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Review

Genetic background of cholesterol gallstone disease

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Abstract

Cholesterol gallstone formation is a multifactorial process involving a multitude of metabolic pathways. The primary pathogenic factor is hypersecretion of free cholesterol into bile. For people living in the Western Hemisphere, this is almost a normal condition, certainly in the elderly, which explains the very high incidence of gallstone disease. It is probably because the multifactorial background genes responsible for the high incidence have not yet been identified, despite the fact that genetic factors clearly play a role. Analysis of the many pathways involved in biliary cholesterol secretion reveals many potential candidates and considering the progress in unraveling the regulatory mechanisms of the responsible genes, identification of the primary gallstone genes will be successful in the near future.
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1. Introduction

Cholesterol gallstone disease is one of the most common disorders of the gastrointestinal tract in the Western Hemisphere. A number of independent risk factors for gallstone disease have been identified: age, gender, pregnancy and parity, chronic medication with estrogens and fibrates, obesity, rapid weight loss, pre-existing disease [diabetes mellitus, Crohn's disease, resection of terminal ileum, Gaucher disease, Down syndrome (DS)] and states associated with gallbladder stasis such as spinal cord injuries, prolonged fasting, long-term total parenteral nutrition, therapy with ocreotide analogues or somatostinoma and low physical activity. None of the listed risk factors explains the family clustering of either gallstone disease or the ethnic influences indicating involvement of genetic factors as a separate additional risk factor. The primary event on the way from gallstone free bile to cholesterol gallstones is supersaturation of bile with cholesterol. Saturation of bile with cholesterol is critically dependent on secretion rates of biliary lipids. Genes controlling the secretion rates are thus the first natural candidates.

Additional defects—gallbladder hypomotility, accelerated cholesterol crystallization rate and mucus hypersecretion—appear simultaneously in patients with cholesterol gallstone. These additional defects may be balanced differently in patients with solitary and multiple stones. Genes involved in control of gallbladder motility, secretion of biliary mucin and possibly non-mucin proteins represent the second source of the potential candidate genes.

The evidence for the genetic background of cholesterol gallstone disease in humans is mostly indirect and is based on geographic and ethnic differences, family and twin studies. Disposition to formation of cholesterol gallstones in humans is multifactorial and no pattern of inheritance fitting to the Mendelian pattern could be demonstrated in most cases. With the exception of multidrug resistance protein 3 (MDR3) and 7α-hydroxylase deficiency reported in the recently published studies of Rosmorduc et al. [1], Jacquemin [2], and Pullinger et al. [3], no gene mutation or polymorphism has been associated with cholesterol gallstones in humans. Fortunately, the mammalian genome is highly conserved and the vast majority of human genes have

Abbreviations: HDL, high-density lipoprotein; SREBP, sterol regulatory element binding protein; LDL, low-density lipoprotein; HMG-CoA, 3-hydroxy methyl-glutaryl-Coenzyme A; SR-BI, scavenger receptor class BI; ABC-transporter, ATP-binding cassette; HNF, hepatocyte nuclear factor; CYP, cytochrome *P*450; RXR, retinoid X receptor; LXR, liver X receptor; PPAR, peroxisome proliferator activating receptor; SHP, short heterodimeric partner; LRH1, liver receptor homolog 1; SCP2, sterol carrier protein 2; MDR, multidrug resistance protein; MRP, multidrug resistance-related protein; CA, cholic acid; CDCA, chenodeoxycholic acid

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their orthologues in other mammals. As a consequence, a similar genetic background might be expected in animal models of numerous human diseases. Mouse models provide comprehensive genetic resources and offer many possibilities for genetic manipulations. In addition, quantitative trait locus analysis may be used for identification of the primary genetic defects responsible for the phenotype of a multifactorial disease under controlled conditions. For these reasons, mouse models became the model of choice for studies of the genetic background of gallstones [4]. The first inventory of candidate mouse *Lith* genes has recently been reviewed by Lammert et al. [5].

2. Scope of the review

In this overview, we will focus on the predominant form of gallstone disease, formation of cholesterol stones. Although in addition to lipids also proteins may play a role in the etiology (see review in Ref. [6]), we will focus on the main biliary components cholesterol, phospholipid and bile acids. We will first discuss the metabolic pathways involved in metabolism of these lipids. Subsequently, we will attempt to couple this information to what is known from human epidemiology and genetic studies. Finally, a brief overview of the genetic diseases associated with gallstones will be presented.

3. Candidate genes involved in regulation of biliary cholesterol secretion

3.1. Recourses of biliary lipids

Since cholesterol in bile is solubilized in a complex mixture of bile salts and phospholipid, the concentration of these factors plays an important role in regulation of cholesterol saturation as given by the biliary cholesterol saturation index (CSI). The increased CSI observed in patients with cholesterol gallstones may result from increased biliary cholesterol secretion but may also be caused by decreased bile salt or phospholipid secretion or by a combination of these parameters.

The main organ for cholesterol metabolism is the liver. It regulates the synthesis of cholesterol, the conversion of cholesterol into bile acids, the secretion of cholesterol into bile either directly or as bile salts, the uptake and hydrolysis/metabolism of plasma cholesterol/esters in the form of high-density lipoprotein (HDL) or low-density lipoprotein (LDL) particles, the esterification of excess of free cholesterol, and the secretion of cholesterol into plasma. The source of cholesterol destined for biliary secretion is as yet not fully known. The major part is thought to be derived from a pre-existing hepatic cholesterol pool; however, this pool is not well defined. One of the sources, although this represents only a small fraction (5–20%) of total biliary cholesterol, is cholesterol from hepatic de novo cholesterol synthesis [7–9]. The second source of biliary cholesterol is cholesterol derived from plasma lipoprotein particles of which HDL seems to be the major supplier [10]. However, *Abca1* knock-out mice which are deficient in HDL show no decrease in biliary cholesterol output, suggesting that either HDL may not be that important or its role can easily be taken over by a parallel mechanism [11]. In contrast to HDL, no direct secretory pathway of LDL or VLDL cholesterol into bile has ever been reported. Bile acids are thought to be preferentially formed from newly synthesized cholesterol; however, depending on the conditions also cholesterol from plasma lipoprotein particles may be used as a precursor for bile acid synthesis [12,13]. Increased biliary cholesterol secretion may result from the increase of de novo synthesis, the decreased conversion of cholesterol into bile acids, the decreased esterification or the increased uptake of cholesterol from plasma lipoprotein particles. In addition, the amount of phospholipids and bile acids as well as the pattern of secreted bile salts is important for regulation of cholesterol secretion into bile. Clearly, defects in genes encoding proteins involved in all these processes may play an important role in the formation of cholesterol gallstones.

3.2. Cholesterol synthesis

Although newly formed cholesterol contributes only partially to biliary cholesterol, the increased cholesterol synthesis may still be one of the mechanisms involved in the formation of cholesterol gallstones. The key enzyme in de novo cholesterol synthesis pathway is 3-hydroxy methylglutaryl-Coenzyme A (HMG-CoA) reductase [14]. Both the gene expression and the activity of the enzyme are regulated by intracellular cholesterol concentrations via cholesterol-derived oxysterols. Oxysterols function as regulators of the activity of the sterol regulatory element binding proteins (SREBPs) (see below). These transcription factors regulate the expression of all genes in the cholesterol biosynthetic pathway [15]. Rodents fed a high-cholesterol diet accumulate cholesterol in the liver. Their cholesterol synthesis is reduced by decreasing the transcription of the genes encoding for HMG-CoA reductase [16], HMG-CoA synthase [17], farnesyl diphosphate synthase [18] and squalene synthase [19]. The activity of HMG-CoA reductase is controlled by regulation of the half-life of the protein and the state of phosphorylation of the protein. In rodents, a relation seems to exist between HMG-CoA reductase expression/activity and gallstone formation. The expression of HMG-CoA reductase in the liver was properly down-regulated in the more gallstone-resistant AKR strain upon feeding a high-cholesterol diet [20], whereas in C57L/J mice which are susceptible for gallstone formation, the expression of HMG-CoA reductase remained unchanged. Furthermore, in hamsters fed a cholesterol gallstone-inducing diet, the activity of HMG-CoA reductase increased enormously even

long before the appearance of gallstones [21]. In humans, however, a relation between the activity of HMG-CoA reductase and biliary cholesterol secretion is less clear. Ahlberg et al. [22] determined the HMG-CoA reductase activity in the liver of 55 patients (10 with adenomyoma of the gallbladder wall, 45 with cholesterol gallstones). Bile of these patients was supersaturated with cholesterol. HMG-CoA reductase activity in gallstone patients, however, was not different from that seen in the gallstone-free controls. In addition, in the gallstone-free control group and in the group of bile acid-treated gallstone patients but not in untreated gallstone patients, the saturation of bile correlated with the activity of HMG-CoA reductase in the liver. Studies of Maton et al. [23] showed that there was no correlation between the activity of HMG-CoA reductase and the biliary cholesterol output in nine patients. Moreover, a similar activity of HMG-CoA reductase was found in patients with gallstones and gallstone-free patients even though the saturation of the gallbladder bile was higher in gallstone patients [24]. A possible caveat in these studies is that the measured in vitro activity of the enzyme may not reflect the activity of HMG-CoA reductase in the intracellular milieu.

3.3. Cholesterol esterifying enzymes

Excess of intracellular cholesterol can be esterified by acyl-CoA cholesterol acyl-CoA transferase (ACAT) [25,26]. Cholesterol esters are then stored in cytosolic droplets or are secreted into the circulation as part of lipoprotein particles. Two *Acat* genes have been identified: *Acat1* and *Acat2* (or *Soat1* and *Soat2*, respectively). Although *Acat1* is expressed in many tissues [26,27], the highest levels in mice were found in macrophages and adrenocortical cells, whereas only low expression was seen in the liver and the small intestine [28,29]. In mice lacking the *Acat1* gene, cholesterol esters were absent in the adrenal glands and peritoneal macrophages [30]. In the liver, however, cholesterol esterification activity was still present suggesting the existence of a second *Acat* gene.

Acat2 was identified in 1998 and the expression was shown to be restricted to liver and intestine [31–33]. *Acat2*-deficient mice [34] had indeed a reduced cholesterol esterifying activity in the small intestine and liver. On a normal diet, *Acat2* $^{-/-}$ mice showed no major differences in cholesterol metabolism compared to wild-type mice. In contrast, a high-cholesterol/high-fat (HC/HF) diet reduced the intestinal cholesterol absorption in *Acat2* $^{-/-}$ mice to 15% of that of wild-type mice. Moreover, the concentrations of cholesterol in gallbladder bile were 30% lower in *Acat2* $^{-/-}$ mice compared to wild-type mice. This was reflected in the formation of gallstones: all wild-type mice but none of the *Acat2*-deficient mice fed the HF/HC diet for 3–6 weeks developed cholesterol gallstones. Even after 3 months, only 3 out of 14 *Acat2*-deficient mice had a few stones [34]. These results in mice seem to contradict the human situation in gallstone formation, since Smith et al. [35] demonstrated that patients with cholesterol gallstones have decreased hepatic ACAT activity. Buhman et al. [34] argue that this is not per se contradicting because *Acat2* deficiency in the intestine lowers cholesterol absorption, which may then decrease the flow of cholesterol to the liver resulting in the decreased biliary cholesterol output. Thus, a specific deficiency of *Acat2* in the liver may increase the risk of gallstone formation. However, in the human liver, ACAT1 rather than ACAT2 seems to be higher expressed [36], and might therefore play a more important role than ACAT2 in esterification of hepatic cholesterol.

3.4. Cholesterol ester hydrolases

Cholesterol esters derived from LDL particles are hydrolyzed by the action of acid lysosomal cholesterol ester hydrolase. Neutral cholesterol ester hydrolase is responsible for hydrolysis of cholesterol esters stored in lipid droplets. In cholesterol-fed rats, the activity of hepatic neutral cholesterol ester hydrolase as well as mRNA levels were decreased [37]. Moreover, inhibition of cholesterol biosynthesis by lovastatin and stimulation of biliary cholesterol secretion by chronic biliary diversion increased both the activity and the expression of the enzyme, whereas chenodeoxycholate feeding decreased this. These data suggest that neutral cholesterol ester hydrolase responds to changes in cholesterol flux through the liver [37]. The mechanism by which the enzyme is regulated is unknown. The increased activity and/or expression of neutral cholesterol ester hydrolase may contribute to the enhancement of biliary cholesterol secretion and hence formation of cholesterol gallstones.

3.5. Bile salt biosynthesis

As already mentioned above, formation of cholesterol gallstones may also be induced by the decreased output of bile salts and/or the shift toward the more hydrophobic bile salt species, thereby changing the CSI in bile. Excess of hepatic cholesterol can be converted into bile salts via two pathways: the classic or neutral pathway and the alternative or acidic pathway. Cholesterol 7α-hydroxylase (CYP7A1) plays a key role in the classic pathway, whereas sterol 27-hydroxylase (CYP27) is the regulatory enzyme in the acidic pathway. The neutral pathway is active exclusively in the liver. The alternative pathway, at least CYP27, is present in almost all tissues.

Sterol 12α-hydroxylase (CYP8B1) controls the ratio of cholic acid (CA) and chenodeoxycholic acid (CDCA) synthesis in both neutral and alternative pathways and thereby regulates the bile salt hydrophobicity index. The biliary cholesterol secretion rate depends on the hydrophobicity of the bile salt. Therefore, via this seemingly indirect way, CYP8B1 may regulate biliary cholesterol output. A fourth cytochrome P450 (CYP) involved in regulation of the rate of cholesterol secretion into bile is oxysterol 7α-hydrox-

ylase (CYP7B1). This enzyme catalyzes the second step in the alternative pathway. In humans, conversion of cholesterol to bile acids accounts for approximately half of the daily elimination of cholesterol from the body. The major portion of the remaining 50% is eliminated as free cholesterol [38,39]. In rats, up to 50% of the total bile acid synthesis may occur via the alternative pathway [40–42]. This is not the case in healthy humans [43] where only less than 10% of the total bile acid synthesis appeared to be synthesized via this pathway [44,45].

The increased delivery of cholesterol to the liver leads to increased bile acid synthesis via the classical pathway [46–48] through up-regulation of CYP7A1 induced by the oxysterol receptor liver X receptor (LXR) in many species [49]. However, hepatic overload of cholesterol, either by dietary cholesterol or by increased hepatic cholesterol synthesis, induces different responses of CYP7A1 in different species. In rodents, cholesterol feeding increases the expression of CYP7A1 and the conversion of cholesterol into bile acids. Rodents therefore are rather resistant to diet-induced hypercholesterolemia [47]. In rabbits [50], hamsters [51], and Green Monkeys [52], CYP7A1 is not induced in response to the high load of dietary cholesterol which leads to the development of hypercholesterolemia and atherosclerosis in these animals. Most humans respond to excess of cholesterol similarly to rabbits and hamsters resulting in increased plasma LDL-cholesterol levels and atherosclerosis, although in some humans, an increase in bile salt synthesis has been reported [53]. This difference in response of CYP7A1 to the high-cholesterol diet between rodents and humans is explained by the lack of binding of LXRα to the human CYP7A1 promoter due to a single nucleotide change in the human LXRE [54]. In *Cyp7a1* $^{-/-}$ mice, the rate of the bile acid synthesis and the size of the bile salt pool were reduced by approximately 60% and 80%, respectively. This significant change affected the intestinal absorption of cholesterol and the fecal excretion of bile salts was decreased as well in these mice. In contrast, both the total fecal cholesterol excretion and the total fecal sterol excretion were increased. However, compensatory increases in sterol synthesis took place in the liver and the intestine. Therefore, the reduced cholesterol absorption did not affect plasma and tissue cholesterol levels [55]. The disruption of *Cyp7a1* did not change the molar ratio of cholesterol to total lipids in gallbladder bile and the effect on the formation of gallstones would probably be minimal. In hamsters overexpressing *Cyp7a1* and fed a high-cholesterol diet, the rate of bile acid synthesis increased [56]. Similarly, *CYP7A1* overexpression in HepG2 cells and primary human hepatocytes increased bile acid synthesis via the classic pathway and suppressed cholesterol synthesis [57], whereas in cholesterol-fed *CYP7A1* transgenic mice lacking the endogenous *Cyp7a1* $^{-/-}$ gene, LXR could not bind the CYP7A1 promoter and failed to increase the expression of CYP7A1 [58]. These studies showed that manipulation with CYP7A1 expression could change non-responsive species into responsive ones and vice versa. The rate of bile acid synthesis and the activity of CYP7A1 protein are tightly feedback inhibited by bile acids returning to the liver via the enterohepatic circulation of bile. Hydrophobic bile acids are the main active bile acid species in this respect [47,59–61]. The *CYP7A1* promoter region contains a bile acid response element (BARE) that has been identified as a hepatocyte nuclear factor 4 (HNF4) binding site [62]. This binding site overlaps with the binding site for several other factors from the family of monomeric nuclear receptors [63]. In both mice and human HepG2 cells, CYP7A1 expression is repressed by bile acids via the activation of the bile acid receptor FXR, the subsequent activation of the short heterodimeric partner (SHP) and following repression of the liver receptor homolog LRH1 [64–66]. In HepG2 cells, HNF4 has been shown to compete with LRH1 for binding on their overlapping binding sites on the CYP7A1 promoter [64]. Gupta et al. [67] reported that besides FXR-mediated down-regulation of CYP7A1 by bile acids, also the Jun kinase signaling pathway is activated and plays an additional role in down-regulation of CYP7A1. Multiple pathways are thus involved in regulation of the expression of CYP7A1 and the control of the rate of the synthesis of bile salts. Recently, Pullinger et al. [3] reported high levels of LDL cholesterol in three patients with a homozygous mutation in CYP7A1. In one patient, the hepatic cholesterol content was increased, whereas fecal excretion of bile salts was absent. In addition, two of the three patients studied had hypertriglyceridemia and premature gallstone disease.

Sterol 27-hydroxylase (CYP27) is an ubiquitously expressed mitochondrial enzyme that catalyzes the first step in the acidic pathway of bile acid synthesis and the 27-hydroxylation of bile acid intermediates in the neutral pathway (CYP7A1) [68]. 27-Hydroxycholesterol can compete with cholesterol in the further metabolization and is also a substrate for CYP7B1, the microsomal oxysterol hydroxylase [69]. In extrahepatic tissues, CYP27 converts cholesterol to 27-hydroxycholesterol and 3β-hydoxy-5-cholestenoic acid which are then effluxed, incorporated into lipoproteins and transported to the liver, where they are probably metabolized via the alternative pathway into bile acids [70–73]. In vitro, 27-hydroxycholesterol inhibits HMG-CoA reductase activity [14,74] and stimulates the activity of ACAT2 [75], resulting in a reduction of the intracellular free cholesterol concentrations. Recently, 27-hydroxycholesterol was identified as a ligand for the nuclear receptor LXR [76] (see below). In rats, CYP27 expression is down-regulated in vivo and in vitro by hydrophobic bile acids [41,77]. In contrast, in rabbits, no effect was seen of bile acids on the expression of CYP27. Cholesterol, however, did stimulate CYP27 expression in rabbits [78] which emphasizes again the differential physiological responses of pathways involved in cholesterol metabolism in the different species.

In humans, mutation of *CYP27* leads to the development of cerebrotendinous xanthomatosis (CTX), a disease marked by progressive neurological dysfunction and accelerated atherosclerosis [79]. In these patients, bile acid synthesis and the total bile acid pool are decreased, whereas cholesterol synthesis is increased. *Cyp27* $^{-/-}$ mice showed a similar decrease in metabolism of cholesterol to bile acids and increase in cholesterol synthesis as seen in humans [80]; however, they did not develop CTX. The expression of CYP7A1 mRNA in *Cyp27* $^{-/-}$ mice was increased enormously, whereas the mRNA levels of CYP7B1 were decreased. Intestinal cholesterol absorption was also decreased which was compensated by an increased synthesis of cholesterol in the liver and the intestine. In gallbladder bile of the *Cyp27* $^{-/-}$ mice, the cholesterol concentration was unchanged, whereas concentrations of bile acids and phospholipids were decreased. The molar ratios of cholesterol to the other biliary lipids in gallbladder bile and the risk for cholesterol gallstone formation in *Cyp27* $^{-/-}$ mice were increased [81]. To our knowledge, no data on gallstones in CTX patients have been reported.

Sterol 12α-hydroxylase (CYP8B1), a microsomal cytochrome *P*450, is responsible for the synthesis of cholic acid. Sterol 12α-hydroxylase acts at a branch point in the bile acid pathway determining the ratio of cholic acid to chenodeoxycholic acid. This may be important for the formation of gallstones because CDCA, in contrast to CA, may reduce the degree of cholesterol saturation in bile. The overall bile acid hydrophobicity index is important for cholesterol and bile acid synthesis, intestinal cholesterol absorption, biliary cholesterol secretion and gallstone formation [82,83]. The expression of CYP8B1 is regulated by a negative feedback control by hydrophobic bile acids [84] in a similar manner to CYP7B1. In contrast, CYP8B1 expression was up-regulated by hydrophilic bile acids, whereas excess of dietary cholesterol decreased the expression of CYP8B1 by 40% [84]. Overexpression of *Cyp8b1* in rats resulted in an increased pool of cholic and deoxycholic acid and a corresponding decrease in the chenodeoxycholic and muricholic acid pool [85], but the overall rate of bile acid synthesis remained unchanged.

Oxysterol 7α hydroxylase (CYP7B1) catalyzes the second step in the alternative pathway. Mice deficient for *Cyp7b1* $^{-/-}$ [86] appeared to have normal bile acid synthesis; however, 25- and 27-hydroxysterols accumulated in serum and in several tissues including the liver. These two oxysterols have been shown to act as ligands for the nuclear receptor LXR [76,87] (see below). The levels of plasma and tissue cholesterol were not changed in the *Cyp7b1* $^{-/-}$ mice and bile of these mice was not analyzed. Recently, Pandak et al. [88] reported that CYP7B1 in primary rat hepatocytes is regulated by bile acids, cholesterol and cAMP in a similar manner to CYP7A1. In contrast, overexpression of CYP7B1 in these cells did not result in increased bile acid synthesis suggesting that under normal conditions, the activity of CYP7B1 is not a rate-limiting step in this pathway. Therefore, a potential role for CYP7B1 in cholesterol gallstone formation remains to be investigated.

3.6. Genes involved in intestinal cholesterol absorption

Cholesterol taken up in the intestine originates from diet and bile. Depending on the species, in most cases, the biliary pool that undergoes enterohepatic recycling is the larger of the two. Until recently, very little was known about the proteins involved in regulation of cholesterol uptake. A role for the HDL receptor SR-B1 was proposed by Schulthess et al. [89]. Recently, this group also implicated CD36 and a third protein factor which together accounted for most of the transport activity in isolated brush border membrane vesicles [90]. In addition to the active transport, there may be still considerable passive diffusion of cholesterol into the intestinal epithelial cells and recent data suggest that the net cholesterol absorption is the result of both uptake and active secretion back to the lumen. The transporters that have been implicated in the secretory route belong to the family of ATP binding cassette (ABC) transporters, that is: ABCA1 [91,92] and ABCG5 and ABCG8 [93–95].

ABCA1 is expressed in the liver but no expression could be detected in enterocytes under normal conditions [96]. The subcellular localization of the protein in enterocytes and hepatocytes has not been reported yet, although in HeLa cells, a GFP-tagged ABCA1 was detected in intracellular vesicles and at the plasma membrane [97], whereas in DBGE cells, basolateral localization of ABCA1 was found [98]. Repa et al. [91] observed an increased intestinal expression of ABCA1 and a simultaneous inhibition of cholesterol absorption in mice fed a retinoid X receptor (RXR) agonist. The strong up-regulation of ABCA1 expression in the intestine observed was mediated via the nuclear receptor LXR and indirectly RXR. In contrast, *Abca1* null mice showed only a slightly reduced cholesterol absorption [92]. It is thus not yet clear whether ABCA1 is really instrumental in the observed inhibition of cholesterol absorption.

The second set of ABC-transporters implicated to have a role in intestinal cholesterol uptake are the recently identified ABC-halftransporters ABCG5 and ABCG8 [93–95]. Mutations in either of these genes cause sitosterolemia, a disease of which one of the hallmarks is the increased absorption of plant sterols and other sterols including cholesterol. Neither subcellular localization nor function of the halftransporters is known at present. Repa et al. [99] showed that ABCG5 and ABCG8 mRNA were homogeneously expressed in enterocytes lining the microvilli.

The multidrug resistance transporter MDR1 has been reported to play a role in intestinal cholesterol absorption. Mdr1a and Mdr1b in mice, but only one MDR1 in humans are expressed in a number of tissues including the apical membrane of the enterocytes. In cell culture experiments, MDR1 was associated with the uptake and trafficking of sterols. Nonspecific inhibitors of MDR1 have been shown

to inhibit cholesterol synthesis and the esterification of cholesterol putatively by blocking the trafficking of sterols from plasma membrane to ER [100–102]. The increased expression of MDR1 was associated with increased accumulation of cholesterol and enhanced kinetics of cholesterol esterification in a rat epithelial cell line [103,104]. Recently, Luker et al. [105] determined that intestinal cholesterol absorption was not affected in *Mdr1a/Mdr1b* double knockout mice. They did find, however, an increased kinetics of cholesterol accumulation in the liver after oral administration of cholesterol. Although the above reports may suggest a role for MDR1 in intestinal and hepatic cholesterol handling, the in vitro and in vivo results are of opposite directions. Therefore, a role for MDR1 in intestinal cholesterol absorption remains to be elucidated. Biliary lipid secretion is not affected in *Mdr1a/b* $^{-/-}$ mice (R. Ottenhoff, personal communication).

3.7. Genes involved intracellular trafficking of cholesterol

One of the proteins reported to have a role in intracellular cholesterol/lipid trafficking and metabolism is sterol carrier protein 2 (SCP2). The intracellular localization of SCP2 is still a matter of controversy. A number of studies localize the protein exclusively in the peroxisomes but others also reported presence of the protein in the cytosol. A role for SCP2 in intracellular lipid transport has been proposed in several studies. Purified SCP2 has been shown to bind cholesterol and to stimulate interorganellar cholesterol transfer [106] as well as the conversion of free cholesterol into cholesterol esters [107] and 7α-cholesterol [108,109] and to stimulate the transfer of sterols among intracellular membranes in vitro [110]. However, SCP2 has been shown to have a similar or higher affinity for fatty acids and fatty acyl CoA than for cholesterol [111,112]. Intracellular cholesterol transport by SCP2 was suggested when Puglielli et al. [113] showed in cultured fibroblasts, using antisense nucleotides, that SCP2 increased the transport rate of newly synthesized cholesterol from the ER to the plasma membrane. Furthermore, overexpression of *Scp2* in rat hepatoma cells enhanced intracellular cholesterol cycling, increased plasma membrane cholesterol content and decreased cholesterol esterification and HDL production [114]. By using in vivo antisense oligonucleotide treatment in rats, a role for SCP2 in the transport of newly synthesized cholesterol in biliary secretion was suggested [115]. In diosgenin-fed rats in which cholesterol synthesis was increased, hepatic SCP2 expression was increased as well [115]. In mouse strains differing in susceptibility to cholesterol gallstone formation fed a lithogenic (gallstone inducing) diet, SCP2 mRNA and protein levels correlated with biliary cholesterol secretion rates [116]. Adenovirus-mediated *SCP2* transfer in C57BL/6 mice [117] resulted in decreased plasma HDL cholesterol levels and increased LDL cholesterol levels. The level of total plasma cholesterol remained unchanged. Hepatic free cholesterol levels were increased (1.6-fold) and cholesterol synthesis was decreased by 40% in Ad*SCP2* mice. The total bile acid pool was slightly increased in infected mice but the composition was not changed. Adenovirus-mediated overexpression of *SCP2* increased the bile flow by 73% due to a 3-fold increase in bile acid-independent bile flow. Biliary output of bile salts and phospholipids increased by 50–60%, whereas cholesterol secretion was increased 2-fold. This led the authors to the conclusion that overexpression of *SCP2* enhanced the enterohepatic circulation of cholesterol and bile acids due to the increased intracellular cholesterol cycling.

Scp2/Scpx knockout mice [118] in a gallstone-susceptible strain (C57BL/6 background) did not show any changes in plasma or liver cholesterol levels; instead, these mice were defective in the peroxisomal oxidation of tetramethyl branched fatty acyl CoAs. Recently, the same group [119] reported that the *Scp2/Scpx* $^{-/-}$ mice have similar biliary cholesterol secretion rates compared to wild-type mice, increased bile flow and decreased bile salt secretion rates on chow diet. Feeding a lithogenic diet resulted in a lower increase in biliary cholesterol secretion in *Scp2* $^{-/-}$ mice compared to wild-type mice but the diet still induced cholesterol gallstone formation in both wild-type and knockout mice. This suggests that the role of SCP2 in gallstone formation is not of major importance although in gallstone patients, higher hepatic levels of SCP2 protein were found [120].

Intracellular cholesterol transport has also been suggested to be mediated by liver fatty acid binding protein (L-FABP) [121,122]. This protein has been shown to bind cholesterol in vitro and to enhance sterol transport between membranes [123], although others could not confirm this [124]. The expression of L-FABP was up-regulated in livers of *Scp-2* knockout mice [119].

Caveolin-1 is the main protein of caveolae, which are plasma membrane invaginations that are highly enriched in cholesterol and sphingomyelin. Caveolins/caveolae have been implicated in cell signaling [125], transcytosis [126,127] and in regulation of intracellular cholesterol transport [128] (reviewed in Refs. [125,129]). In the plasma membrane, caveolin-1 interacts with cholesterol [130,131] and the protein is expressed in a variety of cell types and tissues including the liver where it is present in both the sinusoidal and canalicular membrane of the hepatocyte [132–134]. The transcription of *CAV1* is under the positive control of cholesterol via the SREBP pathway (see below), resulting in increased transcription of *CAV1* when intracellular cholesterol levels are high [135]. In vitro, caveolin-1 has been shown to form a complex with chaperone proteins which may facilitate the transport of cholesterol from the ER to the plasma membrane [136]. In addition, caveolae appeared to be able to mediate cellular cholesterol efflux to plasma [137], whereas in hepatocytes, the scavenger receptor class BI (SR-BI) has been shown to be associated with caveolae indicating their role in cholesterol uptake [138]. A role for caveolin-1 in biliary cholesterol secretion

was suggested from results from studies in inbred mice in which expression of caveolin-1 was reported to be up-regulated during cholesterol gallstone formation [20], whereas expression of caveolin-2 was increased during hypersecretion of cholesterol by diosgenin feeding [139].

Recently, two groups reported the generation of *Cav1* knockout mice [140,141]. Unfortunately, the role of caveolin in biliary secretion was not investigated. The composition of lipoproteins was studied only by Drab et al. [140] and was not significantly changed in *Cav1* $^{-/-}$ mice.

In adenovirus-mediated overexpression of *CAV1* in C57Bl/6J mice [142], caveolin-1 protein was localized on the basolateral membrane of hepatocytes, and induced a ~ 2-fold increase in plasma HDL-cholesterol levels. *CAV1* overexpression inhibited the selective HDL-cholesteryl ester uptake mediated by SR-BI in cultured primary murine hepatocytes infected with AdCAV1. This supported the idea that caveolin-1 may play a role in HDL-cholesterol metabolism.

The Niemann-Pick C1 (NPC1) protein is a transmembrane protein containing a sterol sensing domain homologous to the domains found in HMG-CoA reductase, SCAP and Patched [143]. Defects in the *NPC1* gene result in intracellular accumulation of unesterified cholesterol together with several other lipids in the lysosomal compartment and cholesterol export from the lysosomes may be inhibited [144]. The exact function of NPC1 is not known but it was suggested to be involved in intracellular cholesterol trafficking from the lysosomes to a cholesterol pool, which can be used for bile acid synthesis and biliary cholesterol secretion. A role in transport of cholesterol to the canalicular membrane for biliary cholesterol secretion was suggested when *Npc1* $^{-/-}$ mice fed a high-cholesterol diet failed to up-regulate biliary cholesterol secretion [145].

3.8. Genes involved in cellular uptake of cholesterol

Apolipoprotein AI (ApoAI) is the main lipoprotein on HDL. *Apoa1* knockout mice had low plasma HDL-cholesterol levels [146,147] and their rate of hepatic cholesterol synthesis was 50% lower than in wild-type mice. The total body cholesterol content and hepatic cholesterol concentrations were not changed. In *Apoa1* $^{-/-}$ mice, also the rate of bile acid synthesis was normal and the absolute concentrations of bile acids, phospholipids and cholesterol in gallbladder bile of *Apoa1* $^{-/-}$ mice were not different from wild-type mice [147] despite the lower plasma HDL levels and reduced cholesterol synthesis. In contrast, *Apoa1* overexpressing mice have been reported to have a 2-fold increase in biliary output of bile acid and cholesterol [148]. The similar cholesterol concentrations in gallbladder bile of both wild-type and *Apoa1* knockout mice show that at least that HDL-cholesterol levels do not regulate biliary cholesterol secretion, which is consistent with observations in the *Abca1* null mice.

ApoE is the major protein component of chylomicrons (CM) and serves as the high-affinity ligand for the LDL-receptor and LDL-receptor-related protein [149]. When wild-type mice and *Apoe* knockout mice were fed a 0.02% and 0.5% cholesterol diet, the percentage of intestinal cholesterol uptake in *Apoe* $^{-/-}$ mice was higher than in wild-type mice [150]. In *Apoe* knockout mice, biliary cholesterol and bile salt secretion were not changed on chow diet. Biliary cholesterol secretion increased during either a high-cholesterol or lithogenic diet but the increase was lower in *Apoe* $^{-/-}$ mice compared to wild-type mice [150,151]. Biliary bile salt secretion, however, was not much affected in *Apoe* knockout mice on the lithogenic diet. As a result, the saturation of bile with cholesterol in wild-type mice increased and exceeded the maximal capacity of gallbladder bile to solubilize cholesterol by 61%. In *Apoe* null mice fed the lithogenic diet, the CSI did not change significantly [150] which might explain the decreased gallstone formation compared to the wild-type mice. These reports suggested a role for ApoE in the formation of cholesterol gallstones. Hepatic cholesterol concentrations were increased in *Apoe*-deficient mice compared to wild-type mice; thus, decreased hepatic cholesterol could not be responsible for the decreased secretion of biliary cholesterol in response to a high-cholesterol diet. ApoE might play a role in controlling the availability of dietary cholesterol in the liver (as a component of CMs) for bile secretion, and furthermore, it might regulate the bile cholesterol/bile salt molar ratio, thereby controlling the cholesterol saturation in gallbladder bile. Absence of ApoE could alter intracellular trafficking of cholesterol to specific pools necessary for cholesterol transport to the canalicular membrane [152]. An alternative option could be that ApoE present in bile [153] might play a role in regulating cholesterol crystallization and growth. In humans, contradictory studies have been published for ApoE on this subject [153–156]. Juvonen et al. [153] and Bertomeau et al. [154] found a positive correlation between ApoE4 phenotype and cholesterol crystals in bile, fast cholesterol crystallization in gallbladder bile and a higher cholesterol content in gallstones in patients bearing this polymorphism. However, this correlation could not be confirmed by Van Erpecum et al. [155] and Fischer et al. [156]. Kesaniemi et al. [157] showed that the percentage of dietary cholesterol absorption decreased in the order of ApoE polymorphism E4 > E3>E2, and also showed that the fecal excretion of cholesterol tended to be higher in individuals having the ApoE2 polymorphism compared with E3 or E4 individuals [158].

The SR-BI [159] binds HDL with high affinity [160] and has the highest expression levels in the liver and steroidogenic tissues. At lower levels, it is expressed on the apical surface of the small intestine [161]. The expression of SR-BI in these tissues is coordinately regulated with cholesterol metabolism [162–164]. In the promoter of SR-BI, a number of binding sites for transcription factors have been identified

including C/EBP, SF1 and SREBP1 (reviewed in Refs. [163,165]). SR-BI facilitates the cellular uptake of cholesterol (cholesteryl esters) from HDL. The mechanism by which this occurs is not yet known. The present belief is that only free cholesterol and cholesterol esters are taken up but not the apolipoproteins and that the particle is not internalized. However, recently, Silver et al. [166] reported recently that ApoAI is taken up and recycled. As indicated above, HDL cholesterol has been suggested to be preferentially utilized for direct biliary secretion [167–170] and the generation of *Srb1* knockout and overexpressing mice seemed indeed to imply an important role for SR-BI in biliary cholesterol secretion. Hepatic overexpression of *Srb1* [169] in mice reduced plasma levels of HDL cholesterol and increased biliary cholesterol concentration, whereas bile acid or phospholipid contents were not changed. The opposite characteristics were observed in mice with a disrupted *Srb1* gene [171,172]: total plasma cholesterol levels were elevated and biliary cholesterol secretion was reduced without alteration of biliary bile acid secretion, bile acid pool size or fecal bile excretion [171–173]. This suggested a model in which hepatic SR-BI mediates the transfer of cholesterol from plasma HDL to bile for excretion and in which overactivity of SR-BI may induce increased biliary cholesterol secretion coupled to decreased plasma HDL levels and hence could play a role in gallstone formation.

3.9. Canalicular membrane lipid transporters

Bile formation is regulated by several transmembrane transport proteins belonging to the ABC-transporter family. Since these ABC-transporters are responsible for the secretion of the predominant biliary components over the canalicular membrane, changes in the expression and/or activity of these proteins may result in an alteration of bile composition, which may influence gallstone formation. Until now, three ABC-transporters localized at the canalicular membrane have been found to be involved in the secretion of the main lipid components of bile. The bile salt export pump (BSEP, or SPGP, or ABCB11) regulates the secretion of bile salts into bile. BSEP expression is restricted to the canalicular membrane of the hepatocyte. The second ABC-transporter is Mdr2 in rodents and MDR3 (ABCB4) in humans. Both act as a flippase for the phospholipid phosphatidylcholine. The third important canalicular ABC-transporter is multidrug resistance-related protein 2 (MRP2) (ABCC2, cMoat), which is responsible for biliary secretion of organic anions and glutathione conjugates [174].

The bile acid transporter BSEP has been identified as one of the major *Lith* genes involved in gallstone formation in mice [175] (see below). In mice, the protein may be responsible for transport of the more hydrophobic bile acids. In *Bsep* $^{-/-}$ mice [176], biliary bile salt output was reduced to 30% and plasma and hepatic bile acid concentrations were increased. In contrast, the concentration and output of both phospholipids and cholesterol in bile were both increased. The pattern of biliary bile salts in the *Bsep* knockout mice was shifted to more hydrophilic species. In addition, tetrahydroxylated bile acids were present in bile of knockout mice, which were not found in bile of wild-type mice. To explain the unexpected increase in biliary lipid secretion in *Bsep* $^{-/-}$ mice, Wang et al. [176] postulated that accumulation of hepatic bile acids resulting from the absence of *Bsep* would inhibit bile acid synthesis which could lead to the accumulation of cholesterol in the liver. This in turn may stimulate biliary secretion of cholesterol. Expression levels of MDR2 were, however, not reported and up-regulation of this protein by the increased intrahepatic bile acids [177] seems to be a more likely explanation. In contrast to mice, the homozygous defect of BSEP in humans leads to the complete block of bile salt secretion and severe cholestasis [178]. BSEP is obviously present on the list of human candidate *LITH* genes but the results of the presented animal studies do not necessarily reflect the human situation.

Disruption of the *Mdr2* (*Abcb4*) gene in mice results in the absence of phospholipids into bile. In addition, biliary cholesterol secretion is also very low [179] but bile acid secretion remains unaltered. The expression and activity of MDR2 can be regulated by bile salts [177,180]. In addition, the expression of MDR2 is up-regulated by HMG-CoA reductase inhibitors [181], fibrates, which are ligands of the nuclear receptor peroxisome proliferator activating receptor (PPAR) [182,183], and by peroxisome proliferators in general [184].

The molecular mechanism by which cholesterol is secreted into bile is still an enigma. It has long been thought that the sterol simply follows phospholipids and that transport is not protein mediated. The coupling between phospholipid and cholesterol is, however, certainly not absolute. For instance, dietary supplementation with the plant sterol diosgenin induces a 5- to 7-fold increase in biliary cholesterol secretion [185–187] and in mice, even a higher up-regulation of 15-fold has been reported [134]. Since the lipid composition of the canalicular membrane remains unaltered under these conditions, increased activity of an as yet not identified protein factor must be involved. Good candidates for such an activity are the ABC halftransporters ABCG5 and ABCG8. Patients with sitosterolemia were shown to have mutations in these genes and seem to have impaired biliary sterol secretion [93–95]. It can, however, not be excluded that this effect is secondary to the disturbed handling of plant sterols in these patients. Mouse models for these genes allowing rigorous testing are not yet available.

3.10. Regulatory genes

SREBPs are transcription factors activating genes involved in cholesterol and fatty acid synthesis. Three isoforms of SREBPs have been identified. SREBP1a and

SREBP1c are transcribed from a single gene and only differ in the N-terminal sequence, whereas SREBP2 is transcribed from a second gene. In most cell culture systems, SREBP1a and SREBP2 are the predominantly expressed isoforms, whereas in the liver and other organs, SREBP1c and SREBP2 are most highly expressed. When cells are depleted of sterols, the NH_2-terminal part of the full-length membrane-bound form of each SREBP is cleaved and released from the membrane in two steps [188–190]. Each SREBP can act independently on the same genes [188,191] but the three isoforms have different target gene specificities. All three isoforms tend to stimulate the expression of multiple genes involved in both cholesterol and fatty acid synthesis but the relative degree of stimulation varies and they have differential effects on cholesterol synthesis as opposed to fatty acid synthesis.

The generation of *Srebp1* knockout mice resulted in a high rate of embryonic lethality. The mice that survived showed decreased hepatic fatty acid synthesis and the mRNA levels of these genes tended to be decreased. In contrast, the mRNA levels of genes of cholesterol synthesis were increased due to a compensatory activation of SREBP2. *Srebp2* knockout mice were not viable at all [192].

Mice overexpressing a dominant-positive truncated form of *Srebp1a* [193] developed fatty livers which was accompanied by massive increases in mRNA levels of cholesterol-synthesizing and fatty acid-synthesizing enzymes and of the LDL receptor; however, the plasma lipid levels were not changed in these animals. In *Srebp1c* transgenic mice, the liver was not enlarged and only a modest triglyceride accumulation was observed and similarly, the mRNAs encoding the fatty acid-synthesizing enzymes were only modestly increased. The mRNAs encoding cholesterol biosynthetic pathway enzymes were unchanged [192]. Like overexpression of *Srebp1a*, overexpression of *Srebp2* induced an enormous increase in the expression of genes in the pathways of cholesterol and fatty acid biosynthesis and increased cholesterol (ester) and triglyceride levels in the liver. The ratio of cholesterol synthesis/fatty acid biosynthesis, however, was much higher than in *Srebp1a*-overexpressing mice [194]. From these animal models, it seemed that SREBP2 is more effective than SREBP1a in increasing the amount of mRNAs of genes in cholesterol synthesis, whereas SREBP1a is more effective in activation of fatty acid synthesis. SREBP1c is the least effective of all three in both pathways. In none of the abovementioned SREBP models, however, biliary cholesterol or gallstone formation was investigated.

The LXR subfamily of nuclear receptors consists of two members: LXRα (NR1H3) and LXRβ (NR1H2). Both have a different expression pattern. Whereas LXRβ is ubiquitously expressed, LXRα is most highly expressed in the liver and intestinal tract which are the tissues most involved in cholesterol metabolism. Both receptors form heterodimers with another nuclear receptor, the retinoic X receptor RXR, which is activated by 9-*cis*-retinoic acid. LXR ligands identified thus far are the oxysterols 22*R*-hydroxycholesterol (adrenal glands), 24*S*-hydroxycholesterol (brain) and 24*S*,25-epoxycholesterol (liver) [87] and the recently identified ligand 27-hydroxycholesterol (human macrophages) which is a product of CYP27 action [76,195–197]. Direct target genes of LXRα have proven to be *CYP7A1* [49,196], *SREBP1c* [198,199], *ABCA1* [91,200], *ABCG1* [201], *ABCG5/G8* [93], *CETP* [202], *ApoE* [203], and *ABCG4* [204]. LXRα binding to the CYP7A1 promoter increases the transcription and activity of CYP7A1 in mice leading to the conversion of cholesterol into bile acids. *Lxrα* $^{-/-}$ mice fail to up-regulate CYP7A1 expression on a high-cholesterol diet and accumulate cholesterol in the liver [49]. *Lxrβ* $^{-/-}$ mice in contrast are resistant to increased dietary cholesterol and do not develop fatty livers [205]. In both types of knockout mice, biliary lipids or gallstone formation was not investigated. By regulating the transcriptional activity of key target genes involved in cholesterol catabolism, storage, absorption and transport, LXRα seems to be the bodies' key sensing molecule for maintaining cholesterol homeostasis.

The farnesoid X receptor (FXR, NR1H4) was originally found to be activated by high concentrations of farnesol [206]. Recently, it was shown that a number of bile acids, including chenodeoxycholic, cholic, deoxycholic and lithocholic acids bound and activated FXR in cultured cells [207–209]. Identified target genes of FXR include genes involved in bile acid transport and biosynthesis [210,211] that were previously known to be regulated by bile acids: CYP7A1 and CYP8B1 [212], the basolateral sodium taurocholate cotransporter protein NTCP [213], the hepatic canalicular bile salt transporter BSEP [214,215], the ileal bile acid binding protein I-BABP [207,216,217], the small heterodimeric partner (SHP) [65,66] and phospholipid transfer protein (PLTP) [218,219]. Mice with a targeted disruption of the *Fxr* gene [220] failed to correctly control bile acid biosynthesis and excretion. In a situation of an increased bile acid concentration, FXR is activated and promotes the transcription of SHP. Increased levels of SHP protein inactivate the liver receptor homolog 1 (LRH1) by forming a complex which leads to an inhibition of transcription of CYP7A1 and the other target genes [65,66]. This mechanism has been shown for the target genes CYP7A1 and NTCP [65,66,221]. However, direct regulation of FXR by binding of FXR to the BSEP promoter has also been shown [215].

The liver receptor homologue LRH1 (NR5A2) is a monomeric orphan receptor and is the mammalian homolog of the *Drosophila fushi tarazu* F1 receptor gene [63,222]. It is expressed in liver, pancreas, intestine, and ovary and binds to DNA as monomer. LHR-1 is believed to serve as a tissue-specific competence factor for bile acid synthesis. No ligands have been reported for LRH1 but target genes include: α-fetoprotein [63], SHP [223], CETP [202], CYP7A1 [222], and CYP8B1 [224].

SHP, the small heterodimeric partner (NR0B2) heterodimerizes with several nuclear receptors [225]. It has a

ligand-binding domain but it lacks a DNA binding domain. It is expressed in the liver, intestine, heart, pancreas and adrenal glands [225], and has been shown to be a potent repressor of LRH1 and its target genes. SHP is suggested to be a constitutive repressor and not to have an endogenous ligand [65,66,225–228]. Together, SHP and LRH1 seem to be important factors in the regulation of expression of genes involved in transport and synthesis of bile acids, thereby influencing the CSI in bile.

Peroxisome proliferator-activated receptors (PPARs) were identified as nuclear receptors activated by hypolipidemic compounds, like fibrates, but fatty acids have been found to be the natural ligands. Genes involved in fatty acid metabolism, β-oxidation and ω-oxidation have been found to be activated by PPARα (NR1C1) and specific target genes of PPARα are peroxisomal acyl-CoA oxidase (ACO), CYP4A1 and enoyl-CoA/3-hydroxyacylCoA hydratase/dehydrogenase multifunctional enzyme. Mice with a targeted disruption of the *Pparα* gene lacked peroxisome proliferation and lacked induction of peroxisome proliferator-regulated genes in response to fibrates [229–231]. In addition, *Pparα*-null mice showed increased total and HDL-cholesterol levels [232]. Fibrates are known to lower plasma triglycerides and cholesterol levels [233]. One of these fibrates, bezafibrate, altered the bile acid composition in bile, by inducing an increase in CA and a decrease in CDCA, suggesting the involvement of PPARα in the expression of CYP8B1. Hunt et al. [231] showed that CYP8B1 is up-regulated by PPARα ligands and a concomitant increase in the amount of CA formed was found. CYP7A1 levels were not changed in *Pparα*-null mice. In contrast, administration of fibrates to humans does reduce *CYP7A1* expression, resulting in an increased risk for gallstone formation [233–236]. Post et al. [237] recently showed that fibrates down-regulated the expression of CYP7A1 and CYP27 in mice and that fecal bile acid secretion was decreased and fecal cholesterol secretion increased, both being PPARα dependent.

Numerous functions of the liver are controlled primarily at the transcriptional level by the actions of a limited number of hepatocyte-enriched transcription factors which include the HNF 1α, -1, -3α, -3β, -3γ, -4α, and -6. *Tcf1* (encoding for HNF1α) deletion induced enlarged fatty livers and hypercholesterolemia in mice. In addition, the expression of genes encoding for hepatic bile salt uptake (NTCP, Oatp1 and Oatp2) were decreased resulting in a defect in bile acid uptake and increased plasma bile salt levels. Furthermore, CYP7A1 and HMGR expression levels were elevated resulting in increased synthesis of bile acids and hepatic cholesterol, whereas HDL metabolism was impaired. In addition, FXR expression was down-regulated and the genes were identified as a direct target of HNF1. The *Tcf1* $^{-/-}$ mice also showed elevated expression of genes encoding enzymes involved in the synthesis, catabolism, and transport of fatty acids and of the peroxisomal β-oxidation enzymes (CYP4A3, bifunctional enzyme and thiolase). In contrast, there was a marked reduction of liver fatty acid-binding protein (l-FABP) expression in the livers of *Tcf1* null mice [238,239].

Although the liver-specific deletion of *Hnf3β* does not have large effects on liver gene expression [240], in mice overexpressing liver-specific *Hnf3β*, decreased expression of sinusoidal bile acid uptake transporter NTCP and the canalicular phospholipid transporter MDR2 was seen, but the expression of BSEP and MRP2 did not change.

Mice lacking hepatic *Hnf4α* accumulated lipids in the liver and serum cholesterol and triglyceride levels were enormously decreased, whereas serum bile acid concentrations were increased [241]. Unfortunately, biliary lipids were not measured in this study. HNF4 has been also shown to regulate the expression of CYP8B1 [242,243], CYP7A1 [244], and CYP27 [245].

3.11. Lith genes

Major indications for the genetic basis for cholesterol gallstone formation are the numerous inbred mouse strains with different basal levels of total plasma cholesterol [246] and differential susceptibility to gallstone formation. Total cholesterol levels in plasma (mainly HDL) were the highest in NZB/B1NJ and 129/J strains which had significantly higher total plasma cholesterol levels than the C3H/HeJ strain, whereas the lowest levels were found in the SWR/J strain. The other investigated mouse strains showed intermediate plasma cholesterol levels in the following order: A/J < BALB/cByJ < C57BL/6J < AKR/J < DBA/2J. When C57L/J mice were fed a lithogenic diet, 90% of the mice developed gallstones, whereas gallstones were observed only in 10% of AKR mice [20,247,248]. The susceptible C57L/J mice had early cholesterol supersaturation in bile and larger gallbladders [248]. The resistant AKR mice, however, developed fatty liver and had higher hepatic cholesterol levels on basal diet, which were increased on a lithogenic diet. C57L mice had higher HDL levels than AKR mice, which decreased on a lithogenic diet. HDL levels in AKR mice increased on the lithogenic diet. Both biliary cholesterol and phospholipid secretion rates were higher in the susceptible C57L mice than in the AKR strain. On a lithogenic diet, cholesterol and phospholipid secretion rates increased significantly in both strains, but in AKR mice, this increase was smaller. These mice had also a higher bile flow due to an increased bile acid-independent bile flow. Total bile salt secretion on chow diet was 1.5-fold higher in C57L mice than AKR mice and remained comparable on lithogenic diet. The cholesterol/phospholipid ratio in bile of AKR mice remained constant on both diets, whereas in C57L mice, this ratio increased. The cholesterol/bile acid ratio was also higher in C57L mice on lithogenic diet and increased only slightly in bile of AKR mice. The increased cholesterol/phospholipid ratio in C57L induced an increase in CSI in bile, promoting the formation of gallstones in C57L mice. In bile of gallstone patients, also

higher cholesterol/phospholipid ratios have been reported [249]. The differences between the inbred mouse strains in gallstone formation [250] was the reason for Khanuja et al. [247] to start quantitative trait loci (QTL) analysis to locate chromosomal regions containing genes involved in the differences in gallstone susceptibility. In accordance with gallstone formation being a polygenic disease, multiple QTL regions have been found when the gallstone-susceptible C57L/J mice were crossed with the gallstone-resistant AKR mouse strain [247]. The analysis also revealed that gallstone formation is a dominant trait. At least two strong QTLs have been identified, called *Lith-1* and *-2* [4]. In addition, several other QTLs were identified in this cross but were less significant. Several other crosses have been performed, identifying additional QTLs [251]. A candidate gene for *Lith-1* was later shown to be the bile salt export pump *BsepI* [175], whereas *Lith-2* was associated with *Mrp2* [252] and increased bile flow as well as secretion rates of glutathione and bilirubin conjugates [253]. In C57l mice, expression of SR-BI and ACAT2 was increased 2.5- and 2-fold, respectively, whereas it remained unchanged in AKR mice on the lithogenic diet. HMG-CoA reductase expression failed to down-regulate on the lithogenic diet in C57L mice. The lithogenic diet also increased the cholesterol ester hydrolase mRNA and protein levels in C75L mice, whereas these remained unchanged in AKR mice.

4. Indirect evidence for genetic background of human cholesterol gallstones

4.1. Epidemiology of cholesterol gallstones

The highest prevalence of gallstones was reported in European and particularly Amerindian populations, the lowest prevalence was found in Africans and intermediate prevalence rates were observed in the Far East and India [254,255]. Based on 22 sonographic studies published in the years 1979–1995, Kratzer et al. [254] estimated the mean prevalence rate of cholecystolithiasis in Europe to be 10–12%. A similar value (15.7%) was calculated earlier by Brett and Barker [255] in their critical overview of a series of autopsy studies. Surprisingly, few data have been reported for North American populations, whereas multiple studies were conducted in Latin America [256–261]. In North America, the detailed prevalence data are available only for Hispanics [262,263] and Native Americans [264–267], whereas only a limited number of studies have been performed in North American Caucasians [268–270]. The data obtained by Bortnichak et al. [269] from 5209 subjects involved in the Framingham Heart Study are of particular interest because the association between cholesterol gallstones and increased risk of atherosclerosis in male but not female gallstone patients was reported for the first time in this study.

Native Americans have the highest prevalence of cholelithiasis: 48% overall prevalence was found in Pima Indians [264] and somewhat lower prevalence rates were found in Chileans [257] and in other Native American populations [265,271]. In contrast, the overall prevalence in other American populations was around (non-Caucasians) or below (Caucasians) 20%.

The striking differences in prevalence rates of gallstones between different populations within the same American continents suggest a role of the genetic background in the etiology of the disease. However, most of the differences between epidemiological data may also be (partially) explained by environmental factors or by factors relating to study design (autopsy vs. sonography or cholecystography, prospective vs. retrospective, gender and age distribution, and response rate).

To check the influence of the genetic background more rigorously, prevalence of the disease in migrants coming from the region with a certain prevalence of gallstones integrated into a new environment with a different prevalence of gallstones should be compared with the prevalence in the genetically homogenous acceptor population. Unfortunately, rapid loss of the original genetic background within a few generations makes such studies difficult. In a recent cross-sectional population study of Brasca et al. [261], Italian and Spanish descendants fully integrated into the US society but having all four grandparents of the same Italian or Spanish origin presented higher prevalence rates for all age groups than those reported in Italy and Spain. Interestingly, the difference was significantly higher among the young subjects of both sexes, lower in the intermediate age group and almost no difference was found in subjects older than 60 years. These findings, in accordance with the data obtained from Pima Indians and other high-risk populations, clearly indicate the importance of genetic disposition for early manifestation of the disease.

4.2. Family and twin studies

Surprisingly few genetic studies on gallstone disease in humans have been published until now. In the first study conducted by Huddy in 1925 [272], the incidence of cholelithiasis in family members of 57 non-related affected individuals was 42% as compared with less than 16% in families of 100 controls with no history of "indigestion". Körner [273] found the frequency of gallstones in 74 families of gallstone patients to be five times higher than in families of non-affected controls. In a study of Littler and Ellis [274], 20.8% of 100 patients with gallstones gave a positive family history. Jackson and Gay [275] studied first-degree relatives from 100 patients with gallbladder disease. Definite or suggestive evidence of gallbladder disease was found in 72% of the first-degree relatives. Definite gallbladder disease at least in one parent was found in 33% and only in two cases neither parent had gallbladder disease. A different approach was chosen in a study of van der Linden and Lindelöf [276] who compared the occurrence of gallstone disease in patient's siblings and husbands or wives.

Gallstone disease was found to occur more often among the siblings of gallstone disease patients. The incidence of gallstones in wives of the gallstone patients and wives of their healthy brothers did not differ [277], indicating genetic background of the disease. In their third study, van der Linden and Simondson [278–280] focused on the incidence of gallstones among the parents of young (< 22 years) gallstone patients. A group of 37 patients was compared with two age- and sex-matched control groups: 37 randomly chosen healthy individuals (group I) and 37 patients with normal cholecystography performed because of uncharacteristic abdominal complaints (group II). Either parent was affected in 20 of 37 patients but only in 7 of 37 and 14 of 37 subjects from control groups I and II, respectively. Neither parent had gallstones in 12, 29 and 23 subjects from patient group, control group I and control group II, respectively. Lithogenicity of duodenal bile was investigated in older and younger sisters of 11 gallstone females (mean age 21.1 years) by Danzinger et al. [281]. Four from eight older sisters (mean age 29.4 years), 1 from 10 age-matched controls and 1 from 10 younger sisters (mean age 20.3 years) had lithogenic bile. Gilat et al. [279] studied, in a prospective manner, the frequency of gallstones in 171 first-degree relatives of patients with proven gallstones compared with 200 matched controls. All subjects were studied by oral cholecystography, and their height, weight, blood glucose, cholesterol, and other parameters were measured. Gallstones were found in 20.5% of the first-degree relatives and in 9.0% of the control group. In the most recent study, Sarin et al. [280] evaluated 330 first-degree relatives of gallstone-positive index patients using ultrasonography. Stones were found in 15.5% of first-degree relatives compared to only 3.6% of matched controls and the risk was higher for female relatives. Despite the different design of the presented studies, their results univocally indicate the importance of genetic background of cholelithiasis.

More evidence supporting the role of inherited disposition to gallstones comes from the twin studies. A definite preponderance of concordant cases of gallstones among the uniovular pairs (7 of 18) compared with the biovular pairs of twins of the same sex (3 of 37) was found in a catamnestic investigation of 101 Danish twins with gallstones performed by Harvald and Hauge [282]. From two unselected series of twins investigated by Doig [283], cholelithiasis was present in one or both of 133 pairs. Concordance was demonstrated in 9 of 23 identical pairs but only in 4 of 42 fraternal pairs of the same sex. Unfortunately, in the series of the selected pairs that underwent both clinical and laboratory investigation (see also Doig and Pitman [284]), cholelithiasis occurred only in four monozygous and six dizygous couples. Concordance was seen in two monozygous and one dizygous pairs. An outstanding study of biliary lipid composition in monozygotic and dizygotic pairs of twins was conducted by Antero Kesaniemi et al. [285]. The relative contribution of genetic factors to biliary and serum lipid composition, including cholesterol precursors, squalene and methylated sterols which reflect the activity of cholesterol synthesis was studied in 17 monozygotic and 18 dizygotic middle-aged male pairs of twins. Gallstones were found in seven monozygotic (three concordant pairs) and three dizygotic subjects (all discordant pairs). The pairwise correlations of all measured parameters tended to be higher in the monozygotic than in the dizygotic pairs, suggesting a significant genetic control. However, no target genes were identified.

5. Direct proofs of the role of genes in human gallstone disease

5.1. Single gene defects

The first evidence that human gallstone disease might be caused by a single gene defect came recently from the studies of Rosmorduc et al. [1] and Jacquemin et al. [286]. In the first study, six symptomatic adult patients with a peculiar form of biliary gallstone disease characterized by intrahepatic sludge, gallbladder cholesterol gallstones associated with mild chronic cholestasis and recurrence of symptoms after cholecystectomy were studied. In two of them, hepatic bile analysis showed supersaturation with cholesterol together with low phospholipid concentration. This finding led to the hypothesis that a defect in the *MDR3* gene could constitute the genetic basis for this highly symptomatic and recurrent form of gallstone disease. Indeed, a heterozygous nonsense mutation in two patients, a homozygous missense mutation in three other patients, and a heterozygous missense mutation in one patient were detected. None of these mutations was reported previously in patients with progressive familiar intrahepatic cholestasis type 3 caused by homozygous mutations of *MDR3* gene leading to complete absence of MDR3 protein and secretion of phospholipids into bile [286,287]. None of the studied patients manifested any sign of liver disease in childhood. Jacquemin et al. [286] investigated 31 patients with cholestatic liver disease possessing the features of progressive familiar intrahepatic cholestasis type 3. Mutations of *MDR3* were detected in 17 patients. Gallbladder stones were found in four children and two parents. Both studies showed that defects of *MDR3* might play a more general role in liver diseases such as liver cirrhosis, cholesterol gallstones and familial cholestasis of pregnancy (see also Refs. [288,289]; however, making direct links between liver diseases with common and frequent phenotype and mutations in less conserved regions of the coding sequence that do not affect protein expression without functional studies and further analysis of the genetic background might be premature [290].

A new link between another single gene defect, the CYP7A1 deficiency, and premature cholesterol gallstones associated with hypercholesterolemia resistant to HMG-

CoA inhibitors in two male homoyzgotes, has recently been reported in an elegant study of Pullinger et al. [3].

5.2. Multiple gene defects and polymorphism studies

Direct evidence for the role of genes in human gallstones should come from multiple gene defects and polymorphisms associated with gallstones. No multiple gene mutation has been reported in a gallstone patient to our knowledge. In contrast, putative associations between a series human gene polymorphisms and gallstones have been published. Some of these studies were already discussed in the above sections. A brief overview of the polymorphism studies will be presented in this section.

ApoE polymorphism is the most extensively studied polymorphism in gallstone patients. Controversial data published on the protective role of the *epsilon4* allele against gallstones have already been mentioned [153–156]. In contrast, the *epsilon2* allele seems to act as a protecting factor against gallstones in the middle age according to Niemi et al. [291] whose data are further supported by the earlier work of Kesaniemi et al. [157]. These authors demonstrated that the percentage of dietary cholesterol absorption decreased in the order of ApoE isoforms E4>E3>E2, and also showed that the fecal excretion of cholesterol tended to be higher in individuals having the ApoE2 phenotype compared with E3 or E4 individuals [158].

In a study of polymorphisms at the ApoB, ApoAI, and cholesteryl ester transfer protein gene loci in patients with gallbladder disease conducted by Juvonen et al. [292], CETP polymorphism associated with another HDL lowering factor might be associated with cholesterol gallstone disease. A link between the $X+$ allele of the *ApoB* gene and the increased risk of cholesterol gallstone disease was proposed by Han et al. [293].

Besides the genes involved in biliary secretion of cholesterol, a set of genes controlling emptying of the gallbladder might participate in the etiology of gallstone disease. A search for mutations and/or polymorphisms in the cholecystokinin receptor gene in gallstone patients performed by Nardone et al. [294] was, however, unsuccessful.

In contrast to the previous section, no genetic or biochemically defined risk factor(s) associated with the strong increase of the risk of gallstone disease has been disclosed in any of the studies presented in this section.

5.3. Genetic diseases associated with cholesterol gallstones

Genetic diseases associated with cholesterol gallstones caused by defects of the genes that are not directly involved in biliary lipid secretion will be listed in this section. Increased incidence of radiolucent gallstones has been observed in patients with cystic fibrosis [295–297], DS [298] and Gaucher disease [299].

Patients with cystic fibrosis now commonly survive into adulthood and are at high risk for the development of gallstones. The diagnosis may be obscured by other common gastrointestinal complications of cystic fibrosis. Radiolucent gallstones found on cholecystography were thought to be cholesterol gallstones. This hypothesis was further supported by changes of biliary lipid composition found by Roy et al. [300]. In this study, in 14 patients with cystic fibrosis who had stopped taking pancreatic enzymes for 1 week, the molar percentage of cholesterol and CSI were comparable to values of the cholelithiasis group and almost twice higher than those of controls. In 12 patients with cystic fibrosis taking pancreatic enzymes, the molar percentage of cholesterol and saturation index did not differ from those of controls. The findings of Roy et al. [300] were not confirmed in a later study by Angelico et al. [301] who found non-significant changes in CSI between patients with cystic fibrosis with (1.21 ± 0.28, $n=10$) and without (0.99 ± 0.54, $n=7$) gallstones. Gallstones available from two patients who died of severe bronchopneumopathy contained mainly calcium bilirubinate and protein while their cholesterol content was only 44% and 28% of dry weight. Interestingly, samples of duodenal bile from all 17 patients were checked for microlithiasis and the findings were negative. The authors came to the conclusion that radiolucent gallstones of patients with cystic fibrosis are not of the conventional cholesterol type.

The link between increased risk of gallstones and DS was suggested by Elias Pollina et al. [302] who analyzed 56 cases of cholelithiasis in patients aged 2 months to 15 years. A combination of cholelithiasis with DS was present in four cases from the studied series. Several cases of gallstones in infants with DS were reported in two later studies of Aughton et al. [303] and Aynaci et al. [304]. A systematic prospective study was carried out recently [298]. Of the 126 patients with DS who underwent abdominal ultrasound examination, 6 (4.7%) were found to have cholelithiasis. In contrast, among the 577 age-matched controls, only 1 (0.2%) 7-year-old girl had gallstones. All seven children with cholelithiasis were born at term, were asymptomatic, and did not have identifiable risk factors for gallstones. In the six children with DS and cholelithiasis, abdominal X-ray did not show radiopaque gallstones indicating a high amount of cholesterol in the stones. However, to our knowledge, no data on the composition of bile or gallstones from patients with DS have been published.

The fact that gallstones are often found in patients with Gaucher disease was based on clinical experience but little epidemiological data are available. Perez-Calvo et al. [299] published recently the results of a national study of comorbidity with other common diseases such as dementia, Parkinson disease, ischemic stroke, ischemic heart disease, non-rheumatic valvular disease, hematological and non-hematological malignancies, pulmonary fibrosis, tuberculosis, schizophrenia and gallstones conducted in 67 patients with Gaucher disease, 132 nonaffected heterozygous carriers and 59 healthy relatives. Patients with Gaucher appeared to have the greatest risk of developing gallstone

disease. This increased risk was particularly high among female patients and could not be explained in terms of differences in age. Patients with Gaucher disease have very low HDL cholesterol; the underlying mechanism is not known but increased reverse cholesterol transport leading to increased biliary secretion of cholesterol has been suggested.

6. Conclusions and future prospects

The etiology of cholesterol gallstone disease is clearly multifactorial. This is probably the reason that apart from a few (very rare) exceptions, the genetic background of this extremely common disease has not been elucidated. In this overview, we show that secretion of the main biliary components cholesterol phospholipid and bile salts are all subject to complex regulatory mechanisms and are formed by complex metabolic pathways. Relatively small changes in secretion rates of one or more of these biliary components are enough to induce gallstone formation. Therefore, subtle changes in a number of the steps caused by polymorphisms in the responsible genes may well underlie formation of gallstones. Identification of these polymorphisms is the challenge for the near future.

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