal cholesterol absorption and the prevalence of cholesterol gallstone formation in 15 strains of inbred mice, implying that high efficiency of intestinal cholesterol absorption and

high dietary cholesterol are two independent risk factors for cholesterol gallstone formation.[86] A new finding showed that the potent cholesterol absorption inhibitor ezetimibe prevents the formation of cholesterol gallstones, and facilitates the dissolution of gallstones by forming an abundance of unsaturated micelles in gallstone-susceptible C57 L/J mice carrying *Lith1* and *Lith2* genes.[89] In addition, ezetimibe significantly reduces biliary cholesterol saturation and retards cholesterol crystallization in the bile of patients with gallstones,[89] suggesting that it may act as a potent biliary cholesterol-desaturating agent in patients with gallstones. These findings indicate that ezetimibe is a novel approach to reducing biliary cholesterol content and provides a promising strategy for preventing or treating cholesterol gallstones by inhibiting intestinal cholesterol absorption.[90]

6. Disruption of hepatic lipid secretion leading to the formation of cholesterol-supersaturated bile

Because bile is an aqueous solution and cholesterol is virtually insoluble in water, the mechanisms for cholesterol solubilization in bile are complex. Clinical studies and animal investigations have found that hepatic hypersecretion of biliary cholesterol is the primary defect in the pathogenesis of cholesterol gallstone disease. Hepatic cholesterol hypersecretion into bile may or may not be accompanied by normal, high, or low hepatic secretion rates of biliary bile acids and phospholipids. Cholesterol-supersaturated bile is often defined as a state in which cholesterol cannot be solubilized in bile by biliary bile acids and phospholipids at equilibrium.[91] Therefore, the formation of supersaturated bile is often caused by (i) hepatic hypersecretion of biliary cholesterol; (ii) reduced hepatic bile acid and phospholipid secretion with normal biliary cholesterol secretion; or (iii) a combination of hepatic cholesterol hypersecretion with hyposecretion of these solubilizing lipids.

Many animal studies have provided direct evidence showing that bile acids stimulate secretion of vesicles by the hepatocytes, and these unilamellar vesicles are always detected in freshly collected hepatic bile.[92–97] Accumulated evidence from the genetic study of sitosterolemia has shown that the efflux of biliary cholesterol from the canalicular membrane could be a protein-mediated process.[98–109] This led to the discovery of ABCG5/G8, which plays a critical role in the cellular efflux of cholesterol, and its significance for bile formation has been examined in genetically modified mice.[110–114] Overexpression of ABCG5/G8 in the liver increases the cholesterol content of gallbladder bile. In contrast, the hepatic secretion rate of biliary cholesterol is reduced in Abcg5/g8 double knockout mice and in Abcg5 or Abcg8 knockout mice. In addition, scavenger receptor class B type I (SR-BI), the HDL receptor, is localized mainly in the sinusoidal, and perhaps, in the canalicular membrane of hepatocytes. In transgenic and knockout mice, biliary secretion of cholesterol varies in proportion to the hepatic expression of SR-BI, and the established contribution of SR-BI to the sinusoidal uptake of HDL cholesterol is destined for secretion into bile.[115–117]

Of special note, *Abcg5/g8* has been identified as *Lith9* by QTL studies in mice.[9,10,118] As shown in Fig. 4, *Lith9* is localized on mouse chromosome 17 and is co-localized with a genetic biomarker *D17Mit155* at approximately 55 centimorgans (cM). In the *Lith9* QTL

region, *Abcg5/g8* is a strong candidate for this gallstone gene. Subsequently, *ABCG5/G8* is found to be associated with gallstones in patients (human *LITH9*). Furthermore, many research groups reported that two gallstone-associated variants in *ABCG5/G8*, specifically *ABCG5*-R50C and *ABCG8*-D19H, are involved in the pathogenesis of gallstones not only in Germans and Chileans, but also in Chinese and Indians.[12–19] These studies strongly suggest that *ABCG5*-R50C and *ABCG8*-D19H may play a crucial role in hepatic cholesterol hypersecretion, thus leading to the formation of cholesterol-supersaturated bile in humans.

Sitosterolemia is caused by a mutation in either the *ABCG5* or the *ABCG8* gene alone, but not in both simultaneously, and hepatic cholesterol secretion is reduced, but not completely eliminated in these patients.[105,119–121] To explore the mechanism underlying the effect of ABCG5/G8 on biliary sterol secretion, biliary cholesterol and sitostanol secretion is quantified for 6 h in Abcg8 knockout mice. Mass transport rate of [^{3}H]sitostanol from plasma HDL into bile is significantly faster than that of [^{14}C]cholesterol in wild-type mice; however, reduced amounts of [^{14}C]cholesterol and no [^{3}H] sitostanol are detected in bile of Abcg8 knockout mice.[113] These results clearly demonstrate that the deletion of the *Abcg8* gene alone significantly reduces, but does not eliminate hepatic cholesterol secretion. In addition, biliary cholesterol studies found that hepatic cholesterol output is significantly reduced, but cholesterol is still secreted into bile in mice with the deletion of either *Abcg5* or *Abcg8* alone, or both.[111–113,122,123] Consistent with the human results, these mouse data strongly suggest that an ABCG5/G8-independent pathway could also be involved in regulating hepatic cholesterol secretion in humans and mice.

Thus, it needs to be further investigated whether disruption of the *Abcg5/g8* genes or the *Abcg8* gene alone protects against the formation of cholesterol gallstones in gallstone-susceptible C57BL/6J mice fed a lithogenic diet for 8 weeks.[124] It is surprising to find that although the prevalence of gallstones is significantly reduced in Abag5/g8 double knockout and Abag8 knockout mice, classical parallelogram-shaped cholesterol monohydrate crystals and gallstones are still found in these mice during the 8-week period of the lithogenic diet feeding. In addition, these studies[124] provided clear evidence showing that (i) the ABCG5/G8-independent pathway accounts for 30%–40% of hepatic cholesterol output in the lithogenic state and has an effect on regulating biliary secretion of cholesterol in response to high dietary cholesterol; (ii) in the absence of ABCG5/G8, it plays a pivotal role in biliary cholesterol secretion and the pathogenesis of cholesterol gallstones; (iii) it is able to regulate hepatic secretion of HDL-derived cholesterol, but not sitostanol; and (iv) its activity in the liver is not regulated by the LXR agonist through the LXR signaling pathway. These results support a novel concept that the ABCG5/G8-independent pathway is essential for regulating hepatic cholesterol secretion in the absence of ABCG5/G8 and also plays a determinant role in gallstone formation in mice.

Although biliary phospholipids are possibly derived from the cell membranes of hepatocytes, their compositions differ significantly. The cell membranes of hepatocytes contain high levels of phosphatidylcholine (such as lecithin), phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine, and sphingomyelin. The major source of phosphatidylcholine molecules destined for secretion into bile is hepatic synthesis. However, a fraction of biliary phosphatidylcholines may also originate from the surface phospholipid

coat of HDL particles. A P-glycoprotein member of the multi-drug resistance gene family, ABCB4 plays an important role in regulating hepatic secretion of biliary phospholipids because the deletion of the *Abcb4* gene results in a complete inhibition of biliary phospholipid secretion in mice.[125] ABCB4 may be responsible for the translocation or "flip" of phosphatidylcholines from the endoplasmic (inner) to ectoplasmic (outer) leaflet of the canalicular membrane bilayer, and the action of ABCB4 may form phosphatidylcholine-rich microdomains within the outer membrane leaflet.[126–130] Furthermore, the mutation of the *ABCB4* gene in humans is the molecular defect underlying progressive familial intrahepatic cholestasis, type 3 (PFIC3).[127,131–135] Biliary phospholipids also play a key role in solubilizing excess cholesterol in vesicles. Low phospholipid-associated cholelithiasis (LPAC) is characterized mainly by the occurrence of intrahepatic and gallbladder microlithiasis in young adults associated with *ABCB4* mutations.[136–138] The Abcb4 knockout mouse is an excellent model for studying the pathogenesis of LPAC. Even on a chow diet, Abcb4 knockout mice spontaneously develop gallstones that are composed mainly of needle-shaped anhydrous cholesterol crystals, which form in phospholipid-deficient gallbladder bile with its relative biliary lipid composition that is in the far-left crystallization region of the phase diagram.[130] These studies support the concept that this gene is a monogenic risk factor for this "peculiar" form of cholesterol gallstones and a target for novel therapeutic strategies.

After being secreted into bile and entering the intestine, ~95% of the bile acids are returned to the liver through the enterohepatic circulation via an active transport and absorption by a specific bile acid transporter, apical sodium-dependent bile acid transporter predominantly in the distal ileum.[139–141] As a result, newly synthesized bile acids in the liver contribute only a small fraction (less than 5%) to biliary secretion, which compensate for bile acids that escape intestinal absorption and are lost in the feces. Therefore, biliary bile acids consist of those that are newly synthesized in the liver and those undergoing enterohepatic cycling.[139,142,143] The hepatic secretion of biliary bile acids is determined by ABCB11, a bile acid export pump on the canalicular membrane of hepatocytes.[144–148] Hepatic secretion of bile acids could directly affect phospholipid vesicle secretion,[49,149–151] although the molecular mechanism by which bile acid secretion is coupled to cholesterol and phospholipid secretion is still unclear. The relationship between bile acid secretion and cholesterol secretion has been found to be curvilinear. At low bile acid secretion rates (less than 10 μmol/h/kg), more cholesterol is secreted per molecule of bile acid than at higher rates. Although bile acid secretion rates are not usually low in normal subjects, they could diminish during prolonged fasting, during the overnight period, and with substantial bile acid losses, such as with a biliary fistula or ileal resection when the liver cannot sufficiently compensate with increased bile acid synthesis. In contrast, at high bile acid secretion rates—for example, during and after eating—biliary saturation is less than during the interprandial period.

Recently, genetic analysis in mice supports the candidacy of the G protein-coupled receptor 30 (GPR30), a novel estrogen receptor, for a new gallstone gene *Lith18*.[8,67,152,153] Of special note is that ~50% of cholesterol is converted to bile acids in the liver each day in humans and in mice. Because GPR30 is localized in the endoplasmic reticulum, but not the nucleus, of hepatocytes, GPR30 activation by estrogen possibly through the epidermal growth factor receptor signaling cascade inhibits hepatic cholesterol 7α-hydroxylase and the

classical pathway of bile acid synthesis, thereby leading to the availability of excess cholesterol for hepatic hypersecretion and bile lithogenesis.[154]

7. Cholesterol nucleation and crystallization in supersaturated bile

To systematically study the sequences of cholesterol crystallization, solid cholesterol crystal growth, and gallstone formation, gallbladder bile is carefully investigated at various time points using phase contrast and polarizing light microscopy in mice during the 8-week period of lithogenic diet feeding.[155] Representative photomicrographs of cholesterol crystallization and gallstone formation in mice are shown in Fig. 5. After gallbladder bile becomes supersaturated with cholesterol, i.e., CSI values are greater than 1.0, large amounts of non-birefringent amorphous mucin gel are accumulated in the gallbladder lumen, followed by the formation of numerous liquid crystals. In general, minimally sized, non-birefringent, and scattered small liquid crystals appear first. Non-birefringent aggregated liquid crystals with 1–5 μm of particles in diameter are found subsequently. If CSI values continue to increase in bile, fused liquid crystals are formed, which are birefringent with focal conic Maltese-cross textures and greater than 0.5–1.0 μm in size. In addition, some anhydrous cholesterol crystals are infrequently found. They are denoted as arc-like crystals that are short curved rods and rarely are filamentous, and tubular crystals that often appear to fracture at their ends producing classical cholesterol monohydrate crystals. Typical solid plate-like cholesterol monohydrate crystals are 79.2° and 100.8° angled parallelograms, often with a small notched corner. Mucin gel, a potent pro-nucleating agent, often promotes the growth and agglomeration of solid cholesterol crystals. Amorphous masses of cholesterol monohydrate crystal are defined loosely as agglomerated sheets. Sandy stones are encircled by mucin gel, and individual cholesterol monohydrate crystals are often found to project from the edges of sandy stones. Finally, true gallstones are exhibited with typical round contours and black centers under polarizing light microscopy.

It is well-known that the precipitation of solid cholesterol monohydrate crystals from supersaturated bile is the first irreversible physical-chemical step in gallstone formation. To study the characteristics, metastable intermediates, and kinetics in the phase transitions of bile, a series of phase diagrams that consist of cholesterol, phospholipids, and bile acids are generated for investigating the regions wherein different sequences of metastable intermediates, such as cholesterol crystallization sequences, occur. Five distinct crystallization pathways A to E have been identified in cholesterol-phospholipid-mixed bile acid model bile systems,[156] with each of these cholesterol crystallization pathways illustrating a different sequence of phase transitions. These phase transitions include an anhydrous cholesterol pathway and a liquid crystalline pathway to the formation of classical solid plate-like cholesterol monohydrate crystals.[156,157] Furthermore, five crystallization pathways in model bile systems are carefully investigated as a function of total lipid concentration, CSI value, bile acid composition (hydrophilic-hydrophobic index), cholesterol to phospholipid ratio, cholesterol to bile acid ratio, bile acid to phospholipid ratio, and temperature.[156] These cholesterol crystallization pathways found in model bile systems have been confirmed in native human and mouse gallbladder bile.[155,158–160]

The growth of solid cholesterol crystals starts as soon as cholesterol nucleation and crystallization occurs and this process is greatly accelerated by mucin gel, a potent pro-nucleating agent. Fig. 6 shows three modes of solid cholesterol crystal growth habits as observed by phase contrast and polarizing light microscopy in supersaturated gallbladder bile during the early stage of cholesterol gallstone formation in mice fed the lithogenic diet.[154,161] The first mode of solid cholesterol crystal growth habits is the proportional enlargement patterns that lead to solid cholesterol crystals larger in one direction, length, or width. The second mode is the spiral dislocation growth in which the pyramidal surface contains numerous growth spirals nucleated and crystallized by a screw dislocation. The third mode is the twin crystal growth in which the crystals grow upright and perpendicular to the surface. These solid cholesterol crystal growth habits are found not only in native mouse gallbladder bile, but also in model bile systems.[162] Obviously, these crystal growth modes enlarge solid cholesterol crystals in size and promote the development and evolution of solid cholesterol crystals to microlithiasis and eventually to macroscopic stones. More importantly, in the presence of a heterogeneous pro-nucleating agent, such as mucin gel, higher CSI values promotes more rapid precipitation of solid plate-like cholesterol monohydrate crystals from a phase-separated liquid-crystalline phase in gallbladder bile, followed by growth and agglomeration of these solid cholesterol crystals into mature and macroscopic stones. When CSI values are higher in bile, this process is faster. These findings in mice provide clear evidence showing that these three modes of solid cholesterol crystal growth habits closely recapitulate the early events of cholesterol gallstone formation in humans.

8. Conclusion and future research

Many new findings from physical-chemical, biochemical, genetic, and molecular biological studies of gallstones in humans and animals have clearly demonstrated that interactions of five primary defects lead to the formation of cholesterol gallstones. A novel concept has been established that cholesterol gallstone disease is determined by multiple *Lith* genes, which is a dominant trait. However, no mode of inheritance fitting to the Mendelian pattern is found in most cases. Although hepatic hypersecretion of biliary cholesterol is the primary pathogenic defect, other defects also play a critical role in the pathogenesis of cholesterol gallstone formation, which include unphysiological supersaturation with cholesterol (such as high CSI values in gallbladder bile), accelerated cholesterol nucleation and crystallization, rapid solid cholesterol crystal growth, impaired gallbladder motility, and increased amounts of the absorbed cholesterol delivered to the liver from the small intestine. Obviously, rapid growth and agglomeration of solid cholesterol crystals to form microlithiasis and macroscopic stones is a consequence of both gallbladder mucin hypersecretion and gel formation with impaired gallbladder emptying, leading to the formation of biliary sludge, the precursor of gallstones.

The gallstone (*Lith*) gene map has been updated, which lists all known genetic loci that confer gallstone susceptibility, as well as candidate genes in inbred strains of mice.[87] Understanding molecular genetics of gallstone disease in mice will push for the identification of human *Lith* genes. In addition, genetic analysis of *Lith* genes in mouse models will open the avenue for searching for the orthologous human *Lith* genes and for

exploring their cholelithogenic effects in humans. These studies should lead to the discovery of lithogenic actions of each of the *Lith* genes, providing novel insights into the molecular and cellular mechanisms that determine the formation of cholesterol gallstones. More importantly, the ABCG5/G8-dependent and the ABCG5/G8-independent pathways play critical roles in the regulation of hepatic cholesterol secretion, suggesting that both pathways are potential therapeutic targets for gallstones. Determining the molecular and cellular mechanisms on the formation of cholesterol-supersaturated bile may lead to novel therapeutic approaches through modulating both the ABCG5/G8-dependent and the ABCG5/G8-independent pathways for the prevention and the treatment of cholesterol gallstone disease that affects millions in westernized societies.

Acknowledgements

This work was supported in part by research grants DK101793 and DK106249 (to DQ-HW), both from the National Institutes of Health (US Public Health Service).

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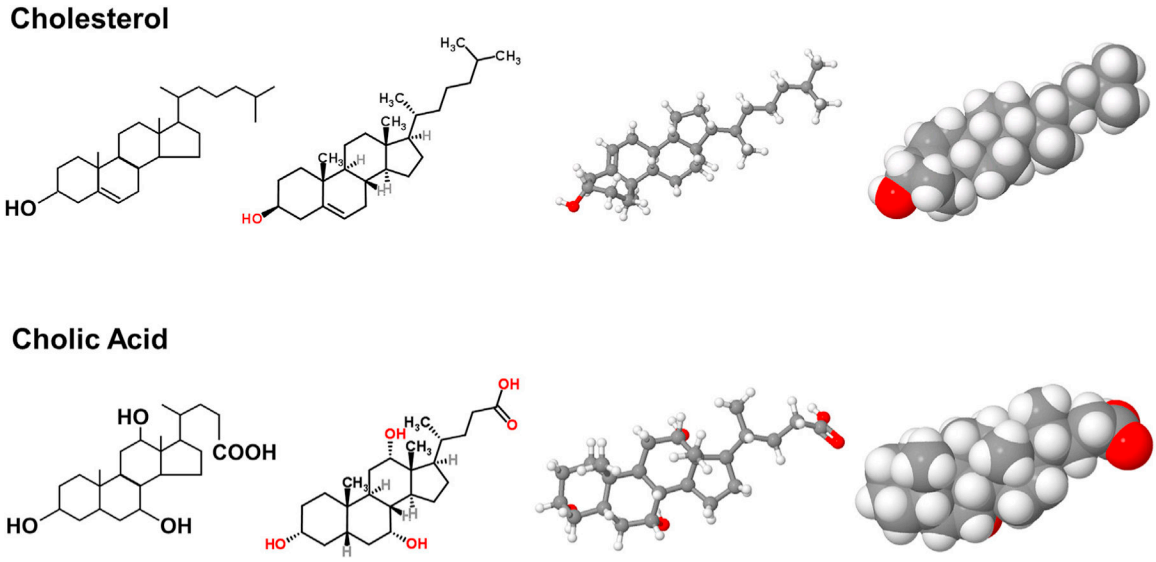

Fig. 1.

From left to right are the molecular structures, the standard chemical formulae, the perspective formulae, and the space-filling models of cholesterol (top panel) and cholic acid (bottom panel), respectively.

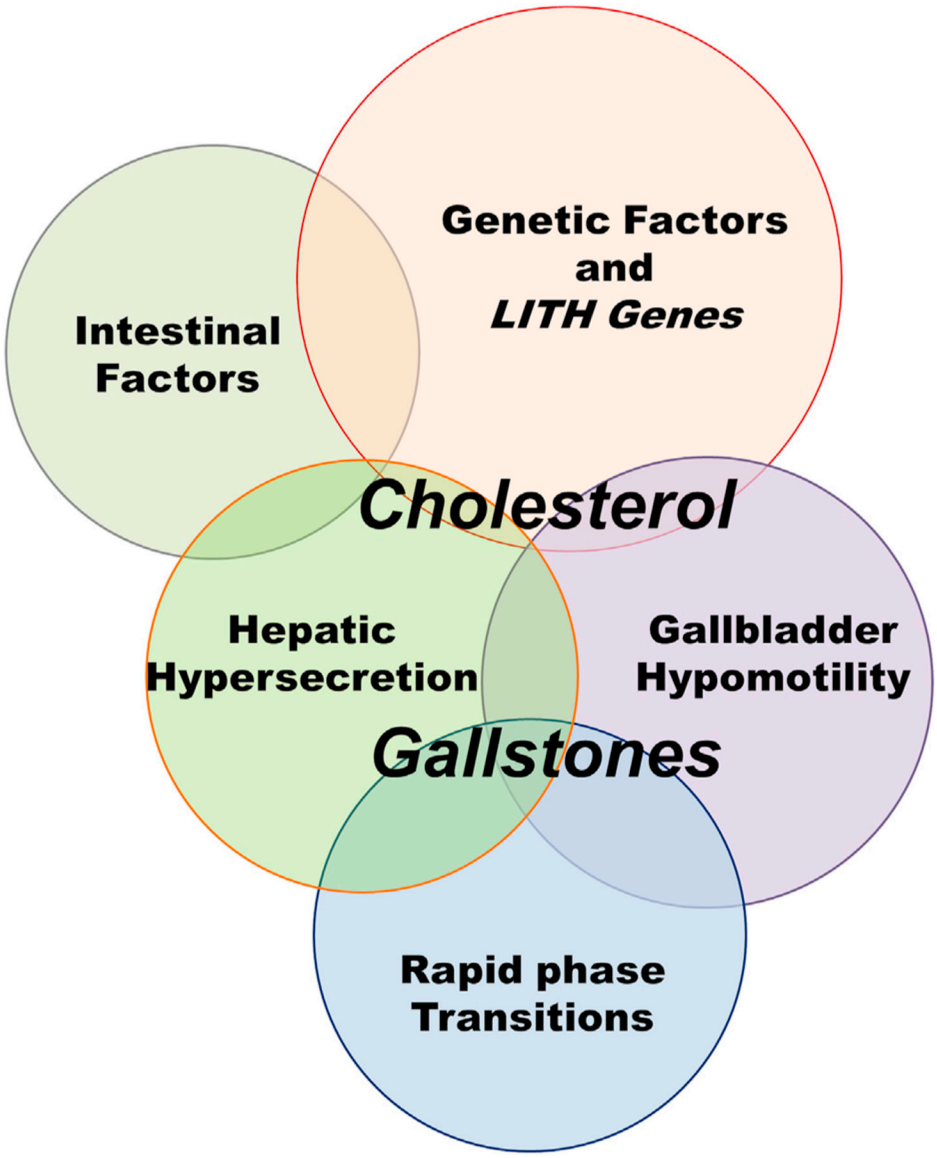

Fig. 2. Venn diagram of five defects that work together to promote cholesterol crystallization and gallstone formation.

Although many candidate *Lith* genes have been identified in mouse models, the identification of human *LITH* genes and their contributions to gallstones are being extensively investigated. Hepatic cholesterol hypersecretion into bile is the primary defect and is the outcome in part of a complex genetic predisposition, thereby leading to the formation of cholesterol-supersaturated bile. Reproduced with slightly modifications and with permission from.[8]

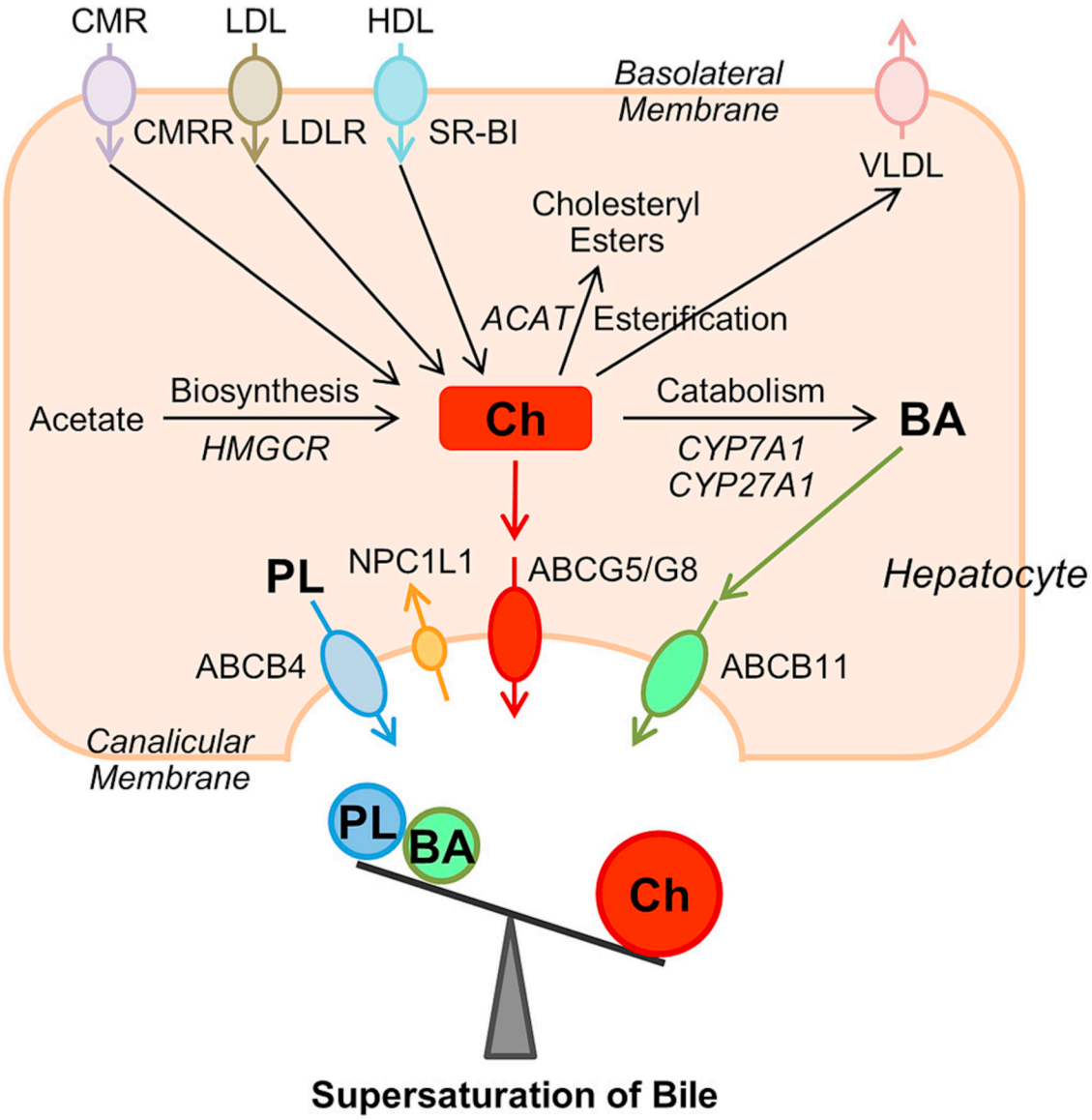

Fig. 3. This diagram shows uptake, biosynthesis, catabolism, and biliary secretion of cholesterol at the hepatocyte level, as well as the formation of supersaturated bile.

Hepatic secretion of biliary cholesterol (CH), bile acids (BA), and phospholipids (PL) across the canalicular membrane is determined by three lipid transporters, ABCG5/G8, ABCB11, and ABCB4, respectively. The Niemann-Pick C1-Like 1 (NPC1L1) protein may play a weak role in taking cholesterol back from hepatic bile into the hepatocyte. Abbreviations: ABC, ATP-binding cassette (transporter); ACAT acyl- coenzyme A: cholesterol acyltransferase; CMR, chylomicron remnants; CMRR, CMR receptor; CYP27A1, sterol 27-hydroxylase; CYP7A1, cholesterol 7α-hydroxylase; HDL, high-density lipoprotein; HMGCR, 3-hydroxy-3-methylglutaryl-coenzyme A reductase; LDL, low-density lipoprotein; LDLR, LDL receptor; SR-BI, scavenger receptor class B type I; VLDL, very-low-density lipoprotein.

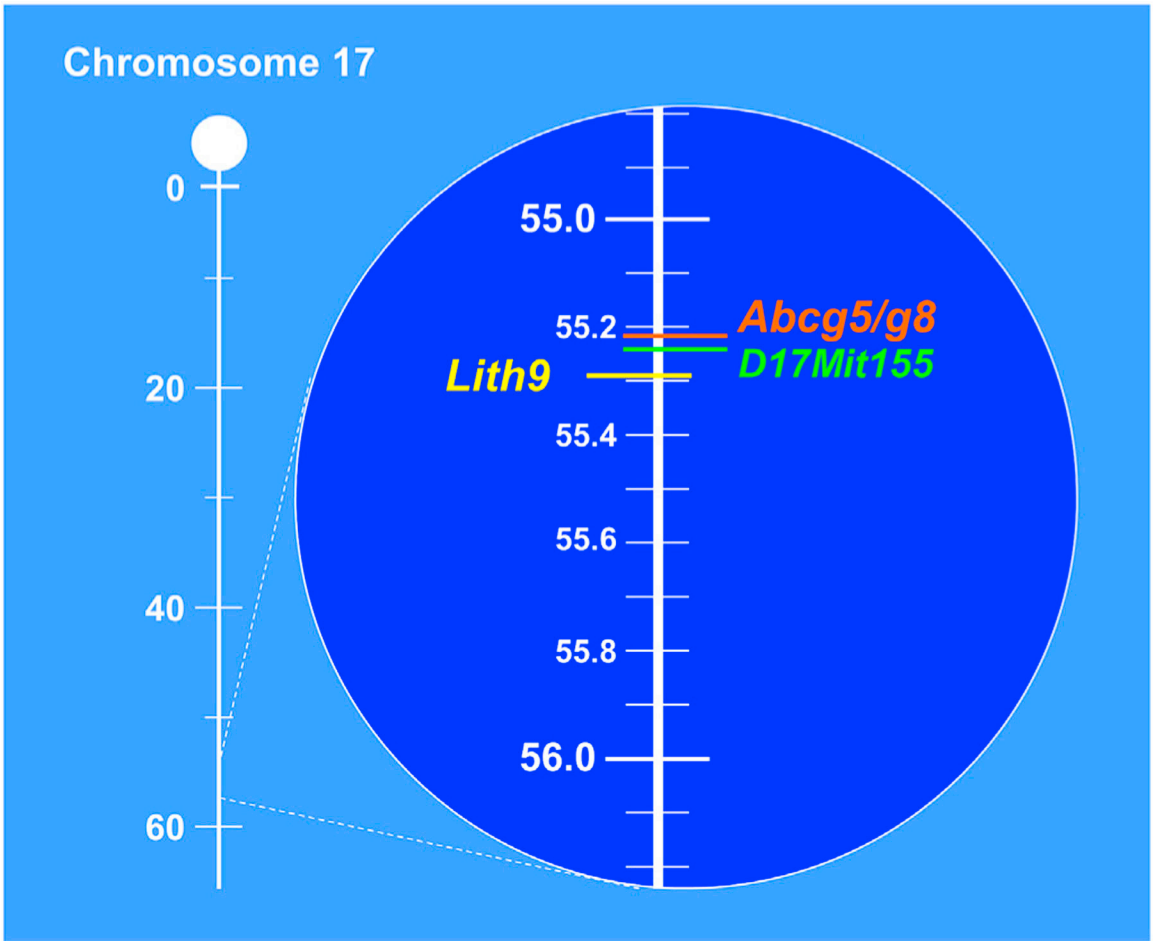

Fig. 4. Composite map of quantitative trait locus (QTL) for *Lith9* gene that is localized on mouse chromosome 17.

A vertical line represents chromosome 17, with the centromere at the top; genetic distances from the centromere (horizontal white lines) are indicated to the left of the chromosomes in centimorgans (cM). Chromosomes are drawn to scale, based on the estimated cM position of the most distally mapped locus taken from Mouse Genome Database. The gallstone QTL (*Lith9* gene) is represented by a horizontal yellow line, as well as the *Abcg5/g8* gene location is indicated by a horizontal orange line. A genetic biomarker, D17Mit155, which is co-localized with *Lith9*, is indicated by a horizontal green line with the marker symbol to the right.

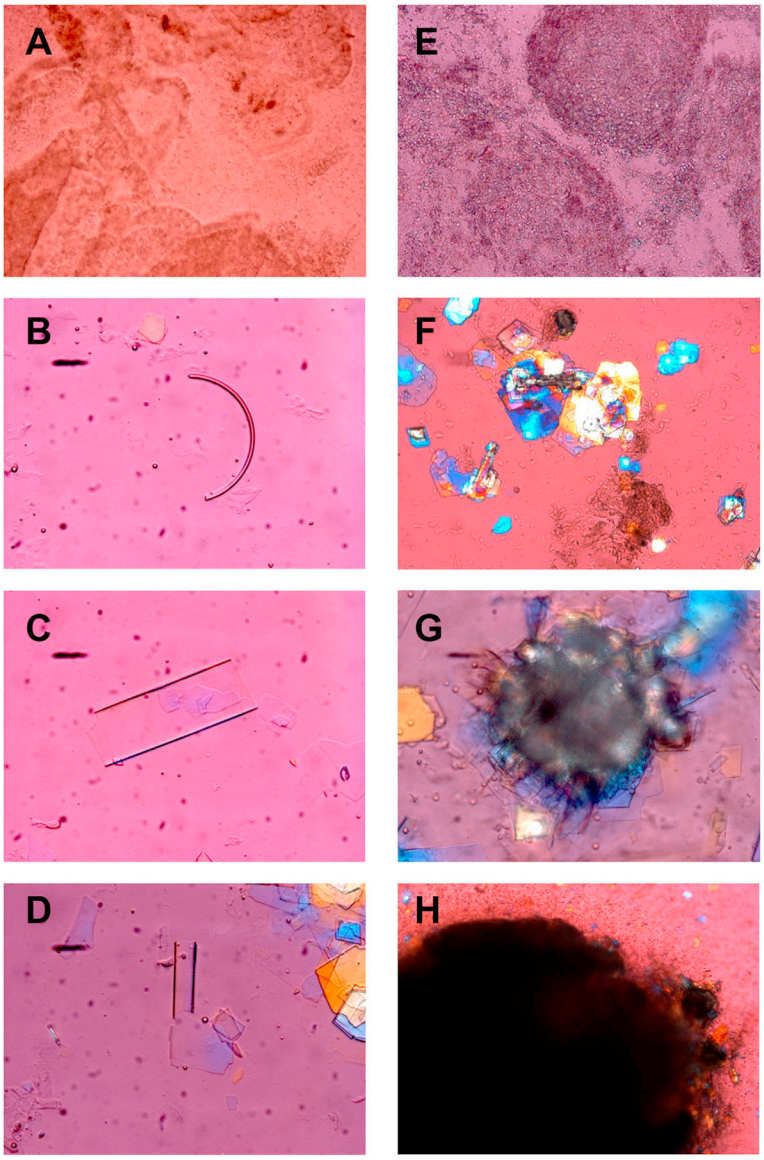

Fig. 5. Representative photomicrographs of cholesterol crystallization and gallstone formation found in gallbladder bile by phase contrast and polarizing light microscopy.

(**A**) Non-Birefringent amorphous mucin gel; (**B**) arc-like (possible anhydrous cholesterol) crystal; (**C**) tubular crystal; (**D**) tubular crystal fracturing at the end to produce plate-like cholesterol monohydrate crystals; (**E**) numerous aggregated non-birefringent liquid crystals and few fused liquid crystals; (**F**) agglomerates of typical cholesterol monohydrate crystals, with 79.2° and 100.8° angles, and often a notched corner; (**G**) disintegrable amorphous sandy stones surrounded by mucin gel, with individual plate-like cholesterol monohydrate crystals projecting from the edges; (**H**) true gallstones displaying rounded contours and black centers from light scattering/absorption. All magnifications are × 800, except F and G × 400 and H × 200, by polarizing light microscopy. Reproduced with slightly modifications and with permission.[161]

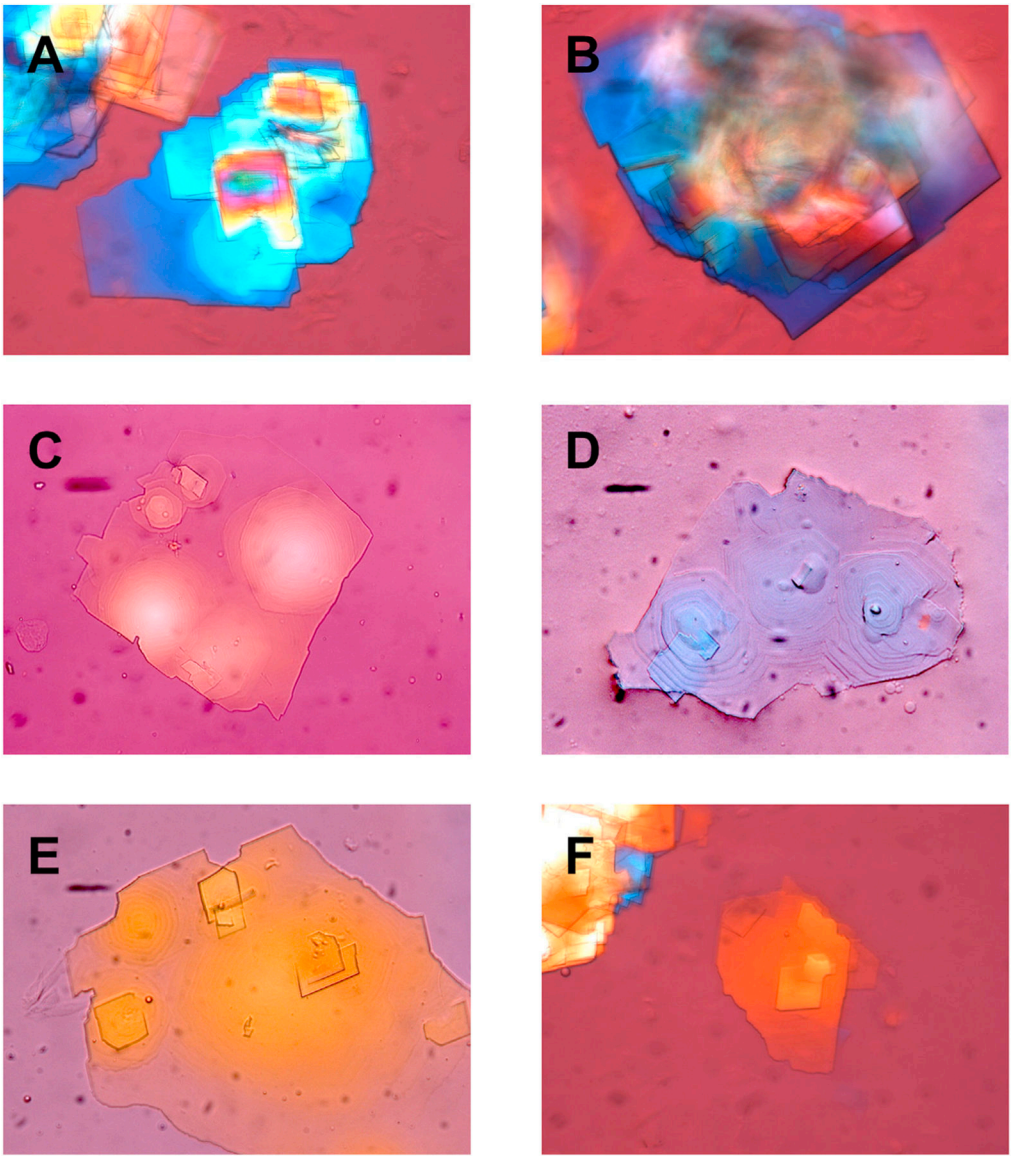

Fig. 6. Three modes of cholesterol crystal growth habits in mice during the 15-day period of lithogenic diet feeding:

(**A** and **B**) proportional enlargement patterns, (**C** and **D**) spiral dislocation growth patterns, and (**E** and **F**) twin crystal growth patterns. The twin crystals grow upright and perpendicular to the surface. These three modes of cholesterol crystal growth habits significantly increase solid cholesterol crystals in size. All magnifications are × 800 using polarizing light microscopy. Reproduced with slight modifications and with permission.[161]