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Studies on cholesterol and bile acid metabolism in Chinese cholesterol gallstone patients

Zhao-Yan Jiang

蒋兆彦

Karolinska Institutet

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Cover picture:

Cholesterol crystals in the gallbladder bile from a patient with cholesterol gallstones observed under polarized microscopy. (*Photographed by ZY Jiang*)

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To you

ABSTRACT

Liver and intestine are important organs for the metabolism of cholesterol and bile acids in the body. Dysfunction in cholesterol and bile acid metabolism may lead to diseases such as cholesterol gallstone (GS) disease and atherosclerosis. The projects presented in this thesis aim to gain further insights into hepatic and intestinal cholesterol and bile acid metabolism and their role in the regulation of lipoprotein metabolism. We found that:

Paper I. Chinese patients with GS disease had higher mRNA expression of hepatic *ABCG5*, *ABCG8* and *LXRα,* which correlated with biliary cholesterol molar percentage and cholesterol saturation index (CSI). The mRNA and protein levels of the hepatic scavenger receptor B type I (SRBI) were increased and a significant correlation was found between the protein levels and the CSI as well. The results suggest that an upregulation of ABCG5/ABCG8 in Chinese GS patients, possibly mediated by increased LXRα, may contribute to the cholesterol supersaturation of bile. The data also indicate the possibility that increased amount of biliary cholesterol may originate from HDL by an enhanced transfer via SRBI.

Paper II. The intestinal mRNA expression of *NPC1L1* and *ACAT2* were significantly higher in Chinese GS patients than in gallstone-free (GSF) controls. Our data suggest that the increased *NPC1L1* and *ACAT2* mRNA levels in GS patients might indicate an upregulation of cholesterol absorption and esterification in the small intestine.

Paper III. Analysis of liver biopsies from Chinese GS and GSF patients revealed strong positive correlations between *NPC1L1* and *SREBP2* and *HNF4α* mRNA. Further studies were carried out to reveal the transcriptional regulation of *NPC1L1* by these transcription factors. We showed a dose-dependent regulation by SREBP2 on the *NPC1L1* promoter activity and mRNA expression in HuH7 cells. Chromatin immuno-precipitation (CHIP) assay confirmed the binding of SREBP2 to the promoter *in vivo*. Moreover, HNF1α increased both *NPC1L1* promoter activity and the gene expression, and an important HNF1α binding site (-158/-144 nt) was identified within the human *NPC1L1* promoter. CHIP assays also confirmed that HNF1α binds to the *NPC1L1* promoter *in vivo*.

Paper IV. In human, hepatic microsomal ACAT2 activity was significantly lower in females than in males. Moreover, the activity of ACAT2 correlated negatively with plasma HDL cholesterol and Apo A1. This is the first description of a gender-related difference of hepatic ACAT2 activity suggesting, a possible role for ACAT2 activity in the regulation of the cholesterol metabolism in humans. The negative correlation between ACAT2 activity and HDL cholesterol or Apo A1 may reflect such regulation. Since ACAT2 activity has been found to be pro-atherogenic, the observed gender-related difference may contribute to the protection of coronary heart disease in females.

Paper V. In this study, we found that female mice had significantly higher hepatic ACAT2 activity and mRNA expression and hepatic cholesteryl esters than male mice, independently of the presence/absence of estrogen receptor α (ERA) or β (ERB). The presence of ERA was associated to a higher mRNA of *Acat2* in mice. Furthermore, the interaction analysis showed that in female mice, presence of ERA was associated with a higher *Acat2* mRNA level. Hepatic ABCA1 protein was also lower in female mice, independently of ERA or ERB. ABCA1 protein correlated positively with the free cholesterol content in the plasma membrane and negatively with hepatic ACAT2 activity. Our data suggest that ERA seems to regulate *Acat2* at the mRNA level but not the activity. The observed relation between ABCA1 protein, free cholesterol in the plasma membrane and hepatic ACAT2 activity suggest a possible regulatory pathway in mice.

LIST OF PUBLICATIONS

I **Increased expression of *LXR alpha*, *ABCG5*, *ABCG8*, and *SR-BI* in the liver from normolipidemic, nonobese Chinese gallstone patients.**

Jiang ZY, Parini P, Eggertsen G, Davis MA, Hu H, Suo GJ, Zhang SD, Rudel LL, Han TQ, Einarsson C.

J Lipid Res 2008; 49(2): 464-72.

II **Increased *NPC1L1* and *ACAT2* expression in the jejunal mucosa from Chinese gallstone patients.**

Jiang ZY, Jiang CY, Wang L, Wang JC, Zhang SD, Einarsson C, Eriksson M, Han TQ, Parini P, Eggertsen G.

Biochem Biophys Res Commun 2009; 379(1): 49-54.

III **HNF1alpha and SREBP2 are important regulators of NPC1L1 in human liver.**

Pramfalk C, Jiang ZY, Cai Q, Hu H, Zhang SD, Han TQ, Eriksson M, Parini P.

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IV **ACAT2 and human hepatic cholesterol metabolism: Identification of important gender-related differences in normolipidemic, non-obese Chinese patients.**

Parini P, Jiang ZY, Einarsson C, Eggertsen G, Zhang SD, Rudel LL, Han TQ, Eriksson M.

Atherosclerosis 2009; 207(1): 266-71.

V **Identification of gender-related difference in hepatic ACAT2 activity in mice and their relation to estrogen receptors.**

Jiang ZY, Degirolamo C, Eriksson M, Inzunza J, Calabresi L, Gomaraschi M, Rudel LL, Gustafsson JA, Parini P.

Manuscript.

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LIST OF ABBREVIATIONS

ABC	ATP binding cassette
ACAT2	Acyl-Coenzyme A: cholesterol acyltransferase 2
ASBT	Apical sodium-dependent bile salt transporter
BA	Bile acid
BSEP	Bile salt export pump
C4	7α-hydroxy-4-cholesten-3-one
CA	Cholic acid
CDCA	Chenodeoxycholic acid
CE	Cholesteryl ester
CHIP	Chromatin immunoprecipitation
CVD	Cardiovascular disease
CYP7A1	Cholesterol 7α-hydroxylase
CYP8B1	Sterol 12α-hydroxylase
CYP27A1	Sterol 27α-hydroxylase
DCA	Deoxycholic acid
DGAT1/2	Diacylglycerol O-acyltransferase homolog 1/2
ER	Estrogen receptor
ESMA	Electrophoretic mobility shift assay
FC	Free cholesterol
FGF	Fibroblast growth factor
HMG-CoA	3-hydroxy-3-methylglutaryl Coenzyme A
GS	Gallstone
GSF	Gallstone-free
HDL	High density lipoprotein
HNF	Hepatocyte nuclear factor
IBABP	Ileum bile acid binding protein
LCA	Lithocholic acid
LDL	Low density lipoprotein
LRH-1	Liver receptor homologue 1
LXR	Liver X receptor
MTTP	Microsomal triglyceride transfer protein
NPC1L1	Niemann-Pick C1-like 1 protein
OATP	Organic anion transporter polypeptide
OST	Organic solute transporter
PCSK9	Proprotein convertase subtilisin/kexin type 9
SHP	Small heterodimer partner
SOAT2	Steroyl O-acyltransferase 2
SRBI	Scavenger receptor B type I
SREBP	Sterol regulatory element binding protein
TG	Triglycerides
TGH	Triglycerides hydrolase
UDCA	Ursodeoxycholic acid

1 INTRODUCTION

1.1 CHOLESTEROL METABOLISM

Cholesterol was first isolated from gallstones in 1769. It is an essential component of the plasma membrane of animal cells, where it maintains the barrier function between cells and environment, modulates fluidity, and creates "rafts" that concentrate signaling molecules. Cholesterol is also the precursor for all steroid hormones and bile acids (BA). Apart from being toxic for cells at high concentration, cholesterol accumulation might lead to diseases, such as cholesterol gallstone (GS) disease in the gallbladder and atherosclerosis in the arterial wall. Liver is an important organ for the regulation of cholesterol metabolism. The origin of cholesterol includes 1) *de novo* synthesis in all cells in the body (endogenous pathway); 2) intestinal absorption of dietary cholesterol (exogenous pathways). The fate of the cholesterol from the liver includes: 1) secretion into bile via canalicular transporters; 2) secretion into plasma at the basolateral membrane; 3) esterification into cholesteryl esters (CE); and 4) conversion into BA.

1.1.1 Synthesis

About 20–25% of total daily cholesterol is synthesized by the liver. This process starts with one molecule of acetyl Coenzyme A and one molecule of acetoacetyl-CoA, which are dehydrated to form 3-hydroxy-3-methylglutaryl Coenzyme A (HMG-CoA) by HMG-CoA synthase [1], as depicted in Figure 1. This molecule is then reduced to mevalonate by the enzyme HMG-CoA reductase. This is an irreversible and rate-limiting step in cholesterol synthesis and the site of action for the HMG-CoA reductase inhibitors (i.e. statins).

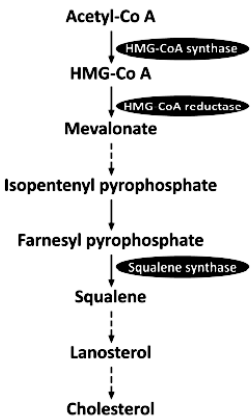

Figure 1 Pathway of cholesterol *de novo* synthesis

1.1.2 Receptor mediated uptake from plasma lipoproteins

In plasma, cholesterol is carried by different lipoprotein classes. According to the density, they are classified into very low density lipoprotein (VLDL), intermediate density lipoprotein (IDL), low density lipoprotein (LDL) and high density lipoprotein (HDL). They all share a common structure of phospholipids and free cholesterol (FC) complexed to apolipoproteins (Apo) at the surface and a core of CE and triglycerides.

LDL receptors clustered in coated pits in the plasma membrane binds to Apo B-100 in the LDL particles (Figure 2). The coated pits with the receptor-ligand complex are then invaginate to form endocytic vesicles. This process of uptake is called endocytosis. Multiple endocytic vesicles can fuse to create larger sacs of an irregular contour, called endosomes. At acidic pH, the LDL dissociates from the receptor, and the latter returns to the surface [2]. Considering the weight of the organ, the liver has the largest number of LDL receptors in the body, while the adrenal glands express the highest affinity LDL-binding [3]. LDL receptors can also recognize Apo E and bind the Apo E-containing lipoproteins with high affinity. Mutations in the gene coding for LDL receptors causes familiar hypercholesterolemia, a disease characterized by an elevation of plasma LDL concentration and premature cardiovascular disease (CVD)[4] and death.

The scavenger receptor B type I (SRBI) is a cell-surface HDL receptor (Figure 2) [5, 6], which also binds to a variety of other ligands, including modified (acetylated or oxidized) lipoproteins [6]. The larger, CE-rich, low density, spherical α-HDL particles bind more tightly to SRB1 than higher density HDLs, lipid-poor pre-β-HDL [7, 8] or lipid-free Apo A1. Thus, SRBI is able to mediate a selective lipoprotein cholesterol uptake [5], whereby cholesterol from plasma HDL is delivered to tissues, such as liver and steroidogenic tissues without degradation of the HDL particle [9, 10].

The liver accounts for 60-80% of the total HDL CE clearance from plasma [11]. The role of the liver in selective HDL CE uptake in CETP-expressing species has also been demonstrated in rabbit and human hepatocytes [11]. Thus, SRBI in liver is important for HDL metabolism and cholesterol homeostasis. Under basal conditions, most hepatic SRBI expression is present in parenchymal cells [12]. In mice, overexpression of *Srbi* result in a decreased in plasma HDL and an increase in biliary cholesterol secretion [13, 14], and *vice versa*, in *Srbi* knockout mice, plasma HDL increase [15] and biliary cholesterol decrease [16, 17]. Disruption of the *Srbi* in mice does not impair either biliary BA secretion, the BA pool size or the fecal BA secretion [16]. These data indicate that hepatic expression

of SRBI is important in controlling the hepatic uptake of HDL cholesterol and the preferential utilization in the liver of HDL cholesterol for biliary cholesterol secretion, and not for BA.

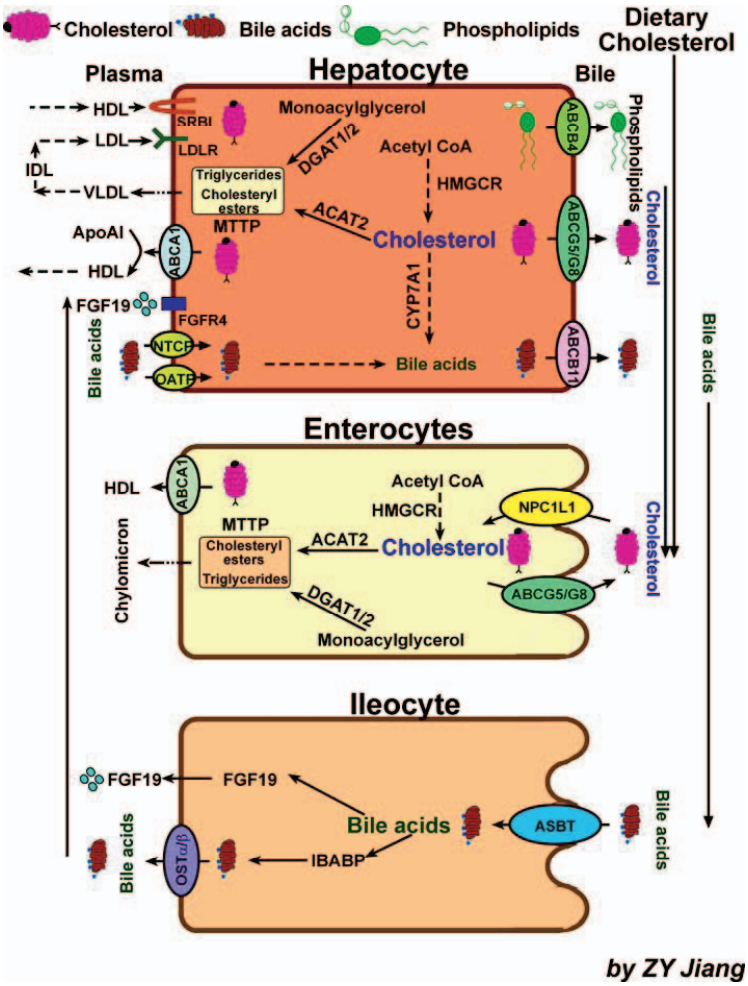

Figure 2 Schematic of the entero-hepatic metabolisms of cholesterol and bile acids.

1.1.3 Basolaterol and canalicular secretion of cholesterol in the liver

ATP binding cassette (ABC) G5 and ABCG8 have also been identified to form functional heterodimers [18] in apical membranes of hepatocytes (Figure 2). Mutations of either *ABCG5* or *ABCG8* genes leads to low biliary secretion of cholesterol and plant sterols [18, 19]. In contrast, in human *ABCG5/ABCG8* transgenic mice, the biliary cholesterol secretion is elevated more than 5 fold [20]. The fact that *Abcg5* knockout mice

have a residual cholesterol secretion [21] suggests the presence of an ABCG5/G8 independent pathway for biliary cholesterol secretion. This hypothesis is also supported by studies in other mouse models [22, 23] as well as observed in humans under certain conditions such as after liver transplantation [24].

In rodents, Niemann-Pick C1 like 1 protein (NPC1L1) is mainly expressed in the intestine [25]. In humans, there is also a high expression of NPC1L1 in the liver [25-27] where it localizes to the canalicular membrane of hepatocytes [27]. Hepatic expression of human *NPC1L1* in mouse liver resulted in a 10-20 fold decrease of the cholesterol concentration in bile, while the concentration of phospholipids or BA was unchanged [27].

In humans, ABCA1 is highly expressed in the liver and tissue macrophages. ABCA1 mediates the transport of cholesterol, phospholipids and other lipophilic molecules across the cellular membrane, where they are removed from the cells by lipid-poor Apo A1 (Figure 2) [28, 29]. The resulting partially lipidated HDL can then acquire additional cholesterol by other processes and mature into more spherical HDL particles. In mice, hepatic expression of ABCA1 accounts for approximately 80% of the HDL formation [30]. It has been proposed that ABCA1 promotes flipping of lipids from the inner to outer membrane leaflet by an ATPase-dependent process. ABCA1 is localized to the plasma membrane and the intracellular compartments [29, 31, 32], where it could potentially facilitate the transport of lipids to either cell surface-bound or to internalized apolipoproteins. Cholesterol deposits in late endosomes and lysosomes are thus important sources of lipids for the ABCA1-mediated cholesterol efflux [33].

1.1.4 Esterification of free cholesterol

Two enzymes are responsible for the esterification of FC into CE in cells: acyl-Coenzyme A: cholesterol acyltransferase (ACAT) 1 and ACAT2 (also known as steroyl O-acyltransferase 2, SOAT2). In the liver, ACAT2 is expressed in hepatocytes and is important for assembling the Apo B-containing lipoprotein (Figure 2), while ACAT1 is expressed in Kupffer cells [34-36]. The CE produced by ACAT2 become part of the neutral lipid core of Apo B-containing lipoproteins (i.e. VLDL and chylomicrons). ACAT2 derived CE (cholesteryl palmitate and cholesteryl oleate) have been shown to be pro-atherogenic [37]. The role of ACAT2 in the development of atherosclerosis has been revealed by target gene depletion of *Acat2* [34, 38, 39] or by liver specific knockdown of *Acat2* by anti-sense oligonucleotides (ASO) in mice [40]. From these studies, it can be concluded that the deficiency of hepatic ACAT2 activity in mice decreases the hepatic CE content, the CE content in the VLDL particles and the formation of atherosclerosis.

Hepatic ACAT2 is regulated by the amount of FC within the cells [41-43]. High dietary cholesterol content increases the hepatic cholesterol load which results in higher hepatic ACAT2 activity which has been shown in cynomolgus monkeys, but not in Africa green monkeys[41]. In human hepatoma cells, after cholesterol loading, the *ACAT2* mRNA expression as well as the ACAT2 activity increase in parallel with an increase of CE [42]. In humans, high dosage statin treatment reduces ACAT2 activity and expression in liver [43]. ACAT2 is responsible for more than 50% of the total ACAT activity in human liver samples [36]. However, the ACAT2 activity in human liver is lower than that the activity in monkey liver and in mouse liver, in the following order: human < Africa green monkey < cynomologus monkey < mouse. In *Acat2*-ASO treated mice, only less than 1% of ACAT2 protein and activity in the liver were preserved [44]. Surprisingly, the hepatic secretion of CE during isolated liver perfusion was unchanged [44]. This may suggest that there is only a small amount of ACAT2 activity needed to drive the packaging of hepatic CE into Apo B-containing lipoproteins.

In humans, the sterol-regulatory element (SRE) could not be identified within the ACAT2 promoter [45]. From our group, it has been shown that hepatocytes nuclear factor (HNF)1α [46] and HNF4α [47] could control the expression of human *ACAT2* gene in hepatocytes. Another study has implicated that HNF1α and caudal type homeobox 2 (CDX2) could synergistically stimulate the intestinal expression of *ACAT2* in Caco2 cells [48].

1.1.5 Reverse cholesterol transport

The concept of reverse cholesterol transport (RCT) was first introduced by Glomset and Norum in 1973[49]. It is currently described as a physiological process by which HDL transport cholesterol from peripheral tissue back to the liver for subsequent secretion into the bile, followed by fecal excretion. Thus, the liver has been thought to play a key role in RCT [50]. RCT is believed to be the major mechanism by which HDL protects against CVD. Cholesterol-loaded foam cells are the cellular hallmark of the atherosclerotic lesion, and increased cholesterol efflux from intimal foam cells may protect against the progression and complication from CVD [51] The cholesterol efflux from the macrophage is the first step in RCT. This mechanism is regulated by specific cellular transporters, such as 1) efflux to lipid-free apolipoproteins, particularly Apo A1, mediated by ABCA1 [52]; 2) efflux to mature HDL particles by ABCG1 [53, 54] and 3) by the SRBI. ABCA1 and ABCG1 mediated flux are unidirectional, leading to net removal of cellular cholesterol and influenced mainly by the nature and quantity of extracellular HDL-acceptors.

However, the role of macrophage SRBI in macrophage cholesterol efflux and protection against atherosclerosis remains contradictory. In mice, there was no decrease seen in the RCT from *Srbi* deficiency macrophage [52], while bone marrow transplantation from *Srbi* deficient mice into *Ldlr*-deficient [55] or *Apoe*-deficient [56] mice resulted in increased atheroslerosis.

The liver is able to take up cholesterol and CE from HDL via its receptor SRBI and in *Srbi* knockout mice the increase of plasma HDL cholesterol in paralleled with a markedly increased in atherosclerosis [55].

1.1.6 Intestinal cholesterol absorption

In Western countries, the daily intake of cholesterol from diet is about 400 mg, while the biliary cholesterol, which originates from the liver and enters the intestine, is about 800 to 1200 mg. The turnover of intestinal epithelium provides a third source of intraluminal cholesterol, contributing with approximately 300 mg cholesterol per day. Although, the entire length of the small intestine has the capability to absorb cholesterol from the lumen, the main sites of cholesterol absorption are the duodenum and proximal jejunum.

1.1.6.1 Cholesterol solubilization in bile salt micelles

Micelles and the cholesterol transporters located in the epithelium are important factors controlling the cholesterol absorption. Cholesterol is only marginally soluble in an aqueous environment and it therefore needs to be partitioned into bile salt micelles prior to transport to the brush border membranes where it can be absorbed. Bile salts, together with fatty acids, mono-acyl-glycerides, lysophospholipids, and FC, form the intestinal mixed micelles [57, 58]. The BA pool size and the BA composition are determinants of intestinal cholesterol absorption. This has been shown in patients with cirrhosis where the cholesterol absorption is positively correlated with the total BA pool [59]. A reduction in intestinal cholesterol absorption can be detected in cholesterol 7α-hydroxylase (*Cyp7a1*) knockout mice because of a marked reduction of BA synthesis and a reduced BA pool [60, 61]. The same is seen in the sterol 27-hydroxylase (*Cyp27a1*) knockout mice [62] in which the BA pool size is reduced despite an increase in *Cyp7a1*. Moreover, in mouse, it has been shown that the efficiency of intestinal cholesterol absorption is inhibited by feeding with hydrophilic BA [63]. Supplementation with cholic acid (CA) increases the hydrophobicity of bile salts in murine bile and markedly increases micellar cholesterol solubility [64], which in turn can explain the increased cholesterol absorption [63] observed in mice challenged with CA. In contrast, in sterol 12α-hydroxylase (*Cyp8b1*) knockout

mice lacking CA, the intestinal cholesterol absorption is decreased by 50% [65]. These data suggest that BA composition is a major determinant of intestinal cholesterol absorption.

1.1.6.2 Intestinal cholesterol transporters

The micelles are the donors of cholesterol and the transporter proteins on the epithelial cells function as the acceptors (Figure 2). Using a genomic-bioinformatic approach, the putative intestinal cholesterol transporter NPC1L1 was identified by Altman *et al* [25]. The compound ezetimibe selectively inhibits the transport of cholesterol and phytosterols across brush border membrane of enterocytes by blocking the transport function of NPC1L1 [66]. Furthermore, Ge *et al* [67] using model cell system showed that cholesterol is internalized into the cells bound to NPC1L1 through clathrin/AP2-mediated endocytosis.

ABCG5 and ABCG8 are co-localized at the apical membrane of the enterocytes and limit the absorption into body of both plant sterols and cholesterol by actively pumping them from the enterocytes back into the intestinal lumen. Mice lacking *Abcg5/Abcg8* genes mimic the major phenotypes of human sitosterolemia [68]. Transgenic mice overexpressing human *ABCG5/ABCG8* genes in the liver and small intestine retain fewer plant sterols in the body and the hepatic cholesterol synthesis is upregulated due to reduced intestinal absorption and increased biliary secretion of cholesterol [20].

Under normal conditions, the main part of the FC in the enterocytes is esterified by ACAT2. After a meal, the CE in chylomicrons represent up to 78% of their total cholesterol content [69-71]. Chylomicrons carry more than 85% of the total cholesterol taken up by the small intestine through the lymph [70, 72]. In *Acat2* deficient mice, when dietary cholesterol content is increased, the intestinal cholesterol absorption was decreased by 60-85% [34, 73].

ABCA1 is expressed on the basolateral surface of intestinal cells where it is suggested to mediated the efflux of cholesterol to HDL particles [74] as supported by the studies in the Caco2 cells [75-77]. In mouse, it has been shown that the ABCA1 protein is present both intracellularly and at the plasma membrane, including the basolateral membrane [74]. Conditional knockout of *Abca1* in mouse intestine reveal that approximately 30% of the steady-state plasma HDL cholesterol is due to the ABCA1 mediated cholesterol secretion [74]. However, in these mice, the total lymphatic lipid transport did not alter in the absence of intestinal *Abca1*, nor were there any changes of the fractional cholesterol absorption [74]. This suggests that intestinal ABCA1 does neither participate in the transport of dietary

cholesterol into lymph, nor influence luminal cholesterol absorption. However, in the absence of *Acat2*, the accumulation of intracellular FC, the oxysterol formation and subsequent transcriptional uprugulation of *Abca1* via liver X receptor (LXR), may partially compensate for the lack of cholesterol esterification [73, 78]. The critical role of intestinal *Abca1* in mediating LXR effect has also been shown by Bruhham *et al* [79]. In their study, they showed that intestinal but not hepatic ablation of *Abca1* abolished LXR agonist GW3965-stimulated elevation in plasma HDL. In addition, in *Npc1l1* knockout mice, the baseline of intestinal *Abca1* mRNA is reduced [80, 81], possibly as consequence of the lower intracellular levels cholesterol. This may also explain why inhibition of NPC1L1 by ezetimibe also lower HDL[82]. Treatment with LXR agonist in these mice results in an activation of the intestinal *Abca1*, but no changes of the steady-state plasma HDL cholesterol was seen [81].

1.2 BILE ACID METABOLISM

1.2.1 Bile acid synthesis

Conversion of cholesterol into BA is a way to eliminate excess cholesterol from the body. In humans, the BA pool consists of the primary BA (CA and chenodeoxycholic acid, CDCA) and the secondary BA (deoxycholic acid, DCA, and lithocholic acid, LCA). Human bile contains mainly CA (30-40%), CDCA (30-40%), DCA (20-30%) and in addition, small amounts of ursodeoxycholic acid (UDCA, 5%) and LCA (<1%) [83, 84]. Primary BA are synthesized from cholesterol exclusively in the liver through two pathways, the classic (neutral) pathway and the alternative (acidic) pathway (Figure 3). Enzymes that catalyze these multistep reactions are located in the endoplasmic reticulum, mitochondria, cytosol, and peroxisomes. The BA can afterwards be conjugated with taurine or glycine.

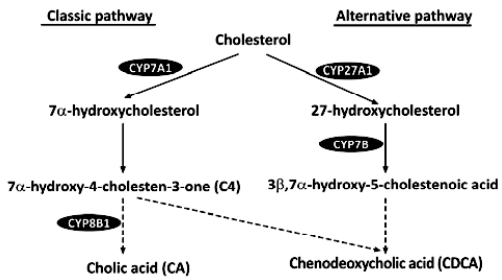

Figure 3 Pathways of bile acid synthesis

1.2.2 Secretion and entero-hepatic cycle of bile acids

BA are transported across the canalicular membrane of the hepatocytes into the bile and stored in the gallbladder. After each meal, the gallbladder empties and the BA are released into the intestinal tract with the bile. 95% of the BA are reabsorbed in the ileum, and transported back to the liver via portal blood for re-excretion into the bile. Only 5% of the BA are lost in feces. This whole process is referred to as the entero-hepatic circulation of the BA (Figure 2). The BA pool size is defined as the total amount of BA circulating in this entero-hepatic circulation. In humans, the BA pool size is around 2.5-3g. In the colon, the bile salts are transformed by the action of bacteria into secondary bile salts. Some of the secondary bile salts are also reabsorbed in the colon. The daily loss of BA in the feces is about 0.2-0.6g and they are compensated by *de novo* synthesis by the liver to maintain the BA pool constant[85]. In humans, BA complete an entero-hepatic cycle about 6-10 times per day. Entero-hepatic cycling serves to reclaim BA and to also act as messengers that carry signals from intestine to liver.

1.2.3 Bile acid transporters

The uptake of BA into the hepatocytes are fulfilled by two BA transporters, the Na^+-dependent taurocholate transporter (NTCP, SLC10A1) and the organic anion transporter polypeptide (OATP). The NTCP is exclusively expressed in the hepatocytes [86], where they are present at the sinusoidal/basolaterol membrane of the hepatocytes [87]. The uptake by the Na^+-dependent bile salt pathway accounts for 80% of the total taurocholate uptake and is considered as the major BA transport system located at the basolateral membrane.

The intracellular transport of BA is not fully understood. Two mechanisms have been proposed for the transcellular transport of BA from the sinusoidal to the canalicular membrane of the hepatocytes: the intracellular trafficking and the vesicle mediated transport of BA [88, 89].

More is known about the excretion of BA from the liver cells into the bile across the canalicular membrane. This occurs against a steep concentration gradient which changes between 100 ~ 1000 fold. At the canalicular membrane, the bile salt export pump (BSEP, also called ABCB11 or sister of P-glycoprotein, SPGP), is mainly responsible for this process (Figure 2) [90]. ABCB11 is solely expressed in the canalicular membrane of the hepatocytes. Mutations in *ABCB11* were first identified in patients with progressive familial intrahepatic cholestasis subtype 2 (PFIC-2) [91, 92].

In the intestine, the main part of the BA absorption is in the terminal ileum (Figure 2) and mediated by the apical sodium-dependent bile salt transporter (ASBT) [93]. ASBT is also expressed in other tissues where it facilitates BA uptake, such as in the proximal renal convoluted tubule cells, in the cholangiocytes and in the gallbladder epithelial cells. Both primary and secondary conjugated and unconjugated BA act as its substrates. The human ASBT efficiently transports conjugated and unconjugated BA but with a preference for the taurine and glycine conjugates over the unconjugated forms. Furthermore, ASBT exhibits a higher affinity for the dihydroxy BA (CDCA and DCA) compared to the trihydroxy BA (CA) [94]. Mice deficient for *Asbt* exhibit intestinal BA malabsorption and the enterohepatic circulation of BA is greatly reduced [95].

After entering the enterocytes, the BA bind to the intestinal bile acid binding protein (IBABP) [89] and are transported to the basolateral membrane for secretion (Figure 2). The organic solute transporter (OST) α and OSTβ form heterodimers and are the major basolateral BA transporters [96]. In mouse lacking *Ostα*, a marked decreases in intestinal BA absorption, serum BA concentration, and BA pool size are seen [96].

1.2.4 FGF15/19 signaling in the regulation of hepatic bile acid synthesis

Fibroblast growth factor (FGF) 19, the human ortholog of mouse FGF15, was first discovered in primary cultures of human hepatocytes after induction of farnesoid X receptor (FXR) [97]. FGF19 binds to FGF receptor 4 (FGFR4) [98], and represses *CYP7A1* through a c-Jun N-terminal kinase (JNK)-dependent pathway [97]. Similarly in mice, FGF15 is induced by the FXR agonists GW4064 or by CA in ileum and inhibits *Cyp7a1* by binding to FGFR4 [99]. In *Fgfr4* transgenic mice, the JNK activity increased, *Cyp7a1* expression and BA pool size decreased [100]. In *Fgfr4* knockout mice, an increase of *Cyp7a1* expression is seen. An increased BA pool size and decreased JNK activity were also observed [101]. In humans, serum FGF19 levels were reduced following treatment with a BA binding resin cholestyramine, in parallel with an increase of BA synthesis as indicated by an increased serum C4 level, and *vice versa*, upon CDCA feeding, the FGF19 level increased and C4 level decreased [102]. Interestingly, FGF19 is absent in the normal liver but highly expressed in the ileum [99], while its receptor FGFR4 is expressed in the liver [99]. Under conditions like extrahepatic cholastasis, *FGF19* mRNA is increased [103]. In *Asbt* knockout mice, due to malabsorption of BA, the FXR activation is lost and its target genes *Fgf15* and small heterodimer partner (*Shp*) are reduced to a level below detection [104]. However, this can be restored by either the synthetic FXR agonist GW4064 or FGF15 [104]. In *Ostα* knockout mice, despite that the basolateral secretion of

BA is impaired at the ileum epithelium, the BA still are able to induce the secretion of FGF15 and inhibit *Cyp7a1* in liver [105]. This suggests that an intact apical absorption of BA into ileum epithelium is requisite for the activation of the FXR/FGF15/FGFR4 signaling pathway on the regulation of BA rather than the basolateral secretion of BA from ileum into portal vein. In addition, FGF15/FGF19 are also shown to play a role in the postprandial timing mechanisms that controls gallbladder motility [106].

1.3 REGULATION OF TRANSCRIPTION FACTORS

1.3.1 Nuclear receptors

Nuclear receptors have a typical modular structure, which contain a highly conserved DNA-binding domain in the N-terminal region and a moderately conserved ligand binding domain in the C-terminal region separated by a variable hinge region [107, 108]. Ligand-independent activation function-1 (AF-1) and ligand-dependent activation function-2 (AF-2) are located in the N-terminal, respectively. In the DNA binding domain, two cysteine-coordinated Zn^{2+} finger motifs are involved in the DNA binding and dimerization. The E region is involved in dimerization and coregulator interaction. After binding to the ligand, nuclear receptors undergo conformation changes, which releases corepressors and recruit co-activators that bind to the AF-2 helix [109, 110].

1.3.1.1 Liver X receptor (LXR)

Both LXR and FXR play a role in coordinating the regulation of BA and cholesterol metabolism in the liver, intestine and peripheral tissues.

LXR has two isoforms, LXRα (NR1H3) and LXRβ (NR1H2). LXRα is expressed in liver, spleen, adipose tissue, lungs and pituitary [111], whereas LXRβ is ubiquitously expressed [112]. Oxysterols are ligand for LXRs [113]. The natural oxysterols include 22(R)-hydroxycholesterol, 24(S)-hydroxycholesterol, 24(S), 25-epoxycholesterol and 27-hydroxycholesterol [114].

LXRα acts as a cholesterol sensor and upregulates the expression of various components in the cholesterol metabolism. In mice, absence of *Lxrα* [115] results in cholesterol accumulation in the liver, accompanied by an increase of plasma LDL cholesterol. The upregulation of *Cyp7a1* and the BA synthesis is important for the clearance of excessive dietary cholesterol [116]. This regulatory mechanism is lost in mice deficient of *Lxrα*. *Lxrα* knockout mice have a marked decrease of the BA pool size and changes in BA composition. This is due to the presence of a LXR response element on the mouse

Cyp7a1 promoter [117]. LXRα does not stimulate the human *CYP7A1* [118] since the promoter region of this gene lacks a LXRα response element.

LXRβ does not have the same function as LXRα in regulating the hepatic response to cholesterol challenge as has been shown in *Lxrβ* knockout mice [119]. The comparison between *Lxrα* and *Lxrβ* knockout mice suggests that these two nuclear receptors, although they share a high degree of amino acid similarity (78%) [120] , do not have a complete overlapping function in the liver.

The regulation of cholesterol and BA metabolism by LXR has also been widely studied by using synthetic LXR agonists such as TO901317 and GW3965. Upon LXR activation, hepatic *Abcg5* and *Abcg8* transporters are induced and promote biliary cholesterol secretion [121]. In parallel, intestinal *Abcg5* and *Abcg8* activation results in a decrease in cholesterol absorption [121]. Hepatic *Abca1* has been shown to be insensitive to LXR activation [122, 123]. While intestinal *Abca1* has been shown to be modulated by LXR [79, 81].

LXR may activate lipogenesis by inducing sterol regulatory element binding protein 1c (SREBP1c) [124] and its target genes which increase of fatty acid synthesis, triglyceride accumulation in liver and hyperglyceridemia. This has been shown in either *LXR* transgenic mice [125] or during the treatment with LXR agonist TO901317 [126]. The development of an intestinal specific LXR agonists, such as GW6340 [127], may avoid such side effect. The LXR agonists also play an important role in RCT [128] and have been proposed to be a pharmaceutical compound in the prevention of atherosclerosis.

1.3.1.2 Farnesoid X receptor (FXR)

FXR (NR1H4) was identified as a BA receptor in 1999 [129, 130]. It is involved in the regulation of BA metabolism. FXR needs to heterodimerize to RXR to exert its transcriptional regulation of target genes. By binding to FXR, BA activate the expression of another nuclear receptor, the SHP. SHP inhibits the transcription of *CYP7A1* and BA synthesis through the interactions with liver receptor homologue 1 (LRH-1)[131, 132] on the promoter sequence of *CYP7A1*. When using liver or intestine specific *Fxr* knockout mice, it has been shown that the FXR mediated repression of BA synthesis by repression of both *Cyp7a1* and *Cyp8b1* expression requires a complementary action of FXR on both liver and intestine [133]. FXR is also able to activate the hepatic canalicular transporters of BA and phospholipids – *ABCB11*[134] and *ABCB4* [135]. CDCA is the most potent endogenous FXR ligand in humans [84], while in mice, CA seems to be the most potent endogenous ligand. Hydrophilic BA such as UDCA and β muricholic acid do not

activate FXR [129]. In mice, *Fxr* depletion leads to an impaired BA and cholesterol metabolism [136, 137] and an accumulation of serum BA, suggesting a defective hepatic BA uptake from the blood and/or a reduced hepatic canalicular BA secretion [136]. Despite this, the *Fxr* knockout mice still exhibit increased intestinal absorption of cholesterol [138] and triglycerides, which is due to an increased BA pool size [133, 137]. In these mice, hepatic uptake of HDL is decreased while synthesis of Apo B-containing lipoproteins is increased [136, 138, 139]. Consequently, the *Fxr* knockout mice have a potentially pro-atherogenic lipoprotein profile that is additionally exaggerated by the increased uptake of dietary cholesterol [136, 139]. However, *Fxr* deficiency alone in mice does not seem to be sufficient to promote the development of atherosclerosis [139]. In fact, loss of *Fxr* reduced atherosclerosis in the *Ldlr* knockout mice [139], where the opposite was found in *Apoe* knockout mice [140].

FXR also regulates the BA transporters in the ileum, activating intracellular trafficking by IBABP [129] and basolateral efflux by OSTα/β [141]. As mentioned above, FXR also induces the expression of FGF15, which in turn represses the hepatic *CYP7A1* expression [99].

1.3.1.3 Estrogen receptors (ERs)

ERα(ERA, NR3A1) and ERβ(ERB, NR3A2) also belong to the nuclear receptor family of transcription factors. Like other nuclear receptors, ERs contains a DNA binding domain, a ligand binding domain and variations at the NH_2-terminal domain in both sequence and length. ERA and ERB are products of separate genes located on different chromosomes [142]. In rat, it has been shown that *Era* mRNA is expressed predominantly in the uterus, mammary gland, testis, pituitary, liver, kidney and skeletal muscle, whereas *Erb* mRNA is expressed in the ovary and prostate [143, 144]. In the liver, *Era* is the predominant ER form expressed as shown by studies in rats [143, 144] and mice[145]. *Erb* mRNA is not expressed in the hepatocytes, but it can be detected in the cholangiocytes [146] and the stellate cells[147]. Furthermore, Alvaro *et al.*[146], by using western blot and immunohistochemistry showed that no ERB protein expression could be detected in either male or female rat hepatocytes. However, in cholangiocytes, ERB was detected. On the contrary, Zhou *et al* [147], by using a different antibody and an autoclave-antigen retrieval technique detected ERB in hepatocytes by immunohistochemistry. Although they showed expression of both ERA and ERB protein in the whole liver extracts from normal rats, they were not able to differentiate the hepatocytes from other cells. Recently by using real-time PCR, Bookout et al showed that *Era* mRNA is expressed in mouse

liver, with no or an undetectable level of *Erb* mRNA [145]. Thus, it seems most likely that mouse liver express ERA but not ERB.

There are several pathways by which estrogen and ERs regulate biological processes:

1) In the classical (genomic) pathway, ligand-activated ERs bind specifically to the estrogen-responsive elements (EREs) through the DNA binding domain and bring co-regulators to the transcription start site[148]. Estrogen also modulates gene expression by interacting with other transcription factors, such as activating protein 1 (AP-1) and stimulating protein 1 (Sp1) [149].

2) In the non-genomic pathway, a ligand activates a possible membrane associated receptor: a classical ER, an ER isoform or another distinct receptor or alternatively; activates a classical ER located in the cytoplasma. After this, a signaling cascade is initiated via a second messenger that affects the ion channels or increases the nitric oxide levels in the cytoplasm which in turn leads to a rapid physiological response without involving gene regulation [150].

3) Growth factor signaling, which activates a kinase that may phosphorylate and activate ERs or associated coregulators in the absence of estrogen ligands [151].

1.3.2 Sterol regulatory element binding proteins (SREBPs)

There are three known sterol regulatory element binding proteins (SREBPs). Each of them shares a similar tripartite structure [152], which consist of 1) an NH_2-terminal transcription factor domain of ~ 480 amino acids; 2) a middle hydrophobic region of ~ 80 amino acids containing two hydrophobic trans-membrane segments; and 3) a COOH-terminal regulatory domain of ~ 590 amino acids. In humans and mice, SREBP1a and SREBP1c are produced from a single gene by alternate transcript start sites. SREBP2 is encoded by a separate gene.

After the NH_2-terminal domain is cleaved, the SREBPs form a mature protein, leaving the membrane and entering the nucleus to activate the transcription of their target genes. SREBP2 can activate the transcription of enzymes involving cholesterol synthesis, such as HMG-CoA synthase, HMG-CoA reductase, and squalene synthase as well as the LDL receptors [153, 154]. These regulatory effects may result in an increase in the cellular cholesterol content. SREBP1c modulates the transcription of genes involved in fatty acids synthesis[155], such as acetyl-Coenzyme A carboxylase[156], fatty acid synthase, and stearoyl-CoA desaturase-1[157].

1.4 CHOLESTEROL AND BILE ACID METABOLISM IN GALLSTONE DISEASE

GS disease is common throughout both industrialized and developing countries[158]. In 1995, the Chinese National Survey reported that GS disease accounted for nearly 10% of all the diagnoses of patients hospitalized in surgical clinics. In the GS patients, the majority of the GS were composed of cholesterol. Diverse genetic and environmental factors contribute to the formation of GS disease [159]. Although a disease of multi-factorial origin, the current consensus view is that supersaturation of biliary cholesterol in bile is the major prerequisite for GS formation.

Much of our knowledge on the pathogenesis of GS has been provided by mouse models challenged with a lithogenic diet[160-165]. These studies suggest that the defects of hepatic cholesterol and BA metabolism contribute to the formation of lithogenic bile and GS. However, limited studies have been focused on the enzymes responsible for hepatic cholesterol and BA synthesis. Early studies attempted to define enzymatic defects in the liver contributing to supersaturation of bile. The activity of HMG-CoA reductase was shown to be increased in GS patients[166]. Conversely, the activity of CYP7A1[166] and ACAT[167] were decreased in GS patients. However, later studies on different populations did not confirm these results[168, 169]. Recently, a study in Chilean Hispanics and Mapuche Indians found an increase in CYP7A1 activity by measuring a surrogate marker – C4 of BA synthesis in the plasma of GS patients[170]. In this population, increased microsomal triglyceride transporter protein (MTTP) activity together with an increased plasma VLDL was also found[171]. The compensatory increase of hepatic BA synthesis is explained by an intestinal loss of BA in GS patients. Recent studies revealed diminished expression of ASBT, IBABP [172, 173] and OSTα/β[174] in German patients with GS disease. On the other hand, a study on Chilean Hispanics with GS disease failed to find an increase in fecal BA excretion rates or differences in mRNA levels of genes for ileal BA transporters. The mRNA expression levels of *CYP7A1* were still increased in GS patients, which indicates that the regulation of BA synthesis is abnormal in Hispanics with GS disease [175]. The discrepancy of studies may be due to the population studied or the criteria of the control patients. Moreover, a subgroup of patients with complex metabolic defects may also exist as GS disease [158, 159], which means that several genetic factors[176-178] may be involved and must be considered.

2 AIM OF THE STUDY

Many of the studies on cholesterol and BA metabolism have been carried out using cell culture and animal models. The aim of this study is to get further insights into cholesterol and BA metabolism in human.

In detail, the respective aim of each paper is:

- Paper I

We attempted to identify the molecular defects in hepatic cholesterol and BA metabolism that may contribute to the formation of GS disease.

- Paper II

To study the expression of intestinal genes crucial for cholesterol absorption in relation with GS disease.

- Paper III

To gain more insights into the mechanisms that participates in the transcriptional regulation of human *NPC1L1* gene in liver.

- Paper IV

To further elucidate the physiological role of hepatic microsomal ACAT2 in human cholesterol metabolism.

- Paper V

To investigate the gender difference in the ACAT2 activity in mice by using estrogen receptor α (ERA) and/or β (ERB) knockouts.

3 METHODS

3.1 SUBJECTS

Chinese patients with/without GS disease were studied. Details concerning the subject recruitment, sample collection are described in the papers I, II, III and IV.

3.2 ANIMALS

Female and male ERα knockout (ERKO), ERβ knockout (BERKO) and ERα/β double knockout (DOKO) mice and the corresponding wild-type mice were used. Details are described in paper V.

3.3 PLASMA LIPIDS AND LIPOPROTEIN PROFILE

Measurement of plasma or serum total cholesterol, triglycerides, HDL cholesterol, LDL cholesterol , Apo A1 and Apo B are described in papers I, II, IV, V. Lipoproteins were separated by size exclusion chromatography as previously described [179].

3.4 PLASMA PLANT STEROLS, LATHOSTEROL AND 7α-HYDROXY-4-CHOLESTEN-3-ONE (C4)

Plasma sitosterol, campesterol and lathosterol were determined by isotope-mass spectrometry using deuterium-labeled sitosterol, campesterol [180] and lathosterol [181] as internal standards. Plasma 7α-hydroxy-4-cholesten-3-one (C4) levels were determined by the LC-MS/MS method with $^{2}H_{6}$-labelled C4 as internal standard as previously described [182].

3.5 BILIARY LIPIDS

Biliary cholesterol, total BA and phospholipids in gallbladder bile were measured as previously described [83]. Cholesterol saturation index (CSI) was calculated using Carey's critical table [183].

3.6 HEPATIC LIPIDS

Lipids were extracted from liver tissue by methanol:chloroform (1:2 v/v). Hepatic total cholesterol, FC and lathosterol were assayed by isotope dilution-mass spectrometry with the use of deuterium-labeled cholesterol and lathosterol as internal standards [181, 184]. Hepatic triglycerides were determined in liver lipid extracts by colorimetric enzymatic methods (TG Roche/Hitachi, Roche Diagnostic GmbH, Mannheim, Germany). Protein content was determined according to Lowry's method. Details are described in paper I, IV and V.

3.7 GENE EXPRESSION

Total RNA from liver, small intestine or cells was extracted by Trizol® (Invitrogen, Carlsbad, USA). cDNA was synthesized using Omniscript (QIAGEN, Valencia, CA, Paper I, III and IV) or High-capacity cDNA Reverse Transcript Kit (Applied Biosystem Inc., Foster City, CA, USA, Paper II and V) and diluted 1:10 with DNase and RNase-free H_2O. Real-time PCR was performed on ABI 7500 with SYBR-Green assay. Data was calculated by the delta-Ct method, expressed in arbitrary units, and was normalized by the signals obtained from the same cDNA for Cyclophilin A.

3.8 PROTEIN EXPRESSION

Protein expression levels were determined by Western-Blot. Details are described in papers I, IV, V.

3.9 ACAT2 ACTIVITY MEASUREMENT

In brief, total ACAT enzymatic activity was determined in hepatic microsomes, including a preincubation with a cholesterol-saturated solution of β-hydroxypropyl cyclodextrin before addition of the ^{14}C-oleoyl Co-A, as described [36]. In a parallel incubation, pyripyropene A (PPPA), a specific ACAT2 inhibitor, was included in the preincubation and reaction mixtures at a concentration of 5 μmol/L in order to separately identify ACAT1 and ACAT2 activities [36]. Details are provided in the paper I, IV, and V.

3.10 COTRANSFECTION

HuH7 and HEK293 cells were cultured as described [46]. Transfections of HuH7 cells were performed using 2 μg promoter construct (or mutated constructs) and 2 μg pSV-β-galactosidase control vector (Promega) with or without increasing concentrations of SREBP2, HNF1α, and HNF4α expression vectors or with 0.5 μg of each expression vector using Lipofectin reagent (Invitrogen, Carlsbad, CA) at a ratio of 3:1 (Lipofectin:DNA). pGL3 empty vector (Promega) was used to adjust for differences in the amount of DNA added to the cells. Transfections of HEK293 cells were performed like those for HuH7 cells, except that Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) at a ratio of 0.25:1 (Lipofectamine:DNA) was used. The pSV-β-galactosidase control vector was used to correct for variation in transfection efficiency. Forty-eight hour after transfection, cell lysates were prepared in reporter lysis buffer (Promega). β-galactosidase and luciferase activities were determined using β-galactosidase or luciferase assay kits, respectively, according to the manufacturer´s instructions

(Promega). All transfection data are expressed as luciferase activity corrected by β-galactosidase activity.

For gene expression analysis, HuH7 cells were transfected with 0, 0.05, 0.1, 0.5, 1, and 2 μg SREBP2, HNF1α, or HNF4α expression vector using Lipofectin reagent (Invitrogen, Carlsbad, CA). Forty-eight hours after transfection, total RNA was prepared using Trizol reagent according to the manufacturer's protocol.

To study the effect of cholesterol on *NPC1L1* gene expression, HuH7 cells were incubated for 12 hours with 0, 0.1, 0.5, 1, and 2 mM LDL cholesterol or with 10% LPDS prior to RNA extraction.

3.11 CHROMATIN IMMUNOPRECIPITATION (CHIP) ASSAY

The CHIP assay was performed using ~200 mg liver from a healthy donor as described [46]. Specific antibodies for HNF1α (sc-6547X; Santa Cruz, CA), SREBP2 (sc-8151X, Santa Cruz, CA) and an IgG antibody (sc-2027, Santa Cruz, CA), as a (baseline) control, were used (4 μg). The primers used for detection of the two SREBP2 binding sites (SRE1 and SRE2), HNF1α binding site to the human NPC1L1 promoter and human exon 7 as correction for DNA loading are listed in paper III.

3.12 ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA)

Nuclear extracts were prepared from HuH7 cells as described by Azzout-Marniche et al.[185]. Forward sequences for primers used to generate double-stranded probes and details of the procedure are described in paper III. For supershift assays, 2 μg HNF1α (sc-6547X; Santa Cruz, CA) or 2 μg SREBP2 (sc-8151X; Santa Cruz, CA) antibodies were added to the binding reaction mixtures.

3.13 STATISTICS

Details are described in papers I-V.

4 RESULTS

4.1 PAPER I

Twenty-two Chinese patients with GS (11 females and 11 males) and 13 gallstone-free patients (GSF: 10 females and 3 males) were investigated.

In bile, a significantly greater molar percentage of biliary cholesterol was present in GS, as well as a significantly higher cholesterol saturation index (CSI: GS *vs* GSF: 1.04±0.08 *vs* 0.71±0.05, $P < 0.01$). Neither the total BA nor the phospholipids in bile differed between the groups. This was in agreement with the concept that supersaturation of biliary cholesterol is the prerequisite for GS formation [159, 176].

Hepatic *ABCG5* and *ABCG8* mRNA levels were significantly higher in GS compared with GSF (Figure 4). The observation is in agreement with recent studies of inbred mice challenged with a lithogenic diet, in which formation of GS was accompanied by an increase of *Abcg5/Abcg8* in liver [160, 186].

Moreover, *ABCG5* and *ABCG8* expression correlated positively with the biliary cholesterol molar percentage (r=0.57 and r=0.54, $P < 0.05$) and the CSI (r=0.54 and r=0.55, $P < 0.05$). Thus, it is likely that the increased expression of ABCG5/ABCG8 may lead to an increased secretion of cholesterol into the bile with a consequent increase in CSI, as also showed in animal models [20].

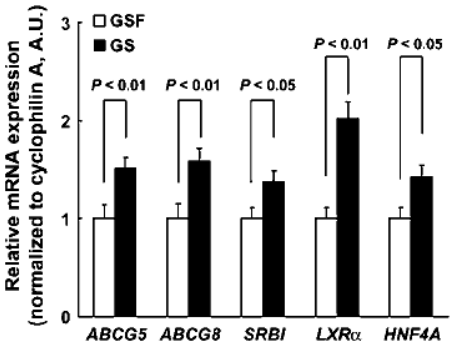

Figure 4 mRNA expressions of hepatic *ABCG5*, *ABCG8*, *SRB1*, *LXRα* and *HNF4A* between GS patients and GSF controls.

Interestingly, the expression of *LXRα* was doubled in GS (Figure 4) and correlated with *ABCG5* (r=0.58, $P < 0.05$), *ABCG8* (r=0.59, $P < 0.05$) and biliary cholesterol molar percentage (r=0.43, $P < 0.05$). The identification of these correlations suggest that in

humans *ABCG5* and *ABCG8* are transcriptional regulated by LXRα as in line with the observation in mouse model [121]. Therefore, this nuclear receptor may play a role in the pathogenesis of GS disease in Chinese patients.

The liver HDL receptor *SRBI* displayed 38% higher levels of mRNA in GS ($P < 0.05$, Figure 4). This was in parallel with a 74% increase of the SRBI protein ($P < 0.05$, Figure 5). In addition, the protein expression of SRBI correlated with both the biliary cholesterol molar percentage (r=0.56, $P < 0.05$) and CSI (r=0.52, $P < 0.05$). Thus, an enhanced uptake of HDL cholesterol may contribute to the increase of biliary cholesterol. This is in line with a previous observation that FC in HDL were in rapid equilibrium with biliary cholesterol [187] and also consistent with the finding that *Srbi* expression in mice regulated biliary cholesterol but not BA or phospholipids [16, 188].

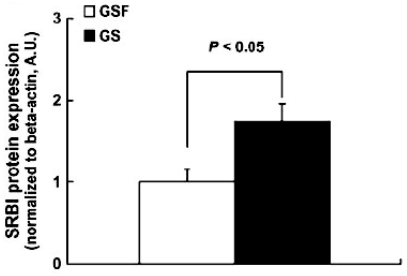

Figure 5 SRB1 proteins between gallstone patients and gallstone-free controls

4.2 PAPER II

In this study, jejunal mucosa from 12 Chinese patients with GS disease and 31 GSF patients were collected and investigated.

In the enterocytes, NPC1L1 is regarded as the major transporter for cholesterol absorption [25]. Using the mucosa of jejunum, we found a 33% increase in the mRNA level of *NPC1L1* in GS compared with GSF ($P < 0.05$, Figure 6), which could signify an enhanced intestinal cholesterol absorption in GS patients.

ABCG5 and ABCG8 are generally proposed to mediate the efflux of cholesterol in the intestinal lumen. However, in this study, no difference was observed between patients and controls for *ABCG5* or *ABCG8* expression.

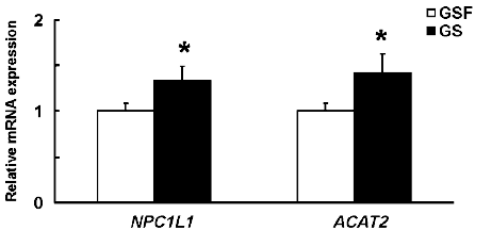

Figure 6 *NPC1L1* and *ACAT2* mRNA levels in the jejunal mucosa were higher in gallstone patients compared with gallstone-free patients.

Interestingly, the mRNA level of *ACAT2* in jejunum of GS patients was also higher (41%, $P < 0.05$, Figure 6), suggesting the existence of a higher rate of intestinal cholesterol esterification in GS. An increase in *NPC1L1* and *ACAT2* in GS patients would be in line with the fact that NPC1L1 facilitates the trafficking of apical membrane cholesterol to ACAT2 as has been shown in Caco2 cells [189].

4.3 PAPER III

By using the liver biopsies from 22 GS and 12 GSF patients, we found a correlation between *NPC1L1* mRNA and *SREBP2* mRNA (r=0.74, $P < 0.05$), *NPC1L1* and *HNF4α* mRNA (r=0.53, $P < 0.05$), but not between *NPC1L1* and *HNF1α* (r=0.06, $P > 0.05$). This prompted us to investigate whether *NPC1L*1 is under transcriptional regulation by these transcription factors in the human liver.

Co-transfection of *SREBP2* and human *NPC1L1* promoter (-1570 to + 137bp) in human hepatoma cells (HuH7) showed a strong dose-dependent up-regulation of human *NPC1L1* promoter activity. An increase in mRNA expression of the *NPC1L1* in HuH7 cells was also observed using 0.5μg *SREBP2* vector. Moreover, cellular cholesterol loading by LDL decreased both *SREBP2* mRNA and *NPC1L1* mRNA; in contrast, cholesterol depletion increased mRNA expression of *NPC1L1*. Two SRE binding sites were identified in the promoter of *NPC1L1* [190]. CHIP assay led to >36 fold enrichment of SRE1 (-91/-81bp) and ~17-fold enrichment of SRE2 (-748/-738bp) sequences present in the human *NPC1L1* promoter (Figure 7). This data collectively suggests that SREBP2 seems to be an important regulator of human *NPC1L1* in the liver which has been shown in intestine by Alrefai *et al.* [190].

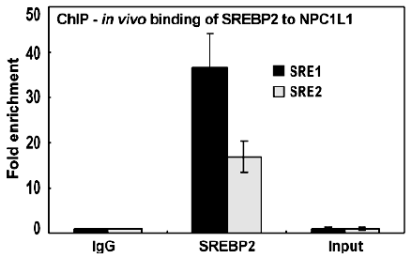

Figure 7 Enrichment of SRE1 and SRE2 sequence present in the human *NPC1L1* promoter by CHIP assay.

We then performed a co-transfection of the human *NPC1L1* promoter with an *HNF4α* expression vector in HuH7 cells. However, we did not observe an induction of *NPC1L1* promoter activity. HNF4α decreased the human *NPC1L1* promoter activity in a dose-dependent manner. This was in contrast to a study by Iwayanagi *et al* [191], who showed that transcription of *NPC1L1* was stimulated by HNF4α with SREBP2 together. We did not find this synergetic regulation, possibly due to the use of a different plasmid, the vector dosage or the different cell line used in this study.

HNF4α and HNF1α can regulate reciprocally, thus we performed a co-transfection using human *NPC1L1* promoter with *HNF1α* expression vector in HuH7 cells. The results showed a dose-dependent regulation by HNF1α on *NPC1L1* promoter activity and *NPC1L1* mRNA as well. This is in line with a report from Odom *et al* [192]. Six cis-elements were found to be located upstream of the ATG start codon. Mutation of the -158/-144 HNF1α binding site almost completely abolished the regulatory effect of HNF1α on *NPC1L1* promoter activity. Immunoprecipitation with an antibody against HNF1α and primers designed to target the region spanning over six HNF1α sites (-769 ~ -119bp) were used in the PCR. This led to >12-fold enrichment of the human *NPC1L1* promoter (Figure 8). EMSA showed a direct binding of HNF1α to the -158/-144bp HNF1α binding site in the *NPC1L1* promoter, but no binding to the other five sites. These data collectively showed that HNF1α can regulate and bind to the *NPC1L1* promoter in human liver and that this effect is mediated by the -158/-144bp HNF1 binding site.

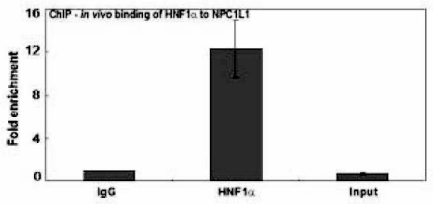

Figure 8 Enrichment of HNF1α sequence present in the human *NPC1L1* promoter by CHIP assay.

4.4 PAPER IV

In this study, 18 females and 22 males with/without GS were included. Some of the patients had been studied in Paper I and an additional 9 male donors of liver transplantation were included. No difference of hepatic ACAT2 activity was observed between GS patients and GSF patients, suggesting that hepatic ACAT2 and esterification of cholesterol did not account for the GS disease in Chinese patients. However, there was a significantly lower hepatic microsomal ACAT2 activity in females (7.24±1.22 pmol/min/mg protein in females *vs* 25.5±5.75 pmol/min/mg protein in males, $P < 0.01$), regardless of the presence of GS disease (Figure 9). In mice, ACAT2 has been shown to be pro-atherogenic[37] and its depletion led to lower hepatic CE formation and atherosclerosis[38]. Warensjo et al [193] also provided strong evidence for the importance of ACAT2 derived CE in relation to human coronary heart disease. Thus the observed gender-related difference of ACAT2 may contribute to the lower incidence of coronary heart disease in women compared to men.

Figure 9 Hepatic ACAT2 activities between females and males.

Plasma HDL and Apo A1 were lower in females than in males ($P < 0.01$). We observed a negative correlation between hepatic ACAT2 activity with plasma HDL (r=-0.57, $P < 0.05$) and Apo A1 (r=-0.49, $P < 0.05$). No gender-related difference was observed for the mRNA and protein level of SRBI. Furthermore, we did not observe a gender related

difference at the mRNA level for *ABCA1*. As we were not able to determine the protein level of ABCA1, we could not rule out whether the elevated HDL levels are also secondary to the expression of the ABCA1 protein. No gender-related difference was found in several other genes involved in the lipid metabolism (cholesterol synthesis, secretion and uptake) in liver.

4.5 PAPER V

In this study, we found that hepatic microsomal ACAT2 activity was significantly higher in female mice (female: 698.2±39.1 pmol/min/mg protein *vs* male: 302.7±14.5 pmol/min/mg protein, $P < 0.01$). This difference was independent of ERA or ERB. The ACAT2 activity in each group and comparison by 2-way ANOVA was shown in Figure 10. Nevertheless, independently of the gender, the presence of ERB was associated to higher ACAT2 activity. Interaction analysis between gender and ERB showed that this isoform of estrogen receptor may regulate hepatic microsomal ACAT2 activity only in female mice,

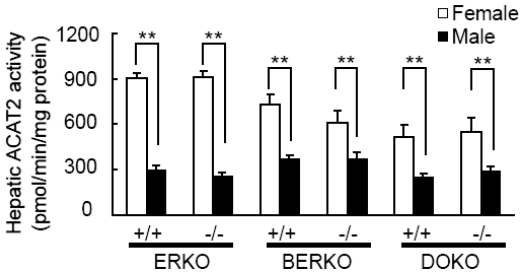

Figure 10 Comparison of hepatic ACAT2 activity between groups.

Gender differences were also observed for *Acat2* mRNA regardless the presence/absence of ERs (female: 1.62±0.07 *vs* male: 1.00±0.04 $P < 0.01$). Independently of the gender, the presence of ERA was associated with higher *Acat2* mRNA than absence of ERA (ERA-presence: 1.41±0.07 *vs* ERA-absence: 1.11±0.06, $P < 0.01$). The interaction analysis between gender and ERA showed that only in female mice, the presence of ERA was associated to higher *Acat2* mRNA (Figure 11).

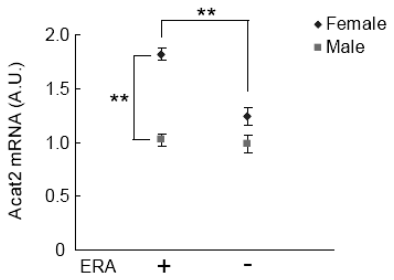

Figure 11 Comparison of hepatic *Acat2* mRNA level by interaction analysis between gender and ERA using multi-factor ANOVA.

Female mice had significantly lower expression of hepatic ABCA1 protein, regardless of ERA or ERB (female: 0.26±0.04 *vs* male: 0.89±0.06, $P < 0.01$). Although hepatic ACAT2 activity and its product CE increased in female mice, hepatic FC was about the same between all the groups. The FC content in the plasma membrane from liver was lower in female mice than male mice regardless of the presence/absence of ERA or ERB (female: 12.15±0.55 g/mg protein *vs* male: 16.11±0.38 g/mg protein, $P < 0.01$). Interestingly, a positive correlation between FC content in membrane and hepatic ABCA1 protein level (r=0.63, $P < 0.05$) and a negative correlation between hepatic ACAT2 activity and hepatic ABCA1 protein (r=-0.47, $P < 0.05$) or membrane FC content (r=-0.45, $P < 0.05$) were observed.

5 GENERAL DISCUSSION

GS disease is common in Western countries with an increasing incidence rate also in China. Supersaturation of cholesterol in the bile is believed to be the prerequisite for GS formation, which may be caused by either an increase of cholesterol and/or a decrease of BA.

In the present study, by using liver biopsies samples from non-obese normo-lipidemic Chinese GS patients, we found an increased mRNA expression of the hepatic canalicular cholesterol transporters *ABCG5* and *ABCG8*. The correlation between the biliary cholesterol supersaturation and the expression of both these genes suggest that an increased expression of ABCG5/ABCG8 may account for the supersaturation of cholesterol in bile. This in turn may be induced by an LXRα-mediated activation. Moreover, we also provide support that the origin of biliary cholesterol in humans may be the cholesterol in plasma taken up by the HDL receptor, because both the mRNA and the protein of SRBI were higher in the GS patients. Our findings are in line with previous observations in humans [194] in which isotope-labeled HDL were used and also with observations from studies in monkey in which their livers were perfused [187]. All these studies together suggest that the biliary cholesterol originates from plasma HDL cholesterol. Further support for this concept comes from studies when genetic modulation of the SRBI expression in mice resulted in a reciprocal regulation of plasma HDL and biliary cholesterol content [13-16].

Interestingly, defects in BA metabolism have also been proposed as a mechanism for the GS disease by studying Chilean GS patients [170]. However, such defects were not observed in the group of Chinese GS patients in our study. In the Chilean GS patients, significantly higher plasma C4 levels were found suggesting an increase of hepatic BA synthesis. This was supposed be due to an increased fecal loss of BA [170]. Nevertheless, a recent study from the same researchers contradicted this hypothesis since no changes in fecal BA excretion were then observed [175]. Neither did they find any changes in the mRNA expression of the ileal BA transporters, despite of an increased *CYP7A1* mRNA expression in the liver. The discrepancy between the two studies from Chile may be due to the fact that two different ethnic groups of patients were investigated: the Mapuche Indians and the Hispanic Chileans.

Our studies also showed that the jejunal mucosa of Chinese GS patients has higher levels of mRNA expression of *NPC1L1* and *ACAT2*. This observation suggests that an

additional mechanism leading to GS formation in Chinese patients may be an increased intestinal cholesterol absorption and esterification.

Considering the results from the different studies it can be concluded that: i) GS disease is a multi-factorial disease, several metabolic defects alone or in combination may all finally cause supersaturation of biliary cholesterol and GS formation; ii) Ethnic differences should also be considered, as the genetic background together with dietary and living habits may affect the development of GS disease; iii) the limitation in human studies should also be emphasized due to the limited number of patients usually studied and due to the different criteria used to select the control subjects.

The role of NPC1L1 and its regulation in human liver is still not fully understood. Our studies showed the existence of a correlation between the mRNA expression of *NPC1L1* with *SREBP2* and *HNF4α* in the liver of the Chinese patients. In addition, we also observed that SREBP2 binds to the SRE sites in the promoter of human *NPC1L1* gene. We also found a regulation of *NPC1L1* by HNF1α and we identified a site in promoter region of human *NPC1L1* (located at -158/-144) to which HNF1α binds.

It has been hypothesized that a lower hepatic esterification of FC may provide more cholesterol available for biliary secretion and hence GS formation. However, during that time, only the total hepatic ACAT activity was measured [167, 168]. Now, ACAT2 has been identified to be the only cholesterol esterifying enzymes in human hepatocytes [36]. In contrast to our expectation, there were no differences in hepatic ACAT2 activity between patients with and without GS. A clear gender-related difference in hepatic ACAT2 activity was instead identified: a difference that was independent of the presence of GS. Since ACAT2 derived CE – cholesteryl palmitate and cholesteryl oleate - are shown to be pro-atherogenic, both in mouse model [37] and in humans [193, 195], a lower hepatic ACAT2 activity should be of benefit for women by less formation of atherogenic CE later loaded onto VLDL particles. Moreover, the negative correlation between hepatic ACAT2 activity and plasma HDL and Apo A1 level suggests that the lower ACAT2 activity in the females may provide an additional athero-protective effect by increasing plasma HDL. Possible mechanisms linking ACAT2 to the HDL metabolism have been identified in our studies on mice, in which a negative correlation between hepatic ACAT2 activity and ABCA1 protein expression in liver membranes was identified.

Estrogen receptors are thought to be of importance for the gender-related difference and we thus chose the ERA and/or ERB knockout mice as a model to investigate whether

gender-related difference in hepatic ACAT2 activity exists in mice and their relation to the ERs. Opposite to women, female mice had higher hepatic ACAT2 activity as well as higher *Acat2* mRNA. In line with the ACAT2 activity, female mice also had higher hepatic CE content. Our results also suggest that ERA may be responsible for the gender-related difference in hepatic *Acat2* mRNA and that this may be more relevant in female mice, as also suggested by the higher hepatic CE content when ERA was present. Because the hepatic CE are the product of hepatic ACAT2, this may indicate a role for ERA on the hepatic ACAT2 activity *in vivo*. The observation of a lack of effects of ERA on the hepatic ACAT2 activity when measured in microsomes in the test tubes may indicate a limitation of this method to evaluate ACAT2 activity. When ACAT2 activity is measured in the test tubes, an information of the maximum activity of this enzyme is provided and this might not always reflect the real enzymatic activity in liver *in vivo*. Thus, the hepatic CE content may provide more reliable information on the hepatic cholesterol esterification in hepatocytes *in vivo*.

In a recent study in non-human primates [196], treatment with conjugated equine estrogen led to a decrease of ACAT2 activity and expression in the liver. These data together with the identification of opposite gender-related difference in hepatic ACAT2 in humans and in mice suggest that estrogen and estrogen receptors may have different regulatory effects in different species. Accordingly, the opposite gender-related differences in BA synthesis, susceptibility to atherosclerosis and GS disease have been observed in humans and mice.

In mice, we also identified a gender-related difference for the hepatic ABCA1 protein expression. Because hepatic ABCA1 protein plays an important role in the regulation of serum HDL cholesterol levels [30], the lower ABCA1 protein expression in female mice may account for the lower HDL cholesterol level observed in these animals. Thus, both the increased CE resulting from the higher ACAT2 activity and the lower HDL cholesterol levels because of lower hepatic ABCA1 protein expression may synergistically contribute to the earlier occurrence of atherosclerosis observed in female mice.

Our studies were based on non-obese, normo-lipidemic patients and in the future it would also be interesting to study patients with other metabolic disorders such as obesity and insulin resistance which are other known risk factors for GS formation.

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