From the
Department of Laboratory Medicine, Division of Clinical Chemistry, Karolinska Institutet, Stockholm, Sweden

FUNCTIONAL STUDY OF NUCLEAR RECEPTORS AND BILE ACIDS IN THE MODULATION OF CHOLESTEROL HOMEOSTASIS

Xiaoli Hu

胡小莉

Stockholm 2013

All previously published papers were reproduced with permission from the publisher.

Published by Karolinska Institutet.
Printed by larserics
Tryck | Bokbinderi | Atelje | Storbild

© Xiaoli Hu, 2013
ISBN 978-91-7549-290-2

The only real failure is the failure to try, and the measure of success is how we cope with disappointment, as we must… We get up in the morning, we do our best, nothing else matters.

---The Best Exotic Marigold Hotel

ABSTRACT

Cholesterol carries multiple biological functions in the body, and imbalanced cholesterol metabolism leads to atherosclerosis and cardiovascular diseases. The present thesis aims to extend the knowledge of cholesterol metabolic regulation mediated by nuclear receptor LXRs and bile acids, two major players in the homeostasis of body cholesterol.

In the first paper, we aim to understand how liver X receptor (LXR) regulates cholesterol metabolism in the intestine, in particular to compare the effects of the two isoforms, LXRα and LXRβ on dietary cholesterol absorption and serum lipoprotein profiles. We find that selective activation of LXRβ enhances dietary cholesterol absorption in mice, which is accompanied by increased apoB lipoprotein cholesterol in the circulation. We also find that LXRα and LXRβ compensate for each other in the transcriptional regulation of intestinal Abcg5, Abca1 and Npc1l1. Furthermore, the hepatic enzymes Cyp7a1 and Cyp8b1 are differently modulated upon systemic LXR isoform activation. Given the contribution of the hydrophobic bile acid profile in the intestine, these changes together with the net differences in biliary cholesterol output may partially explain the isoform mediated changes in cholesterol absorption. Our findings reinforce the non-redundant function of LXRα and LXRβ, and suggest that selective activation of LXRβ as anti-atherogenic therapy may lead to undesired metabolic adverse effects.

Bile acid synthesis represents the crucial elimination pathway for excess cholesterol. The negative feedback regulation by end-product hydrophobic bile acids has been well established, involving the activation of nuclear receptor FXR, and a subsequent upregulation of SHP and Fgf15 for the suppression of bile acid synthesis in mice (Fgf19 as human counterpart). However, the role of hydrophilic bile acids in such context has largely been ignored. By using a cholic acid (CA) deficient mouse model and different bile acid-modulating regimes, we define MCAs as FXR antagonistic bile acids, which counteract the FXR activation by hydrophobic bile acids. By modulating the enterohepatic circulation of bile acids, the positive feedback mechanism regulates bile acid homeostasis without employing the hormonal effect of Fgf15, although such an effect is likely to exist. This finding is of fundamental importance for the understating of bile acid metabolism in both humans and mice, as the Fgf15/19 negative feedback mechanism is believed to operate in both species.

Paper III explores the therapeutic potential of CA depletion on systemic cholesterol overloading by using second generation antisense oligonucleotides (ASOs). Several ASOs targeting Cyp8b1, the enzyme responsible for CA production, have been used in the study. In mice, we observe a significant reduction of the CA fraction in the biliary bile acid profile under ASO treatment. This reduction is accompanied by resistance to liver cholesterol accumulation and an athero-protective lipoprotein profile upon cholesterol overloading. The data suggest the feasibility of using second generation ASOs as therapeutic target for cholesterol homeostasis, although a careful systematic study is needed to address the clinical aspect in human subjects.

LIST OF PUBLICATIONS

I. **LXRβ activation increases intestinal cholesterol absorption, leading to an atherogenic lipoprotein profile**
Hu X, Steffensen KR, Jiang Z-Y, Parini P, Gustafsson J-Å, Gåfvels M, and Eggertsen G
J Intern Med. 272(5): 452-464 (2012).

II. **Muricholic bile acids are potent regulators of bile acid synthesis via a positive feedback mechanism**
Hu X, Bonde Y, Eggertsen G, and Rudling M
J Intern Med. Accepted July 2013.

III. **Second generation antisense oligonucleotides prevent accumulation of liver cholesterol by inhibiting cholic acid synthesis**
Hu X, Graham M, Parini P, and Eggertsen G
Manuscript (2013)

CONTENTS

LIST OF ABBREVIATIONS

ABC	ATP-binding cassette
ABCA1	ATP-binding cassette, subfamily A, member 1
ABCG5	ATP-binding cassette, subfamily G, member 5
ABCG8	ATP-binding cassette, subfamily G, member 8
ACAT2	Acyl coenzyme A: cholesterol acyltransferase 2
apo	Apolipoprotein
ASBT	Apical sodium-dependent bile salt transporter
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
BSEP	Bile salt export pump
CA	Cholic acid
CDCA	Chenodeoxycholic acid
cDNA	Complementary DNA
CETP	Cholesteryl ester transfer protein
CYP7A1	Cholesterol 7α-hydroxylase
CYP8B1	Sterol 12α-hydroxylase
CYP27A1	Sterol 27-hydroxylase
DNA	Deoxyribonucleic acid
DCA	Deoxycholic acid
FGF15	Fibroblast growth factor 15
FGF19	Fibroblast growth factor 19
FGFR	Fibroblast growth factor receptor
FPLC	Fast protein liquid chromatography
FXR	Farnesoid X receptor
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GC	Gas chromatography
GC-MS	Gas chromatography-mass spectrometry
HDL-C	High density lipoprotein cholesterol
HL	Hepatic lipase
HMGCoAR	3-hydroxy-3-methylglutaryl-CoA reductase
HNF4α	Hepatic nuclear factor 4α
HPRT	Hypoxanthine-guanine phosphoribosyltransferase
IBABP	Ileal bile acid binding protein
IDL	Intermediate density lipoprotein
KO	Knock out
LCA	Lithocholic acid
LCAT	Lethicin: cholesterol acyltransferase
LC-MS	Liquid chromatography-mass spectrometry
LDL-C	Low density lipoprotein cholesterol
LDLr	LDL receptor
LRH-1	Liver receptor homolog-1
LXR	Liver X receptor
LXRE	Liver X receptor element
LPL	Lipoprotein lipase

MCA	Muricholic acid
NPC1L1	Niemann-Pick C1 Like 1
NTCP	Na^+-taurocholate cotransporter polypeptide
OST	Organic solute transporter
PCR	Polymerase chain reaction
PCSK9	Proprotein subtilisin kexin type 9
RCT	Reverse cholesterol transport
RXR	Retinoid X receptor
SHP	Small heterodimer partner
SRBI	Scavenger receptor class B type I
SREBP	Sterol regulatory element binding protein
UDCA	Ursodeoxycholic acid
VLDL-C	Very low density lipoprotein cholesterol
WT	Wild type

1 INTRODUCTION

1.1 CHOLESTEROL METABOLISM

Cholesterol is an essential structural component of the mammal cell responsible for the establishment of membrane permeability and fluidity. In addition, cholesterol also serves as a precursor for the biosynthesis of steroid hormones and bile acids (Figure 1). The whole body cholesterol homeostasis is balanced by the *de novo* synthesis, dietary intake and excess cholesterol excretion. According to the literature, dietary cholesterol intake constitutes around 1/4 of the daily total body cholesterol turnover, while the rest is contributed by *de novo* synthesis [1]. Almost all cell types have the ability to synthesize cholesterol, which is a long energy demanding process. These steps are precisely regulated by the body in relation to demand, as a high level of cholesterol in the circulation is associated with the progression of atherosclerosis and other cardiovascular diseases.

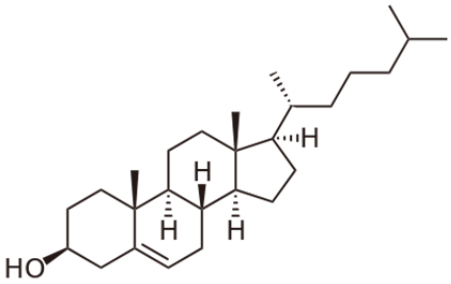

***Figure 1**. Chemical structure of cholesterol*

1.1.1 *De novo* cholesterol synthesis

The synthesis of cholesterol starts from acetyl-CoA, a substance derived from the oxidative reaction of fatty acids or pyruvate. Several enzymes are involved in cholesterol synthesis, of which the rate-limiting enzyme is 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCoAR). It converts 3-hydroxy-3-methylglutaryl Coenzyme A (HMGCoA) to mevalonic acid. This is an irreversible step in the cholesterol synthesis process, and also represents the target site of action for drug design. Commercially available drug statins inhibit HMGCoAR for the treatment of atherosclerosis [2].

Due to its crucial nature in the course of cholesterol synthesis, HMGCoAR is subjected to strict regulations under multiple stages. The transcriptional regulation of the enzyme involves sterol regulatory element binding protein (SREBP), which can be translocated to the nucleus when the cellular cholesterol level goes down, and therefore upregulates the HMGCoAR transcriptional level [3, 4]. In addition, the enzyme is also subjected to short term posttranslational regulation such as biodegradation or phosphorylation. Studies show that rising levels of sterols increase the susceptibility of HMGCoAR to ER-associated degradation and proteolysis [5, 6]

1.1.2 Intestinal cholesterol absorption

The cholesterol absorption is important for the whole body cholesterol maintenance. Cholesterol entering the gut originates from various sources, e.g. the bile, diet, trans-intestinal cholesterol efflux and cell membrane debris. All food containing animal fat contains cholesterol at various levels, as animal fat is a mixture of predominant triglycerides with little phospholipids and cholesterol. Due to its hydrophobic nature, cholesterol forms micelles with triglycerides, plant sterols, bile acids, free fatty acids, and other lipids when entering the gut [7, 8]. Cholesterol with biliary origin is mostly unesterified, which entails more efficient absorption by the intestine.

The crucial step of cholesterol uptake takes places in the proximal small intestine, in particular the enterocytes, which express several transporters to facility the cholesterol absorption. It has been demonstrated that Neimann-Pick C1 Like 1 (NPC1L1) is important for the uptake of cholesterol and plant sterols at the brush border membranes of the enterocytes [9-11]. After entering the enterocytes, most of the free cholesterol is then esterified by acyl coenzyme A: cholesterol acyltransferase 2 (ACAT2), and assembled into chylomicrons with a large amount of triglycerides [12, 13]. The chylomicrons are then transported to the liver and peripheral tissues via blood circulation for energy supply or storage. The free cholesterol in the enterocyte can also be assembled into high-density lipoprotein (HDL), a process which requires ATP-binding cassette transporter ABCA1 located on the basolaterol side of the enterocyte. A study using intestinal specific Abca1 knockout mice has demonstrated that the small intestine contributes up to 30% of the total plasma HDL [14]. Unlike free cholesterol, most of the plant sterols are exported back to the intestinal lumen via the apical co-transpoter ABCG5/G8. Mutations in either gene cause sitosterolemia, a rare recessive disease with high levels of neutral sterol in the plasma and the tissue [15]. In contrast, overexpression of ABCG5/G8 by different approaches significantly reduces the cholesterol absorption in mice [16]. Reducing cholesterol absorption is regarded as a promising approach to treat hypercholesterolemia. The drug ezitimibe, a synthetic NPC1L1 inhibitor, has been approved for such indications.

1.1.3 Lipoproteins and apopipoproteins in the blood

The common structure of lipoprotein particles represents a biochemical assembly with a hydrophilic surface and lipophilic core. Non-polar molecules such as the triglycerides, esterified-cholesterol and other hydrophobic molecules are packed inside the particle, while the polar ends of apolipoproteins, phospholipids and free cholesterol are located on the surface. Each lipoprotein particle has one or several apolipoproteins embedded on the surface, acting as ‘’hallmarks’’ for the recognition of particles by relevant lipoprotein receptors during metabolism.

Lipoproteins can be classified by their hydrate density or electrophoretic mobility. Due to the fact that the density of the non-polar core is usually low while the polar surface high, the size of the lipoprotein particles is therefore inversely correlated to the density. Chylomicrons and very low-density lipoprotein (VLDL) represent the two largest

lipoprotein particles in the blood, followed by smaller particles such as the intermediate density lipoproteins (IDL), the low density lipoproteins (LDL) and the HDL.

The presence of apolipoproteins is essential in the blood due to their crucial roles in lipid transportation and atherogenic processes. A large number of apolipoproteins has been identified and carefully studied [17, 18]. The subtypes of apolipoproteins contain apoAs, Bs, Cs, D, E, etc. From the evolutionary point of view [19], the smaller proteins, i.e. apoAs, apoCs and apoE share similar sequences in the coding regions and their genomic structures. ApoB, on the other hand, differs from the other apolipoproteins in the genomic sequences by having 29 exons including two extremely long ones. It exists in two different forms, the apoB-100 and apoB-48, which is a truncated protein that appears only in the small intestine. It represents 48% of the amino acid sequences in apoB-100 due to the introduction of a stop codon in the mRNA sequences. Therefore lipoproteins containing apoB-48 can be identified as of intestinal origin in humans. According to the established theory, the major 3D structure of apoB is mainly a beta-sheet, which allows the irreversible association of the apolipoprotein to the lipid droplet. In contrast, the other apolipoproteins mainly have alpha-helices as the 3D structure, which are then associated to the lipid droplets reversibly. Accordingly, apoBs are mostly found in the LDL or IDL, whereas HDL mainly contains apoAs and apoE.

1.1.4 Lipoprotein metabolism

The exogenous pathway
The exogenous pathway, specifically the metabolism of chylomicrons, represents the metabolism of cholesterol, triglycerides and free fatty acids, which enter the gut via the bile or the diet. After each meal, micelles (a mixture of cholesterol, triglycerides, phospholipids and bile acids) are formed and are taken up by transporters located on the enterocytes. Within the enterocyte, free cholesterol forms cholesteryl esters with fatty acids, and is subsequently packed into nascent chylomicrons with apolipoproteins (mainly apoB-48, apoC-I, apoC-III), phospholipids and triglycerides to be secreted into the lymph. In the circulation, chylomicrons acquire apoC-II and apoE from HDL and become mature. The acquisition of apoC-II enables chylomicrons to activate lipoprotein lipase, which is located on the luminal side of endothelial cells, and catalyzes the hydrolysis of triglycerides into free fatty acids and monoacylglycerol. These products are taken up by adipose tissue and muscle cells for energy supply or storage. The leftover part of the chylomicrons, namely chylomicron remnants, remains in the circulation until they interact with the chylomicron remnant receptor in the liver via apoE, and taken up by the hepatocytes for the further degradation by the lysosome.

The endogenous pathway
The endogenous pathway starts with the assembly of the nascent VLDL in the liver, which contains esterified cholesterol, triglycerides, phospholipids and apoB-100. The nascent VLDL is released from the hepatocyte into the circulation, where it acquires apoC-II and apoE from the HDL, eventually becoming mature. Similar to chylomicrons, VLDL particles also undergo the hydrolysis of lipoprotein lipase via apoC-II, and release monoacylglycerol and free fatty acids for the energy storage by

peripheral tissues. After the hydrolysis, the VLDL remnants (also called intermediate-density lipoprotein, IDL) are further hydrolyzed by the hepatic lipase and become LDL. The LDL particle is enriched with cholesterol and contains only apoB-100 as apolipoprotein. In humans, it represents the major cholesterol transportation particle. It circulates in the blood stream and can be recognized by the LDL receptor (LDLr) before entering the cell by endocytosis. Most of the LDL particles are then degraded by the lysosome and lipid components are further hydrolyzed. As lipoproteins accumulate in the intima of the arterial vessels, they can be oxidized by the oxygen free radicals generated by either macrophages or endothelial cells. These oxidized LDL particles, however, can also be taken up by the macrophages which later become foam cells and form the fatty streaks of the plaque inside the intima of the arteries. A high level of LDL in the circulation is a known risk for atherosclerosis. As every LDL particle contains only one molecule of apoB-100, the level of apoB-100 in the circulation is a helpful marker for the evaluation of the LDL particles and therefore useful for predicting the development of atherosclerosis.

Reverse cholesterol transportation

The reverse cholesterol transportation (RCT) represents the multi-step process of cholesterol net movement from the peripheral tissue to the liver for the elimination via bile. It was first described by Glomset in 1973 [20], and was regarded as a protective event against cardiovascular disease by the synthesis and metabolism of HDL. The procedures of RCT include cellular cholesterol efflux, HDL remodeling and HDL transportation from the peripheral tissue to the liver. HDL contains mainly apoA-I as apolipoprotein, with apoA-II present in some HDL particles, although its role is less well defined [21, 22]. Intercellular free cholesterol is effluxed by the ABCA1 and interacts with apoA-I, which facilities the esterification of cholesterol by lecithin:cholesterol acyltransferase (LCAT). Nascent HDL matures as the amount of cholesteryl esters accumulate via remodeling in the circulation. These mature HDL particles are then interacting with hepatocytes through its receptor, the scavenger receptor class B type I (SRBI). SRBI thereafter mediates non-endocytotic cholesterol transport into the hepatocyte.

1.2 BILE ACID METABOLISM

Bile acids are important cholesterol derivatives during the metabolic process. The synthesis and excretion of bile acids represents a major path to eliminate excess cholesterol in the body. In most of the early studies, bile acids have been recognized merely as detergent facilitating lipid absorption in the gut. Recent studies revealed crucial functions of bile acids as signaling molecules and their important roles in governing lipid metabolism, energy expenditure, glucose metabolism, carcinogenic balance, etc.

1.2.1 Synthesis of primary bile acids

About 500 mg of cholesterol are converted daily into bile acids in the adult human liver [23]. The synthesis takes place exclusively in the liver. The intermediate products of the synthesis are named as primary bile acids, with various chemical structures indicating

the different biological functions. In humans, the most predominant primary bile acids are cholic acid (CA) and chenodeoxycholic acid (CDCA) (Figure 2), whereas in mice and rats, the primary bile acids mainly consist of CA and muricholic acids (MCA).

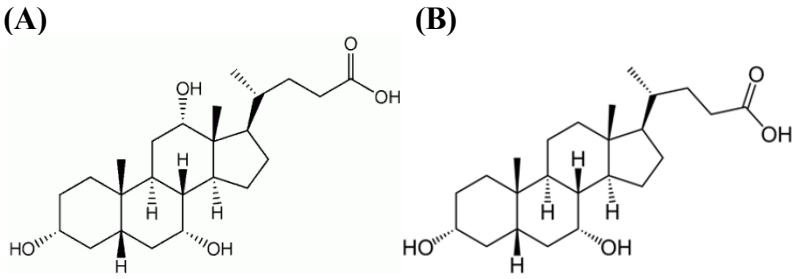

***Figure 2**. Chemical structures of (A) cholic acid and (B) chenodeoxycholic acid.*

Two major pathways have been identified for the bile acid synthesis, namely the classic pathway (or the neutral pathway) and the alternative pathway (or the acidic pathway). The crucial steps in the classic pathway involve the participation of several enzymes, of which the rate-limiting one is cholesterol 7α-hydroxylase (CYP7A1). This enzyme belongs to the cytochrome P-450 super family, is expressed exclusively in the liver, and is subjected to strict regulation by a number of nuclear receptors and other signaling pathways.

Cyp7a1-deficient mice reveal a complex phenotype and high mortality rate in their early life stage, indicating the crucial function of this enzyme in the murine metabolic system [24]. However, the above observation seems to vary according to experimental condition and genetic background, as another study using mice with disrupted Cyp7a1 observed a much lower mortality rate and milder clinical phenotype [25]. In addition, an elevated level of serum cholesterol was detected in these mice. These observations, however, seem to be more representative to the human Cyp7a1 deficient condition. A common variation of the human CYP7A1 gene is the single nucleotide polymorphism (SNP) in the promoter region of the gene, resulting in an A/C transversion and subsequently higher level of LDL-cholesterol [26, 27].

In this way, cholesterol can either be converted to CA or CDCA before entering the enterohepatic circulation. The P-450 cytochrome enzyme sterol 12α-hydroxylase (CYP8B1) is crucial for the synthesis of CA. It is exclusively expressed in the liver and regulated by the sterol regulatory element binding proteins (SREBPs) [28, 29]. In a mouse model with genetic disruption of Cyp8b1, the CA synthesis was completely abolished, which resulted in a dramatic increase of MCA and CDCA [30]. This change resulted in reduced dietary cholesterol absorption, increased liver bile acid synthesis, an expanded bile acid pool, and increased resistance to atherosclerotic formation when cross-bred with apoE knockout mice [31-33]. In humans, no individual with CYP8B1 deficiency has been described and a polymorphism found in the coding region of the gene does not seem to correlate with the difference in the ratio between CA and CDCA [34]. A report described a change of the CA/CDCA ratio in human bile in cystic fibrosis patients, but no correlation was found between the ratio and the disease onset [35]. The alternative (acidic) pathway also plays an important role for the bile acid

synthesis. It contributes to about 25% of the bile acids in rodents, but less in humans [23]. The key enzyme of this step represents the sterol 27-hydroxylase (CYP27A1). Mice with disrupted Cyp27a1 display a reduced total bile acid pool, and a predominance of CA in the total bile acid composition [36]. Human CYP27A1 deficiency is the cause of cerebrotendinous xanthomatosis (CTX), which is characterized by dementia, ataxia, cataracts and xanthomas in the central nerve system and peripheral tendons [37].

1.2.2 Enterohepatic circulation and secondary bile acid synthesis

The enterohepatic circulation is important in maintaining a constant bile acid pool size, which is measured as 4 mg in mice and 2 to 4 g in humans [38-40]. These bile acids circulate several rounds in each meal, facilitating the absorption of lipids in the food while act as signaling factors for the metabolism of glucose and energy [41-44].

The hepatocytes have the ability to transport bile acids efficiently from the portal blood into the bile, a step that is essential for the bile acid enterohepatic circulation. The transportation process is driven by the Na^+-taurocholate co-transporting polypeptide (NTCP). This membrane glycoprotein is the founding protein of the SLC10 superfamily for solute carrier proteins, which consists of two bile acid carriers, SLC10A1 (NTCP) and SLC10A2 (ASBT) [45]. The high expression level of NTCP on the sinusoidal membrane of the hepatocytes enables the transporter to extract bile acids efficiently from the portal blood, with the highest affinity to the conjugated bile acids and weaker affinity to unconjugated or sulfated bile acids [46-48]. On the canaliculi side of the hepatocytes, the bile salt export pump (BSEP) is located. This ABC transporter acts as canalicular bile acid exporter and effluxes bile acids into the bile. After each meal, the contraction of the gallbladder empties the bile from the biliary tract into the gut where the bile acids meet with the apical sodium bile transporter (ASBT) in the distal ileum. There, about 95% of the BAs are reabsorbed, a step that is crucial for the maintaining of the total BA pool size [49, 50]. These bile acids are then taken into the enterocytes and exported by the organic solute transporters (OSTs), which form a dimer between the subtypes OSTα and OSTβ, into the blood [51, 52], transported back to the liver via the portal blood, and thereby complete a round of enterohepatic circulation.

Not all the BAs are reabsorbed in the distal ileum. Less than 5% of the leftover BAs continue to the large intestine, where they are converted by the bacteria into secondary BAs. In humans and rodents, the secondary BAs formed by the colon bacteria are consisted of deoxycholic acid (DCA), lithocholic acid (LCA) and a small amount of ursodeoxycholic acid (UDCA). The percentage of DCA is much higher in humans (about 25% of total bile acid pool) as compared to rodents, whose DCA fraction is less than 1% due to the re-hydroxylation ability in their liver. Secondary BAs are more lipophilic and therefore exhibit higher cellular toxicity than the primary BAs.

1.3 REGULATION OF CHOLESTEROL METABOLISM

Since the discovery of nuclear receptors, significant progress has been made in the ligand identification and physiological function dissection. Among them are the liver X receptor (LXR) and farnesoid X receptor (FXR), whose natural ligands were previously unknown. It is now clear that the ligands for LXRs are mainly cholesterol derivatives, while bile acids account for the ligands of FXR. These two nuclear receptors play crucial roles in the regulation of genes related to lipid homeostasis.

1.3.1 Nuclear receptors

Nuclear receptors are a group of proteins located within the cell nucleus, of which most can bind directly to DNA. A common character they share is the ability to bind ligands such as steroids and other lipid molecules. Upon the ligand binding, nuclear receptors commence conformational changes that result in the transcriptional signaling change, leading to the up or down-regulation of certain target genes. Recent studies reveal that transcriptional regulation by nuclear receptors also requires the participation of co-activators, which are recruited after the ligand binding procedure [53, 54]. The common structure of the nuclear receptors consists of an N-terminal domain, regions of DNA binding, hinging, ligand binding, and a C-terminal domain (Figure 3) [55, 56].

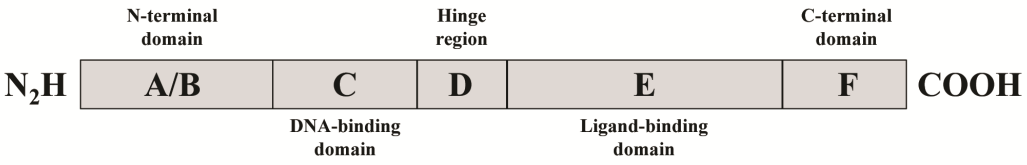

Figure 3. *Schematic diagram of a typical nuclear receptor with its functional domains.*

1.3.2 LXRs, cholesterol homeostasis and synthetic LXR agonists

LXRs are transcriptional factors which belong to the nuclear receptor superfamily. It was first identified as orphan receptor, of which the natural ligand was unknown. LXR forms a heterodimer with the retinoid X receptor (RXR) and therefore can also be activated by RXR agonists. Upon ligand binding, LXR experiences conformational changes resulting in the release of co-suppressors and recruiting of co-activators. Thereafter, the transcriptional regulation of the target genes is initiated. Natural ligands for LXRs includes cholesterol metabolites, oxysterols such as 24(S),25-epoxycholesterol, 24(S)-hydroxycholesterol, and 27-hydroxycholesterol [57-59]. These molecules are believed to act as endogenous LXR activators in tissues like liver, intestine and macrophages. LXR has two subtypes, LXRα (NR1H3) and LXRβ (NR1H2), which share approximately 77% identity in DNA and ligand-binding domains. These two subtypes however have different expression frequencies in various tissues [60, 61]. LXRα is expressed in lipid-metabolic tissues such as the liver, intestine, macrophage, etc; while LXRβ is more ubiquitously expressed and governs a multitude of metabolic functions.

Genetically modified animal models have been created to study the impact of LXRs in relation to the lipid metabolites. In the macrophage, LXRα regulates cholesterol efflux transporters upon activation. Genes such as ABCA1, ABCG1, apoC and apoE were significantly upregulated under LXR stimulation, and therefore facilitate the intracellular sterol efflux for the return as HDL to the liver [62]. Activation of LXRs also affects the intestinal cholesterol absorption, although the exact mechanism has not been completely clear. When LXRα knockout mice were cross-bred with apoE knockout mice, the new strain showed an attractive phenotype for the study of atherosclerosis, as the number of atherosclerotic lesions was significantly decreased in the double knockout mice [31]. A novel insight on how LXRs regulate lipid homeostasis was added when a new LXR target gene was identified, namely the inducible degrader of LDL receptor (IDOL, also known as MYLIP) [63]. The E3 ubiquitin ligase functions as a degrader for the LDL particles and LDL receptor, thereby affecting the LDL levels in the blood. The identification of IDOL reinforces the crucial role of LXR in preserving cellular lipid homeostasis under cholesterol overloading conditions.

A large number of synthetic LXR agonists has been created by the pharmaceutical industry aiming to treat atherosclerosis and other indications. The first generation of the LXR pan-agonist, TO-901317 and GW3965 have been synthesized by Amgen (Tularik) and GlaxoSmithKline, respectively, and widely used in preclinical studies. The beneficial effects of these two agonists are shown in many animal models of different indications ranging from cardiovascular diseases, neuro-degenerative diseases, inflammatory diseases and other metabolic disorders. However, such beneficial effects are clearly counteracted by the significant adverse effects associated with the products, as mice treated with first generation LXR agonists developed hypertriglyceridemia [64], a known risk factor for cardiovascular disease. This phenotype has largely been attributed to the up-regulation of sterol regulatory element binding protein 1c (SREBP1c), a direct target gene of LXR. The activation of SREBP1c leads to massive induction of its downstream genes related to fatty acid synthesis, including fatty acid synthase (FAS), acetyl-CoA carboxylase, and stearoyl CoA desaturase-1 (SCD-1) [65]. Later on, Wyeth developed LXR-623, another LXR agonist which preserved the beneficial effects of its first generation, but without the elevation of hepatic lipogenesis. However, the clinical trial for LXR-623 was forced to be discontinued due to the severe neurological side effects during the clinical trial [66].

1.3.3 FXR and bile acid metabolism

The original name of FXR (NR1H4) comes from the observation that farnesol acts as the ligand for this nuclear receptor [67]. In the subsequent studies, it has become more and more clear that bile acids are the actual natural ligands for FXR [68, 69]. FXR is mainly expressed in the liver, intestine, kidney and adrenal glands, whereas low levels of expression are also discovered in adipose tissue and heart. Similar to LXRs, FXR also forms a heterodimer with RXR and responds to RXR agonists. The transcriptional regulation of the target genes starts when bile acids bind to FXR. In addition, FXR can also exert indirect regulation of certain genes via the induction of small heterodimer partner (SHP). It is important to dissect the physiological role of pure FXR activation,

as bile acids also act as ligands for other nuclear receptors such as pregnane X receptor (PXR), constitutive androstane receptor (CAR), vitamin D receptor and TGR5. For this reason, it is important to use FXR-deficient mice in order to draw conclusions for FXR-mediated metabolic effects observed by exogenous bile acid activation.

High levels of bile acids are cytotoxic; therefore it is crucial to maintain the proper bile acid levels in the body. To achieve this task, FXR regulates the flux of bile acid in various feedback or feedforward loops, covering the bile acid synthesis, uptake, transportation and reabsorption. FXR knockout mice also show several severe metabolic phenotypes, which adds to the evidences that FXR is crucial for bile acid flux and overall metabolic homeostasis. The regulation of bile acid synthesis by FXR is mainly through the FXR-SHP activation, which then inhibits the liver receptor homolog-1 (LRH-1), LXR and hepatocyte nuclear factor 4α (HNF4α), factors all necessary for the preservation of CYP7A1 expression [70-72]. In addition, it has been claimed that bile acid-FXR signaling also induces the fibroblast growth factor 19 (FGF19, Fgf15 as homolog in rodents), which is synthesized in the distal intestine and exerts hormonal effects in the liver by repressing CYP7A1 expression. Although the hormonal function of Fgf15 has been predicted long ago, it is not until recently that the protein has been detected in the blood for the first time in mice [73]. This could either be due to instability or the fast degradation rate of the protein, which raises the question of its real physiological function in rodents. Nevertheless, FXR activation leads to suppression of CYP7A1 in the liver, resulting in a feedback regulatory effect of bile acid synthesis. In addition to the bile acid synthesis, FXR modulates bile acid flux by transcriptional regulation of ASBT and OSTα/β [74]. The FXR-mediated regulation of bile acid synthesis implied the crucial role of FXR as lipid regulator, as bile acid synthesis represents an important way for cholesterol elimination.

1.3.4 Bile acids as ligands for G-protein coupled receptors (TGR5)

In addition to their lipid solubility function, bile acids are also signaling molecules which exert genomic and non-genomic regulations on the body metabolism. One such function involves the activation of TGR5, a G protein-coupled receptor (also known as M-BAR, GPBAR1). The mechanistic stimulation of TGR5 by bile acids includes the accumulation of intracellular cAMP and subsequently activation of the mitogen-activated protein kinase (MAPK) pathway. The potency of bile acids on TGR5 activation is positively correlated to the hydrophobicity of the molecule, which is ranked as LCA > DCA > CDCA > CA according to an early study [75].

As the expression of TGR5 has been found in several tissues including the gall bladder, distal small intestine and the colon, numerous studies on its physiological functions have been conducted. Watanabe et al [44] have demonstrated that the bile acid-TGR5 signaling pathway stimulates energy expenditure by acting in the brown adipose tissue in mice via inducing 2-iodothyronine deiodinase (D2), an enzyme which converts thyroxine (T4) into tri-iodothyronine (T3). This finding was confirmed in mice with high-fat diet feeding and was further applied into the prevention of diet-induced obesity. Another physiological effect of the bile acid-TGR5 pathway is related to the glucose metabolism. It has been shown that TGR5 stimulates the secretion of glucagon-

like peptide 1 (GLP1) [76], a member of the incretin family. This protein functions as a regulator of the insulin secretion in response to the meal. The physiological significance of this finding for human implication is still under investigation. The natural expression of TGR5 in the liver is almost negligible according to the previous studies, however the homozygous $TGR5^{-/-}$ mice showed a 20% reduction in their bile acid pool size [77]. The exact mechanism for this change is still unclear, as CA feeding was unable to exert a regulatory effect on any of the enzymes (e.g. Cyp7a1, Cyp8b1, Cyp27a1, etc.) responsible for bile acid production in these mice.

1.4 CHOLESTEROL METABOLIC DISEASES AND POTENTIAL TREATMENT

1.4.1 LXRs, FXR and atherosclerosis

The therapeutic effects of LXR agonists on atherosclerosis have been demonstrated by various animal models. The activation of LXR by GW3965 showed strong reduction of atherosclerosis in apoE- and LDLr-knockout mice [78]. This observation has been further extended to the other widely used agonist TO-901317 [79]. According to earlier studies, LXR activation led to the alteration of plaque composition, which subsequently contributed to the attenuation of inflammatory reactions. In addition, reduced levels of adhesion molecules and changing in fibrous cap thickness were also observed in the studies. Notably, the reduced lesion development did not happen simultaneously with the lowering of blood lipid levels, suggesting that the efflux of cholesterol via RCT can occur without changing the static lipid level. The atherogenic protective effect of LXRs was further confirmed by the loss-of-function study using LXR double knockout mice. It was demonstrated that such mice developed atherosclerotic lesions enriched with foam cells even under normal chow diet [80]. Despite the beneficial effect of LXR pan activation, an LXRβ selective activation is still of great pharmacological interest due to the massive hepatic lipogenic effect under LXRα stimulation. It has been demonstrated that selective LXRβ activation in mice is sufficient to attenuate atherosclerosis development with marginal effects on blood triglyceride levels [81], which provided a solid scientific rationale for such approaches.

Compared to LXR agonists, the benefit of FXR activation against atherosclerosis seems more controversial and is largely dependent on the experimental conditions. Several studies have shown an atheroprotective effect by creating FXR and LDLr-double knockout mice [82, 83]. In contrast, FXR and apoE-double knockout mice developed severe atherosclerosis with a high mortality rate when fed with atherogenic diet [84]. Nevertheless, the atheroprotective effect of FXR stimulation seems to be dependent on the improvement of the blood lipid profile [85, 86]. On the other hand, the existence of FXR expression on smooth muscle cells of the cardiovascular vessels [87] and endothelial cells [88] provides evidence that FXR might actually regulate atherogenic events via modulating plaque formation or inflammatory effects.

1.4.2 Bile acids, glucose metabolism and type 2 diabetes

Dyslipidemia, together with obesity, increased blood glucose level and hypertension form the criteria of the clinical diagnosis of metabolic syndrome, which is a known risk factor for type 2 diabetes [89]. Recent medication for dyslipidemia includes statin, bile acid sequestrants, fibrates, nicotinic acid, etc. The link between bile acid modulation and lipid homeostasis is mainly due to the signaling pathway of two receptors, namely the FXR and TGR5, both of which require bile acids as ligands. Numerous studies have provided solid evidence that bile acid-TGR5 activation reduces the blood glucose level and improves glucose tolerance in high fat-treated mice [41, 90]. It has been shown that TGR5 stimulation by the synthetic ligand INT-777 induces the production of GLP-1 in the small intestine, thereby enhancing the postprandial insulin secretion. A similar conclusion has been drawn by administering INT-777 to the TGR5 knockout mice, and no elevation of GLP-1 has been observed [41]. Another aspect about TGR5 stimulation is the increased energy expenditure through the induction of deiodinase in the brown adipose tissue, thereby improving the diet-induced weight gain and obesity [44]. This observation also provides evidence that activation of TGR5 might be a promising target for the treatment of metabolic syndrome.

The link between FXR and glucose metabolism has been proven by several studies showing that FXR depletion leads to insulin resistance in the peripheral tissues [91-93]. Other studies have also shown that FXR is regulating several genes which are involved in the gluconeogenesis pathway, including glucose 6-phosphatase (G6Pase) and phosphoenolpyruvate carboxykinase (PEPCK). However, the exact mechanism for the regulation has not been clarified, also the results seem to be inconsistent and highly dependent on the experimental conditions [94-96]. In line with this notion, the beneficial effect of FXR agonist on glucose homeostasis is still unclear and partly controversial. Therefore, whether FXR stimulation should be considered as a potential therapy for alteration in glucose metabolism needs to be validated with more solid experimental evidence.

1.4.3 Antisense oligonucleotides and cardiovascular diseases

ASOs are short synthetic single-stranded nucleotide polymers, which usually contain 15-25 nucleotides in length. These single-stranded polymers have the ability to hybridize with the target RNA via Watson-Crick base pairing, and activate the endogenous RNase H to hydrolyze the target RNA strand. This results in the reduction of mRNA expression and subsequently protein levels of the target gene [97-99]. Since the approval of the first generation ASO, there has been a growing interest to improve the pharmacokinetic and pharmacodynamic properties of the first generation ASOs. Among all the attempts, adding a 2'-O-methoxyethyl (MOE) or a 2'-O-methyl (Me) group to the ASO strand has been proven to provide higher biological stability, greater in vivo potency and less toxicity [100]. Because of the ability to inhibit specific targets with a broad range of selection on the target, the ASO technique has also been used for the design of drugs against dyslipidemia and cardiovascular diseases. Targets involved in preclinical or clinical studies include apoB-100, PCSK9 and Lp(a) [101].

2 AIMS

The general aim of this thesis is to investigate and better understand the regulatory effect of bile acids and nuclear receptors on cholesterol homeostasis under the cholesterol overloading condition.

The specific aim for each project is described as:

Paper I — To characterize the cholesterol metabolic profile induced by selective activation of LXRβ, in particular to study the regulatory effect mediated by LXRβ on intestinal cholesterol absorption and the blood lipoprotein profile.

Paper II — To evaluate the regulatory effect of muricholic acid on bile acid synthesis, and to re-characterize the phenotype of $Cyp8b1^{-/-}$ mice regarding bile acid metabolism.

Paper III — To explore the therapeutic potential of CA depletion on systemic cholesterol overloading by using second generation antisense oligonucleotides.

3 MATERIALS AND METHODS

3.1 ANIMALS AND TREATMENTS

Paper I

Male mice on a C57BL/6J background (6-9 weeks of age, Taconic A&B, Denmark) were used for the experiment and housed in a pathogen-free facility with a regular light/dark circle of 12h/12h. Each experimental group contained 4-6 individuals. Mice were either fed with a standard diet (SDS, Germany) or 0.2% cholesterol-enriched diet (wt/wt, Harlan Teklad, USA) with free access to water for 2 weeks before the termination. When necessary, the LXR agonist GW3965 was given at 40mg/kg/day for 4 days by oral gavage. All mice were fasted for 4 hours before the termination for the collection of gallbladder bile. All animal experiments were approved by the ethical committee of Karolinska Institutet.

Paper II

Inbred female mice on a C57BL/6J background (8-10 weeks of age, genotype: $Cyp8b1^{-/-}$ and the litter mates $Cyp8b1^{+/+}$) were housed in a pathogen-free facility under a light/dark circle of 12h/12h with free access to standard food and water unless otherwise specified. For each treatment group, 6 mice were used and received single doses of saline (100 μL) with or without AMP (100mg/kg/day) by gavage. Another four groups of $Cyp8b1^{-/-}$ mice (n=3-6) were used and given oral gavage of 200μL saline solution with AMP (100mg/kg/day) and supplemented with CA, CDCA or DCA at a concentration of 9 μmol/kg/day. All gavages were given at 9am for 3 continuous days, with a boosting dose at 5pm on day 3. Before the end of the experiments, all mice were fasted for 4 hours with free access to water. All animal experiments were approved by the ethical committee of Karolinska Institutet.

Paper III

Male mice on a C57BL/6J background (around 6 weeks of age, Taconic A&B, Denmark) were housed in a pathogen-free facility under a light/dark circle of 12h/12h with free access to water and food during the treatment with the ASOs. Five or six mice were used for each treatment group. The ASO substances were given by intra-peritoneal injection (IP) twice per week at 50mg/kg/week (wt/wt) for six weeks. For dietary studies, mice were fed with a 0.2% cholesterol diet, (wt/wt, Harlan Teklad, USA) for 2 weeks in parallel to the ASO treatment before the sacrifice. All mice were fasted for 4 h with free access to water before the sacrifice. All animal experiments were approved by the ethical committee of Karolinska Institutet.

3.2 GENE EXPRESSION

Total RNA was purified from individual samples of liver or small intestine, and the concentration of RNA was determined. After that, 1μg of total RNA was used for the synthesis of cDNA. The relative expression levels of target genes were determined by

real-time PCR (7500, Applied Biosystems, USA) and calculated in relation to the house-keeping genes HPRT or cyclophilin.

3.3 PROTEIN EXPRESSION

Total protein was purified from individual or pooled samples of liver or small intestine. For Western blotting, 4-12% gradient bis-tris gels were used for the separation of the protein. After blotting, the membrane was incubated with primary and secondary antibodies. Results were recorded using a Molecular Imager® camera (Bio-Rad, USA). Densitometry analyses were performed for quantification of the results. Detailed information of the antibodies can be obtained from method part of paper I-III.

3.4 DIETARY INTESTINAL CHOLESTEROL ABSORPTION

Mice were given 100μL peanut oil mixed with 1 μCi[4-^{14}C] cholesterol (Amersham Bioscience, USA) and 2 μCi [5,6-^{3}H] β-sitostanol (American Radiolabel Chemical Inc., USA) by oral gavage. After the gavage, each mouse was caged separately for 24 hours to allow the fecal collection. The lipid in the feces was then purified using the Folch method (chloroform: methanol 2:1, v/v).

3.5 FECAL NEUTRAL STEROIDS AND BILE ACIDS

Neutral steroids and bile acids were purified from 0.5g feces homogenates for each mouse using hexane and ether methods, respectively [102]. The final extracts were then analyzed by GC.

3.6 LIVER CHOLESTEROL AND BILE ACIDS

Cholesterol and bile acids were purified from 1g of liver tissue with Folch and ether, respectively. The final extracts were then analyzed by GC-MS.

3.7 BILIARY CHOLESTEROL AND BILE ACIDS

Cholesterol and bile acids were purified from 2μL of gallbladder bile using Folch and ether, respectively. The final extracts were then analyzed by GC-MS.

3.8 SERUM LIPOPROTEIN CHOLESTEROL

Serum lipoprotein cholesterol was analyzed by FPLC using size-exclusion chromatography [102].

3.9 STATISTICS

Statistical analyses were performed by STATISTICA version 9 and GraphPad Prism 5.04 Software. Detailed methods can be obtained in each paper.

4 RESULTS AND COMMENTS

4.1 PAPER I: LXRBETA ACTIVATION INCREASES INTESTINAL CHOLESTEROL ABSORPTION, LEADING TO AN ATHEROGENIC LIPOPROTEIN PROFILE

It has been demonstrated that the inhibition of intestinal cholesterol absorption alone or with other lipid lowering drugs in humans leads to clear atheroprotective effects [103-105]. Studies have been focused on the mechanism of how LXR stimulation leads to reduced intestinal cholesterol absorption, however little is known about the individual role of LXRα and LXRβ on the regulation of cholesterol absorption. In this study, we have investigated the significance of LXRα and LXRβ on the absorption of cholesterol in mice, and evaluated the subsequent effects on cholesterol and bile acid metabolism.

Separate activation of LXRβ induces dietary cholesterol absorption and elevates serum apoB-containing particles in LXRα$^{-/-}$ mice.

A major concern of using LXR agonists in treating atherosclerosis is the dramatic elevation of serum triglycerides when LXRα is activated. Therefore, a dissected LXRβ agonist might be of great potential value in terms of therapeutic purpose. In line with this notion, we investigated the effect of selective LXRβ activation by using the synthetic LXR pan agonist GW3965 in LXRα$^{-/-}$ mice. As observed in the study, a significant enhancement of dietary cholesterol absorption was shown only in LXRα$^{-/-}$ mice after 4 days of 0.2% cholesterol diet with or without GW3965. This is in sharp contrast with the other two genotypes used in the same study, the WT and LXRβ$^{-/-}$ mice, which showed marginal effects of dietary cholesterol absorption under the same treatment. Results from fecal neutral sterol measurements confirmed the absorption data, as the fecal neutral sterol output was reduced solely in the LXRα$^{-/-}$ mice under 0.2% cholesterol or/and GW3965. However, changes on fecal neutral sterols can also be due to the different cholesterol output through the bile, therefore we investigated whether that could be the explanation. According to the experiment, no changes were found between the three genotypes on biliary cholesterol excretion. Taken together, these results suggested an increased dietary cholesterol absorption in the gut, which led to a decrease in fecal lipid output.

The above observations are accompanied by elevated total and apoB-lipoprotein cholesterol in the serum when mice were challenged by 0.2% cholesterol and/or GW3965. According to our observation, the total lipoprotein cholesterol was almost doubled in all genotypes of mice when treated with 0.2% cholesterol and GW3965, although the individual lipoprotein fraction differed between the genotypes. It seemed that activation of LXRs and LXRα mainly resulted in the increase of the HDL fraction, while LXRβ activation resulted in LDL cholesterol induction in the blood. Based on the above two observations, we suggested that the separate activation of LXRβ should be used with caution as adverse effects might appear. Consequently, it is of great

importance that the physiological effects of LXRα and LXRβ are carefully evaluated in order to avoid the side effects while maximizing the advantage for the therapeutic purpose.

Compensatory effects of LXRα and LXRβ in the regulation of cholesterol metabolic genes.

This study also reveals the compensatory effect of LXRα and LXRβ in the regulation of the intestinal genes ABCG5 and NPC1L1. The loss of either subtype did not jeopardize the regulatory effect on the other subtype, as both LXRα$^{-/-}$ and LXRβ$^{-/-}$ mice fed with 0.2% cholesterol with GW3965 showed a downregulation of NPC1L1 and an upregulation of ABAG5. Interestingly, the 0.2% cholesterol diet alone did not markedly affect Npc1l1 gene regulation, and when compared to the dietary cholesterol absorption, the downregulation of NPC1L1 by 0.2% cholesterol+GW3965 did not correlate to the changes in cholesterol absorption. An explanation for the discrepancy could be that the transcriptional level of NPC1L1 might not reflect the real activity of this cholesterol transporter. Studies have revealed that the crucial step of Npc1l1 in cholesterol uptake is the shuttling of the protein from the endocytic recycling compartment to the plasma membrane, and internalization before transporting cholesterol into the cell [106, 107]. Therefore, the rate of cholesterol absorption would be affected not only by the expression level of NPC1L1, but also by the modifications of the other steps involved in this process (Figure 4).

LXRα and LXRβ modify bile acid composition and excretion in response to the ligand stimulation.

Bile acids are crucial for the regulation of the dietary cholesterol absorption due to their amphipathic nature which facilitates lipid solubility. The ratio between the hydrophobic bile acid CA and its hydrophilic counterpart β-MCA is closely related to the cholesterol absorption rate. Generally speaking, the higher the ratio of CA/β-MCA, the more cholesterol gets absorbed in the intestine. In line with this notion, we analyzed the biliary bile acid composition of the mice as differences of cholesterol absorption have been detected. Under 0.2% cholesterol diet, the ratio between CA and β-MCA went down in WT and LXRβ$^{-/-}$ mice, indicating a more hydrophilic bile acid pool during the feeding, while the ratio in LXRα$^{-/-}$ showed only moderate changes. When GW3965 was given in addition, the bile acid composition in WT and LXRβ$^{-/-}$ mice showed further hydrophilic properties in comparison to the LXRα$^{-/-}$ mice. Thus, the bile acid composition of LXRα$^{-/-}$ mice had the highest hydrophobicity among all genotypes, which was in line with its highest cholesterol absorption rate. It is of particular interest that LXRα$^{-/-}$ mice might have a more hydrophobic bile acid profile, as the fecal bile acid excretion of these mice contained more DCA and less MCA than the other two genotypes.

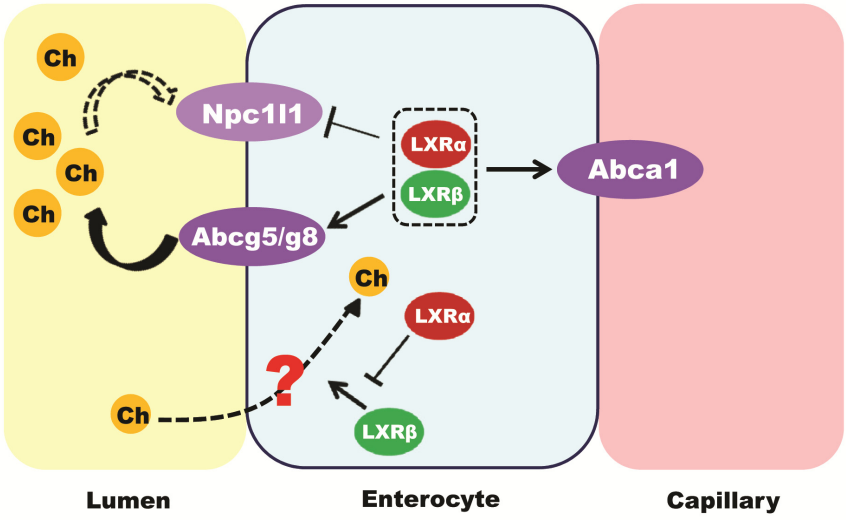

***Figure 4**. Proposed mechanisms of the effects of LXRα and LXRβ on the regulation of cholesterol transport processes in the small intestine. LXRα and LXRβ similarly regulate the expression of cholesterol transporters, reducing the mRNA of Npc1l1 while elevating Abcg5/g8 and Abca1. These changes lead to the reduction of cholesterol uptake and enhanced cholesterol excretion. Meanwhile, selective activation of LXRβ increases cholesterol uptake through an unknown pathway, which is counteracted by LXRα.*

4.2 PAPER II: MURICHOLIC BILE ACIDS ARE POTENT REGULATORS OF BILE ACID SYNTHESIS VIA A POSITIVE FEEDBACK MECHANISM

Several animal models have been used to investigate the individual role of bile acids on their activation of FXR and other regulatory factors, but the exact role of each bile acid on their own homeostatic regulation remains to be elucidated [30, 108-110]. In this study, we used ampicillin (AMP) to eliminate the production of secondary bile acids in wild type (WT) and Cyp8b1$^{-/-}$ mice (KO) in order to gain a close look at the individual bile acids and their regulatory functions. In addition, we also fed KO mice with various bile acids in parallel to the AMP treatment, so as to compare the impact of exogenous bile acid feeding on the bile acid regulation.

Effects of ampicillin on bile acid synthesis and excretion in WT and KO mice.

WT mice fed with AMP dramatically induced their Cyp7a1 expression for 4 fold as compared to the control group. The expression level of Cyp7a1 mRNA in the WT-AMP group was similar to that in the KO group, regardless of the treatment. Analysis on Cyp7a1 protein activity confirmed the above observation. We then analyzed the two major bile acid regulatory factors, SHP and Fgf15. In the intestine of WT mice, both receptors were downregulated at the mRNA level with AMP treatment. The expression levels of both receptors in the KO mice, regardless of the treatment, were similar to the

ones in AMP-treated WT mice. Marginal changes on SHP were observed in the liver. These data suggest that the synthesis of bile acids in the liver was significantly increased in WT+AMP mice.

In accordance with the above observation, we continued to investigate the fecal excretion of bile acids in WT+AMP mice, as induced liver bile acid synthesis is likely to result in an enhanced output on fecal bile acid excretion. Surprisingly, the excretion of fecal bile acid was almost completely eliminated in AMP treated mice for both the WT and the KO group. This is in drastic contrast to the situation in the non-treated groups, as KO mice showed an almost doubled bile acid excretion in the feces as compared to the WT mice. Such result could only be due to the dramatic increase in bile acid reabsorption in the distal ileum, as the hepatic bile acid synthesis has been enhanced after the AMP treatment. As confirmed by Western-blotting, the Asbt expression in AMP treated mice was much higher than in the non-treated group, indicating enhanced bile acid reabsorption. Taken together, these data highly suggest that the total mass flow of bile acid in the enterohepatic circulation is increased several fold in AMP treated mice, and that this phenotype is most likely due to an impaired FXR-signaling.

The liver bile acid analysis revealed an increase of the total bile acid in the WT+AMP group, which was similar to the levels of KO mice with or without AMP. The increased amount of bile acid is mainly due to the elevation of hydrophilic bile acids (MCAs and UDCA), whereas the changes on hydrophobic bile acids (CDCA, DCA and LCA) and CA were marginal. It is worth noticing that DCA can only be detected in the WT+AMP group. Based on the above data, it is obvious that the differences in CA cannot be explained by the upregulation of bile acid synthesis in the AMP groups. Therefore the question remains whether loss of DCA or increase of MCA concentration is responsible for the bile acid synthesis changes.

Effects of bile acid administration on bile acid synthesis and excretion in KO mice.

In order to gain a deeper understanding of the regulatory effect of individual bile acids, we treated the KO mice with CA, CDCA, or DCA in parallel to the AMP gavage. The advantage of such an approach is the elimination of the endogenous primary bile acid CA, and the secondary bile acids DCA and LCA. As expected, the Cyp7a1 mRNA levels were suppressed by 81% and 82% in the CA and DCA treated groups, respectively. In contrast, the CDCA treatment was less effective as it reduced Cyp7a1 mRNA only by 59%. The Cyp7a1 mRNA changes might be due to the hepatic SHP, as inverse correlations were seen between the hepatic SHP and Cyp7a1 in all treatment groups. Intestinal expression of SHP showed elevated expression levels in all bile acid treated groups, a pattern also seen for intestinal Fgf15 mRNA. Finally, Asbt mRNA revealed 45% reductions in the CA and CDCA groups while a stronger 65% reduction was observed in the DCA group. The Asbt protein expression was suppressed by 40% in the CA and the DCA groups while it was 60% suppressed in the CDCA group.

The liver total bile acid concentration has been reduced by 30-34% in CA and DCA-treated mice, whereas the amount has been increased by 30% in CDCA-treated mice as compared to WT mice. An 89% reduction of CDCA was found in both CA and DCA treated groups. These observations indicate that the potent FXR activator CDCA is unlikely to explain the Cyp7a1 suppression in these two groups. In addition, DCA was detectable in the DCA-treated group, which had similar Cyp7a1 expression as the CA group. This observation further eliminates the possibility that DCA is the explanation for the induction of bile acid synthesis. By contrast, total hydrophilic bile acids (α-, β-MCAs and UDCA) were reduced by 39% and 34% in the CA- and DCA groups, respectively, while a 22% increase was detected in the CDCA group. Indeed, the α-MCA content was down by 68% in both CA and DCA treatment, while a 3-fold elevation of α-MCA was detected in the CDCA-treated group. These results suggest a general significance of the regulatory effect of MCA on bile acid synthesis, in particular within the positive feedback which counteracts FXR signaling (Figure 5).

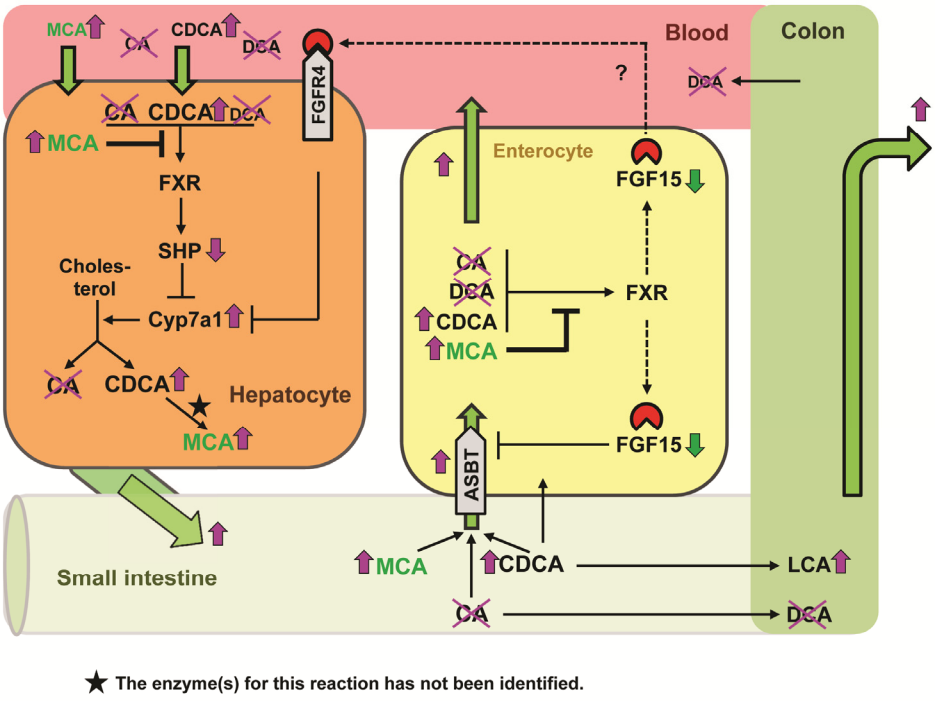

Figure 5: *Schematic diagram of BA metabolism in mice. The bile acid flow in Cyp8b1$^{-/-}$ mice is illustrated with green arrows, while magenta arrows show the increase or decrease of the bile acids. As Cyp8b1$^{-/-}$ mice lack CA and DCA, CDCA and MCA production is increased in the liver, which then enter the small intestine. As the absorption of these bile acids in the ileum increases in parity, presumably via Asbt-dependent and -independent mechanisms, MCAs exert FXR-antagonistic effects in the enterocytes, which override the agonistic effects of CDCA, thereby strongly reducing ileal FGF15 expression. This de-represses Asbt expression, which increases BA absorption enlarging the BA pool that will be further enriched with MCAs from hepatic conversion of CDCA to MCAs. Likewise, in the hepatocyte, MCAs will exert FXR-antagonistic effects de-repressing Cyp7a1 expression, thereby increasing hepatic BA synthesis. In parallel, reduced FGF15 signaling from the gut to liver may contribute to de-repress hepatic Cyp7a1 expression, although evidence for that is circumstantial.*

4.3 PAPER III: SECOND GENERATION ANTISENSE OLIGONUCLEOTIDES PREVENT ACCUMULATION OF LIVER CHOLESTEROL BY INHIBITING CHOLIC ACID SYNTHESIS

In paper II, it was found that the hydrophilic bile acid MCA exerts positive feedback regulation on bile acid synthesis, and might counteract the agonistic effect of hydrophobic bile acids such as CA or DCA. In this study, we modulate the CA production by using second generation antisense oligonucleotides (ASOs) against Cyp8b1, the key enzyme for CA production in order to evaluate their pharmacological potential as a target for maintaining cholesterol homeostasis.

Effects of ASOs on cholesterol and bile acid metabolic parameters in mice under normal diet.

A total of ten different ASOs were used for the evaluation of their respective efficacy. The effects of ASOs were measured initially by the level of Cyp8b1 mRNA after 6 weeks of treatment. According to the results, the most effective ASO, ASO-81, suppressed Cyp8b1 mRNA by 75% compared to the control group. A similar reduction in Cyp8b1 expression was also shown at the protein level. Importantly, no liver toxicity was detected with ASO-81, indicating that the compound was well tolerated during the treatment.

The analysis of the biliary bile acid composition revealed that the CA fraction was reduced in the ASO-81 treated group by almost 50% as compared to the control group. In contrast, the proportion of MCA and CDCA was increased in ASO-81 treated mice. This resulted in a dramatic reduction of the CA/β-MCA ratio in the ASO-81 group (1.0 *vs* 3.3 in the control group). These results clearly indicate the potency of ASO-81 in eliminating the CA production in mice. Similar to Cyp8b1$^{-/-}$ mice, the ASO-81 treated mice were characterized by a less hydrophobic bile acid profile with a higher percentage of MCA and UDCA in the bile acid pool. Such a bile acid profile is highly in favor for the reduction of cholesterol absorption, which is crucial in maintaining a good balance of cholesterol homeostasis.

The expression of genes involved in bile acid metabolism was also affected by the ASO treatment. Downregulation on ileal SHP and Fgf15 mRNA was detected in the ASO-81 treated group, while induction on ileal ASBT was found in parallel. These results suggest the increase of bile acid reabsorption through the enterohepatic circulation during the ASO treatment. Similar results were found in the Cyp8b1$^{-/-}$ mice, of which the CA production was depleted genetically.

Effects of ASO-81 on liver cholesterol accumulation and serum lipoprotein cholesterol profiles after dietary cholesterol challenge.

As Cyp8b1$^{-/-}$ mice showed marked resistance to cholesterol overload due to the relatively higher bile acid turnover and low cholesterol absorption, we evaluated the

effects of ASO-81 on liver cholesterol metabolism under 0.2% cholesterol feeding. The treatment reduced the liver cholesterol content by 50% compared to the control group. This was mainly due to the reduction of esterified cholesterol in the liver. Simultaneously, a slight increase of free cholesterol was observed. In line with the reduced liver cholesterol content, we observed a 70% decrease of VLDL cholesterol in the serum under the ASO-81 treatment compared to the control group, while no major changes were found in the LDL cholesterol fraction. A mild increase of HDL-cholesterol was also detected with ASO-81 treatment. This is in line with the liver cholesterol finding, as reduced levels of cholesterol in the apoB-containing lipoproteins could be recognized as an expression for a state of relative 'cholesterol deficiency'.

In the Cyp8b1$^{-/-}$ mice, an important phenotype is the upregulation of Cyp7a1 due to the lack of feedback suppression by CA elimination. Compellingly, treatment of ASO-81 did not completely phenocopy the Cyp8b1$^{-/-}$ mice, and one striking difference represented the consistent level of hepatic Cyp7a1 in the ASO-81 mice. Despite the upregulation of bile acid regulatory factors, namely the SHP and Fgf15 expression, the liver bile acid synthesis remained consistent compared to the control group. This observation could be explained by the CA in the ASO-81 treated mice, which is different from the Cyp8b1$^{-/-}$ mice. Another explanation might be due to the complex regulatory pathways of Cyp7a1, and the FXR-mediated feedback mechanism only represents one such approach.

5 GENERAL DISCUSSION AND FUTURE PERSPECTIVES

The present thesis extends the knowledge of cholesterol metabolism regulation by nuclear receptor LXRs and bile acids, two major players in the homeostasis of cholesterol in the body. Although such topics have been intensively studied at a broader scope, lots of detail mechanisms still remain unclear.

It has been more than a decade since the discovery of LXRs as oxysterol receptors by the *in vitro* study [57]. An early study has demonstrated that impaired synthesis of 24S-, 25- and 27-hydroxycholesterol resulted in disrupted LXR signaling upon cholesterol feeding in mice [111]. However, this theory was recently challenged by the creation of mice transgenic for human cholesterol 24-hydroxylase with enhanced production of 24-hydroxycholesterol [112]. In this model, LXR signaling was not dramatically enhanced despite the dramatic elevation of its natural endogenous ligand. Nevertheless, numerous studies have been intensively conducted onwards in order to clarify the exact metabolic role of LXRs in lipid metabolism. Although the expression of both LXRs has been found in many tissues, it is important to note that the two subtypes regulate gene transcription in a tissue-specific and subtype-specific manner. For this reason, animal models with specific knockout have been proven to be more useful. One major function of LXRs, in particular LXRα, is to maintain the homeostasis of lipid metabolism. A subtype specific knockout of LXRα in mice was first described in 1998 by Peet et al [113]. In this animal model, the natural resistance to cholesterol overloading was disrupted when mice were fed with 2% cholesterol diet. This observation was attributed to the lack of Cyp7a1 upregulation by LXRα in the liver, which is otherwise upregulated upon high cholesterol challenge. Interestingly however, LXRβ knockout mice failed to display a similar phenotype under the same cholesterol loading condition, indicating the different physiological functions of the two subtypes [114]. Later on, the biological functions of LXRs were extended to cholesterol uptake, absorption, excretion and reverse transportation as more genes have been discovered under direct LXRs governance [63, 115-117].

The massive improvement of LXR stimulation on lipid homeostasis has aroused great interest in translating the nuclear receptor into a potential therapeutic target against atherosclerosis. However, the initial attempt by using first generation LXR synthetic agonists such as TO-901317 and GW3965 was largely disappointing due to the strong elevation of lipogenesis by the activation of LXRα, resulting in adverse effects such as tryglyceridemia and fatty liver disease [64]. These observations give the scientific rationale for the creation of a selective activation on LXRβ, especially when results have shown strong atheroprotective effects of LXRβ without induction of lipogenesis [81, 118, 119]. In line with the notion, we used selective LXRα or LXRβ knockout mice and compared the separate activation of either subtype on cholesterol and bile acid metabolism by using the synthetic agonist GW3965. We demonstrated that selective activation of LXRβ might generate effects such as enhanced cholesterol absorption with elevated apoB-lipoprotein cholesterol in the circulation. In addition, such an approach also led to decreased fecal neutral sterol output without affecting the

biliary sterol excretion. A more hydrophobic profile on bile acid excretion was also discovered by selective LXRβ activation [120]. These observations raised the concern that selective LXRβ activation should be used with caution as adverse effects might exist.

Another approach to avoid massive lipogenesis is the tissue selective activation of LXRα, which has been created and thoroughly investigated by Lo Sasso et al [121]. In their study, the authors demonstrated tissue specific effects of LXRβ on ABCG5 regulation, without induction of the hepatic target genes of LXRs. It further unraveled the key role of the small intestine in the context of cholesterol homeostasis by showing the reduced absorption of cholesterol during the intestinal LXRα specific induction. Taken together, the numerous experimental data provide a solid scientific rationale for the development of LXR agonists for an atheroprotective purpose. Meanwhile, safety concerns and a more careful dissection of isoform specific activation remain highlighted before the mature translation of the LXR agonists from bench to bedside.

In addition to the nuclear receptor LXRs, another major player in the homeostasis of cholesterol is the bile acids, governing the elimination pathway of excess cholesterol. Manipulation of the bile acid production generates a significant impact on Cyp7a1 and bile acid synthesis due to the signaling intensity change of the FXR pathway. Interestingly, studies on humans and mice showed a different affinity of bile acids to FXR. While CA acts as the strongest ligand for FXR in mice, human FXR reacts strongly to CDCA [122, 123]. In mice with genetic depletion of CA production ($Cyp8b1^{-/-}$ mice), the bile acid synthesis was dramatically elevated resulting in an increased total bile acid pool size [30]. The phenotype was ascribed to the elimination of CA, a strong agonist for FXR at least in mice. However, the limitation of such an explanation is that the effect of secondary bile acids such as DCA has been largely ignored. In addition, whether the significant increase of MCAs in these mice contributes to the phenotype is unknown.

We investigated this possibility by using antibiotics to eliminate the secondary bile acid production by WT and $Cyp8b1^{-/-}$ mice. We found that CA elimination could not be the possible explanation for the upregulation of bile acid synthesis in $Cyp8b1^{-/-}$ mice, as similar levels of Cyp7a1 were detected in WT+AMP and KO mice, whose CA levels differed dramatically. By giving exogenous bile acids to AMP-treated $Cyp8b1^{-/-}$ mice, we could systematically exclude DCA as the potential explanation for the phenotype. Instead, we found that by counteracting the FXR stimulation, the increase in hydrophilic bile acids (MCAs and UDCA) was the real driving force for the elevated bile acid synthesis in $Cyp8b1^{-/-}$ mice. This observation indicates that by modulating the enterohepatic circulation of bile acids, the positive feedback mechanism regulates bile acid homeostasis without employing the hormonal effect of Fgf15, although such effect is most likely to exist. This finding is of fundamental importance for the understating of bile acid metabolism in both humans and mice, as the Fgf15/19 negative feedback mechanism is believed to operate in both species. Our proposed positive feedback mechanism of MCA in the context of bile acid synthetic regulation could possibly be extended to the germ-free and bile duct ligated rodents. The finding is in line with the recent publication from Sayin et al that addresses the importance of MCA for the bile acid metabolism in mice [124]. In their study, they reveal the profound systematic

effect of gut microbiota not only on local secondary bile acid production, but also on the synthesis of bile acids in the liver.

More studies on intestinal bacteria reveal the close relation between gut microbiota and other lipid metabolic diseases. The imbalanced bacterial population caused by the high-fat diet is a trigger for the development of obesity [125, 126], diabetes [127, 128] and hypercholesterolemia [129] in rodents. In humans, an imbalanced gut microbiota has been reported in patients with diabetes and inflammatory bowel diseases [130, 131]. A recent study by Islam et al demonstrated that CA feeding in rats caused a bacterial repopulation similar to the one found in the high-fat fed rats [132]. This observation improves our understanding on the relation between gut bacteria, bile acids and metabolic diseases.

The thorough characterization of cholesterol metabolism by using various animal models opens up the gate for the profound understanding of human disease conditions, in particular diseases related to lipid metabolism such as atherosclerosis. New therapeutic pharmaceuticals combine modern drug design technology with a specific target on atherosclerosis, creating products which are potentially useful for the treatment of atherosclerosis. One such approach is to use ASOs, a synthetic DNA chimeric compound. Clinical trials on the efficacy and safety of such drugs have been carried out to evaluate their potential therapeutic value. To date, the most advanced ASO in the clinical development for dyslipidemia is a 2'-O-methoxyethyl phosphorothioate 20-mer ASO with the common name mipomersen. Its primary function is to inhibit the synthesis of apoB-100 in the liver, which then results in a reduced serum LDL cholesterol level in animal models and clinical trials [133-135]. Another promising novel ASO target is PCSK9, the use of which also lowers the blood LDL cholesterol as shown in preclinical studies [136, 137]. We also followed a similar approach by using an ASO targeting Cyp8b1 in mice, as an early study from our group showed that eliminating CA production was associated with an atheroprotective effect in apoE knockout mice [31]. We observed a dramatic reduction of the CA percentage in the mice treated with ASO-81, although the elimination of CA by ASO was not as drastic as in the Cyp8b1$^{-/-}$ mice. The phenotype observed in the treated mice also included the prevention of liver cholesterol accumulation under high cholesterol loading, and a reduced serum apoB cholesterol profile. The above observations reveal the potential role of such an approach for the development of atheroprotective therapeutics, although further studies are still needed, including sequence screening and efficacy evaluation for the human counterpart of ASO-Cyp8b1. Other potential targets including apoC-III and Lp(a) for the ASO drug design are also under development.

In summary, the regulation of cholesterol homeostasis represents several complex interactions between nuclear receptors and bile acids. A thorough understanding of how each pathway is regulated is of benefit for the development of novel therapeutic approaches for dyslipidemia, cardiovascular disease and diabetes. With the help of modern drug design technology, a combined expertise of medicine and pharmacology would help to design a specific drug with minimized adverse effects, which will eventually refine the treatment of diseases such as atherosclerosis and other dyslipidemia-related disorders.

6 CONCLUSIONS

In the present thesis, we intended to gain a deeper understanding of how cholesterol and bile acids regulate cholesterol homeostasis. We identify the individual function of LXRα and LXRβ in the regulation of cholesterol absorption and serum lipid profile, and find that the separate activation of LXRβ as a therapeutic approach for an anti-atherogenic purpose should be used with caution as it enhances the cholesterol absorption in the small intestine and raises the apoB-lipoprotein cholesterol in the circulation (paper I). In addition, the phenotype of Cyp8b1$^{-/-}$ mice in terms of bile acid metabolism has been reinvestigated in the thesis, to which a novel positive feedback mechanism of MCAs on bile acid synthesis has been proposed (paper II). The finding of MCAs as important bile acid synthesis regulators provides new insights into the bile acid homeostasis, and mouse models such as the Cyp8b1$^{-/-}$ mice become a more attractive experimental tool due to the massive increase of their MCAs. In line with this notion, we also explore the potential of creating such mouse models from WT background with second generation ASOs against Cyp8b1 mRNA. To our knowledge, this is the first report for such an approach with successful results in generating Cyp8b1 knockdown animal models (paper III). The above findings in this thesis reveal a highly potent mode of interaction between nuclear receptors and bile acids in the regulation of cholesterol homeostasis, and are therefore likely to be important in understanding the lipid metabolism in both preclinical and clinical conditions.

7 ACKNOWLEDGEMENTS

The past five years of this PhD journey have been an extraordinary experience in my life both within and outside academia. Therefore I take this opportunity to express my sincere gratitude to everyone and everything that helped me, in one way or another, in the path of growing up during this time. In particular, I would like to thank:

My supervisor, Dr. **Gösta Eggertsen**, for all the time you spent with me for project discussions, for manuscript corrections, and for exchanging all the brilliant scientific inspirations. In addition, thank you for all the help you offered during my first year of arrival, for the Chinese dinner cooking, for the mushroom picking, for everything you did to speed up my integration into this lovely country.

My co-supervisor, Dr. **Paolo Parini**, for involving me in your weekly group meeting, for project discussions, critical reading of my manuscripts, for sharing your knowledge on medical statistics, and for the entire positive attitude. Also, thank you for being patient with me and tolerating my stubbornness during the discussions.

My co-supervisor, Dr. **Mats Gåfvels**, for teaching me the importance of being independent. It is proven to be so valuable for my future development both within and outside academia. Also, thank you for correcting my thesis☺.

To Dr. **Rachel Fisher**, thank you for being my mentor. Also, thank you for inviting me to your nice apartment and for the wonderful afternoon cake!

To Dr. **Knut Steffensen** and Dr. **Mats Rudling**, thank you for the fruitful collaborations and for believing in me.

To all the present and past officemates: **Vera Tillander, Tina Kannisto, Diba Ahmed,** Dr**. Naama Kenan Modén, Osman Ahmed, Ahmed Saeed, Treska Hassan,** Dr. **Zhao-Yan Jiang,** Dr**. Yufang Zheng,** Dr**. Davide Gnocchi,** Dr. **Lisa-Mari Mörk, Dara Deegan,** Dr**. Marjan Shafaati,** Dr**. Katharina Slätis,** Dr. **Ann Båvner.** The weekdays would have been harder without your company ☺. As I wrap thing up on my table, the giant pile of bottles just becomes more visible in the corner-- It's time for cake and ice cream again!

To my labmates: **Maria Olin, Mikaela Bodell, Ninawa Aho**. Thank you for all the technical support on methodology questions, practical help in finding reagents, and for all the pipetting chats, which made the tedious experiments more interesting.

To my animal roommates: **Lilian Larsson,** Dr**. Matteo Pedrelli,** Dr**. Maura Heverin, Zeina Ali.** Thank you for being nice to my mice by giving them a spacious growing environment, really appreciate that!

To all the other colleagues in and outside the division: Dr**. Ingemar Björkhem,** Dr**. Stefan Alexson,** Dr**. Anders Helander,** Dr**. Ulf Diczfalusy, Anita Lövgren**

Sandblom, Dr**. Ulla Andersson, Hanna Nylén,** Dr**. Camilla Pramfalk,** Dr**. Malgorzata Strozyk,** Dr**. Jenny Flygare,** Dr**. Anna-Klara Rundlöf,** Dr**. Ylva Bonde**. Thank you for all the help and for all the good time together.

To our wonderful secretary, **Jenny Bernström,** thank you for all the administrative help which saved me lots of time and effort!

To my dear friends: **Ying, Sonal, Sameer, Devesh, Gökce, Esra, Nina, Melroy, Bea, Ashwin** for the kind supports and suggestions you gave when I needed them. And for all the wonderful moments and fun we shared during the past 5 years.

To my parents, **Dongping** and **Caiyu**. Thank you for allowing me to realize my potential, and for teaching me the value of education and hardworking. I would have not reached this far without your constant contribution.

To my dear husband, **Erwin**. Life has never been sweeter since we are together. Thank you for being who you are, for always standing by my side with care and support, and for making me laugh even during the bad days. I feel so blessed to have you in my life!

To life. Tomorrow the sun will always rise and shine.

8 REFERENCES

1 Dietschy JM, Turley SD, Spady DK. Role of liver in the maintenance of cholesterol and low density lipoprotein homeostasis in different animal species, including humans. *Journal of lipid research* 1993; **34:** 1637-59.

2 Taylor F, Ward K, Moore TH, Burke M, Davey Smith G, Casas JP, Ebrahim S. Statins for the primary prevention of cardiovascular disease. *The Cochrane database of systematic reviews* 2011**:** CD004816.

3 Gill S, Chow R, Brown AJ. Sterol regulators of cholesterol homeostasis and beyond: the oxysterol hypothesis revisited and revised. *Progress in lipid research* 2008; **47:** 391-404.

4 Horton JD, Goldstein JL, Brown MS. SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver. *The Journal of clinical investigation* 2002; **109:** 1125-31.

5 Istvan ES, Palnitkar M, Buchanan SK, Deisenhofer J. Crystal structure of the catalytic portion of human HMG-CoA reductase: insights into regulation of activity and catalysis. *The EMBO journal* 2000; **19:** 819-30.

6 Goldstein JL, Brown MS. Regulation of the mevalonate pathway. *Nature* 1990; **343:** 425-30.

7 Staggers JE, Hernell O, Stafford RJ, Carey MC. Physical-chemical behavior of dietary and biliary lipids during intestinal digestion and absorption. 1. Phase behavior and aggregation states of model lipid systems patterned after aqueous duodenal contents of healthy adult human beings. *Biochemistry* 1990; **29:** 2028-40.

8 Hernell O, Staggers JE, Carey MC. Physical-chemical behavior of dietary and biliary lipids during intestinal digestion and absorption. 2. Phase analysis and aggregation states of luminal lipids during duodenal fat digestion in healthy adult human beings. *Biochemistry* 1990; **29:** 2041-56.

9 Altmann SW, Davis HR, Jr., Zhu LJ*, et al.* Niemann-Pick C1 Like 1 protein is critical for intestinal cholesterol absorption. *Science* 2004; **303:** 1201-4.

10 Jia L, Ma Y, Rong S*, et al.* Niemann-Pick C1-Like 1 deletion in mice prevents high-fat diet-induced fatty liver by reducing lipogenesis. *Journal of lipid research* 2010; **51:** 3135-44.

11 Davis HR, Jr., Hoos LM, Tetzloff G, Maguire M, Zhu LJ, Graziano MP, Altmann SW. Deficiency of Niemann-Pick C1 Like 1 prevents atherosclerosis in ApoE-/- mice. *Arteriosclerosis, thrombosis, and vascular biology* 2007; **27:** 841-9.

12 Fielding CJ, Renston JP, Fielding PE. Metabolism of cholesterol-enriched chylomicrons. Catabolism of triglyceride by lipoprotein lipase of perfused heart and adipose tissues. *Journal of lipid research* 1978; **19:** 705-11.

13 Klein RL, Rudel LL. Cholesterol absorption and transport in thoracic duct lymph lipoproteins of nonhuman primates. Effect of dietary cholesterol level. *Journal of lipid research* 1983; **24:** 343-56.

14 Brunham LR, Kruit JK, Iqbal J*, et al.* Intestinal ABCA1 directly contributes to HDL biogenesis in vivo. *The Journal of clinical investigation* 2006; **116:** 1052-62.

15 Berge KE, Tian H, Graf GA*, et al.* Accumulation of dietary cholesterol in sitosterolemia caused by mutations in adjacent ABC transporters. *Science* 2000; **290:** 1771-5.

16 Yu L, Li-Hawkins J, Hammer RE, Berge KE, Horton JD, Cohen JC, Hobbs HH. Overexpression of ABCG5 and ABCG8 promotes biliary cholesterol

secretion and reduces fractional absorption of dietary cholesterol. *The Journal of clinical investigation* 2002; **110:** 671-80.
17 Bashtovyy D, Jones MK, Anantharamaiah GM, Segrest JP. Sequence conservation of apolipoprotein A-I affords novel insights into HDL structure-function. *Journal of lipid research* 2011; **52:** 435-50.
18 Luo CC, Li WH, Moore MN, Chan L. Structure and evolution of the apolipoprotein multigene family. *Journal of molecular biology* 1986; **187:** 325-40.
19 Patsch W, Gotto AM, Jr. Apolipoproteins: pathophysiology and clinical implications. *Methods in enzymology* 1996; **263:** 3-32.
20 Glomset JA. The metabolic role of lecithin: cholesterol acyltransferase: perspectives from pathology. *Advances in lipid research* 1973; **11:** 1-65.
21 Rader DJ. Molecular regulation of HDL metabolism and function: implications for novel therapies. *The Journal of clinical investigation* 2006; **116:** 3090-100.
22 Castellani LW, Lusis AJ. ApoA-II versus ApoA-I: two for one is not always a good deal. *Arteriosclerosis, thrombosis, and vascular biology* 2001; **21:** 1870-2.
23 Russell DW. The enzymes, regulation, and genetics of bile acid synthesis. *Annual review of biochemistry* 2003; **72:** 137-74.
24 Ishibashi S, Schwarz M, Frykman PK, Herz J, Russell DW. Disruption of cholesterol 7alpha-hydroxylase gene in mice. I. Postnatal lethality reversed by bile acid and vitamin supplementation. *The Journal of biological chemistry* 1996; **271:** 18017-23.
25 Erickson SK, Lear SR, Deane S*, et al.* Hypercholesterolemia and changes in lipid and bile acid metabolism in male and female cyp7A1-deficient mice. *Journal of lipid research* 2003; **44:** 1001-9.
26 Nakamoto K, Wang S, Jenison RD, Guo GL, Klaassen CD, Wan YJ, Zhong XB. Linkage disequilibrium blocks, haplotype structure, and htSNPs of human CYP7A1 gene. *BMC genetics* 2006; **7:** 29.
27 Wang J, Freeman DJ, Grundy SM, Levine DM, Guerra R, Cohen JC. Linkage between cholesterol 7alpha-hydroxylase and high plasma low-density lipoprotein cholesterol concentrations. *The Journal of clinical investigation* 1998; **101:** 1283-91.
28 del Castillo-Olivares A, Gil G. Differential effects of sterol regulatory binding proteins 1 and 2 on sterol 12 alpha-hydroxylase. SREBP-2 suppresses the sterol 12 alpha-hydroxylase promoter. *The Journal of biological chemistry* 2002; **277:** 6750-7.
29 Yang Y, Eggertsen G, Gafvels M, Andersson U, Einarsson C, Bjorkhem I, Chiang JY. Mechanisms of cholesterol and sterol regulatory element binding protein regulation of the sterol 12alpha-hydroxylase gene (CYP8B1). *Biochemical and biophysical research communications* 2004; **320:** 1204-10.
30 Li-Hawkins J, Gafvels M, Olin M*, et al.* Cholic acid mediates negative feedback regulation of bile acid synthesis in mice. *The Journal of clinical investigation* 2002; **110:** 1191-200.
31 Slatis K, Gafvels M, Kannisto K*, et al.* Abolished synthesis of cholic acid reduces atherosclerotic development in apolipoprotein E knockout mice. *Journal of lipid research* 2010; **51:** 3289-98.
32 Wang J, Gafvels M, Rudling M, Murphy C, Bjorkhem I, Einarsson C, Eggertsen G. Critical role of cholic acid for development of hypercholesterolemia and gallstones in diabetic mice. *Biochemical and biophysical research communications* 2006; **342:** 1382-8.

33 Wang J, Einarsson C, Murphy C, Parini P, Bjorkhem I, Gafvels M, Eggertsen G. Studies on LXR- and FXR-mediated effects on cholesterol homeostasis in normal and cholic acid-depleted mice. *Journal of lipid research* 2006; **47:** 421-30.
34 Abrahamsson A, Gafvels M, Reihner E, Bjorkhem I, Einarsson C, Eggertsen G. Polymorphism in the coding part of the sterol 12alpha-hydroxylase gene does not explain the marked differences in the ratio of cholic acid and chenodeoxycholic acid in human bile. *Scandinavian journal of clinical and laboratory investigation* 2005; **65:** 595-600.
35 Becker M, Staab D, Leiss O, von Bergmann K. Biliary lipid composition in patients with cystic fibrosis. *Journal of pediatric gastroenterology and nutrition* 1989; **8:** 308-12.
36 Rosen H, Reshef A, Maeda N*, et al.* Markedly reduced bile acid synthesis but maintained levels of cholesterol and vitamin D metabolites in mice with disrupted sterol 27-hydroxylase gene. *The Journal of biological chemistry* 1998; **273:** 14805-12.
37 Bjorkhem I, Leitersdorf E. Sterol 27-hydroxylase deficiency: a rare cause of xanthomas in normocholesterolemic humans. *Trends in endocrinology and metabolism: TEM* 2000; **11:** 180-3.
38 Dawson PA, Lan T, Rao A. Bile acid transporters. *Journal of lipid research* 2009; **50:** 2340-57.
39 Hulzebos CV, Renfurm L, Bandsma RH*, et al.* Measurement of parameters of cholic acid kinetics in plasma using a microscale stable isotope dilution technique: application to rodents and humans. *Journal of lipid research* 2001; **42:** 1923-9.
40 Hofmann AF, Molino G, Milanese M, Belforte G. Description and simulation of a physiological pharmacokinetic model for the metabolism and enterohepatic circulation of bile acids in man. Cholic acid in healthy man. *The Journal of clinical investigation* 1983; **71:** 1003-22.
41 Thomas C, Gioiello A, Noriega L*, et al.* TGR5-mediated bile acid sensing controls glucose homeostasis. *Cell metabolism* 2009; **10:** 167-77.
42 Thomas C, Pellicciari R, Pruzanski M, Auwerx J, Schoonjans K. Targeting bile-acid signalling for metabolic diseases. *Nature reviews Drug discovery* 2008; **7:** 678-93.
43 Thomas C, Auwerx J, Schoonjans K. Bile acids and the membrane bile acid receptor TGR5--connecting nutrition and metabolism. *Thyroid : official journal of the American Thyroid Association* 2008; **18:** 167-74.
44 Watanabe M, Houten SM, Mataki C*, et al.* Bile acids induce energy expenditure by promoting intracellular thyroid hormone activation. *Nature* 2006; **439:** 484-9.
45 Hagenbuch B, Dawson P. The sodium bile salt cotransport family SLC10. *Pflugers Archiv : European journal of physiology* 2004; **447:** 566-70.
46 Hata S, Wang P, Eftychiou N*, et al.* Substrate specificities of rat oatp1 and ntcp: implications for hepatic organic anion uptake. *American journal of physiology Gastrointestinal and liver physiology* 2003; **285:** G829-39.
47 Mita S, Suzuki H, Akita H, Stieger B, Meier PJ, Hofmann AF, Sugiyama Y. Vectorial transport of bile salts across MDCK cells expressing both rat Na+-taurocholate cotransporting polypeptide and rat bile salt export pump. *American journal of physiology Gastrointestinal and liver physiology* 2005; **288:** G159-67.
48 Mita S, Suzuki H, Akita H, Hayashi H, Onuki R, Hofmann AF, Sugiyama Y. Vectorial transport of unconjugated and conjugated bile salts by monolayers of LLC-PK1 cells doubly transfected with human NTCP and BSEP or with rat Ntcp and Bsep. *American journal of physiology Gastrointestinal and liver physiology* 2006; **290:** G550-6.

49 Shneider BL. Intestinal bile acid transport: biology, physiology, and pathophysiology. *Journal of pediatric gastroenterology and nutrition* 2001; **32:** 407-17.
50 Weinman SA, Carruth MW, Dawson PA. Bile acid uptake via the human apical sodium-bile acid cotransporter is electrogenic. *The Journal of biological chemistry* 1998; **273:** 34691-5.
51 Wang W, Seward DJ, Li L, Boyer JL, Ballatori N. Expression cloning of two genes that together mediate organic solute and steroid transport in the liver of a marine vertebrate. *Proceedings of the National Academy of Sciences of the United States of America* 2001; **98:** 9431-6.
52 Seward DJ, Koh AS, Boyer JL, Ballatori N. Functional complementation between a novel mammalian polygenic transport complex and an evolutionarily ancient organic solute transporter, OSTalpha-OSTbeta. *The Journal of biological chemistry* 2003; **278:** 27473-82.
53 Jakobsson T, Venteclef N, Toresson G*, et al.* GPS2 is required for cholesterol efflux by triggering histone demethylation, LXR recruitment, and coregulator assembly at the ABCG1 locus. *Molecular cell* 2009; **34:** 510-8.
54 Venteclef N, Jakobsson T, Ehrlund A*, et al.* GPS2-dependent corepressor/SUMO pathways govern anti-inflammatory actions of LRH-1 and LXRbeta in the hepatic acute phase response. *Genes & development* 2010; **24:** 381-95.
55 Giguere V. Orphan nuclear receptors: from gene to function. *Endocrine reviews* 1999; **20:** 689-725.
56 Castrillo A, Tontonoz P. Nuclear receptors in macrophage biology: at the crossroads of lipid metabolism and inflammation. *Annual review of cell and developmental biology* 2004; **20:** 455-80.
57 Janowski BA, Willy PJ, Devi TR, Falck JR, Mangelsdorf DJ. An oxysterol signalling pathway mediated by the nuclear receptor LXR alpha. *Nature* 1996; **383:** 728-31.
58 Lehmann JM, Kliewer SA, Moore LB*, et al.* Activation of the nuclear receptor LXR by oxysterols defines a new hormone response pathway. *The Journal of biological chemistry* 1997; **272:** 3137-40.
59 Fu X, Menke JG, Chen Y*, et al.* 27-hydroxycholesterol is an endogenous ligand for liver X receptor in cholesterol-loaded cells. *The Journal of biological chemistry* 2001; **276:** 38378-87.
60 Prufer K, Boudreaux J. Nuclear localization of liver X receptor alpha and beta is differentially regulated. *Journal of cellular biochemistry* 2007; **100:** 69-85.
61 Fan X, Kim HJ, Bouton D, Warner M, Gustafsson JA. Expression of liver X receptor beta is essential for formation of superficial cortical layers and migration of later-born neurons. *Proceedings of the National Academy of Sciences of the United States of America* 2008; **105:** 13445-50.
62 Calkin AC, Tontonoz P. Transcriptional integration of metabolism by the nuclear sterol-activated receptors LXR and FXR. *Nature reviews Molecular cell biology* 2012; **13:** 213-24.
63 Zelcer N, Hong C, Boyadjian R, Tontonoz P. LXR regulates cholesterol uptake through Idol-dependent ubiquitination of the LDL receptor. *Science* 2009; **325:** 100-4.
64 Schultz JR, Tu H, Luk A*, et al.* Role of LXRs in control of lipogenesis. *Genes & development* 2000; **14:** 2831-8.
65 Repa JJ, Liang G, Ou J*, et al.* Regulation of mouse sterol regulatory element-binding protein-1c gene (SREBP-1c) by oxysterol receptors, LXRalpha and LXRbeta. *Genes & development* 2000; **14:** 2819-30.

66 Katz A, Udata C, Ott E*, et al.* Safety, pharmacokinetics, and pharmacodynamics of single doses of LXR-623, a novel liver X-receptor agonist, in healthy participants. *Journal of clinical pharmacology* 2009; **49:** 643-9.
67 Forman BM, Goode E, Chen J*, et al.* Identification of a nuclear receptor that is activated by farnesol metabolites. *Cell* 1995; **81:** 687-93.
68 Parks DJ, Blanchard SG, Bledsoe RK*, et al.* Bile acids: natural ligands for an orphan nuclear receptor. *Science* 1999; **284:** 1365-8.
69 Makishima M, Okamoto AY, Repa JJ*, et al.* Identification of a nuclear receptor for bile acids. *Science* 1999; **284:** 1362-5.
70 Goodwin B, Jones SA, Price RR*, et al.* A regulatory cascade of the nuclear receptors FXR, SHP-1, and LRH-1 represses bile acid biosynthesis. *Molecular cell* 2000; **6:** 517-26.
71 Brendel C, Schoonjans K, Botrugno OA, Treuter E, Auwerx J. The small heterodimer partner interacts with the liver X receptor alpha and represses its transcriptional activity. *Mol Endocrinol* 2002; **16:** 2065-76.
72 De Fabiani E, Mitro N, Anzulovich AC, Pinelli A, Galli G, Crestani M. The negative effects of bile acids and tumor necrosis factor-alpha on the transcription of cholesterol 7alpha-hydroxylase gene (CYP7A1) converge to hepatic nuclear factor-4: a novel mechanism of feedback regulation of bile acid synthesis mediated by nuclear receptors. *The Journal of biological chemistry* 2001; **276:** 30708-16.
73 Yu XX, Watts LM, Manchem VP, Chakravarty K, Monia BP, McCaleb ML, Bhanot S. Peripheral reduction of FGFR4 with antisense oligonucleotides increases metabolic rate and lowers adiposity in diet-induced obese mice. *PloS one* 2013; **8:** e66923.
74 Kim I, Ahn SH, Inagaki T*, et al.* Differential regulation of bile acid homeostasis by the farnesoid X receptor in liver and intestine. *Journal of lipid research* 2007; **48:** 2664-72.
75 Maruyama T, Miyamoto Y, Nakamura T*, et al.* Identification of membrane-type receptor for bile acids (M-BAR). *Biochemical and biophysical research communications* 2002; **298:** 714-9.
76 Katsuma S, Hirasawa A, Tsujimoto G. Bile acids promote glucagon-like peptide-1 secretion through TGR5 in a murine enteroendocrine cell line STC-1. *Biochemical and biophysical research communications* 2005; **329:** 386-90.
77 Maruyama T, Tanaka K, Suzuki J*, et al.* Targeted disruption of G protein-coupled bile acid receptor 1 (Gpbar1/M-Bar) in mice. *The Journal of endocrinology* 2006; **191:** 197-205.
78 Joseph SB, McKilligin E, Pei L*, et al.* Synthetic LXR ligand inhibits the development of atherosclerosis in mice. *Proceedings of the National Academy of Sciences of the United States of America* 2002; **99:** 7604-9.
79 Verschuren L, de Vries-van der Weij J, Zadelaar S, Kleemann R, Kooistra T. LXR agonist suppresses atherosclerotic lesion growth and promotes lesion regression in apoE*3Leiden mice: time course and mechanisms. *Journal of lipid research* 2009; **50:** 301-11.
80 Schuster GU, Parini P, Wang L*, et al.* Accumulation of foam cells in liver X receptor-deficient mice. *Circulation* 2002; **106:** 1147-53.
81 Bradley MN, Hong C, Chen M*, et al.* Ligand activation of LXR beta reverses atherosclerosis and cellular cholesterol overload in mice lacking LXR alpha and apoE. *The Journal of clinical investigation* 2007; **117:** 2337-46.
82 Zhang Y, Wang X, Vales C, Lee FY, Lee H, Lusis AJ, Edwards PA. FXR deficiency causes reduced atherosclerosis in Ldlr-/- mice. *Arteriosclerosis, thrombosis, and vascular biology* 2006; **26:** 2316-21.

83 Guo GL, Santamarina-Fojo S, Akiyama TE, Amar MJ, Paigen BJ, Brewer B, Jr., Gonzalez FJ. Effects of FXR in foam-cell formation and atherosclerosis development. *Biochimica et biophysica acta* 2006; **1761:** 1401-9.
84 Hanniman EA, Lambert G, McCarthy TC, Sinal CJ. Loss of functional farnesoid X receptor increases atherosclerotic lesions in apolipoprotein E-deficient mice. *Journal of lipid research* 2005; **46:** 2595-604.
85 Hartman HB, Gardell SJ, Petucci CJ, Wang S, Krueger JA, Evans MJ. Activation of farnesoid X receptor prevents atherosclerotic lesion formation in LDLR-/- and apoE-/- mice. *Journal of lipid research* 2009; **50:** 1090-100.
86 Mencarelli A, Renga B, Distrutti E, Fiorucci S. Antiatherosclerotic effect of farnesoid X receptor. *American journal of physiology Heart and circulatory physiology* 2009; **296:** H272-81.
87 Bishop-Bailey D, Walsh DT, Warner TD. Expression and activation of the farnesoid X receptor in the vasculature. *Proceedings of the National Academy of Sciences of the United States of America* 2004; **101:** 3668-73.
88 He F, Li J, Mu Y*, et al.* Downregulation of endothelin-1 by farnesoid X receptor in vascular endothelial cells. *Circulation research* 2006; **98:** 192-9.
89 Porez G, Prawitt J, Gross B, Staels B. Bile acid receptors as targets for the treatment of dyslipidemia and cardiovascular disease. *Journal of lipid research* 2012; **53:** 1723-37.
90 Sato H, Genet C, Strehle A*, et al.* Anti-hyperglycemic activity of a TGR5 agonist isolated from Olea europaea. *Biochemical and biophysical research communications* 2007; **362:** 793-8.
91 Ma K, Saha PK, Chan L, Moore DD. Farnesoid X receptor is essential for normal glucose homeostasis. *The Journal of clinical investigation* 2006; **116:** 1102-9.
92 Zhang Y, Lee FY, Barrera G*, et al.* Activation of the nuclear receptor FXR improves hyperglycemia and hyperlipidemia in diabetic mice. *Proceedings of the National Academy of Sciences of the United States of America* 2006; **103:** 1006-11.
93 Cariou B, van Harmelen K, Duran-Sandoval D*, et al.* The farnesoid X receptor modulates adiposity and peripheral insulin sensitivity in mice. *The Journal of biological chemistry* 2006; **281:** 11039-49.
94 Duran-Sandoval D, Cariou B, Percevault F*, et al.* The farnesoid X receptor modulates hepatic carbohydrate metabolism during the fasting-refeeding transition. *The Journal of biological chemistry* 2005; **280:** 29971-9.
95 Stayrook KR, Bramlett KS, Savkur RS*, et al.* Regulation of carbohydrate metabolism by the farnesoid X receptor. *Endocrinology* 2005; **146:** 984-91.
96 Yamagata K, Daitoku H, Shimamoto Y, Matsuzaki H, Hirota K, Ishida J, Fukamizu A. Bile acids regulate gluconeogenic gene expression via small heterodimer partner-mediated repression of hepatocyte nuclear factor 4 and Foxo1. *The Journal of biological chemistry* 2004; **279:** 23158-65.
97 Yu RZ, Grundy JS, Geary RS. Clinical pharmacokinetics of second generation antisense oligonucleotides. *Expert opinion on drug metabolism & toxicology* 2013; **9:** 169-82.
98 Wagner RW. Gene inhibition using antisense oligodeoxynucleotides. *Nature* 1994; **372:** 333-5.
99 Altmann KH, Fabbro D, Dean NM, Geiger T, Monia BP, Muller M, Nicklin P. Second-generation antisense oligonucleotides: structure-activity relationships and the design of improved signal-transduction inhibitors. *Biochemical Society transactions* 1996; **24:** 630-7.
100 Manoharan M. 2'-carbohydrate modifications in antisense oligonucleotide therapy: importance of conformation, configuration and conjugation. *Biochimica et biophysica acta* 1999; **1489:** 117-30.

101 Visser ME, Witztum JL, Stroes ES, Kastelein JJ. Antisense oligonucleotides for the treatment of dyslipidaemia. *European heart journal* 2012; **33:** 1451-8.
102 Hu X, Steffensen KR, Jiang ZY, Parini P, Gustafsson JA, Gafvels M, Eggertsen G. LXRbeta activation increases intestinal cholesterol absorption, leading to an atherogenic lipoprotein profile. *Journal of internal medicine* 2012; **272:** 452-64.
103 Bays HE, Neff D, Tomassini JE, Tershakovec AM. Ezetimibe: cholesterol lowering and beyond. *Expert review of cardiovascular therapy* 2008; **6:** 447-70.
104 Sudhop T, Lutjohann D, Kodal A*, et al.* Inhibition of intestinal cholesterol absorption by ezetimibe in humans. *Circulation* 2002; **106:** 1943-8.
105 Brown WV. Cholesterol absorption inhibitors: defining new options in lipid management. *Clinical cardiology* 2003; **26:** 259-64.
106 Ge L, Wang J, Qi W*, et al.* The cholesterol absorption inhibitor ezetimibe acts by blocking the sterol-induced internalization of NPC1L1. *Cell metabolism* 2008; **7:** 508-19.
107 Xie C, Li N, Chen ZJ, Li BL, Song BL. The small GTPase Cdc42 interacts with Niemann-Pick C1-like 1 (NPC1L1) and controls its movement from endocytic recycling compartment to plasma membrane in a cholesterol-dependent manner. *The Journal of biological chemistry* 2011; **286:** 35933-42.
108 Campana G, Pasini P, Roda A, Spampinato S. Regulation of ileal bile acid-binding protein expression in Caco-2 cells by ursodeoxycholic acid: role of the farnesoid X receptor. *Biochemical pharmacology* 2005; **69:** 1755-63.
109 Zhang Y, Hong JY, Rockwell CE, Copple BL, Jaeschke H, Klaassen CD. Effect of bile duct ligation on bile acid composition in mouse serum and liver. *Liver international : official journal of the International Association for the Study of the Liver* 2012; **32:** 58-69.
110 Miyata M, Takamatsu Y, Kuribayashi H, Yamazoe Y. Administration of ampicillin elevates hepatic primary bile acid synthesis through suppression of ileal fibroblast growth factor 15 expression. *The Journal of pharmacology and experimental therapeutics* 2009; **331:** 1079-85.
111 Chen W, Chen G, Head DL, Mangelsdorf DJ, Russell DW. Enzymatic reduction of oxysterols impairs LXR signaling in cultured cells and the livers of mice. *Cell metabolism* 2007; **5:** 73-9.
112 Shafaati M, Olin M, Bavner A*, et al.* Enhanced production of 24S-hydroxycholesterol is not sufficient to drive liver X receptor target genes in vivo. *Journal of internal medicine* 2011; **270:** 377-87.
113 Peet DJ, Turley SD, Ma W, Janowski BA, Lobaccaro JM, Hammer RE, Mangelsdorf DJ. Cholesterol and bile acid metabolism are impaired in mice lacking the nuclear oxysterol receptor LXR alpha. *Cell* 1998; **93:** 693-704.
114 Alberti S, Schuster G, Parini P*, et al.* Hepatic cholesterol metabolism and resistance to dietary cholesterol in LXRbeta-deficient mice. *The Journal of clinical investigation* 2001; **107:** 565-73.
115 Repa JJ, Turley SD, Lobaccaro JA*, et al.* Regulation of absorption and ABC1-mediated efflux of cholesterol by RXR heterodimers. *Science* 2000; **289:** 1524-9.
116 Repa JJ, Berge KE, Pomajzl C, Richardson JA, Hobbs H, Mangelsdorf DJ. Regulation of ATP-binding cassette sterol transporters ABCG5 and ABCG8 by the liver X receptors alpha and beta. *The Journal of biological chemistry* 2002; **277:** 18793-800.
117 Venkateswaran A, Laffitte BA, Joseph SB, Mak PA, Wilpitz DC, Edwards PA, Tontonoz P. Control of cellular cholesterol efflux by the nuclear oxysterol

receptor LXR alpha. *Proceedings of the National Academy of Sciences of the United States of America* 2000; **97:** 12097-102.
118 Quinet EM, Savio DA, Halpern AR*, et al.* Liver X receptor (LXR)-beta regulation in LXRalpha-deficient mice: implications for therapeutic targeting. *Molecular pharmacology* 2006; **70:** 1340-9.
119 Lund EG, Peterson LB, Adams AD*, et al.* Different roles of liver X receptor alpha and beta in lipid metabolism: effects of an alpha-selective and a dual agonist in mice deficient in each subtype. *Biochemical pharmacology* 2006; **71:** 453-63.
120 Hu X, Steffensen KR, Jiang ZY, Parini P, Gustafsson JA, Gafvels M, Eggertsen G. LXR beta activation increases intestinal cholesterol absorption, leading to an atherogenic lipoprotein profile. *Journal of internal medicine* 2012; **272:** 452-64.
121 Lo Sasso G, Murzilli S, Salvatore L*, et al.* Intestinal specific LXR activation stimulates reverse cholesterol transport and protects from atherosclerosis. *Cell metabolism* 2010; **12:** 187-93.
122 Ellis E, Axelson M, Abrahamsson A*, et al.* Feedback regulation of bile acid synthesis in primary human hepatocytes: evidence that CDCA is the strongest inhibitor. *Hepatology* 2003; **38:** 930-8.
123 Howard WR, Pospisil JA, Njolito E, Noonan DJ. Catabolites of cholesterol synthesis pathways and forskolin as activators of the farnesoid X-activated nuclear receptor. *Toxicology and applied pharmacology* 2000; **163:** 195-202.
124 Sayin SI, Wahlstrom A, Felin J*, et al.* Gut microbiota regulates bile acid metabolism by reducing the levels of tauro-beta-muricholic acid, a naturally occurring FXR antagonist. *Cell metabolism* 2013; **17:** 225-35.
125 Turnbaugh PJ, Backhed F, Fulton L, Gordon JI. Diet-induced obesity is linked to marked but reversible alterations in the mouse distal gut microbiome. *Cell host & microbe* 2008; **3:** 213-23.
126 Hildebrandt MA, Hoffmann C, Sherrill-Mix SA*, et al.* High-fat diet determines the composition of the murine gut microbiome independently of obesity. *Gastroenterology* 2009; **137:** 1716-24 e1-2.
127 Cani PD, Neyrinck AM, Fava F*, et al.* Selective increases of bifidobacteria in gut microflora improve high-fat-diet-induced diabetes in mice through a mechanism associated with endotoxaemia. *Diabetologia* 2007; **50:** 2374-83.
128 Cani PD, Delzenne NM. The role of the gut microbiota in energy metabolism and metabolic disease. *Current pharmaceutical design* 2009; **15:** 1546-58.
129 Martinez I, Wallace G, Zhang C*, et al.* Diet-induced metabolic improvements in a hamster model of hypercholesterolemia are strongly linked to alterations of the gut microbiota. *Applied and environmental microbiology* 2009; **75:** 4175-84.
130 Larsen N, Vogensen FK, van den Berg FW*, et al.* Gut microbiota in human adults with type 2 diabetes differs from non-diabetic adults. *PloS one* 2010; **5:** e9085.
131 Frank DN, St Amand AL, Feldman RA, Boedeker EC, Harpaz N, Pace NR. Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases. *Proceedings of the National Academy of Sciences of the United States of America* 2007; **104:** 13780-5.
132 Islam KB, Fukiya S, Hagio M*, et al.* Bile acid is a host factor that regulates the composition of the cecal microbiota in rats. *Gastroenterology* 2011; **141:** 1773-81.
133 Crooke RM, Graham MJ, Lemonidis KM, Whipple CP, Koo S, Perera RJ. An apolipoprotein B antisense oligonucleotide lowers LDL cholesterol in

hyperlipidemic mice without causing hepatic steatosis. *Journal of lipid research* 2005; **46:** 872-84.
134 Kastelein JJ, Wedel MK, Baker BF*, et al.* Potent reduction of apolipoprotein B and low-density lipoprotein cholesterol by short-term administration of an antisense inhibitor of apolipoprotein B. *Circulation* 2006; **114:** 1729-35.
135 Akdim F, Tribble DL, Flaim JD*, et al.* Efficacy of apolipoprotein B synthesis inhibition in subjects with mild-to-moderate hyperlipidaemia. *European heart journal* 2011; **32:** 2650-9.
136 Graham MJ, Lemonidis KM, Whipple CP, Subramaniam A, Monia BP, Crooke ST, Crooke RM. Antisense inhibition of proprotein convertase subtilisin/kexin type 9 reduces serum LDL in hyperlipidemic mice. *Journal of lipid research* 2007; **48:** 763-7.
137 Frank-Kamenetsky M, Grefhorst A, Anderson NN*, et al.* Therapeutic RNAi targeting PCSK9 acutely lowers plasma cholesterol in rodents and LDL cholesterol in nonhuman primates. *Proceedings of the National Academy of Sciences of the United States of America* 2008; **105:** 11915-20.