Review Article

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

Helen H. Wang a, Tiangang Li b, Piero Portincasa c, David A. Ford d, Brent A. Neuschwander-Tetria, Patrick Tso e, David Q.-H. Wang a, *

a Department of Internal Medicine, Division of Gastroenterology and Hepatology, Saint Louis University School of Medicine, St. Louis, MO, USA
b Department of Pharmacology, Toxicology and Therapeutics, Kansas University Medical Center, Kansas City, KS, USA
c Clinica Medica “A. Muri”, Department of Biomedical Sciences and Human Oncology, University of Bari “Aldo Moro” Medical School, Bari, Italy
d Department of Biochemistry and Molecular Biology, Saint Louis University School of Medicine, St. Louis, MO, USA
e Department of Pathology and Laboratory Medicine, University of Cincinnati College of Medicine, Cincinnati, OH, USA

A R T I C L E   I N F O

Article history:
Received 23 November 2016
Received in revised form 23 January 2017
Accepted 3 February 2017

Keywords:
Bile flow
Bile acid
Biliary secretion
Lith gene
Micelle
 Vesicle

A B S T R A C T

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

© 2017 Published by Elsevier B.V. on behalf of The Third Affiliated Hospital of Sun Yat-Sen University. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

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. 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). Modern chemical analysis of the bile of Asian black bears (Ursus thibetanus or Sele-narctos 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. 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. However, the therapeutic effect of UDCA is not always achieved in clinical practice because of a high recurrence rate of gallstones. 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.

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, 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. 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 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. 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 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.

After cholesterol was found to be an alcohol by Berthelot in 1859, 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 identified that the empirical formula of cholesterol was C27H46O, indicating that cholesterol was not a straight-chain compound with a double bond, since it did not have enough hydrogen atoms to bond 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, Taintor isolated a substance from rye seeds infected with ergot, which closely resembled cholesterol. This compound was named ergosterin (now called ergosterol). Furthermore, the empirical formulae of cholic acid (C24H40O4), which was found by Mylius in 1886, 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 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, Wieland first described a brief history of how three bile acids (including cholic, deoxycholic, and lithocholic acids) were discovered and then, summarized and chemical experiments of bile acids. Based on his experimental results, he proposed a possible chemical structure of bile acid. In his Nobel lecture, 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 cholic acid. Unfortunately, the steroid nucleus of bile acid and cholesterol shown in their Nobel lectures was incorrect. 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. 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. 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. 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 isomers of the 3,6,7-trihydroxy bile acids in rat bile. Subsequently, William Elliott synthesized these bile acids and investigated their chemical and chromatographic properties. 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 α configuration. The hydrogen atoms at C-9 and C-14 are in β 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.

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. 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. Furthermore, the vesicles that are composed primarily of phospholipids also greatly promote the solubility of cholesterol in bile.

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. 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. 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. 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. 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.
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 are48: (i) it is a major route for the elimination of bile acids. Three important physiological functions of bile formation are48: (ii) it represents an important pathway for the removal of drugs, toxins, and waste products 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 cholesterol 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. 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. 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. 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. 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. The administration of estrogen to postmenopausal

Please cite this article in press as: Wang HH, et al., New insights into the role of Lith genes in the formation of cholesterol-supersaturated bile, Liver Research (2017). http://dx.doi.org/10.1016/j.livres.2017.05.005
women and estrogen therapy to men with prostatic carcinoma display similar lithogenic effects, leading to hepatic cholesterol hypersecretion and biliary lithogenicity. 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. 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. 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.

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 its transport in chylomicrons and the chylomicrons, and subsequently, chylomicron remnants after chylomicrons are hydrolyzed by plasma lipoprotein lipase and hepatic lipase. 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. 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. 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. 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. 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 gallbladder bile. 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.

6. Disruption of hepatic lipase 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. 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. Accumulating 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. 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. 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 1 (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.

Of special note, Abcg5/g8 has been identified as LITH9 by QTL studies in mice. 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. 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. 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 [3H]sitostanol from plasma HDL into bile is significantly faster than that of [14C]cholesterol in wild-type mice; however, reduced amounts of [3H]cholesterol and no [3H]sitostanol are detected in bile of Abcg8 knockout mice. These results clearly demonstrate that the deletion of the Abcg8 gene alone significantly reduces, but does not eliminate hepatic cholesterol absorption.

Please cite this article in press as: Wang HH, et al., New insights into the role of LITH genes in the formation of cholesterol-supersaturated bile, Liver Research (2017), http://dx.doi.org/10.1016/j.livres.2017.05.005.
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 ABCC5/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 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 provided clear evidence showing that (i) the ABCC5/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 ABCC5/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 sitosterol; 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 ABCC5/G8-independent pathway is essential for regulating hepatic cholesterol secretion in the absence of ABCC5/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.125–127 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.128–130 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 of a mixed monohydrate cholesterol crystal in the liver and gallbladder.139,140 The hepa tic secretion of biliary bile acids is determined by ABCB11, a bile acid export pump on the canalicular membrane of hepatocytes. Hepatic secretion of bile acids could directly affect phospholipid vesicle secretion.141–144 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 Lith1b.145–149 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.
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. 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-

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.

Please cite this article in press as: Wang HH, et al., New insights into the role of Lith genes in the formation of cholesterol-supersaturated bile, Liver Research (2017), http://dx.doi.org/10.1016/j.livres.2017.05.005
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 frequently 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 pronucleating 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, 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. 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. These cholesterol crystallization

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.
pathways found in model bile systems have been confirmed in native human and mouse gallbladder bile.\textsuperscript{153,156–160}

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.\textsuperscript{162} 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.\textsuperscript{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 human and mouse gallbladder bile, but also in model bile systems.\textsuperscript{162} Obviously, these crystal growth modes enlarge solid cholesterol crystals in size and promote the development and evolution of solid cholesterol crystals into mature and macroscopic stones. More importantly, in the presence of a heterogeneous pro-nucleating agent, such as mucin gel, higher CSI values promote more rapid precipitation of solid plate-like cholesterol mono-hydrate 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.\textsuperscript{17} 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.

Conflict of interest

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

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).

References

1. Wang DQ, Carey MC. Therapeutic uses of animal bile in traditional Chinese medicine: an ethnopharmacological, biophysical chemical and medicinal review. World J Gastroenterol. 2014;20:9952–9975.

2. Portincasa P, Caiafa AD, Bonfante L, Wang DQ. Therapy of gallstone disease: what it is, what it what it will be. World J Gastrointest Pharmacol Ther. 2012;3:7–20.

3. Portincasa P, Wang DQ, Gallstones. In: Podolsky DK, Camilleri M, Fitz JG, Kalloo AN, Shanahan F, Wang TC, eds. Yamada’s Textbook of Gastroenterology. 6 vol. 2. Hoboken, New Jersey: Wiley-Blackwell; 2015:1808–1834.

4. Portincasa P, Wang DQ, Gallstones. In: Podolsky DK, Camilleri M, Fitz JG, Kalloo AN, Shanahan F, Wang TC, eds. Yamada’s Atlas of Gastroenterology. 5. Hoboken, New Jersey: Wiley-Blackwell; 2016:335–353.

5. Di Caia A, Wang DQ, Wang HH, Bonfante L, Portincasa P. Targets for current pharmacologic therapy in cholesterol gallstone disease. Gastroenterol Clin North Am. 2010;39:245–264. viii–ix.

6. Gurusamy KS, Davidson BR. Surgical treatment of gallstones. Gastroenterol Clin North Am. 2010;39:229–244. viii.

7. Khanuja B, Cheah YC, Hunt M, et al. Lith1, a major gene affecting cholesterol gallstone formation among inbred strains of mice. Proc Natl Acad Sci U. S. A. 1995;92:7729–7733.

8. Wang HH, Portincasa P, Afdhal NH, Wang DQ. Lith genes and genetic analysis of cholesterol gallstone formation. Gastroenterol Clin North Am. 2010;39:185–207 (vii–viii).

9. Wittenburg H, Lyons MA, Li R, et al. Association of a lithogenic ABCG5/ABCg8 allele on Chromosome 17 (Lith9) with cholesterol gallstone formation. Gut. 2007;56:1591–1596.

10. Wittenburg H, Lyons MA, Li R, Churchill GA, Carey MC, Paijen B, EXR and ABCG5/ABCg8 as determinants of cholesterol gallstone formation from quantitative trait locus mapping in mice. Gastroenterology. 2003;125:868–881.

11. Wittenburg H, Lyons MA, Li R, et al. QTL mapping for genetic determinants of lipoprotein cholesterol levels in combined crosses of inbred mouse strains. J Lipid Res. 2006;47:1780–1790.

12. Brunhage F, Acalovschi M, Tiriizu S, et al. Increased gallstone risk in humans conferred by common variant of hepatic ATP-binding cassette transporter for cholesterol. Hepatology. 2007;46:793–801.

13. Wang Y, Jiang ZY, Fei J, et al. ATP binding cassette G8 T400K polymorphism may affect the risk of gallstone disease among Chinese males. Clin Chim Acta. 2007;384:80–85.

14. Jiang ZY, Parini P, Eggertsen G, et al. Increased expression of LXRP alpha, ABCG5, ABCg8, and SR-BI in the liver from normolipidemic, nonobese Chinese gallstone patients. J Lipid Res. 2008;49:464–472.

15. Kuo KK, Shin SJ, Chen ZC, Yang YH, Yang JF, Hsiao PJ. Significant association of ABCG5 604Q and ABCG8 D19H polymorphisms with gallstone disease. Br J Surg. 2008;95:1005–1011.

16. Rudkowska I, Jones JP. Polymorphisms in ABCG5/8 transporters linked to hypercholesterolemia and gallstone disease. Nutr Rev. 2008;66:343–348.

17. Katsika D, Magnusson P, Krawczyk M, et al. Gallstone disease in Swedish twins: risk is associated with ABCG8 D19H genotype. J Intern Med. 2010;268:279–285.

18. von Kampen O, Buch S, Nothegel M, et al. Genetic and functional identification of the likely causative variant for cholesterol gallstone disease at the ABCG5/8 Lithogenic locus. Hepatology. 2013;57:2407–2417.
Donovan JM, Carey MC. Separation and quantitation of cholesterol. J Chromatogr. 1973;69:213–224.

54. Bourges M, Small DM, Dervichian DG. Biopolymers of lipidic associations. II. The ternary systems: cholesterol-lecithin-water. Biochim Biophys Acta. 1967;137:395–401.

55. Brecher P, Chobanian J, Small DM, Chobanian AV. The use of phospholipid vesicles in vitro studies on cholesterol ester hydrolysis. J Lipid Res. 1970;11:239–247.

56. Carey MC. Aqueous bile salt-cholesterol systems: equilibrium aspects. Hepatology. 1984;4:1515–1545.

57. Gantz DL, Wang DQ, Carey MC, Small DM. Cryoelectron microscopy of a nucleating model bile in vitro: ice formation of primordial vesicles. Biochim Biophys Acta. 1999;1465:349–355.

58. Hay DW, Cahalane MJ, Tomovezyka N, Carey MC. Molecular species of lecithin in human gallbladder bile. J Lipid Res. 1993;34:759–768.

59. Wang DQ, Adhahl NH. Gallstone disease. In: Feldman M, Friedman LS, Brandt L, eds. Sleisenger and Fordtran’s Gastrointestinal and Liver Disease. 9. Philadelphia: Elsevier Saunders; 2010:1089–1120.

60. Mendez-Sanchez N, Chavez-Tapia NC, Motola-Kuda D, et al. Metabolic syndrome as a risk factor for gallstone disease. World J Gastroenterol. 2005;11:1653–1657.

61. Hofmann AF, Hagey LK. Key discoveries in bile acid chemistry and biology and their clinical applications: history of the last eight decades. J Lipid Res. 2014;55:1533–1595.

62. Beutel JD. Crystal structures of vitamin D and related compounds. Nature. 1932;129:277–278.

63. Rosenheim O, King H. The chemistry of the sterols, bile acids, and other cyclic constituents of natural fats and oils. Annu Rev Biochem. 1934;3:87–110.

64. Cherayil GD, Hsia SL, Matschiner JT, et al. Bile acids. XVII. Metabolism of alpha-1-linoleic acid. J Biol Chem. 1958;233:1337–1340.

65. Lammert F, Gurusamy K, Ko CW, et al. Gall Nat Rev Dis Prim. 2016:2:16024.

66. Wang HH, Portincasa P, Wang DQ. Molecular pathophysiology and physical properties of the bile acids. J Lipid Res. 1987;28:1383–1395.

67. Wang HH, Portincasa P, Wang DQ. Overexpression of estrogen receptor alpha (ERalpha) genes in the formation of cholesterol-supersaturated bile, the pancreas, and the intestine. J Clin Invest. 1991;87:237–246.

68. Cirillo DJ, Wallace RB, Rodabough RJ, et al. Effect of estrogen therapy on gallbladder disease. JAMA. 2005;293:330–339.

69. Honore LH. Increased incidence of symptomatic cholesterol cholelithiasis in perimenopausal women receiving estrogen replacement therapy: a retrospective study. J Reprod Med. 1980;25:187–190.

70. Thijis C, Knipschild P. Oral contraceptives and the risk of gallstone disease: a meta-analysis. Am J Public Health. 1993;83:1113–1120.

71. Henriksen P, Emarsson K, Eriksson A, Kelter U, Angelin B. Estrogen-induced gallstone formation in males. Relation to changes in serum and biliary lipids during hormonal treatment of prostatic carcinoma. J Clin Invest. 1989;84:811–816.

72. Wang HH, Adhahl NH, Wang DQ. Overexpression of estrogen receptor alpha increases hepatic cholelithogenesis, leading to biliary hypersecretion in mice. J Lipid Res. 2006;47:778–786.

73. Wang DQ. Regulation of intestinal cholesterol absorption. Annu Rev Physiol. 2006;68:221–248.

74. Wang DQ, Zhang L, Wang HH. High cholesterol absorption efficiency and rapid biliary secretion of chylomicron remnant cholesterol enhance cholelithogenesis in gallstone-susceptible mice. Biochim Biophys Acta. 2005;1733:269–277.

75. Wang DQ, Adhahl NH. Gallstone disease. In: Feldman M, Friedman LS, Brandt L, eds. Sleisenger and Fordtran’s Gastrointestinal and Liver Disease. 10. Philadelphia: Elsevier Saunders; 2014:1100–1133.
Kozarsky KS, Donohue MH, Rigotti A, Iqbal SN, Edelman ER, Krieger M. Overexpression of the HDL receptor SR-BI alters plasma HDL and bile cholesterol levels. Lipids. 2009;44:223–417.

Wittenburg H, Lyons MA, Paigen B, Carey MC. Mapping cholesterol gallstone susceptibility (Lith genes) in inbred mice. Dig Liver Dis. 2003;35(Suppl 3):32–47.

Martens TA. Phytosterolemia, xanthomatosis and premature athero- sclerotic arterial disease: a case with high plant sterol absorption, impaired sterol elimination and low cholesterol synthesis. Eur J Clin Invest. 1980;10:35–39.

Behnke D, Bjorkhem I, Beil UF, von Bergmann K. Sterol absorption and balance in phytosterolemia evaluated by deuterium-labeled sterols: effect of sitosterol treatment. J Lipid Res. 1995;36:1763–1773.

Gould RG, Jones RJ, LeRoy GV, Wissler RW, Taylor CB. Absorbability of beta- sitosterol in humans. Metabolism. 1969;18:162–165.

Plosch T, Bloks VW, Terawasa Y, et al. Sirotosterolemia in ABC transporter G5-deficient mice is aggravated on activation of the liver-X receptor. Gastroenterology. 2004;126:290–301.

Wang HH, Li X, Patel SB, Wang DQ. Evidence that the adenosine triphosphate-binding cassette G5/G8-independent pathway plays a determining role in cholesterol gallstone formation in mice. Hepatology. 2016;64:853–864.

Smit JJ, Schinkel AH, Oude Elferink RP, et al. Homozygous disruption of the murine mdr2 P-glycoprotein gene leads to a complete absence of phospholipid from bile and to liver disease. Cell. 1997;91:451–462.

Oude Elferink RP, Beuers U. Targeting the ABCB4 gene to control cholesterol homeostasis. Expert Op Ther Targets. 2011:15:1173–1182.

Oude Elferink RP, Paulusma CC. Function and pathophysiological importance of ABCB4 (MDR3) and ABCG8 (MRP3, Pgamers A1). Biochim Biophys Acta. 2013;1833:38–53.

Langheim S, Yu L, von Bergmann K, et al. ABCG5 and ABCG8 require MDR2 for secretion of cholesterol into bile. J Lipid Res. 2005;46:1732–1738.

Dikkers A, Tietge UJ. Biliary cholesterol secretion: more than a simple ABC. J Lipid Res. 2010;51:594–5945.

Lammert F, Wang HH, Hillebrands B, et al. Spontaneous cholecytost-e and hepatolithiasis in Mdr2/-/- mice: a model for low phospholipid-associated cholelithiasis. Hepatology. 2004;39:17–128.

Davitz-Spaull A, Gonzales E, Bausan C, Jacobin M. The spectrum of liver diseases related to ABCB4 gene mutations: pathophysiology and clinical aspects. Semin Liver Dis. 2010;30:134–146.

Gonzales E, Davitz-Spaull A, Bausan C, Buffet C, Maurice M, Jacobin E. Liver diseases related to MRD3 (ABCB4) gene deficiency. Front Biosci. 2009;14: 4242–4256.

Stapelbroek JM, van Erpecum KJ, Klomp LW, Hovenh RH. Liver disease associated with canulcal transport defects: current and future therapies. J Hepatol. 2010;52:258–271.

Paulusma CC, Elferink RP, Janssen PL. Progressive familial intrahepatic chole- lystasis type I. Semin Liver Dis. 2010;30:117–124.

Janssen PL, Sturm E. Genetic cholestasis, causes and consequences for hepato-biliary transport. Liver Int. 2003;23:315–322.

Rosmoroduc O, Hermel B, Borel PY, Parc R, Taboury J, Poupon R. ABCB4 gene mutation-associated cholelithiasis in adults. Gastroenterology. 2003;125:1262–1269.

Rosmoroduc O, Hermel B, Poupon R. MRD3 gene defect in adults with symptomatic intrahepatic and gallbladder cholesterol cholelithiasis. Gastroenterology. 2010;139:1459–1467.

Rosmoroduc O, Poupon R. Low phospholipid associated cholelithiasis: associa- tion with mutation in the MD3/ABCB4 gene. Orphanet J Rare Dis. 2007;2:29.

Carey MC, Gahalane MJ. Enterohepatic circulation. In: Arias IM, Jakoby WB, Popper H, Schachter D, Shafritz DS, eds. The Liver: Biology and Pathobiology. 2. New York: Raven Press; 1988:573–616.

Hofmann AF, Hagey LR. Bile acids: chemistry, pathochemistry, biology, pathobiology, and therapeutics. Cell Mol Life Sci. 2008:65:2461–2483.

Hofmann AF. The enterohepatic circulation of bile acids in mammals: form and functions. Front Biosci. 2009;14:2584–2598.

Hofmann AF, Bile acids and the enterohepatic circulation. J Hepatol. 2005;42:322.

Hofmann AF, Bile acids transport: molecular characterization, function, and regulation. Physiol Rev. 2003;83:623–671.

Gonzales E, Bausan C, Jacobin M. Liver diseases related to MRD3 (ABCB4) gene deficiency. Front Biosci. 2009;14:2584–2598.

Hofmann AF, Bile acids and the enterohepatic circulation. J Hepatol. 2005;42:322.

Hofmann AF, Bile acids transport: molecular characterization, function, and regulation. Physiol Rev. 2003;83:623–671.

Gelfand TF, Stieger B, Hagenbuch B, et al. The sister of P-glycoprotein represents the canalicular bile salt export pump of mammalian liver. J Biol Chem. 1998;273:10046–10050.

Wang Z, Sale M, Liu ZM, et al. Targeted inactivation of sister of P- glycoprotein gene (spgp) in mice results in nonprogressive but persistent intrahepatic cholelithiasis. Proc Natl Acad Sci U S A. 2001:98:1101–1106.

Wang T, Pan T, Liu L, et al. Severe cholestasis induced by cholic acid feeding of knockout mice of sister of P-glycoprotein. Hepatology. 2003;38: 1489–1499.
149. Cohen DE, Leighton LS, Carey MC. Bile salt hydrophobicity controls vesicle secretion rates and transformations in native bile. Am J Physiol. 1992;263:G386–G395.

150. Cohen DE, Leonard MR, Carey MC. In vitro evidence that phospholipid secretion into bile may be coordinated intracellularly by the combined actions of bile salts and the specific phosphatidylcholine transfer protein of liver. Biochemistry. 1994;33:9975–9980.

151. Fuchs M, Carey MC, Cohen DE. Evidence for an ATP-independent long-chain phosphatidylcholine translocator in hepatocyte membranes. Am J Physiol. 1997;273:G1312–G1319.

152. Lyons MA, Korstanje R, Li R, et al. Single and interacting QTLs for cholesterol gallstones revealed in an intercross between mouse strains NZB and SM. Mamm Genome. 2005;16:152–163.

153. Lyons MA, Wittenburg H. Cholesterol gallstone susceptibility loci: a mouse map, candidate gene evaluation, and guide to human LITH genes. Gastroenterology. 2006;131:1943–1970.

154. de Bari O, Wang TY, Liu M, Portincasa P, Wang DQ. Estrogen induces two distinct cholesterol crystallization pathways by activating ERalpha and GPR30 in female mice. J Lipid Res. 2015;56:1691–1700.

155. Wang DQ, Paigen B, Carey MC. Phenotypic characterization of Lith genes that determine susceptibility to cholesterol cholelithiasis in inbred mice: physical-chemistry of gallbladder bile. J Lipid Res. 1997;38:1395–1411.

156. Wang DQ, Carey MC. Complete mapping of crystallization pathways during cholesterol precipitation from model bile: influence of physical-chemical variables of pathophysiologic relevance and identification of a stable liquid crystalline state in cold, dilute and hydrophilic bile salt-containing systems. J Lipid Res. 1996;37:606–630.

157. Konikoff FM, Chung DS, Donovan JM, Small DM, Carey MC. Filamentous, helical, and tubular microstructures during cholesterol crystallization from bile. Evidence that cholesterol does not nucleate classic monohydrate plates. J Clin Invest. 1992;90:1155–1160.

158. Wang DQ, Carey MC. Characterization of crystallization pathways during cholesterol precipitation from human gallbladder biles: identical pathways to corresponding model biles with three predominating sequences. J Lipid Res. 1996;37:2539–2545.

159. Wang DQ, Cohen DE, Lammert F, Carey MC. No pathophysiologic relationship of soluble biliary proteins to cholesterol crystallization in human bile. J Lipid Res. 1999;40:415–425.

160. Wang HH, Afdhal NH, Gendler SJ, Wang DQ. Evidence that gallbladder epithelial mucin enhances cholesterol cholelithogenesis in MUC1 transgenic mice. Gastroenterology. 2006;131:210–222.

161. Wang HH, Portincasa P, Liu M, Tso P, Samuelson LC, Wang DQ. Effect of gallbladder hypomotility on cholesterol crystallization and growth in CCK-deficient mice. Biochim Biophys Acta. 2010;1801:138–146.

162. Toor EW, Evans DF, Cussler EL. Cholesterol monohydrate growth in model bile solutions. Proc Natl Acad Sci U. S. A. 1978;75:6230–6234.