From the
DEPARTMENT OF LABORATORY MEDICINE
DIVISION OF CLINICAL CHEMISTRY
Karolinska Institutet, Stockholm, Sweden

Regulation of the hepatic ACAT2 expression and roles of HNF1α and HNF4α in cholesterol metabolism

Camilla Pramfalk

Karolinska Institutet

Stockholm 2009

Published by Karolinska Institutet.

ISBN 978-91-7409-679-8

Printed by
REPROPRINT AB
Stockholm 2009
www.reproprint.se
Gårdsvägen 4, 169 70 Solna

Lord grant me the serenity to accept the things I cannot change,
the courage to change the things I can,
and the wisdom to know the difference

From The Serenity Prayer by Reinhold Niebuhr

ABSTRACT

Acyl-Coenzyme A:cholesterol acyltransferases (ACATs) 1 and 2 are integral membrane proteins located in rough endoplasmatic reticulum that catalyzes the formation of cholesteryl esters (CEs) from cholesterol and long-chain fatty acids. ACAT1 is present in most tissues, whereas ACAT2 is confined to enterocytes and hepatocytes. Disparities in tissue expressions, together with animal studies, suggests that ACAT2-derived CEs are incorporated into hepatic and intestinal apoB-containing lipoproteins and secreted into plasma, whereas ACAT1 is involved in esterification of cholesterol in other cells (e.g. macrophages) and thereby prevents apoptosis. Hepatic nuclear factors (HNFs) 1 and 4 are involved in diverse metabolic pathways (e.g. glucose, cholesterol, and fatty acid metabolism) and are highly expressed in liver, pancreas, and kidney. The overall aim of this thesis was to gain more insight into the molecular mechanisms that participate in the hepatic regulation of ACAT2 and the roles of HNF1α and HNF4α in cholesterol metabolism.

In **Paper I** we aimed to investigate a possible transcriptional regulation by cholesterol of the human ACAT2 gene. In addition, we aimed to appraise the use of two human hepatoma cell lines, HuH7 and HepG2, as model systems in studies of ACAT. We showed a dose-dependent increase of ACAT2 mRNA expression, an increased enzymatic activity of ACAT2, and increased esterified cholesterol mass upon cholesterol loading. These results suggested that ACAT2, but not ACAT1, is transcriptionally regulated by cholesterol in humans. Additionally, we showed that cell differentiation affects the mRNA expression of ACAT1 and ACAT2 in HuH7, but not in HepG2 cells. Since HuH7 cells required much lower concentrations of cholesterol to obtain similar results as HepG2 cells, and were more sensitive to cholesterol depletion, HuH7 cells may represent a better system for sterol-studies of ACAT.

In **Paper II** we aimed to characterize mechanisms that control the liver-specific expression of the human ACAT2 gene. We identified an important HNF1 binding site, located -871 to -866 bp upstream of the transcription start site, which serves as a positive regulator of the ACAT2 gene expression and showed that this site is functionally active both *in vitro* and *in vivo*. The transcription factors HNF1α and HNF1β, which binds to this site, play an important part in the regulation of the human ACAT2 promoter.

Paper III: Maturity onset diabetes of the young (MODY) is a group of syndromes characterized by autosomal dominant inheritance, early onset diabetes, and β-cell dysfunction. Mutations of the genes encoding HNF1α and HNF4α cause MODY3 and MODY1, respectively. ACAT2 is thought to be responsible for production of CEs in hepatic very low density lipoprotein (VLDL) assembly. We identified HNF1α as an important regulator of ACAT2. HNF4α is an upstream regulator of HNF1α. Thus we hypothesized that MODY3 and possibly MODY1 subjects may have lower VLDL esterified cholesterol. Unexpectedly, we found that MODY1 subjects had lower VLDL and low density lipoprotein (LDL) esterified cholesterol levels, whereas MODY3 subjects had similar lipoprotein composition as controls. Hence, we characterized the role of HNF4α in the transcriptional regulation of ACAT2 and identified HNF4α as an important regulator of the hepatocyte-specific expression of ACAT2. These studies suggested that the lower levels of esterified cholesterol in VLDL- and LDL-particles in MODY1 subjects may – at least in part – be due to lower ACAT2 activity in these patients.

Paper IV: Niemann-Pick C1 like 1 (NPC1L1) is highly expressed in human liver and intestine. NPC1L1 is a key regulator of intestinal cholesterol absorption but its hepatic function is not well defined. Thus, we aimed to gain more insight into the hepatic expression of the human NPC1L1 gene. Gene expression analyses were performed in liver samples from Chinese patients with or without cholesterol gallstone disease. Strong positive correlations between NPC1L1 and sterol regulatory element binding protein 2 (SREBP2) and between NPC1L1 and HNF4α were observed. HNF4α is an upstream regulator of HNF1α. Thus, we further investigated possible roles of SREBP2, HNF4α, and HNF1α in the hepatic regulation of NPC1L1. We identified an important HNF1 binding site located -158 to -144 bp upstream of the transcription start site of the human NPC1L1 promoter. Also, we showed that SREBP2 and HNF1α are important transcription factors for the hepatic NPC1L1 promoter activity that can bind to and regulate its expression in humans. Moreover, it is possible that HNF4α may function by transactivating NPC1L1 via binding to other transcription factors, including HNF1α.

Collectively, these studies imply that ACAT2 is under metabolic control and that HNF1α and HNF4α participates in several important processes in cholesterol metabolism. HNF1α may participate in hepatic cholesterol esterification, uptake of free cholesterol (FC) in hepatocytes, and in bile acid synthesis. HNF4α may participate in esterification of cholesterol in high density lipoprotein (HDL), affect plasma levels of esterified cholesterol and triglycerides in VLDL- and LDL-particles, and indirectly participate in the regulation of uptake of FC in hepatocytes.

Keywords: ACAT2, HNF, MODY, NPC1L1, SREBP, cholesterol metabolism

LIST OF PUBLICATIONS

I. **Cholesterol regulates ACAT2 gene expression and enzyme activity in human hepatoma cells**
Pramfalk C, Angelin B, Eriksson M, and Parini P
Biochem Biophys Res Commun. 364(2):402-409 (2007)

II. **Control of ACAT2 liver expression by HNF1**
Pramfalk C, Davis MA, Eriksson M, Rudel LL, and Parini P
J Lipid Res. 46(9):1868-1876 (2005)

III. **Control of ACAT2 liver expression by HNF4α: lesson from MODY1 patients**
Pramfalk C, Karlsson E, Groop L, Rudel LL, Angelin B, Eriksson M, and Parini P
Arterioscler Thromb Vasc Biol. 29(8):1235-1241 (2009)

IV. **HNF1α and SREBP2 are important regulators of NPC1L1 in human liver**
Pramfalk C, Jiang Z-Y, Cai Q, Hu H, Zhang S-D, Han T-Q, Eriksson M, and Parini P
J Lipid Res. (2009)

CONTENTS

LIST OF ABBREVIATIONS

ABCA1	ATP-binding cassette transporter A1
ACAT	Acyl-Coenzyme A:cholesterol acyltransferase
Apo	Apolipoprotein
ASO	Antisense oligonucleotide
BA	Bile acid
C4	7α-hydroxy-4-cholesten-3-one
CDX2	Caudal type homeobox 2
CE	Cholesteryl ester
CETP	Cholesteryl ester transfer protein
ChIP	Chromatin immunoprecipitation assay
CYP7A1	Cholesterol 7α-hydroxylase
DMEM	Dulbecco´s Modified Eagle Medium
EMSA	Electrophoretic mobility shift assay
ER	Endoplasmatic reticulum
FC	Free cholesterol (unesterified cholesterol)
FFA	Free fatty acid
FH	Familial hypercholesterolemia
FXR	Farnesoid X receptor
GS	Gallstone patients
GSF	Gallstone-free patients
HDL	High density lipoprotein
HMG-CoA	3-hydroxy-3-methylglutaryl-Coenzyme A
HNF	Hepatocyte nuclear factor
IDL	Intermediate density lipoprotein
LCAT	Lecithin:cholesterol acyltransferase
LDL	Low density lipoprotein
LDLr	Low density lipoprotein receptor
LPDS	Lipoprotein deficient serum
LPL	Lipoprotein lipase
LRP	Low density lipoprotein receptor-related protein
MI	Myocardial infarction
MODY	Maturity onset diabetes of the young
MTP	Microsomal triglyceride transfer protein
NPC1L1	Niemann-Pick C1 like 1
PL	Phospholipid
PPPA	Pyripyropene A
RCT	Reverse cholesterol transport
SCAP	SREBP cleavage-activating protein
SEC	Size-exclusion chromatography
SHP	Small heterodimer partner
SOAT	Sterol O-acyltransferase
SRBI	Scavenger receptor class B type I
SRE	Sterol regulatory element
SREBP	Sterol regulatory element binding protein
T1D	Type 1 diabetes mellitus
T2D	Type 2 diabetes mellitus
TCF	Transcription factor
TESS	Transcription Element Search Software

TG	Triglycerides
TICE	Transintestinal cholesterol efflux
TMD	Transmembrane domain
TNFα	Tumor necrosis factor α
VLDL	Very low density lipoprotein

1 INTRODUCTION

1.1 CHOLESTEROL

Cholesterol ($C_{27}H_{45}OH$) is a hydrophobic compound that consists of four fused hydrocarbon rings. Secretion of cholesterol is essential for maintaining cholesterol homeostasis. The ring structure of cholesterol cannot be metabolized to CO_2 and H_2O, and cholesterol is therefore secreted in the bile as such or after its conversion to bile acids (BAs). Mammalian cells acquire exogenous cholesterol from the diet; in addition, almost all cells in the body can synthesize cholesterol *de novo*. Collectively, the extrahepatic tissues synthesize as much cholesterol as the liver does [1].

The liver plays a crucial role in regulating whole-body cholesterol homeostasis. Low intestinal cholesterol absorption upregulates hepatic cholesterol synthesis and turnover, whereas high intestinal cholesterol flux to the liver suppresses cholesterol synthesis; consequently, cholesterol homeostasis may be regulated at levels of cholesterol synthesis, cholesterol absorption, or biliary cholesterol excretion.

Cholesterol is known to have a number of important biological functions: it is an essential component of cellular membranes; it serves as a precursor of steroid hormones and BAs; and it plays a role in transcriptional gene regulation.

The majority of cholesterol exists unesterified as an essential component of cellular membranes. The quantity of cholesterol in membranes in part determines the degree of fluidity (more cholesterol allows phospholipids to be packed more closely, resulting in increased membrane rigidity), which exerts an influence on the properties of many kinds of proteins, cytoskeletal anchors, and receptors. Overaccumulation of free (unesterified) cholesterol can be toxic to cells. To prevent accumulation, cholesterol is converted to cholesteryl esters (CEs), in which the sterol moiety is covalently attached to a long-chain fatty acid (to the 3-position hydroxyl group), and which mainly are stored as cytosolic lipid droplets. The synthesis of CEs is catalyzed by three enzymes: lecithin:cholesterol acyltransferase (LCAT) which acts solely in the plasma to esterify cholesterol associated to lipoproteins, and acyl-Coenzyme A:cholesterol acyltransferase 1 (ACAT1) and 2 (ACAT2) [2] which both act intracellularly but with different tissue distributions and functions.

CEs can exist in several physical states, including crystalline, liquid-crystalline (smectic and cholesteric), and liquid. CEs in a liquid state hydrolyzes faster than when in a liquid-crystalline state; saturated esters undergo liquid-crystalline melting between 70-80°C, monounsaturated esters melt between 40-50°C, and cholesteryl linoleate (18:2) melts at 34°C [3]. Increased rate of clearance from the cell correlates with an increased cellular triglyceride (TG) content and a more fluid CE physical state [4].

1.2 LIPOPROTEINS

In the blood stream, the transport of both TG and cholesterol occurs in lipoproteins. Lipoproteins are particles with a highly hydrophobic core (TG and CE) and a relatively hydrophilic outer surface monolayer [phospholipid (PL) and free cholesterol (FC)]. Each lipoprotein particle is associated with one or more specific proteins, the apolipoproteins (apo). These proteins have hydrophobic domains, which dip into the core and anchor the protein to the particle, and hydrophilic domains that are exposed at the surface.

Lipoproteins consist of a heterogeneous group of particles with different lipid and protein compositions, and different sizes. Lipoproteins can be isolated by diverse techniques including electrophoresis (i.e. agarose and polyacrylamide gels), gel filtration [size-exclusion chromatography (SEC)], ultra centrifugation (i.e. density gradient), precipitation, and nuclear magnetic resonance spectroscopy.

Based on density, lipoproteins are traditionally separated into five main groups: chylomicrons, very low density lipoproteins (VLDL), intermediate density lipoproteins (IDL), low density lipoproteins (LDL), and high density lipoproteins (HDL).

TABLE I. Characteristics of the human lipoproteins

	Density (g/mL)	*Diameter (nm)*	*Major apolipoproteins*	*Composition (% by weight)*			
				TG	*Cholesterol*	*PL*	*Protein*
Chylomicrons	<0.930	80-1200	B48, AI, AII, C, E	85-95	2-5	3-8	1-2
VLDL	0.930-1.006	30-80	B100, AI, C, E	50	22	19	8
IDL	1.006-1.019	25-35	B100, C, E	20	38	23	19
LDL	1.019-1.063	15-25	B100	11	47	22	21
HDL	1.063-1.210	5-15	AI, AII, C, E	6	15-22	23-30	55

There are three main pathways responsible for the synthesis, secretion, and transport of lipoproteins within the body: the exogenous (dietary) pathway, the endogenous (hepatic) pathway, and reverse cholesterol transport (RCT).

1.2.1 The exogenous pathway

Depending on diet, humans ingest ~100 g lipids each day which primarily consists of TG, cholesterol, PL, and plant sterols. Daily, 1200-1700 mg cholesterol enters the lumen of the small intestine, but only ~300-500 mg of the cholesterol is of dietary origin; the rest comes mainly from the bile, and a minor part from the turnover of intestinal mucosal epithelium [5]. In addition to biliary cholesterol secretion, animal studies have suggested a direct transintestinal pathway for cholesterol excretion [called transintestinal cholesterol efflux (TICE)] [2, 6].

The digestion of lipids starts in the oral cavity through exposure to salivary lipases, and continues in the stomach by salivary and gastric lipases [7]. In the duodenum emulsification is enhanced by BA and lecithin, and small lipid droplets are formed. Pancreatic lipase hydrolyses these particles, converting TG to free fatty acids (FFAs) and monoglycerides. These FFAs and monoglycerides – together with BA, cholesterol, lecithin, and fat-soluble vitamins – form micelles that are absorbed by enterocytes. Micelle formation is essential for absorption of the hydrophobic lipids, and BAs are vital in this process. After absorbtion, FC and fatty acids are re-esterified in the enterocytes by ACAT2, and packaged with FC, TG, PL, and apoB48 into chylomicrons; the assembly occurs mainly in the endoplasmatic reticulum (ER). The chylomicrons enter the circulation, and acquire apoC and apoE. ApoCII is an activator of lipoprotein lipase (LPL), which attacks the TG-rich core in the capillary walls of adipose tissue and muscle. This results in hydrolysis of TG, liberating FFA and glycerol. These FFAs are taken up and are either re-esterified or oxidized for energy production. Also, some FFA reaches the liver and after uptake stimulates the hepatic production of VLDL (spillover effect). The excess surface lipids and apolipoproteins are transferred to HDL. ApoB48 and apoE remains on a smaller particle, chylomicron remnant, containing CE and small amounts of TG in its core, which is rapidly cleared by the liver via the low density lipoprotein receptor (LDLr) and the LDLr-related protein (LRP). A delayed removal of chylomicron remnants may promote atherosclerosis [8]. The half-life of chylomicrons is ~15 min.

1.2.2 The endogenous pathway

ApoB is required for the assembly and secretion of chylomicrons and VLDL[9]. Each lipoprotein particle contain only one apoB molecule. Through RNA editing event (in humans this process only occurs in the intestine, but in rodents it also occur in the liver) that converts Gln^{2153} to a stop codon, a truncated form (apoB48) containing 48% of the protein is produced. Thus in humans, chylomicrons carry apoB48 whereas VLDL and LDL carry apoB100. ApoB100, but not apoB48 (lacks the LDLr binding domain), is a ligand for the LDLr, whereas chylomicrons depend upon apoE for binding to the LDLr or to the LRP. A large portion of newly synthesized apoB protein is subjected to rapid co-translational degradation. The rate-limiting step for apoB secretion is the exit from the rough ER [10]. Microsomal triglyceride transfer protein (MTP) is required for apoB lipoprotein assembly and secretion [11].

The assembly of VLDL involves stepwise lipidation of apoB100 by MTP in the ER to form pre-VLDL, which, by additional lipidation, is converted to a TG-poor VLDL particle that exits the ER [12]. The TG-poor VLDL particles can be secreted from the cells as $VLDL_2$ or they can be further lipidated to form TG-rich $VLDL_1$ particles. The latter process is highly dependent on the accumulation of TG in cytosolic lipid droplets and it is the availability of neutral lipids that principally controls the rate of VLDL production. Interestingly, insulin infusion suppresses $VLDL_1$, with little effect on $VLDL_2$, production [13]. Also, $VLDL_2$ is increased in patients with familial hypercholesterolemia (FH) [14].

VLDL particles (containing TG, CE, FC, PL, apoB100, apoC, and apoE) are secreted by the liver. In the circulation, VLDL is subjected to lipolysis by LPL. After a meal, there is a competition between VLDL and chylomicrons for LPL [15]. LPL has higher affinity for chylomicrons, resulting in a longer half-life of VLDL (1-2 h). In plasma, the TG in VLDL is hydrolyzed into FFA and monoglycerides by LPL and its cofactor apoCII. This results in the production of smaller VLDL remnants (IDL). Some of the IDL particles are removed through the interaction of apoE with the LDLr, or the TG in IDL can be further hydrolyzed by hepatic lipase to produce LDL (half-life ~2-3 d). LDL is normally removed by the interaction of apoB100 with the LDLr. If LDL is oxidized, it can enter macrophages through scavenger receptors (e.g. CD36 and SR-A) [8, 16].

1.2.3 Reverse cholesterol transport (RCT)

RCT is a metabolic pathway in which peripheral cholesterol is returned to the liver for excretion in the bile and ultimately the feces [17]. HDL has a central role in this process. ApoAI is the main HDL protein (~70%) and a LCAT cofactor [18]. ApoAI is synthesized in the liver (~70%) and intestine (~30%) and can be secreted into plasma in its free form [18]. ApoAII is synthesized in the liver but its physiological role is yet not clear [19]. RCT starts with uptake of FC from peripheral cells by interaction of apoAI with ATP-binding cassette transporter A1 (ABCA1). The FC is then esterified by LCAT. As larger amounts of CE become incorporated into the particle, HDL becomes larger, forming HDL_3 and HDL_2. The CE in these spherical particles may be taken up by the liver through scavenger receptor class B type I (SRBI) or transferred by cholesteryl ester transfer protein (CETP) from HDL to apoB-containing lipoproteins.

HDL, besides having a central role in RCT, is also thought to protect against atherosclerosis by maintaining normal macrophage lipid homeostasis, acting like an antioxidant, inhibiting platelet aggregation, and having anti-inflammatory properties and modulating immune function [17].

1.2.4 The LDLr

The LDLr plays a critical role in the regulation of plasma LDL levels by mediating ~ two thirds of LDL clearance. Loss of LDLr function leads to decreased LDL catabolism and elevated LDL levels [20]. LDLr levels are affected by diet, hormones, and by mutations in the LDLr locus that leads to FH. The LDLr, present on the surface on all cells, binds lipoproteins containing apoB or apoE which is then internalized by endocytosis. The CE content is hydrolyzed in lysosomes, liberating FC. The LDLr is then recycled back to the cell surface.

1.3 REGULATION OF CHOLESTEROL METABOLISM

Transcription factors are *trans*-acting DNA-binding proteins that bind to a specific *cis*-acting DNA sequence; thereby interacts with the transcriptional machinery and enable selective gene expression and regulation. Also, binding of different proteins to cognate DNA-binding sites enables combinatorial control of gene expression. Moreover, protein-protein interactions between transcription factors and coactivators or corepressors form a multiprotein complex that enables regulated gene expression.

Intracellular cholesterol homeostasis is regulated by end-product repression of transcription of genes that control the *de novo* synthesis and the receptor-mediated uptake of cholesterol from plasma lipoproteins (e.g. LDLr). The rate-limiting reaction of the cholesterol biosynthesis pathway is production of mevalonate by 3-hydroxy-3-methylglutaryl-Coenzyme A (HMG-CoA) reductase [21]. The membrane bound transcription factors sterol regulatory element binding proteins (SREBPs) plays a central role in this regulation. SREBP2 is known to up-regulate genes involved in cholesterol biosynthesis and uptake (e.g. HMG-CoA reductase and LDLr).

1.3.1 The SREBP pathway

The precursor forms of SREBPs are integral membrane proteins residing in the ER. SREBP1a and 1c are produced from a single gene through the use of alternative promoters and are more selective for lipogenic genes; SREBP2 is produced from a separate gene and is more selective for cholesterogenic genes [22]. SREBPs are produced as membrane-bound precursors that require cleavage to release their amino-terminal domain into the nucleus to activate target genes [23]. SREBP precursor and SREBP cleavage-activating protein (SCAP) form a complex on the rough ER membrane. When sterol is depleted, the SREBP-SCAP complex targets to Golgi where site-1 and site-2 proteases cleaves SREBP. SCAP goes back to rough ER. Nuclear SREBP enters the nucleus and activates transcription of genes by binding to SREs or E-boxes. When sterol is abundant, the SREBP-SCAP complex is retained at the rough ER through interaction of SCAP with a retention-protein (INSIG1 or INSIG2) and no cleavage occurs. SCAP is regarded as a cholesterol sensor and is prerequisite for cleavage of SREBP.

1.3.2 Cholesterol synthesis inhibition

Statins are a group of synthetic inhibitors of HMG-CoA reductase, the rate-limiting enzyme in cholesterol synthesis, which lowers plasma total and LDL cholesterol. At higher degrees of HMG-CoA reductase inhibition, the concentrations of VLDL cholesterol and TG are reduced [24], whereas HDL cholesterol may vary depending on the statin [25, 26]. Several trials [27] have shown that statins decrease the mortality from coronary artery disease and decrease the incidence of myocardial infarction (MI), stroke, and peripheral vascular disease. Also, the risk reduction was directly proportional to the degree to which LDL cholesterol was lowered. Thus, cholesterol-lowering is now recommended for a wide range of subjects at cardiovascular risk but may also be used in primary prevention [28]. Statins are considered as very effective and safe cholesterol-lowering drugs, although adverse effects, such as elevation of liver enzymes and rhabdomyolysis are recognized. Atorvastatin, fluvastatin, simvastatin, and rosuvastatin are all examples of statins available on the market.

1.4 ACYL-COENZYME A: CHOLESTEROL ACYLTRANSFERASE (ACAT)

ACATs are integral membrane proteins located in the rough ER that catalyses the formation of CEs from cholesterol and long-chain fatty acids [29]. There are two known genes encoding the two ACAT enzymes, ACAT1 and ACAT2, known by international convention as sterol O-acyltransferase 1 and 2 (SOAT1 and SOAT2), respectively [30].

1.4.1 Tissue distribution

It is generally agreed that ACAT2 is present in the human intestine. However, there were conflicting data regarding whether ACAT2 is expressed in adult human hepatocytes [31, 32]. It has been shown in mice [30, 33], nonhuman primates [34, 35], and hamsters [36] that ACAT1 is expressed in most tissues whereas the expression of ACAT2 is confined to enterocytes and hepatocytes. Previously, Chang *et al.* [32] showed that ACAT2 is present in fetal, but not adult, human hepatocytes; in the intestine, the ACAT2 expression is more concentrated at the apical half of the villi, whereas ACAT1 is uniformly distributed along the vilus-crypt axis. However, these studies were performed in organs removed from bodies 2-21 h postmortem and significant degradations occurred in several samples. In a previous study, using real-time RT PCR, homogenate from human liver and duodenum showed that ACAT1 mRNA levels are nine times more abundant than ACAT2 in the liver, whereas ACAT2 transcripts are three times more abundant than ACAT1 in the duodenum [37]. Another study showed that ACAT1 expression is higher in Kupffer cells than in hepatocytes [38]. Thus, the high levels of ACAT1 in liver homogenate may origin from the strong ACAT1 expression in Kupffer cells.

Sakashita *et al.* [39] showed that fully differentiated macrophages express both ACAT1 and ACAT2 under various pathologic conditions (e.g. in atherosclerotic aorta and during cholestasis in the gall bladder). In peripheral blood mononuclear cells, low mRNA levels of ACAT1 and ACAT2 were detected [37]; however, whether the two ACAT enzymes were functionally active or not in these cells was not investigated.

Immunostaining, using a specific ACAT2 antibody, in liver tissues from 14 subjects, freshly isolated and snap-frozen in liquid nitrogen, showed strong signals from all hepatocytes [31]. Also, in pooled human livers, ACAT2 activity accounted for >50% of total ACAT activity in all, except one, of the pools [31]. Interestingly, the enzymatic activity varied widely among individual samples and between species; for example, the ACAT2 mRNA expression and activity is ~10-times higher in African green monkeys than in humans [31]. Thus, it seems more than reasonable to conclude that ACAT2 is confined to enterocytes and hepatocytes also in humans.

1.4.2 Structure of ACAT

Computer models predicted that ACAT1 and ACAT2 spans the ER membrane 8 and 7 times, respectively, whereas, experimental studies suggested that ACAT1 contain five [40] or seven [41] transmembrane domains (TMDs) while ACAT2 contain two [42] or five TMDs [40]. However, the techniques used for these studies may have influenced the outcome of the predicted model. Due to difficulties with purification, X-ray crystallography or nuclear magnetic resonance spectroscopy has yet not been performed. Also, the active site of ACAT1 was proposed to be located on the cytoplasmic side of the ER whereas the active site of ACAT2 was proposed to be located on the luminal side of ER [40]; contrary, another study suggested opposite locations of the active sites [42].

ACAT1 and ACAT2 share strong homology near the carboxyl terminus but not in the amino terminus. In mice, ACAT2 is 44% identical to ACAT1 [43]; in African green monkeys, ACAT2 is highly homologous to ACAT1 with a 57% identity over the carboxyl terminal 425 amino acids [44]. However, the first 101 amino acids of ACAT2 have no sequence homology with the first 138 amino acids of ACAT1 [44].

1.4.3 Cellular function

Overexpression of either ACAT1 or ACAT2 in rat hepatoma cells, McA-RH7777, resulted in increased CE synthesis and secretion and total cellular CE mass; these effects were associated with decreased intracellular degradation and increased secretion of apoB as VLDL. However, overexpression of ACAT2 had a greater impact upon assembly and secretion of VLDL from the cells than overexpression of ACAT1 [45]. Another study reported that levels of ACAT2 expression in concert with FC availability determine the CE content of apoB-containing lipoproteins [46].

ACATs preferentially uses 18:1 (oleic acid) and 16:0 (palmitic acid) as fatty acid substrates [2, 47]. Both ACAT1 and ACAT2 are located in the ER and catalyze intracellular cholesterol esterification. However, their different tissue distributions suggest divergent roles in lipoprotein metabolism. Hepatocytes and enterocytes are specialized in lipoprotein assembly and secretion, and the lipoprotein assembly process occurs within the lumen of ER. ACAT2, exclusively expressed in these cells, is believed to be responsible for CE production for storage and secretion in the lipid core of VLDL and chylomicrons, whereas ACAT1 may function to produce CEs as cytoplasmic lipid droplets in macrophages and other cell types. Interestingly, humans with hepatic steatosis have increased levels of palmitoleic and oleic acid in the liver [48] suggesting a role of ACAT1 and/or ACAT2 in this process.

Linoleic acid percentages (in CE, PL, and TG) were lower in patients with an acute MI as the first manifestation of coronary heart disease than in controls [49] and atherosclerotic patients with low concentrations of linoleic acid in their plasma-CEs run an increased risk of sudden death from MI or cerebrovascular incidents [50]. Also, patients suffering from coronary artery disease had higher proportion of oleic acid in the aortic tissue compared to healthy controls [51]. In the Uppsala Longitudinal Study of Adult Men (ULSAM), high serum proportions of palmitoleic and oleic acid predicted both cardiovascular disease and total mortality [52]. This study is in agreement with the Atherosclerosis Risk in Communities (ARIC) study in which average carotid intima-media thickness was positively associated with saturated and monounsaturated fatty acid composition, and inversely with polyunsaturated fatty acid composition in CEs [53]. Collectively, these studies provide strong evidence for the importance of ACAT2-derived CEs in coronary heart disease.

1.4.4 Gene and promoter studies

The human ACAT1 gene is about 200 kb in length, contains 18 exons (exons X_a, X_b, and 1-16), and is located in two different chromosomes (1 and 7) with each chromosome containing a distinct promoter (P1 and P7) [54]. Four human ACAT1 mRNAs (7.0, 4.3, 3.6, and 2.8 kb) that shares the same coding sequence have been identified [55]. The 4.3 kb mRNA contains an additional exon X_a and X_b immediately upstream from the exon 1 sequence [55]. Exons 1-16 are located in chromosome 1 whereas the X_a sequence is located in chromosome 7 [56]. No TATA box or CCAAT box were found in the P1 sequence [55]. In the P7 sequence no TATA box, but two copies of CCAAT boxes were found [55]. The normal ACAT1 protein (50 kDa) is translated from the ACAT1 mRNA transcribed only from chromosome 1 [57]. Additionally, a 56 kDa ACAT1 protein is produced from ACAT1 sequences located on both chromosomes 1 and 7 [56]. In mice, the ACAT1 gene is also located in chromosome 1 but it does not contain the optional exon X_a present in the human ACAT1 gene [57]. ACAT1 encodes a protein of 550 amino acid in humans [58] and a protein of 540 amino acids in mice [59].

The mouse ACAT2 gene maps to chromosome 15 whereas the human ACAT2 gene has been mapped to chromosome 12 [43]. The human ACAT2 gene spans slightly over 18 kb and contains 15 exons [54, 60]. No TATA box or CCAAT box adjacent to the transcription start sites were found; accordingly, multiple transcription start sites located at the 5´-flanking region were identified [54] (a common feature of genes with TATA-less promoter). Similar to the human ACAT1 promoters [55] no SREs could be found [54]. The human ACAT2 and insulin-like growth factor binding protein 6 (IGFBP-6) genes are located in a head-to-tail manner and the distance between them is less than 1.3 kb [54]. The human ACAT2 mRNA encodes a single 46 kDa protein on SDS-PAGE [57]. Human, monkey, and mouse ACAT2 cDNAs are predicted to encode 522, 526, and 525 amino acid proteins, respectively [44].

In cultured differentiating human monocytes, tumor necrosis factor α (TNFα) enhanced the expression of the ACAT1 gene, increased the CE accumulation, and promoted lipid-laden cell formation [61].

Caudal type homeobox 2 (CDX2) is expressed in a differentiation-dependent manner in Caco-2 cells and can efficiently bind to the mouse and the human ACAT2 promoter regions [62]. In the human ACAT2 promoter four binding sites for CDX2 and one for HNF1α were identified. In Caco-2 cells, it was shown that CDX2 and HNF1α synergistically stimulates the intestinal expression of ACAT2 [63].

1.4.5 Animal studies

ApoE is a structural component of all TG-rich lipoproteins. ApoE-/- mice are atherosclerosis-susceptible due to impaired clearance of apoB48-containing lipoproteins. These mice have higher plasma cholesterol levels (~8-fold on a chow diet and ~14-fold on a high-fat diet) compared to controls; at 10 weeks of age they have developed atherosclerotic lesions in the aorta and pulmonary arteries [64]. LDLr-/- mice have increased plasma cholesterol levels (~2-fold on a chow diet and ~12-fold on a high-fat diet) [65]. In contrast to apoE-/- mice, the LDLr-/- mice slowly develop atherosclerotic lesions on a chow diet while a high-fat diet rapidly results in atherosclerotic lesions throughout the aorta [65].

ACAT1-/- studies in mice

ACAT1 deficiency in mice (ACAT1-/-ApoE-/- and ACAT1-/-LDLr-/-) led to extensive deposition of unesterified cholesterol in skin and brain and did not prevent the development of atherosclerotic lesions, despite lower serum cholesterol levels [66]. In a study by Fazio *et al.* [67], LDLr-/- mice reconstituted with ACAT1 deficient macrophages developed larger atherosclerotic lesions; the lesions had reduced number of macrophages and more FC compared to controls. Yagyu *et al.* [68] showed that ACAT1 deficiency (on ApoE-/- or LDLr-/- backgrounds) resulted in extensive cutaneous xanthomatosis and increased FC content in the skin; however, aortic fatty streak lesion size and CE content were moderately reduced. Bone marrow transplantation of apoE-/- mice showed that the presence or absence of macrophage ACAT1 did not affect the extent of atherosclerosis in mice receiving apoE+/+ marrow, but increased lesion size in mice receiving apoE-/- marrow [69].

ACAT2-/- in mice

ACAT2 deficiency in mice resulted in complete resistance to diet-induced hypercholesterolemia and cholesterol gallstone formation [30]. Interestingly, ACAT2 deficiency in mice fed a chow diet had no effect on plasma total cholesterol, and the mice displayed similar lipoprotein morphology and diameters as wild-type, yet the mice did not developed gallstones [30]. ACAT2 deficiency in apoE-/- mice resulted in ~70% reduction in plasma CE and, in contrast to control mice, showed nearly absence of atherosclerotic lesions after 30 weeks on a chow diet [70].

Mice (on a apoB100-only, LDLr-/- background) treated with antisense oligonucleotide (ASO) against hepatic ACAT2 had ~50% reduced VLDL and LDL cholesterol without any change in HDL cholesterol, and ~70% reduction in hepatic CE mass without any reciprocal accumulation of unesterified cholesterol [2]. Another study, in which apoB100-only, LDLr-/-, ACAT2-/- mice fed diets enriched in polyunsaturated, saturated, or monounsaturated fats, showed that these mice were protected from atherosclerosis regardless of the type of dietary fat that was fed [71]. In control mice, diets enriched with saturated and monounsaturated fatty acids resulted in larger LDL particles compared to polyunsaturated; these effects were lost when ACAT2 were absent [71].

Deletion of both LCAT and ACAT2 in mice leads to complete absence of plasma CE and absence of atherosclerotic lesions [72]. Deletion of LCAT solely lead to higher plasma cholesterol, modified CE composition, and to increased atherosclerotic lesions because circulating CEs were solely ACAT2-derived [72]. The study also suggests that LCAT and ACAT2, but not ACAT1, have the ability to synthesize plasma CEs [72]. In contrast, LCAT deficiency in different mouse models resulted in reduced aortic atherosclerosis [73]. LDLr-/- mice deficient in ACAT2, LCAT, or both ACAT2 and LCAT showed that: ACAT2 -/- mice had decreased CE and increased TG in plasma levels of VLDL; LCAT-/- mice had decreased CE and increased PL in plasma levels of LDL [74]; and deficiency of both ACAT2 and LCAT had similar effect on VLDL as ACAT2-deficiency alone but depleted LDL of core lipids and enriched the particle in surface lipids [74]. This suggests that ACAT2 is essential for incorporation of CE into the core of VLDL whereas LCAT adds CE to LDL.

ACAT2 deficient mice (ACAT2-/- mice or mice treated with ASO against hepatic ACAT2) had increased fecal neutral sterol loss without changes in biliary sterol secretion [2]. Instead the unesterified cholesterol seemed to exit the liver directly into the plasma, and shunted to the proximal small intestine for direct excretion [2]. This study suggested a non-biliary pathway for sterol excretion (TICE), which also have been suggested by others [6, 75].

Nonhuman primates

African green monkeys fed monounsaturated and polyunsaturated fats for 5 years had lower plasma LDL cholesterol than monkeys fed saturated fat [76]. However, the LDL particle sizes were larger, enriched with cholesteryl oleate, and coronary artery atherosclerosis (measured by intimal area) was more extensive in monkeys fed monounsaturated and saturated than polyunsaturated fat [76]. When livers from these monkeys were isolated and perfused, the CE secretion by the liver (mostly cholesteryl oleate) was positively correlated to the extent of coronary artery atherosclerosis [47].

1.4.6 Cholesterol regulation of ACAT

As mentioned, SREBPs regulates several genes involved in cholesterol and fatty acid metabolism by binding to SRE or E-box motifs within promoters. Since no SRE or E-box motifs have been shown to be present within the ACAT1 [55, 77] or ACAT2 [54] promoters, these genes are generally thought not to be transcriptionally regulated by cholesterol. ACAT1 have been shown to display sigmoidal kinetics with cholesterol as its substrate, implying that ACAT1 is an allosteric enzyme regulated by cholesterol [78]. In one study [79], performed before the identification of the two ACAT enzymes, ACAT was shown not to be transcriptionally regulated by cholesterol in HepG2 cells; however, the primers used in the study targeted ACAT1. Experiments performed in various cells showed that neither FFA or cholesterol affected ACAT2 transcription, whereas certain FFA modulated ACAT1 mRNA levels in a cell-specific manner [80]. Cynomolgus monkeys on a high-cholesterol diet expressed increased hepatic ACAT2 mRNA levels [81]. Female rats fed a high-fat and sucrose diet for 20 months had higher LDL and VLDL cholesterol and TG levels; ACAT2 protein and ACAT activity were higher and the ACAT2 mRNA expression showed an insignificant increase compared to controls [82]. Patients treated with 80 mg/d atorvastatin for four weeks, prior to elective cholecystectomy because of uncomplicated gallstone disease, had ~50% reduced hepatic ACAT2 activity, protein expression, and mRNA expression as well as decreased plasma VLDL and LDL cholesterol [83].

Humans ingest 300-500 mg dietary cholesterol and 250-500 mg plant and shellfish-derived sterols each day [84]. Sitosterol, derived from plant and vegetables, is the most abundant dietary non-cholesterol sterol. In the small intestine, 40-50% of cholesterol but only 5% of the ingested sitosterol is absorbed [85]. Whereas ACAT1 only have a slightly greater efficiency for cholesterol than sitosterol esterification, ACAT2 showed a strong preference for esterification of cholesterol compared to sitosterol [86].

1.4.7 Effects of age and gender on ACAT

Livers of 4-24 months old mice, without exogenous cholesterol feeding were analyzed; with aging, there were an increase in cholesterol content and in ACAT activity in liver microsomes [87]. Also, ACAT2 mRNA expression increased whereas the LDLr expression decreased with age [87]. The intestinal mRNA expressions of NPC1L1 and ACAT2 were higher in gallstone patients (GS) than in gallstone-free patients (GSF); also, the intestinal ACAT2 activity was 40-fold higher than the liver activity in GSF [88]. In normolipidemic, non-obese GS or GSF, plasma HDL cholesterol was higher whereas plasma TG was lower in females than in males [89]. Also, females had lower hepatic ACAT2 activity (~70%) regardless of the presence of gallstone disease, than males. Moreover, significant negative correlations between the hepatic ACAT2 activity and plasma levels of HDL cholesterol and apoAI were reported [89].

1.4.8 ACAT inhibition

Inhibition of ACAT has for nearly two decades been regarded as an attractive target to lower levels of plasma CEs. Hypothetically, an unspecific inhibition of ACAT would have the potential to both lower plasma lipids and to reduce foam cell formation. However, inhibition of ACAT1 in mice lead to increased atherosclerotic lesion size whereas inhibition of ACAT2 was atheroprotective. No inhibitor that selectively inhibits ACAT1 or ACAT2 is yet available on the market.

Pyripyropene A (PPPA) was found from the culture broth of Aspergillus fumigates FO-1289 [90]. PPPA is a highly selective *in vitro* inhibitor of ACAT2 (>2000-fold compared to ACAT1) [91]. It has been shown in mammalian cells that PPPA can effectively transverse the plasma membrane and inhibit ACAT2 [92].

Pactimibe (CS-505) is an unspecific ACAT inhibitor. In the CAPTIVATE study [93], patients heterozygous for FH were randomized into pactimibe or placebo treatment in addition to standard lipid-lowering therapy. The study was terminated prematurely after a mean follow-up of 15 months. Levels of LDL cholesterol and apoB were modestly increased, whereas the annual progression of the relative mean carotid intima-media thickness revealed an increase, and major cardiovascular events occurred more frequent in pactimibe-treated subjects [93]. From this study, the authors draw the wrong conclusion that ACAT2-inhibition have no beneficial effect on lipid levels [93] since pactimibe is an unspecific inhibitor.

In another study, patients with coronary disease were randomized into pactimibe or placebo treatment and intravascular ultrasonography was performed at baseline and after 18 months of treatment [94]. The percent atheroma volume was similar between the groups, whereas the normalized total atheroma volume showed regression in the placebo but not in the pactimibe group. Also, the combined incidence of adverse cardiovascular outcomes were similar between the two groups [94].

Avasimibe (CI-1011) is another unspecific ACAT inhibitor. In the Avasimibe and Progression of Lesions on UltraSound (A-PLUS) study, avasimibe treatment for 24 months had no effect on the progression of coronary atherosclerosis as assessed by intravascular ultrasound; however, dose-related reductions in plasma TG (up to 16%) as well as increases in LDL cholesterol (up to 11%) were observed [95].

Thus, the studies above are in agreement with what would be expected from unspecific ACAT inhibition, as previously predicted [96]. Inhibition of ACAT1 has indeed negative effects on the atherosclerotic plaque by its destabilization, resulting from the increased FC that leads to cytotoxicity, cell death, and subsequent increased inflammation. These negative effects on the atherosclerotic plaque would likely blunt the expected positive effect of ACAT2 inhibition on lipoprotein metabolism.

1.4.9 LCAT versus ACAT

LCAT, a glycoprotein that is secreted by the liver into the blood, forms CEs in HDL by transferring polyunsaturated fatty acids from phosphatidylcholine to cholesterol [97]. The most potent activator of LCAT is apoAI. Subjects with LCAT mutations have low plasma HDL levels but they usually do not develop coronary heart disease [97]. LCAT derived CEs are enriched in polyunsaturated (cholesteryl linoleate) fatty acids. In contrast, ACAT-derived CEs are enriched in saturated (cholesteryl palmitate) and monounsaturated (cholesteryl oleate) fatty acids [74, 96]. LCAT acts solely in the plasma to esterify cholesterol associated to lipoproteins, and ACAT1 and ACAT2 [2] acts intracellularly but with different tissue distributions and functions.

1.5 HEPATOCYTE NUCLEAR FACTOR 1 (HNF1)

HNF1 is a dimeric protein functionally composed of three domains: an amino-terminal dimerization domain, a DNA-binding domain, and a carboxyl-terminal domain that is essential for transactivation of target promoters. HNF1α and HNF1β share strong homologies in both the amino-terminal dimerization domain and the internal DNA-binding domain (~75 and 93% identity, respectively) but differ in their carboxyl-terminal region (~47% identity). These homologies enable the two proteins to form heterodimers and bind to the same DNA sequences [98, 99].

1.5.1 Tissue distribution

HNF1α and HNF1β are expressed in polarized epithelia of different organs, including the liver, digestive tract, pancreas, and kidney [99, 100]. The expression of HNF1β overlaps with that of HNF1α with the exception of lung, where only HNF1β is expressed; conversely, HNF1β is very weakly expressed in liver, where HNF1α constitutes more than 95% of the total HNF1-protein [99, 100].

A significant difference between HNF1α and HNF1β is the onset of their expressions during embryonic development [101, 102]. During liver and renal development, HNF1β is expressed from the first stages of organogenesis, whereas HNF1α is turned on later, when differentiation is more advanced [101, 102].

1.5.2 Gene and promoter studies

HNF1α is regarded as an important regulator of the transcriptional network in liver development and liver-specific gene expression. Promoters that are under the control of HNF1 generally have additional binding sites nearby for other transcription factors that participate in the overall activation of transcription [98]. Regulatory regions may also have multiple HNF1 sites, particularly those belonging to genes expressed in the liver [98]. HNF4α is an essential positive regulator of HNF1α [103] but not of HNF1β [104].

HNF1 negatively regulates its own expression and that of other HNF4-dependent genes that lack HNF1 binding sites in their promoter region [105]. This repression is exerted by a direct interaction of HNF1 with the AF2, the main activation domain of HNF4 [105]. Cholesterol 7α-hydroxylase (CYP7A1) is the rate-limiting enzyme in the conversion of cholesterol to BAs in the liver and elimination in the bile fluid [106]. HNF1α is a positive regulator of the human, but not the rat, CYP7A1 promoter [107]. HNF1α can also interact with GATA4, GATA5, and CDX2 transcription factors [108]. In human hepatocytes, HNF1α is bound to at least 222 target genes [109]. The human HNF1α gene, named transcription factor 1 (TCF1), is located on chromosome 12 whereas the human HNF1β gene, named TCF2, is located on chromosome 17; both genes consist of 10 exons [101].

1.5.3 Animal studies

HNF1β-/- mice embryos die at day 7 after conception, because of a defect in extraembryonic visceral endoderm differentiation [110, 111]. Liver-specific inactivation of the HNF1β gene resulted in a severe phenotype, including growth retardation, jaundice, and epithelial abnormalities [112].

TCF1-/- mice [113] develop hepatomegaly and central lobular hypertrophy at 5-7 weeks of age. Newborn animals, however, had no morphological or biochemical evidence of liver disease. TCF1-/- mice had type 2 diabetes (T2D), dwarfism, renal Fanconi syndrome, hepatic dysfunction and hypercholesterolemia, a defect in BA transport, increased BA and liver cholesterol synthesis, and impaired HDL metabolism [113]. Almost all of the cholesterol in the plasma of TCF1-/- mice was carried by the HDL fraction and an "abnormal" fraction with intermediate buoyancy between HDL and LDL peaks [113]. However, HNF1+/- heterozygous mice do not exhibit any insulin secretion defect or glucose intolerance [114].

1.6 HEPATOCYTE NUCLEAR FACTOR 4 (HNF4)

The HNF4 subfamily belongs to the nuclear receptor superfamily and is composed of HNF4α, HNF4β, HNF4γ, and many splice variants. In 1998 [115], it was reported that fatty acyl-CoA thioesters are ligands of HNF4α.

1.6.1 Tissue distribution

HNF4α is expressed in various organs, including liver, kidney, pancreas, and small intestine [116]. HNF4γ is expressed in the kidney, pancreas, small intestine, but not in the liver [117]. In frogs, HNF4β is expressed in the liver, kidney, stomach, intestine, lung, ovary, and testis [118].

1.6.2 Gene and promoter studies

The human HNF4α gene, named TCF14, was mapped to chromosome 20 [101]. HNF4α is produced from two promoters (P1 and P2). The expression of HNF4α mainly initiates at P1 in adult liver and kidney [119]. HNF4α transcription is driven almost exclusively by P2 in the endocrine pancreas [119]. HNF4α mRNA is dependent on HNF1α specifically in differentiated pancreatic cells, but not in the liver [120]. This is due to the fact that the pancreatic HNF4α gene is almost exclusively driven by P2, which is bound and controlled by HNF1α, whereas in the liver and most other tissues HNF4α is predominantly driven by P1, which does not require HNF1α [120-122].

In human hepatocytes, HNF4α was bound to 1575 genes [109]. HNF4 regulates MTP gene expression either directly or indirectly through elevated HNF1 levels [123]. In one study, HNF4α was shown to serve as coactivator for SREBP2 [124]. Another study [125] showed that HNF4α is a crucial modulator of NPC1L1,and that it acts synergistically with SREBP2 on the NPC1L1 promoter (however, HNF4α alone did not affect NPC1L1 promoter activity).

HNF4 DNA-binding activity is modulated post-translationally by phosphorylation. *In vivo* phosphorylation of HNF4 depends on the diet; it is decreased by a carbohydrate-rich diet and is increased by fasting. Long-chain fatty acids directly modulate the transcriptional activity of HNF4α by binding as their acyl-CoA thioesters to the ligand-binding domain of HNF4α [101].

1.6.3 Animal studies

Mouse embryos lacking HNF4α die before completing gastrulation due to visceral endoderm dysfunction [126]. Conditional liver-specific disruption of the HNF4α gene in mice resulted in hepatomegaly, hepatocyte hypertrophy, and abnormal glycogen and lipid deposition in liver [127]. Also, total cholesterol, LDL and HDL cholesterol, and TG levels were dramatically reduced [127]. Conversely, serum BA concentrations were markedly elevated, and CYP7A1 expression was reduced [127]. Surprisingly, HNF4α +/- mice do not exhibit any insulin secretion defect or glucose intolerance and are perfectly normal [102, 128].

1.7 MATURITY ONSET DIABETES OF THE YOUNG (MODY)

MODY is defined as a monogenic form of T2D, characterized by autosomal dominant inheritance, young age at onset (usually before the age of 25 years), and pancreatic β-cell dysfunction [129]. Heterozygous mutations leading to MODY have been identified in six genes: HNF4α (MODY1) [130], glucokinase (MODY2), HNF1α (MODY3) [131], insulin promoter factor IPF-1 (PDX1) (MODY4), HNF1β (MODY5), and neurogenic differentiation factor 1 (NeuroD1)/BETA2 (MODY6). Moreover, additional MODY genes are likely to exist (MODY-X). Most MODY patients have heterozygous mutations, but not all [132]. One of the outstanding questions in the genetics of MODY is why heterozygous mutations in genes like HNF1α or HNF4α cause a phenotype essentially restricted to β-cells, whereas homozygous mutations give rise to phenotypes affecting numerous other cell types [133].

1.7.1 Prevalence

The exact prevalence of MODY is unknown, but has been estimated to ~ 2-5% of all cases of T2D [134]. The relative prevalence of the different subtypes of MODY has been shown to vary greatly in different populations. In general, MODY2 represents 8-63% and MODY3 represents 21-64% of all MODY cases; the other four types of MODY are extremely rare forms [134]. Mutations in HNF4α may account for 2-5% of subjects with MODY, although only 26 families worldwide has been described [135]. Diagnostic genetic services for diabetes frequently only offer HNF1α and glucokinase testing; HNF4α is unfortunately rarely tested [136].

1.7.2 Diagnosis and treatment

Genetic testing is recommended in any young adult with apparent type 1 diabetes (T1D) and a diabetic parent, and who is antibody-negative at diagnosis (e.g. GAD), especially if there is preservation of C-peptide levels in both the child and the parent. Genetic testing is also recommended in patients with apparent young-onset T2D, lack of obesity, absence of acanthosis nigricans or polycystic ovarian syndrome, and elevated or normal HDL cholesterol and reduced or normal TG-levels. An important reason for genetic diagnosis is that, in many cases, treatment with low-dose oral sulfonylurea is highly effective [137]. Depending on glycemic levels, oral hypoglycemic agents or insulin can be used.

1.7.3 MODY1

Due to few cases worldwide, little is known about MODY1 patients. MODY1 patients, carrying a 2-bp deletion (K99fsdelAA) in exon 3 of the HNF4α gene, had significantly lower TG and apoCIII than nondiabetic family members without the mutation [138]. The reduction in TG correlated with the reduction in apoCIII and apoB. The K99fsdelAA mutation results in a frameshift and a premature stop codon, which in turn leads to truncation of the HNF4α protein to 122 instead of 465 amino acids [138].

The R154X mutation in exon 4 of the HNF4α gene results in the synthesis of a truncated protein of 153 amino acids with an intact DNA-binding domain, but lacking the ligand binding and transactivation domain [139]. Subjects carrying this mutation do not display lower TG or apoCIII levels. In a previous study [140], the R154X mutation decreased the transcriptional activity of HNF4α more pronounced in β-cells compared with non-β-cells. The R303H mutation is less well characterized and results in a G→A substitution in codon 303 of exon 8. Another small study, showed that MODY1 patients had significant reductions in serum apoAII, apoCIII, Lp(a), and TG levels compared to controls [141]. The lowered TG levels may result from increased LPL activity since apoCIII is an inhibitor of LPL [141]. Pearson *et al.* [136] reported that MODY1 patients had reduced HDL cholesterol, apoAI, and apoAII compared to controls.

1.7.4 MODY3

More than 200 different HNF1α mutations have been reported [142]. They include missense, nonsense, deletion, insertion, and frameshift mutations [143]. The clinical expression of MODY3 is highly variable from one family to another or even within the same family [142]. HNF1α mutation carriers may be normoglycemic while their siblings may be hyperglycemic at a comparable age [142]. The severity and the course of insulin secretion defect also vary since ~one-third of the patients are treated with insulin after 15 years of diabetes duration, whereas others control their diabetes by diet or oral hypoglycemic agents [142]. Part of the variability of the clinical expression in MODY3 patients may be explained by the type and the location of the HNF1α mutations [142].

Microangiopathic and neuropathic complications are as common in MODY3 patients as in T1D and T2D patients and these complications were determined by the degree of glycemic control [144]. In addition, coronary heart disease was more frequent in MODY3 than in T1D patients but lower than in T2D patients [144]. The age at diagnosis is determined in part by the location of the mutation [137].

1.7.5 MODY5

The predominant phenotype of MODY5 (HNF1β) mutations is developmental renal disease [137]. Other clinical features that have been described include pancreas dysplasia and insufficiency, dyslipidemia, genital abnormalities, and mental retardation [145]. MODY5 patients are more insulin resistant than MODY3 patients [137]. Some mutations in the HNF1β gene are associated with an increased risk for prostate cancer and may protect against T2D [137]. MODY5 carriers are not sensitive to oral sulfonylurea and early insulin therapy is required [137].

1.8 NIEMANN-PICK C1 LIKE 1 (NPC1L1)

Niemann-Pick type C (NPC) is a rare autosomal recessive lipidosis, in which patients exhibit progressive neurodegeneration and hepatosplenomegaly, leading to death during early childhood [146]. It is characterized by the accumulation of LDL-derived unesterified cholesterol in the endosomal/lysosomes system [147]. Mutations in two genes can cause NPC disease: NPC1 and NPC2 [146]. The NPC1L1 protein shares 42% identity and 51% similarity with NPC1 [148], and the gene is mapped to chromosome 7 [148].

1.8.1 Tissue distribution

In humans, NPC1L1 is predominantly expressed in the intestine and in the liver [149]; in the intestine, NPC1L1 is mainly expressed in the jejunum at the brush-border membrane [150]. NPC1L1 in mice [149] is predominantly expressed in small intestine with minimal liver expression, suggesting that there are significant differences between the expression of human and mouse NPC1L1.

1.8.2 Ezetimibe

Ezetimibe has been shown to effectively reduce the plasma phytosterol levels in sitosterolemic patients [151] and mice [152]. It can also completely reverse xanthomatosis when used in combination with low-dose cholestyramine therapy in sitosterolemic patients [151]. NPC1L1 mediates intestinal cholesterol absorption from micelles in the intestinal lumen and may also promote cholesterol re-uptake from micelles in the canalicular bile. It has been hypothesized that the pharmacological efficacy of ezetimibe may be partially attributed to blocking of canalicular reuptake in humans and species that express NPC1L1 in liver [153]. Consequently, ezetimibe might predispose some individuals to gallstone formation by increasing cholesterol saturation of bile [153].

1.8.3 Animal studies

NPC1L1-/- mice on a high cholesterol diet showed no elevation in LDL but a significant decrease in total and HDL cholesterol and also in TG levels in relation to controls on a high cholesterol diet [149]. Livers from these mice were normal but smaller, indicating that inactivation of the NPC1L1 protein has a protective effect against diet-induced hypercholesterolemia [149]. Mice deficient in NPC1L1 had ~70% reduction in sterol absorption, with the residual being insensitive to ezetimibe, suggesting that NPC1L1 is critical for the uptake of cholesterol across the plasma membrane of enterocytes [154]. Mice deficient in NPC1L1 protein, in the apoE-/- mouse model, had decreased dietary and biliary cholesterol absorption and increased liver and intestinal cholesterol synthesis [155]. Miniature pigs treated with ezetimibe showed ~80% decreased cholesterol absorption and ~65% increased plasma lathosterol [156]. Transgenic mice expressing human NPC1L1 in hepatocytes had a 10- to 20-fold decrease in biliary cholesterol, which was associated with 30-60% increase in plasma cholesterol, mainly because of the accumulation of apoE-rich HDL [157], which returned into normal with ezetimibe treatment [157].

1.8.4 Cell experiments

NPC1L1 proteins traffic between the plasma membrane and intracellular compartments through the endocytic recycling pathway in cultured hepatoma cells [158]. The endocytic recycling of NPC1L1 proteins is regulated by cellular cholesterol availability, and acute cholesterol depletion relocates NPC1L1 to the cell surface, resulting in increased uptake of FC by NPC1L1 [158]. NPC1L1 mediates the selective unidirectional cellular transport of FC, which occurs in a K+-sensitive manner in hepatoma cells [159]. Deficiency of NPC1L1 in Caco-2 cells decreased ACAT activity and mRNA expression [150].

Human NPC1L1 mRNA expression was decreased by 25-hydroxycholesterol but increased in response to cellular cholesterol depletion by mevinolin in Caco-2 cells [160]. Also, the NPC1L1 promoter was transactivated by overexpression of SREBP2. Two SREs were found in the promoter, located -35/-26 bp and the other located -657/-648bp upstream of the transcription start site. Mutation in the -657/-648 bp site alone attenuated the response to sterol but was not sufficient to abolish it. However, mutation in the -35/-26 bp site alone resulted in reduction in the basal activity of the promoter and completely abrogated its regulation by sterols [160]. HNF4α, reported to interact with SREBP2 in regulating genes related to cholesterol metabolism, was recently shown to bind to the NPC1L1 promoter and transactivate the promoter activity along with SREBP2 in HepG2 cells [125]. HNF4α specific-knock down reduced the mRNA level of NPC1L1 and abolished the cholesterol-dependent regulation of NPC1L1. Thus, the transcription of NPC1L1 was stimulated by HNF4α together with SREBP2, but not by HNF4α alone [125].

A previous study [161] showed that NPC1L1 mRNA was higher in the intestine of diabetes patients than in controls, but animal and human studies have not demonstrated an increase in dietary cholesterol absorption in T2D; instead, these patients have an increase in intestinal cholesterol synthesis.

2 METHODOLOGY

2.1 CELL CULTURE

HepG2 cells were grown in Dulbecco´s Modified Eagle Medium (DMEM) supplemented with 10 % FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, and 2 mM L-glutamine. HuH7 and HEK293 cells were grown in DMEM supplemented with 10 % FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin. Cells were maintained in 75 cm^2 tissue culture flasks and passaged when reaching ~90% confluence. In all cell experiments, cells were seeded in six-well tissue culture plates so that they reached ~70% confluence after 24 hours.

2.2 RNA ISOLATION AND REAL-TIME RT-PCR

Total RNA was prepared using TRIzol reagent (phenol and guanidine isothiocyanate) according to the manufacturer´s protocol (Invitrogen). To control for degradation and contamination, samples were separated on an agarose gel. One microgram RNA was transcribed into cDNA using Omniscript reverse transcriptase according to the manufacturer´s protocol (QIAGEN). The cDNA was diluted 1:10 in DEPC-H_2O. Real-time RT-PCR was performed in triplicate with 2.5 µL cDNA, 6.25 µL SYBRGreen Mastermix, forward and reverse primers. Arbitrary units were calculated by linearization of the C_T values. All values were normalized to GAPDH mRNA concentration.

2.3 MUTAGENESIS

Mutageneses were performed using QuikChange® site-directed mutagenesis kit according to the manufacturer´s protocol (Stratagene). In brief, the procedure utilizes a plasmid with an insert of interest and two primers containing the mutation. The primers are extended during temperature cycling by *Pfu Turbo* DNA polymerase. Incorporation of the primers generates a mutated plasmid containing staggered nicks. *Dpn* I restriction enzyme is added to digest the parental DNA template. The nicked vector DNA, containing the mutation, is then transformed into XL1-Blue supercompetent cells, which repair the nicks in the mutated plasmid.

For primer design, the mutagenic primers must contain the mutation, flanked by ~10-15 bases on each side of the mutation, and anneal to the same sequence on opposite strands of the plasmid. Primers should be 25-45 bases in length, and T_M of the primers should be ≥78°C. Also, the primers optimally should have at least 40% GC-content and terminate in one or more C/G bases.

2.4 TRANSFECTION

The cell membrane represents a major barrier to the intracellular delivery of macromolecules (e.g. plasmids and oligonucleotides). Cationic lipids, such as Lipofectamine, Fugene-6, and Lipofectin are commonly used for the introduction of polynucleic acids into cells. These liposome formulas are thought to perform at least three functions. First, the cationic lipid coats and partially condenses the plasmid. Second, the presence of cationic lipid at levels that give rise to an overall positive charge leads to enhanced association of the cationic lipid:plasmid complex with negatively charged cell surfaces, leading to cellular uptake via endocytosis [162-164] . Third, following uptake, the cationic lipid plays a role in destabilizing the endosomal membrane, thus facilitating cytoplasmic delivery of the plasmid. This is achieved partly by destabilizing endosomal or plasma membranes by inducing non-bilayer lipid structures [165, 166]. In general, the liposomes used for complex formation contain at least two kinds of lipid molecules. The key component is the cationic lipid, which serves as the condensing agents of the negatively charged DNA strands, whereas the neutral helper lipid plays a crucial role in determining the structure of the lipid phases [167, 168].

2.4.1 Transfection experiments

Careful optimisation of the transfection conditions is an important step for efficient transfection. Cell densities, transfection agent, amount of DNA, and incubation time are examples of steps that need to be optimised for each cell type. For each cell line used in this thesis, we optimised the transfection conditions using different amounts of a plasmid containing enhanced green fluorescent protein along with two different volumes of various transfection reagents according to the manufacturer´s protocols. The percent transfected cells were visualized using a fluorescent microscope. Transfections were then performed as described in Paper II, with the optimised condition that generated ~50% (HepG2) to ~90% (HuH7 and HEK293) transfection efficiency.

2.4.2 β-galactosidase activitity

Cells were transfected with β-galactosidase vector to correct for variations in transfection efficiency. β-galactosidase activities were measure in cell lysates using a β-Galactosidase Assay kit according to the manufacturer´s protocol (Promega). In brief, equal volumes (50 μL) of cell lysate and assay buffer were mixed and incubated for ~30 min. The assay buffer contains *o*-nitrophenyl-β-D-galactopyranoside. During the incubation, β-galactosidase hydrolyzes the colourless substrate to *o*-nitrophenol, which is yellow. The reactions were terminated by addition of sodium carbonate and the absorbance read at 420 nm.

2.4.3 Luciferase activity

Luciferase activities were measured in cell lysates from transfected cells using a Luciferase Assay System kit according to the manufacturer´s protocol (Promega). Twenty microliter cell lysates were applied to a 96-well plate (Costar No.3912). The luminometer was programmed for addition of 100 µL reagent per well at a speed of 200 µL/sec, shaking for 3 sec with an amplitude of 2 nm, and an integration time of 10 seconds. Light is produced by converting the chemical energy of luciferin oxidation through an electron transition, forming the product molecule oxyluciferin. Firefly luciferase catalyzes luciferin oxidation using $ATP{\cdot}Mg^{2+}$ as cosubstrate.

2.5 ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA)

The gel shift assay is used to study sequence-specific DNA-binding proteins such as transcription factors. The assay is based upon that protein:DNA complexes migrate more slowly through a non-denaturating polyacrylamid gel than free DNA fragments or double-stranded oligonucleotides. The assay is performed by incubation of purified protein or a nuclear extract with a ^{32}P end-labelled DNA fragment containing the putative binding site. The samples are separated on a non-denaturating polyacrylamid gel and bands are detected by autoradiography. The specificity of the DNA-binding protein for the putative binding site is established by competition experiments using labelled mutated oligonucleotides. For supershift assays, a specific antibody that bind to the putative binding site is added and the antibody:protein:DNA complexes migrates more slowly than the protein:DNA complexes.

EMSA and supershift assays were performed as described in Paper II. Double-stranded oligonucleotides were designed with the *cis*-element of interest in the middle flanked by ~10 bp on each side.

2.6 CHROMATIN IMMUNOPRECIPITATION ASSAY (CHIP)

ChIP assay is a powerful tool to determine whether proteins (e.g. transcription factors) are associated with a specific genomic region in living cells or tissues, i.e. to characterize protein:DNA interactions that occurs in the cell nucleus *in vivo*. The ChIP assays were performed as described in Paper II. In brief, proteins are cross-linked to DNA using formaldehyde. To isolate chromatin, cells are lysed and crude extracts are sonicated to shear the DNA. The protein:DNA complexes are immunoprecipitated using a specific antibody against the protein of interests. After extensive washing, the cross-linking is reversed at 66°C for at least 5 hours and the DNA fragments are purified and identified by PCR using specific primers designed to target the binding region of interests.

2.7 LIPOPROTEIN SEPARATION

Separation of lipids in serum or plasma by ultra centrifugation involves sequential isolation of lipoproteins after adjustment of the density (e.g. using potassium bromide). It takes several days to isolate all subclasses and requires significant sample volumes. For cell experiments, LDL, HDL, and lipoprotein deficient serum (LPDS) were prepared as described in Paper I.

Gel filtration chromatography is a technique in which an aqueous solution is used to transport the sample through the column. In SEC, particles of different size will elute through a stationary phase at different rates. The automated system used in Paper III have previous been described in detail [169]. Ten microliter serum was automatically injected and carried over the separation column (Superose®-6 PC 3.2/30 column) by the elution buffer (150 mM NaCl, 10 mM Tris-HCl, 0.02% sodium azide, pH 7.4) delivered by a pump at a flow-rate of 40 $\mu L/min^{-1}$. The eluate was continuously mixed with cholesterol, FC, or TG reagents, using a T-tube, delivered by a second pump at a flow-rate of 40 $\mu L/min^{-1}$. The mixture went through a reaction coil immersed in a water-bath at +37°C. The absorbance was measured at 500 nm and data were collected every 20 second. Chromatograms were integrated by the D-7000 HPLC System Manager Software. The lipid content in the respective lipoprotein fractions was calculated as the product of the area percent and the lipid content determined from the injected sample. CE was calculated by subtracting values of free from total cholesterol.

2.8 ACAT ASSAY

The enzymatic activities of ACAT1 and ACAT2 were measured in microsomes from HepG2 and HuH7 cells treated with LDL, HDL, FC, and 10% LPDS as described in Paper I. ACAT1 and ACAT2 activities were separated by PPPA, a highly specific ACAT2 inhibitor [91].

2.9 CHOLESTEROL CONTENT OF CELLS

Cholesterol contents in HepG2 and HuH7 cells treated with LDL, HDL, and FC were measured as described in Paper I.

2.10 STATISTICAL ANALYSIS

Statistical analyses were performed as described in the respective papers.

3 AIM OF THE STUDY

The overall aim of this thesis was to gain more insight into the molecular mechanisms that participate in the hepatic regulation of ACAT2 and the roles of HNF1α and HNF4α in cholesterol metabolism.

The specific aims were:

I. To examine a possible transcriptional regulation by cholesterol of the human ACAT2 gene. Also, to appraise the use of HepG2 and HuH7 cells as model systems in the study of ACAT and evaluate whether cell differentiation could influence its expression.

II. To characterize mechanisms involved in the hepatic transcriptional regulation of ACAT2 in humans.

III. To further investigate the roles of HNF1α and HNF4α in lipoprotein metabolism, particularly their association to ACAT2-derived CEs *in vivo*.

IV. To gain more insight into the hepatic expression of the human NPC1L1 gene, particularly the roles of HNF1α and HNF4α in its regulation.

4 RESULTS

4.1 Cholesterol regulates ACAT2 gene expression and enzyme activity in human hepatoma cells (Paper I)

One of the aims in this study was to characterize similarities and differences between the two human hepatoma cell lines HuH7 and HepG2, and appraise the use of these cells as model systems in studies of ACATs. HepG2 cells are commonly used in studies of cholesterol metabolism whereas HuH7 cells are rarely utilized. By following the expression of albumin, which is considered to be a marker for the mature hepatocyte [170, 171], we studied whether differentiation affected the mRNA expressions of ACAT1 and/or ACAT2. In HuH7 cells, we showed that increased differentiation decreased the ACAT1 and increased the ACAT2 mRNA expressions; also, a strong positive correlation between albumin and ACAT2 mRNA was present. Contrary, HepG2 cells had stable expressions of ACAT1 and ACAT2 that were not affected by differentiation.

In contrast to several other cholesterol-regulated genes, no SRE or E-box motifs are present within the ACAT1 [55, 77] or ACAT2 [54] promoters. Thus, these genes are not thought to be transcriptionally regulated by cholesterol. However, cynomolgus monkeys on a high-cholesterol diet had increased hepatic ACAT2 mRNA levels [81]. Also, in a study which at that time was unpublished data by Parini *et al*. [83], patients treated with 80 mg/d atorvastatin for four weeks had decreased hepatic ACAT2 mRNA levels compared to controls. Thus, we hypothesized that cholesterol may exert transcriptional regulation on the human ACAT2 gene. We loaded and starved HuH7 and HepG2 cells with cholesterol to study its effect on ACAT1 and ACAT2 mRNA expressions, enzymatic activities, and on the cellular cholesterol mass. The LDLr mRNA expression was used as control for the cholesterol loading/starvation. We showed that the ACAT2 mRNA expression and enzymatic activity increased with increasing concentrations of LDL and FC in both HepG2 and HuH7; although HuH7 cells required much lower concentrations to obtain similar effects. In contrast, the expression of ACAT1 was almost unaltered.

By working as an acceptor molecule, HDL removes excess cholesterol from cells. Incubation of HuH7 cells with HDL cholesterol decreased the ACAT2 mRNA expression and enzymatic activity; in contrast, incubation of HepG2 cells with 1 mM HDL increased the ACAT2 activity but did not affect the mRNA expression. We do not know the reason for this discrepancy, but it may – at least in part – be due to sub-optimal HDL-loading in the HepG2 cells. Also, cholesterol-depletion using LPDS decreased the ACAT2 mRNA expression in both cell lines, suggesting that low intracellular cholesterol levels lead to decreased ACAT2 transcription. Moreover, incubation of the cells with LDL and FC increased esterified cholesterol mass whereas incubation with LPDS decreased the esterified cholesterol mass.

In summary, we showed that cell differentiation affects the mRNA expressions of ACAT1 and ACAT2 in HuH7, but not in HepG2 cells. We also showed a dose-dependent increase of ACAT2 mRNA expression, an increased enzymatic activity of ACAT2, and increased esterified cholesterol mass upon cholesterol loading. These results suggested that human ACAT2, but not ACAT1, is transcriptionally regulated by cholesterol.

4.2 Control of ACAT2 liver expression by HNF1 (Paper II)

In this study we aimed to characterize mechanisms involved in ACAT2 transcriptional regulation in human liver. Nearly 1400 bp of the 5´-flanking sequence upstream to the start codon ATG of the human ACAT2 gene (from -1305 to +86) was cloned into a pGL3 empty vector. This promoter construct was used as template to create four deletion constructs, termed p-1196 (-1196 to +86), p-1044 (-1044 to +86), p-782 (-782 to +86), and p-269 (-269 to +86), which were used for transfection studies in HuH7 and HepG2 cells. We showed that the p-1044 construct conferred maximum luciferase activity in both cell lines, although HepG2 cells displayed higher basal activity than HuH7 cells. Also, the activity declined appreciably when comparing the p-1044 with the p-782 construct, suggesting presence of potential positive regulatory elements in this region. Moreover, the promoter activity increased >4-fold when comparing the p-1044 with the full-length (p-1305) promoter construct, suggesting the presence of potential repressor elements in this region. We chose to characterize the positive regulatory element in detail, without further studies of the potential repressor elements. The liver-specificity of these findings was test using the human kidney-derived cell line HEK293, which displayed >20-fold lower activity than HepG2 cells and showed a completely different expression pattern.

We screened this region (-1044 to -782) to search for potential positive regulatory elements using Transcription Element Search Software (TESS) (http://www.cbil.upenn.edu) and identified two c*is*-elements, HNF1 and C/EBP, which displayed a 100% match. Deletion of the putative binding site for HNF1 decreased the activity 5- to 6-fold in both HuH7 and HepG2 cells whereas deletion of the C/EBP element had no significant effect. To investigate whether this HNF1 binding site was functional, EMSA and supershift assays were performed. Nuclear extracts were prepared from HuH7 cells. Incubation with antibodies raised against either HNF1α or HNF1β showed a supershift by HNF1α, but not by HNF1β. This might be attributable to the fact that EMSA experiments commonly reveal the most abundant and/or highest affinity interacting protein [172], which in this case might be HNF1α. Thus, we also performed ChIP assay using human liver and showed that both HNF1α and HNF1β are associated with the human ACAT2 promoter *in vivo*.

Co-transfections, using expression vectors for HNF1α and HNF1β along with the ACAT2 promoter, showed that both HNF1α and HNF1β could regulate the human ACAT2 promoter in HuH7 cells; however, HNF1α, but not HNF1β, caused a minor increase in HepG2 cells. This might be explained by the previously reported presence of intermediate to high endogenous levels of HNF1 in these cells [173, 174]. Co-transfections in HEK293 cells – which do not express HNF1α [175], HNF1β [175], or ACAT2 [54] – showed that both HNF1α and HNF1β could increased the ACAT2 promoter activity. To investigate whether HNF1α and HNF1β could regulate the ACAT2 promoter through another *cis*-element, we transfected the cells with the HNF1-mutated promoter construct along with HNF1α and HNF1β expression vectors. A complete loss of HNF1-dependent stimulation was seen in both HuH7 and HepG2 cells, indicating that deletion of this HNF *cis*-element prevents activation by HNF1α and HNF1β.

In summary, we identified an important HNF1 binding site located -871 to -866 bp upstream of the transcription start site of the human ACAT2 promoter. This site serves as a positive regulator of the ACAT2 gene expression and is functionally active both *in vitro* and *in vivo*. Interestingly, mutation of this HNF1 binding site also decreased the basal ACAT2 promoter activity. The transcription factors HNF1α and HNF1β, which binds to this site, are important regulators of the human ACAT2 promoter.

4.3 Control of ACAT2 liver expression by HNF4α: lesson from MODY1 patients (Paper III)

ACAT2 is thought to incorporate CEs into hepatic and intestinal apoB-containing lipoproteins that are secreted into plasma. We previously identified HNF1α and HNF1β as important regulators of the human ACAT2 promoter. Also, HNF4α is an upstream regulator of HNF1α [130]. Thus, we hypothesized that MODY3 (mutations in the HNF1α gene, TCF1) and possibly MODY1 (mutations in the HNF4α gene, TCF14) subjects may have lower VLDL esterified cholesterol levels compared to controls. Surprisingly, analysis of lipids in lipoprotein fractions from patients with MODY3 did not differ from controls. Instead, MODY1 patients had lower VLDL and LDL esterified cholesterol levels compared to controls; in addition, MODY1 subjects had dramatically lower VLDL TG levels. These findings prompted us to investigate the role of HNF4α on the human ACAT2 promoter activity.

Co-transfections in HuH7 cells, using the human ACAT2 promoter along with an expression vector for HNF4α, revealed a strong dose-dependent regulation by HNF4α on the ACAT2 promoter. To identify the region that conferred this strong regulatory effect, HuH7 cells were co-transfected with the deletion constructs (described in Paper II) of the ACAT2 promoter along with the HNF4α expression vector. The strong induction by HNF4α was most pronounced in the p-1044 construct, although it pertained to the p-269 construct. The liver-specificity of these findings was tested in HEK293 cells, and showed that HNF4α did not induce the ACAT2 promoter activity as efficiently as in HuH7 cells (4-fold in HEK293 versus >50-fold in HuH7cells).

We screened the sequence using TESS to search for putative HNF4 binding sites as potential positive regulators in the human ACAT2 promoter region. Two HNF4 *cis*-elements were found, located -247 bp and -311 bp upstream of the ATG start codon. We performed mutagenesis on these HNF4 elements, with or without mutation of the previously identified -866 bp HNF1 binding site (Paper II). Co-transfections of these mutated constructs along with the HNF4α expression vector showed that deletion of the -247 bp HNF4 binding site only modestly decreased the induction by HNF4α. Deletion of the -311 bp HNF4-binding site decreased the induction ~30%; the decrease was of greater magnitude when the -866 bp HNF1 binding site also was mutated (~50%), suggesting an interaction between HNF1α and HNF4α. In contrast to HNF1, deletion did not completely abolish the stimulatory effects of HNF4α; hence, we were not able to identify one single element responsible for the regulation of the ACAT2 promoter by HNF4α.

ChIP assays were performed using human liver to assess whether HNF1α and HNF4α interacts with each other when binding to the ACAT2 promoter. Incubation with antibodies against either HNF1α or HNF4α revealed that both HNF1α and HNF4α can bind to the -866 bp HNF1, to the -247 bp HNF4, and to the -311 bp HNF4 binding site *in vivo*. To further investigate the possible protein-protein interaction between HNF1α and HNF4α, nuclear extracts from human liver were immunoprecipitated with antibodies against either HNF1α or HNF4α and immunoblotted using primary antibodies against HNF1α and HNF4α. These experiments showed that HNF1α can bind to HNF4α and vice versa in the human liver.

The MODY1 subjects in our study carried three different mutations in the HNF4α gene; the K99fsdelAA, the R154X, and the R303H mutation. To test the functional consequences of these mutations on the human ACAT2 promoter, we introduced the mutations into the HNF4α expression vector and used in co-transfections along with the human ACAT2 promoter in HuH7 cells. The K99fsdelAA mutation reduced the basal activity and completely abolished the transactivation potential of HNF4α overexpression on the ACAT2 promoter activity. Both the R154X mutation and the R303H mutation reduced the transactivation potential of HNF4α overexpression on the ACAT2 promoter, although the R303H mutation reduced it to a lesser extent.

In summary, we identified HNF4α as an important regulator of the hepatocyte-specific expression of the human ACAT2 promoter. The results suggests that the lower levels of esterified cholesterol in VLDL-and LDL-particles in MODY1 subjects may – at least in part – be due to lower ACAT2 activity in these patients.

4.4 HNF1α and SREBP2 are important regulators of NPC1L1 in human liver (Paper IV)

The exact function of NPC1L1 in the human liver is currently not well defined. Thus, the aim of this study was to gain more insight into mechanisms that participates in the transcriptional regulation of hepatic NPC1L1. Gene expression analyses were performed in liver samples from Chinese patients with or without cholesterol gallstone disease. No significant differences were observed in NPC1L1, SREBP2, and HNF1α mRNA expressions between the two groups of patients; though, gallstone patients had 43% higher HNF4α mRNA expression. Strong positive correlations between NPC1L1 and SREBP2 and between NPC1L1 and HNF4α were observed. However, no significant correlation was observed between NPC1L1 and HNF1α. These results prompted us to investigate whether SREBP2, HNF4α, and HNF1α may participate in the hepatic regulation of NPC1L1 in humans.

Co-transfections in HuH7 cells, using a human NPC1L1 promoter construct (-1570 to +137 bp) and an expression vector for SREBP2, showed a strong dose-dependent regulation by SREBP2 on the promoter activity. Also, SREBP2 overexpression increased NPC1L1 mRNA. To study the effect on the endogenous NPC1L1 gene expression under more physiological conditions, HuH7 cells were depleted or loaded with cholesterol. Loading of the cells with LDL cholesterol decreased NPC1L1 and SREBP2 mRNA expressions, whereas cholesterol depletion resulted in an insignificant trend toward increased NPC1L1 mRNA. Two SREs, SRE1 (-91/-81 bp) and SRE2 (-748/-738 bp), were previously identified in the human NPC1L1 promoter [160]. We performed ChIP assay using human liver with a specific antibody against SREBP2 and showed that SREBP2 can bind to these two SREs in the NPC1L1 promoter *in vivo*.

Unexpectedly, co-transfections in HuH7 cells using the human NPC1L1 promoter along with an HNF4α expression vector decreased the promoter activity whereas HNF4α overexpression had no effect on NPC1L1 mRNA. A previous study [125] reported that the transcription of NPC1L1 was stimulated by HNF4α together with SREBP2, but not by HNF4α alone. To test a possible synergism in the activation of the NPC1L1 promoter, we performed co-transfections in HuH7 cells with both SREBP2 and HNF4α expression vectors. However, no further activation of the promoter activity occurred.

HNF4α is an upstream regulator of HNF1α, and both contains binding sites for each other in their promoter regions [109, 176]. Hence, we also wanted to investigate whether HNF1α participate in the regulation of NPC1L1 despite the lack of correlation. Co-transfections in HuH7 cells using the human NPC1L1 promoter along with an HNF1α expression vector revealed a dose-dependent regulation by HNF1α on the promoter activity; also, HNF1α overexpression increased NPC1L1 mRNA. Mutation of one (-158/-144) of the six putative HNF1 binding sites in the human NPC1L1 promoter almost completely abolished the regulatory effect of HNF1α on the promoter activity. In human liver, ChIP assay was performed. Primers were designed to span over the six putative HNF1 binding sites, due to the proximity between these sites. These experiments showed that HNF1α is able to bind to the NPC1L1 promoter *in vivo*. EMSA and supershift assays were performed to be able to distinguish which of the six putative HNF1 binding sites is responsible for the transactivation by HNF1α. These experiments revealed a direct binding of HNF1α to the -158/-144 bp HNF1 binding site, but not to the other five HNF1 sites.

In summary, we identified an important HNF1 binding site located -158 to -144 bp upstream of the transcription start site of the human NPC1L1 promoter. Also, we showed that SREBP2 and HNF1α are important transcription factors for the hepatic NPC1L1 promoter activity that can bind to and regulate its expression in humans.

5 CONCLUSIONS AND FUTURE PERSPECTIVES

In this thesis we aimed to gain more insight into the molecular mechanisms that participate in the hepatic regulation of ACAT2 and the roles of HNF1α and HNF4α in cholesterol metabolism. We found that ACAT2, but not ACAT1, mRNA expression and enzymatic activity increased upon cholesterol loading in human hepatoma cell lines, suggesting that human ACAT2 is transcriptionally regulated by cholesterol. HNF1 and HNF4 are involved in diverse metabolic pathways (e.g. glucose, cholesterol, and fatty acid metabolism) and we identified HNF1α, HNF1β, and HNF4α as important transcription factors in the regulation of the human hepatic ACAT2 promoter. Importantly, these findings suggest that ACAT2 may be subjected to metabolic control. We also identified HNF1α as an important regulator of the NPC1L1 promoter, whereas the role of HNF4α was inconclusive. Collectively, these studies also imply new and important roles for HNF1α and HNF4α in cholesterol metabolism. HNF1α may participate in hepatic cholesterol esterification and uptake of FC in hepatocytes; also, HNF4α may participate in esterification of cholesterol in HDL, affect plasma levels of esterified cholesterol and TG in VLDL- and LDL-particles, and indirectly participate in the regulation of uptake of FC in hepatocytes.

We identified HNF1α and HNF1β as important regulators of the human ACAT2 promoter, which led us to postulate that MODY3, MODY5, and possibly MODY1 subjects may have alterations in CE content of apoB-containing lipoproteins. However, MODY5 subjects were unavailable and, contrary to our initial prediction, the MODY3 subjects in our study had similar lipoprotein composition as controls. Homologous inactivation of the TCF1 gene in mice only weakly affected the transcription of most hepatic genes initially thought to be under strict HNF1α control [177]. HNF1β is weakly expressed in human liver, where HNF1α constitutes more than 95% of the total HNF1-protein [99, 100]. Levels of HNF1β protein were increased in livers of HNF1-/- mice. Thus, it was suggested that HNF1β may compensate for the loss of HNF1α function in the liver [177]. Moreover, HNF4α is produced from two promoters (P1 and P2): P1 is more active in extrapancreatic tissues (e.g. liver and kidney), whereas P2 is more active in pancreas [119]. P2, but not P1, is bound and controlled by HNF1α [120-122]. Consequently, HNF1α controls the transcription of HNF4α in pancreas whereas the roles are opposite in the liver [103, 120]. Thus, it is possible that mutations in HNF4α may have bigger impact in the liver than mutations in HNF1α as suggested from the results of our study.

Moreover, various mutations may have variable effects on the ability of HNF4α to transactivate target genes [128, 178, 179]. MODY1 subjects have been shown to have lower TG, HDL cholesterol, apoCIII, apoB, apoAI, apoAII, and Lp(a) plasma levels [136, 138, 141]. Collectively, these studies suggest that the type and the location of the HNF4α mutation affect cholesterol and TG levels in various ways. More than 200 different HNF1α mutations have been found [142]. Of the five MODY3 subjects in our study, one carried the Pro291fins3 mutation (insertion in exon 4), two the L1071 mutation (substitution of leucine with isoleucine in exon 1), and two the insTGGGGGT (insertion in the 5´untranslated region) mutation. Hence, it is possible that MODY3 subjects with other mutations in the TCF1 gene may have alterations in lipoprotein composition.

Due to the findings in MODY1 subjects, we characterized the role of HNF4α in the transcriptional regulation of ACAT2. We showed a dose-dependent regulation of HNF4α on the human ACAT2 promoter activity and two putative *cis*-elements were identified. However, we were not able to identify one single transacting element in the ACAT2 promoter region that was responsible for the induction by HNF4α. This has also been reported when the putative HNF4 binding site was mutated in the fatty acid binding protein-1 (FABP1) promoter [176], and suggested an interaction between HNF4α and HNF1α. HNF1α and HNF4α can bind directly to one another and a cooperative interaction can occur for target genes with binding sites for both HNF1α and HNF4α, resulting in greater activation; also, HNF1α can bind to the DNA and HNF4α bind to HNF1α, or vice versa, thereby affecting regulation [174, 180]. If the target gene contains an HNF1, but no HNF4, binding site, HNF4α bind to HNF1α and co-activate the gene [181]. If the gene contains an HNF4, but no HNF1, binding site, HNF1α can inhibit the activation of HNF4α [182]. Thus, despite deletion of both HNF4 binding sites, HNF4α may still be able to activate the ACAT2 promoter through direct or indirect binding to HNF1. In addition to the important -866 HNF1 binding site, two putative HNF1 binding sites (located at -220 and -276 bp) are located in the human ACAT2 promoter. Although these were not shown to be important for the hepatocyte-specific expression of ACAT2 in Paper II, they may still take part in the metabolic regulation especially when the -866 HNF1 binding site was deleted. ChIP experiments confirmed that both HNF1α and HNF4α can bind to the -866 HNF1 binding site and to the two HNF4 binding sites in the human ACAT2 promoter. Also, we showed a protein-protein interaction between HNF1α and HNF4α in human liver, supporting the concept of a cooperative interaction between these two transcription factors in the regulation of the human ACAT2 promoter. Furthermore, HNF4α is an upstream regulator of HNF1α, but not of HNF1β, and we showed that HNF1α, but not HNF1β, can bind to the two HNF4 binding site in the human ACAT2 promoter region. Thus a clear difference between HNF1α and HNF1β exists in the regulation of the human ACAT2 promoter.

The MODY1 subjects in our study had reduced VLDL and LDL esterified cholesterol and dramatically lower VLDL TG compared to controls. Previous studies [141, 143] reported that MODY1 subjects have lower plasma levels of TG and apoCIII, an inhibitor of LPL [141], which may contribute to the lower VLDL TG levels in our MODY1 subjects. Moreover, conditional liver-specific disruption of HNF4α in mice [127] resulted in lower serum TG levels and decreased expression of apoB and MTP, two important genes involved in hepatic VLDL secretion. Odom *et al.* [109] reported that both HNF1α and HNF4α regulates the human MTP gene; also that HNF4α regulates apoB and apoCIII. Thus, HNF4α may influence VLDL secretion from the liver by affecting the expression of several important proteins taking part in VLDL assembly. Furthermore, the MODY1 subjects in our study had higher HDL FC compared to controls. LCAT forms CEs in HDL by transferring polyunsaturated fatty acids from phosphatidylcholine to cholesterol [97]. By esterifying HDL FC, LCAT is thought to promote RCT by maintaining a FC gradient between HDL and peripheral tissues [183]. LCAT is not considered as a target of HNF4α. However, the higher levels of FC in HDL in the MODY1 patients, together with similar levels of CEs in HDL, suggests that the activity of LCAT may be lower in the MODY1 patients and that LCAT expression may be under HNF4α control.

In additional MODY1 and MODY3 subjects (Table II), we also investigated serum biochemical markers for cholesterol and BA synthesis (unpublished data). Lathosterol and lanosterol, regarded as markers for hepatic and whole-body cholesterol synthesis [184], showed no significant differences between either MODY1 or MODY3 subjects compared to controls. Thus, in contrast to T2D patients which have been shown to have higher cholesterol synthesis [185] , mutations in HNF4α or HNF1α did not result in altered cholesterol synthesis. Serum levels of 7α-hydroxy-4-cholesten-3-one (C4), a BA precursor that strongly reflects BA synthesis [186], showed that MODY3 subjects had almost 40% higher C4 levels ($p<0.01$) whereas MODY1 subjects had a similar trend that did not reach statistical significance. A previous study reported that impaired farnesoid X receptor 1 (FXR1) expression in TCF1-/- mice resulted in decreased levels of small heterodimer partner 1 (SHP1), leading to increased CYP7A1 activity and increased BA synthesis [113]. Also, previous studies [187, 188] reported that FXR is decreased in animal models of diabetes and that FXR null mice exhibit impaired glucose tolerance and insulin sensitivity. Thus, the higher BA synthesis in the MODY3 subjects may be due, at least in part, to decreased FXR activity. These experiments imply an additional important role for HNF1α in BA synthesis.

TABLE II. Clinical characteristics of control, MODY1 and MODY3 subjects.

	Controls	MODY1	MODY3
Subjects (Male/Female)	15 (7/8)	12 (4/8)	19 (8/11)
Age (years)	37.4 ± 0.4	38.4 ± 4.3	38.4 ± 3.3
BMI (kg/m^2)	23.4 ± 0.9	25.0 ± 1.0	23.4 ± 1.1
Cholesterol (mmol/L)	5.19 ± 0.2	5.08 ± 0.3	5.49 ± 0.3
Triglycerides (mmol/L)	0.95 ± 0.1	1.09 ± 0.2	1.12 ± 0.1
Glucose (mmol/L)	5.35 ± 0.1	8.20 ± 1.0	8.86 ± 1.6
Lathosterol/Cholesterol (μmol/mmol)	0.585 ± 0.06	0.515 ± 0.08	0.519 ± 0.04
Lanosterol/Cholesterol (nmol/mmol)	42.91 ± 2.27	38.82 ± 3.67	49.92 ± 4.86
C4/Cholesterol (nmol/mmol)	7.30 ± 0.36	8.88 ± 1.11	9.98 ± 0.54 *

* $p<0.01$ MODY3 versus control

NPC1L1 is expressed in several tissues with high expression levels in the intestine of both mice and humans [149]. NPC1L1 is also highly expressed in the human, but not in the mouse, liver [149]. NPC1L1 have been shown to transport FC in hepatoma cells [158, 159], and we identified HNF1α as an important regulator of its expression. Hence, HNF1α participates in the regulation of both uptake of FC and cholesterol esterification in human hepatocytes. Although we did not detect any regulation by HNF4α on the NPC1L1 promoter activity or mRNA expression, the strong correlation between NPC1L1 and HNF4α suggests that HNF4α may have an indirect role by binding to other transcription factors, including HNF1α.

Whereas human hepatocytes *in vivo* only express ACAT2 [31], we found that HepG2 and HuH7 cells expresses both ACAT1 and ACAT2. In addition, we recently tested the human hepatoma cell line Hep3B and found that these cells also express both ACAT1 and ACAT2 (unpublished data). The mechanism leading to the silencing of ACAT1 expression in hepatocytes *in vivo* is not known. One may speculate that the simultaneous expressions of ACAT1 and ACAT2 may be associated to the pathophysiological condition of these cells, since they are hepatocellular carcinoma cell lines. In concert, HuH7, HepG2, and Hep3B cells expresses α-fetoprotein (AFP) [189, 190] which is highly expressed in the fetal liver but decline rapidly after birth [191]; elevated AFP is commonly seen in human hepatocellular carcinomas and used as a diagnostic marker for detection and to monitor cancer therapy [191].

The studies in this thesis implied new and important roles for HNF1α and HNF4α in cholesterol metabolism and suggested that ACAT2 may be subjected to metabolic control. Future research that would further elucidate the present findings may include:

- Studies of other human hepatocyte-derived cell lines, in order to find a cell model that resembles the human hepatocyte *in vivo* and accordingly only express ACAT2, and not ACAT1.

- Investigation of the presence of potential repressor element(s) in the region -1305 to -1044 bp upstream of the transcription start site in the human ACAT2 promoter, since the promoter activity increased when comparing the p-1044 with the p-1305 promoter construct.

- Investigate whether ACAT2 is transcriptionally regulated by cholesterol in humans, as suggested in our study and, indirectly, in the study in which subjects treated with high or low doses of statins showed a dose-dependent decrease in hepatic ACAT2 mRNA levels [83]. Changes in mRNA abundance do not necessarily imply that rate of gene transcription is altered. To be able to conclude whether ACAT2 is transcriptionally regulated by cholesterol or not, cells can be loaded or depleted (e.g. using cyclodextrin or mevinolin) of cholesterol in the presence or absence of a transcription inhibitor (e.g. actinomycin D) and an inhibitor of protein synthesis (e.g. cycloheximide). Furthermore, investigation of the molecular mechanism leading to the upregulation of ACAT2 by cholesterol.

- Investigate the effects of HNF1β gene mutations on cholesterol and TG metabolism. For example, the experiments performed in Paper III may be repeated in MODY5 subjects.

- Investigate whether MODY3 subjects with other mutations have alterations in apoB-containing lipoproteins. For example, the experiments performed in Paper III may be repeated in MODY3 patients with other mutations in the TCF1 gene; these mutations may then be introduced into an HNF1α expression vector and used along with the human ACAT2 promoter in co-transfection studies.

- Investigate whether LCAT may be under control of HNF4α.

- Further investigate the role of HNF1α and HNF4α in BA synthesis.

- Investigate whether NPC1L1 -/- mice have decreased expression of HNF1α and/or HNF4α: and whether TCF1-/- mice or conditional liver-specific disruption of the HNF4α gene in mice results in lower NPC1L1 expression in the intestine.

6 CONCLUDING REMARKS

In this thesis we aimed to gain more insight into the molecular mechanisms that participate in the hepatic regulation of ACAT2 and the roles of HNF1α and HNF4α in cholesterol metabolism. We found that ACAT2 mRNA expression and enzymatic activity increased upon cholesterol loading in human hepatoma cell lines, suggesting that human ACAT2 is transcriptionally regulated by cholesterol. In addition, we identified HNF1α, HNF1β, and HNF4α as important transcription factors in the regulation of the hepatic ACAT2 promoter, and that HNF4α can bind and regulate its expression directly or indirectly via HNF1α. We also identified HNF1α as an important regulator of the NPC1L1 promoter although the role of HNF4α was inconclusive.

Collectively, these studies imply that ACAT2 is under metabolic control and that HNF1α and HNF4α participate in several important processes in cholesterol metabolism. HNF1α may participate in hepatic cholesterol esterification, uptake of FC in hepatocytes, and in BA synthesis. HNF4α may participate in esterification of cholesterol in HDL, affect plasma levels of esterified cholesterol and TG in VLDL- and LDL-particles, and indirectly participate in the regulation of uptake of FC in hepatocytes.

7 SVENSK SAMMANFATTNING

Kolesterol är ett fett som har en mängd viktiga funktioner i kroppen t ex som beståndsdel i cellmembran och som utgångsmaterial vid bildandet av hormoner och gallsyror. Det mesta av det kolesterol som vi behöver kommer från kroppens egna produktion av kolesterol, men det finns även i födan. Eftersom kolesterol inte kan lösas i blodet, som är vattenbaserat, transporteras det i lipoproteiner. Dessa kan,baserat på densitet, indelas i kylomikroner, VLDL, IDL, LDL och HDL. Eftersom höga nivåer av fritt kolesterol är giftigt för cellerna omvandlas det till kolesterolestrar som sedan kan lagras som fettdroppar inuti cellerna. ACAT1 och ACAT2 är två enzym som katalyserar reaktionen i vilken kolesterol och långa fettsyrakedjor omvandlas till kolesterolestrar. ACAT1 finns i de flesta celler, medan ACAT2 endast finns i tarmceller (enterocyter) och leverceller (hepatocyter). Idag tror man att kolesterolestrar som bildats av ACAT2 packas in i kylomikroner och VLDL, medan ACAT1 bildar kolesterolestrar i en mängd andra celler (t ex makrofager) och därmed förhindrar celldöd. Olika djurstudier har visat att genom att minska eller ta bort ACAT2, men inte ACAT1, kan uppkomst av åderförkalkning (ateroskleros) minskas eller förhindras.

HNF1 och HNF4 är så kallade transkriptionsfaktorer vilket innebär att de behövs för att en gen ska kopieras till RNA, dvs för att transkriptionen ska starta. HNF1 och HNF4 finns i flera olika organ t ex lever, bukspottskörteln (pancreas) och njurarna. De deltar bla. i glukos-, kolesterol- och fettsyrametabolism.

Huvudsyftet med denna avhandling var att undersöka vad som reglerar uttrycket av ACAT2 i levern. Då vi fann att HNF1α och HNF4α är viktiga för regleringen av ACAT2 ämnade vi även undersöka deras inverkan på det kolesterol som packas in i lipoproteiner (t ex VLDL) samt om de deltar i regleringen av NPC1L1 i levern.

Artikel I: ACAT1 och ACAT2 anses inte vara transkriptionellt reglerade av kolesterol. Genom att svälta och ladda två humana levercell-linjer (HuH7 och HepG2) med kolesterol, kunde vi visa att kolesterol reglerar genuttrycket och den enzymatiska aktiviteten av ACAT2, men inte av ACAT1. Detta tyder på kolesterol transkriptionellt reglerar ACAT2, men inte ACAT1. Vi utvärderade även HepG2 och HuH7 cellers lämplighet som modellsystem för studier av ACAT1 och ACAT2. HuH7 celler var känsligare för svält och behövde lägre kolesterolkoncentrationer för att erhålla samma resultat som HepG2 celler, vilket tyder på att HuH7 celler är en bättre modell för kolesterolstudier av ACAT.

Artikel II: I denna del av projektet ämnade vi karaktärisera mekanismer som kontrollerar det lever-specifika uttrycket av ACAT2. Vi identifierade ett viktigt HNF1-element som fungerar som en positiv regulator för genuttrycket av ACAT2 och visade att detta element är funktionellt både *in vitro* och *in vivo*. Mutation av detta HNF1-element minskade även det basala uttrycket av ACAT2. HNF1α och HNF1β, som båda binder till detta element, har därmed viktiga roller i regleringen av ACAT2.

Artikel III: MODY är en grupp syndrom som karaktäriseras av autosomal dominant ärftlighet, tidig uppkomst av diabetes (oftast före 25 års ålder) samt av dysfunktionella β-celler (insulinproducerande celler i pancreas). MODY orsakas av mutationer i enstaka gener; mutation i genen för HNF1α orsakar MODY3 och mutation i genen för HNF4α orsakar MODY1. ACAT2 anses ansvara för bildandet av de kolesterolestrar som packas in i VLDL. Då vi tidigare fann att HNF1α är viktig för regleringen av ACAT2, och då tidigare studier har visat att HNF4α är viktig för regleringen av HNF1α, ämnade vi undersöka om patienter med MODY3 och möjligen MODY1 hade lägre kolesterolestrar i VLDL. Vi kunde visa att patienter med MODY1, men inte MODY3, hade lägre nivåer av kolesterolestrar i VLDL och LDL. Vi karaktäriserade HNF4α´s roll i regleringen av ACAT2 och visade att HNF4α är viktig för regleringen av ACAT2-uttrycket i levern. Detta tyder på att de lägre nivåerna av kolesterolestrar i VLDL och LDL hos patienter med MODY1, åtminstone till viss del, beror på lägre ACAT2-aktivitet i dessa patienter.

Artikel IV: NPC1L1 finns framförallt i lever och tarm hos människor. NPC1L1 är viktig för regleringen av kolesterol-upptaget i tarmen, men dess roll och reglering i levern hos människa är för närvarande oklar. Genom korrelation mellan genuttrycken för NPC1L1, HNF4α, HNF1α och SREBP2 i lever från kinesiska patienter med och utan gallstenssjukdom kunde vi visa starka positiva korrelationer mellan NPC1L1 och HNF4α samt mellan NPC1L1 och SREBP2. Då HNF4α är en regulator av HNF1α ämnade vi vidare undersöka om SREBP2, HNF4α och HNF1α deltar i regleringen av NPC1L1 i levern. Vi identifierade ett viktigt HNF1-element och visade att SREBP2 och HNF1α är viktiga transkriptionsfaktorer som kan binda till och reglera uttrycket av NPC1L1 i levern hos människan. HNF4α verkar mer fungera som en indirekt regulator och påverka genom att binda till andra transkriptionsfaktorer som t ex HNF1α.

Dessa studier tyder på att ACAT2 är metabolt reglerad och att HNF1α och HNF4α deltar i flera viktiga processer i kolesterolmetabolismen.

8 FINANCIAL SUPPORT

This work was supported by the Swedish Research Council, the Stockholm County Council (ALF), the National Institutes of Health, the Swedish Medical Association, the Karolinska Institutet, the National Natural Science Foundation of China, and by the Åke Wiberg, Fernström, Throne Holst, and Swedish Heart-Lung Foundations. Paolo Parini is a recipient of a research grant from AstraZenica AB, Sweden.

9 ACKNOWLEDGEMENTS

I would like to thank my colleagues, family and friends who helped and supported me and, in various ways, have contributed to this thesis.

I want to start to thank my supervisor **Paolo Parini** for accepting me as a PhD student, for your great knowledge and passion for science, for scientific and non-scientific discussions and argue, for arranging nice parties and social activities! Thanks for all your help and support, friendship and for always trying to improve things (e.g. me, you, manuscripts)! But most of all – thanks for putting up with me! ☺

To my co-supervisor **Mats Eriksson** for support, great scientific knowledge, for always has the patient in focus and for being such an optimistic person who always sees possibilities!

To my former co-supervisor **Bo Angelin**, for your great scientific knowledge, your great ability to improve manuscripts and for letting me stays at NOVUM!

To **Suzanne Lind** for accepting to be my mentor!

To the former and the present head of the department of Clinical chemistry, **Ingemar Björkhem** and **Stefan Alexson,** respectively, for creating a good scientific environment.

To **Lawrence L Rudel, Matt A Davis**, **Ella Ekholm (Karlsson)**, and **Leif Groop** for nice collaborations!

All the members of the Lipoparini group: **Lilian Larsson, Lisa-Mari Nilsson, Matteo Pedrelli, and Zhao-Yan Jiang**. Thanks for nice times spent inside and outside the lab! A special thanks to Lilian for the nice trip to Istanbul, for joining me on several hour-walks ☺ and never complaining!

To colleagues and friends at Clinical chemistry and Metabolic lab: Ulla Andersson, **Ingela Arvidsson**, **Lisbeth Benthin** (thanks for help with scary centrifuges ☺, lab work, and cakes), **Åse Bergstedt, Jenny Bernström**, **Ylva Bonde**, **Ann Båvner, Gösta Eggertsen, Lena Emtestam, Cecilia Gälman, Elisabeth Kjellén, Johanna Lundberg, Maria Ohlin, Lena Persson, Mats Rudling, Catharina Sjöberg, Katharina Slätis, Ewa Steninger, Sabine Süllow Barin,** and **Jin Wang**.

All former and present colleagues and friends at NOVUM! A special thanks to **Manuela Matasconi** for nice times spent inside and outside the lab, for friendship, trance-nights in Stockholm ☺, and for showing me Rome! To **Thomas Lundåsen** for support, friendship, and for sharing office with me during several years! To **Beatrice Sjöberg**, my new "office-mate" and friend! To **Lisen Linnerborg** for introducing me to the lab, for help, support, and friendship! To **Anders Ström, Tomas Jakobsson, Patrick Müller, Knut Steffensen, Maria Nilsson, Christophoros Thomas**, **Lise-Lotte Vedin, Tiina Skoog,** and **Viljar Jaks**. Also, to **Inger, Inger, Inger, and Ewa** for being nice and helping peoples that facilitates my life on the lab!

To **Stephen James** and **Moustapha Hassan** for introducing me into the world of science and for encouraging me to take the leap!

To **Swedish Heart-Lung foundation** for financing part of my PhD period and for travel grants.

To my mother **Birgitta** – for all your help in my life! For never dig into problems, always trying to see the bright side of life, for your generous and compassion side, and for teaching us the importance of love (and to wash hands and cloths) ☺! Also, thanks for being such a wonderful grandmother!!
To my father **Per** – for always encourage me to study and for ”kunskapens rot äro bitter, dess frukter äro söta”. For love and support throughout the years!
To my mother's husband **Evert** – for everything you have done for me and my children, and for giving us the benefit of having you in our family! Without all your help it would not have been possible for me to study!
To my little sister **Gunilla** – for having the strength and courage to fulfill her dreams and to live her life the way she wants and believes in. To her husband **Magnus**, for help with computers and other stuff and for contributing to make me “The aunt of three little princesses”: **Amanda**, the wise and good girl with the amazing voice, that beats me on Singstar ☺; **Matilda**, my god-daughter, little miss sunshine ☼, the funny, crazy, passionate, and intelligent girl; And **Agnes**, the little charming and crazy girl (jag är klok som en bok)! Thanks for love and support through life!
To my baby-brother **Martin**! From being a little spoiled, screaming, candy-stealing ☺ bastard you have become a really nice guy!
To my big brother **Tobbe** – for being an avant-gardes and showing the importance of education!

To **Ewa** – my best friend! For ALWAYS helping, listening, caring, and supporting me! What would I be without you?!? LOVE YOU!
To **Andreas** – for all the things I learned from you and all the things you made me feel!
To **Patrick B** – for love and friendship!
To **Micke** and **Smail** – for being such nice, warm, caring, compassionate, and wise persons; and for being my friends!
To **Fredrik**, who passed away way too early! I miss you!!
To **Ingela** – for help when I started to study, for long discussions, and for friendship!
To **Tïesto** – especially for “Forever today”, the best song ever written; a song that heals!!

Finally, but certainly not the least, thanks to my children **Carl-Johan** and **William.** To **Calle** for your good heart, compassion, sense of justice, intelligence, ambition, strength, and ability to love! To **Wille** for harboring a soul of passion, love, honesty, sense of justice, and compassion that hopefully will burst into life again in the future!

10 REFERENCES

1. Dietschy JM, Woollett LA, Spady DK. The interaction of dietary cholesterol and specific fatty acids in the regulation of LDL receptor activity and plasma LDL-cholesterol concentrations. *Ann N Y Acad Sci.* 1993;676:11-26.
2. Brown JM, Bell TA, 3rd, Alger HM, Sawyer JK, Smith TL, Kelley K, Shah R, Wilson MD, Davis MA, Lee RG, Graham MJ, Crooke RM, Rudel LL. Targeted depletion of hepatic ACAT2-driven cholesterol esterification reveals a non-biliary route for fecal neutral sterol loss. *J Biol Chem.* 2008;283:10522-10534.
3. Rothblat GH, Rosen JM, Insull W, Jr., Yau AO, Small DM. Production of cholesteryl ester-rich, anisotropic inclusions by mammalian cells in culture. *Exp Mol Pathol.* 1977;26:318-324.
4. Adelman SJ, Glick JM, Phillips MC, Rothblat GH. Lipid composition and physical state effects on cellular cholesteryl ester clearance. *J Biol Chem.* 1984;259:13844-13850.
5. Turley SD, Dietschy JM. Sterol absorption by the small intestine. *Curr Opin Lipidol.* 2003;14:233-240.
6. van der Velde AE, Vrins CL, van den Oever K, Kunne C, Oude Elferink RP, Kuipers F, Groen AK. Direct intestinal cholesterol secretion contributes significantly to total fecal neutral sterol excretion in mice. *Gastroenterology.* 2007;133:967-975.
7. Iqbal J, Hussain MM. Intestinal lipid absorption. *Am J Physiol Endocrinol Metab.* 2009;296:E1183-1194.
8. Kwiterovich PO, Jr. The metabolic pathways of high-density lipoprotein, low-density lipoprotein, and triglycerides: a current review. *Am J Cardiol.* 2000;86:5L-10L.
9. Blasiole DA, Davis RA, Attie AD. The physiological and molecular regulation of lipoprotein assembly and secretion. *Mol Biosyst.* 2007;3:608-619.
10. Borchardt RA, Davis RA. Intrahepatic assembly of very low density lipoproteins. Rate of transport out of the endoplasmic reticulum determines rate of secretion. *J Biol Chem.* 1987;262:16394-16402.
11. Jamil H, Chu CH, Dickson JK, Jr., Chen Y, Yan M, Biller SA, Gregg RE, Wetterau JR, Gordon DA. Evidence that microsomal triglyceride transfer protein is limiting in the production of apolipoprotein B-containing lipoproteins in hepatic cells. *J Lipid Res.* 1998;39:1448-1454.
12. Adiels M, Olofsson SO, Taskinen MR, Boren J. Overproduction of very low-density lipoproteins is the hallmark of the dyslipidemia in the metabolic syndrome. *Arterioscler Thromb Vasc Biol.* 2008;28:1225-1236.
13. Malmstrom R, Packard CJ, Caslake M, Bedford D, Stewart P, Yki-Jarvinen H, Shepherd J, Taskinen MR. Effects of insulin and acipimox on VLDL1 and VLDL2 apolipoprotein B production in normal subjects. *Diabetes.* 1998;47:779-787.
14. James RW, Martin B, Pometta D, Fruchart JC, Duriez P, Puchois P, Farriaux JP, Tacquet A, Demant T, Clegg RJ, et al. Apolipoprotein B metabolism in homozygous familial hypercholesterolemia. *J Lipid Res.* 1989;30:159-169.
15. Brunzell JD, Hazzard WR, Porte D, Jr., Bierman EL. Evidence for a common, saturable, triglyceride removal mechanism for chylomicrons and very low density lipoproteins in man. *J Clin Invest.* 1973;52:1578-1585.
16. Beaven SW, Tontonoz P. Nuclear receptors in lipid metabolism: targeting the heart of dyslipidemia. *Annu Rev Med.* 2006;57:313-329.
17. Krimbou L, Marcil M, Genest J. New insights into the biogenesis of human high-density lipoproteins. *Curr Opin Lipidol.* 2006;17:258-267.
18. Lewis GF, Rader DJ. New insights into the regulation of HDL metabolism and reverse cholesterol transport. *Circ Res.* 2005;96:1221-1232.

19. Santos-Gallego CG, Ibanez B, Badimon JJ. HDL-cholesterol: is it really good? Differences between apoA-I and HDL. *Biochem Pharmacol.* 2008;76:443-452.
20. Bajari TM, Strasser V, Nimpf J, Schneider WJ. LDL receptor family: isolation, production, and ligand binding analysis. *Methods.* 2005;36:109-116.
21. Ikonen E. Cellular cholesterol trafficking and compartmentalization. *Nat Rev Mol Cell Biol.* 2008;9:125-138.
22. Shimano H. Sterol regulatory element-binding proteins (SREBPs): transcriptional regulators of lipid synthetic genes. *Prog Lipid Res.* 2001;40:439-452.
23. Chang TY, Chang CC, Ohgami N, Yamauchi Y. Cholesterol sensing, trafficking, and esterification. *Annu Rev Cell Dev Biol.* 2006;22:129-157.
24. Lewis JH, Mortensen ME, Zweig S, Fusco MJ, Medoff JR, Belder R. Efficacy and safety of high-dose pravastatin in hypercholesterolemic patients with well-compensated chronic liver disease: Results of a prospective, randomized, double-blind, placebo-controlled, multicenter trial. *Hepatology.* 2007;46:1453-1463.
25. Pedersen TR, Faergeman O, Kastelein JJ, Olsson AG, Tikkanen MJ, Holme I, Larsen ML, Bendiksen FS, Lindahl C, Szarek M, Tsai J. High-dose atorvastatin vs usual-dose simvastatin for secondary prevention after myocardial infarction: the IDEAL study: a randomized controlled trial. *Jama.* 2005;294:2437-2445.
26. Lawrence JM, Reid J, Taylor GJ, Stirling C, Reckless JP. The effect of high dose atorvastatin therapy on lipids and lipoprotein subfractions in overweight patients with type 2 diabetes. *Atherosclerosis.* 2004;174:141-149.
27. Baigent C, Keech A, Kearney PM, Blackwell L, Buck G, Pollicino C, Kirby A, Sourjina T, Peto R, Collins R, Simes R. Efficacy and safety of cholesterol-lowering treatment: prospective meta-analysis of data from 90,056 participants in 14 randomised trials of statins. *Lancet.* 2005;366:1267-1278.
28. Shepherd J, Cobbe SM, Ford I, Isles CG, Lorimer AR, MacFarlane PW, McKillop JH, Packard CJ. Prevention of coronary heart disease with pravastatin in men with hypercholesterolemia. West of Scotland Coronary Prevention Study Group. *N Engl J Med.* 1995;333:1301-1307.
29. Chang TY, Chang CC, Cheng D. Acyl-coenzyme A:cholesterol acyltransferase. *Annu Rev Biochem.* 1997;66:613-638.
30. Buhman KK, Accad M, Novak S, Choi RS, Wong JS, Hamilton RL, Turley S, Farese RV, Jr. Resistance to diet-induced hypercholesterolemia and gallstone formation in ACAT2-deficient mice. *Nat Med.* 2000;6:1341-1347.
31. Parini P, Davis M, Lada AT, Erickson SK, Wright TL, Gustafsson U, Sahlin S, Einarsson C, Eriksson M, Angelin B, Tomoda H, Omura S, Willingham MC, Rudel LL. ACAT2 is localized to hepatocytes and is the major cholesterol-esterifying enzyme in human liver. *Circulation.* 2004;110:2017-2023.
32. Chang CC, Sakashita N, Ornvold K, Lee O, Chang ET, Dong R, Lin S, Lee CY, Strom SC, Kashyap R, Fung JJ, Farese RV, Jr., Patoiseau JF, Delhon A, Chang TY. Immunological quantitation and localization of ACAT-1 and ACAT-2 in human liver and small intestine. *J Biol Chem.* 2000;275:28083-28092.
33. Meiner V, Tam C, Gunn MD, Dong LM, Weisgraber KH, Novak S, Myers HM, Erickson SK, Farese RV, Jr. Tissue expression studies on the mouse acyl-CoA: cholesterol acyltransferase gene (Acact): findings supporting the existence of multiple cholesterol esterification enzymes in mice. *J Lipid Res.* 1997;38:1928-1933.
34. Anderson RA, Joyce C, Davis M, Reagan JW, Clark M, Shelness GS, Rudel LL. Identification of a form of acyl-CoA:cholesterol acyltransferase specific to liver and intestine in nonhuman primates. *J Biol Chem.* 1998;273:26747-26754.
35. Lee RG, Willingham MC, Davis MA, Skinner KA, Rudel LL. Differential expression of ACAT1 and ACAT2 among cells within liver, intestine, kidney, and adrenal of nonhuman primates. *J Lipid Res.* 2000;41:1991-2001.
36. Lee JY, Carr TP. Dietary fatty acids regulate acyl-CoA:cholesterol acyltransferase and cytosolic cholesteryl ester hydrolase in hamsters. *J Nutr.* 2004;134:3239-3244.

37. Smith JL, Rangaraj K, Simpson R, Maclean DJ, Nathanson LK, Stuart KA, Scott SP, Ramm GA, de Jersey J. Quantitative analysis of the expression of ACAT genes in human tissues by real-time PCR. *J Lipid Res.* 2004;45:686-696.
38. Sakashita N, Miyazaki A, Takeya M, Horiuchi S, Chang CC, Chang TY, Takahashi K. Localization of human acyl-coenzyme A: cholesterol acyltransferase-1 (ACAT-1) in macrophages and in various tissues. *Am J Pathol.* 2000;156:227-236.
39. Sakashita N, Miyazaki A, Chang CC, Chang TY, Kiyota E, Satoh M, Komohara Y, Morganelli PM, Horiuchi S, Takeya M. Acyl-coenzyme A:cholesterol acyltransferase 2 (ACAT2) is induced in monocyte-derived macrophages: in vivo and in vitro studies. *Lab Invest.* 2003;83:1569-1581.
40. Joyce CW, Shelness GS, Davis MA, Lee RG, Skinner K, Anderson RA, Rudel LL. ACAT1 and ACAT2 membrane topology segregates a serine residue essential for activity to opposite sides of the endoplasmic reticulum membrane. *Mol Biol Cell.* 2000;11:3675-3687.
41. Lin S, Cheng D, Liu MS, Chen J, Chang TY. Human acyl-CoA:cholesterol acyltransferase-1 in the endoplasmic reticulum contains seven transmembrane domains. *J Biol Chem.* 1999;274:23276-23285.
42. Lin S, Lu X, Chang CC, Chang TY. Human acyl-coenzyme A:cholesterol acyltransferase expressed in chinese hamster ovary cells: membrane topology and active site location. *Mol Biol Cell.* 2003;14:2447-2460.
43. Cases S, Novak S, Zheng YW, Myers HM, Lear SR, Sande E, Welch CB, Lusis AJ, Spencer TA, Krause BR, Erickson SK, Farese RV, Jr. ACAT-2, a second mammalian acyl-CoA:cholesterol acyltransferase. Its cloning, expression, and characterization. *J Biol Chem.* 1998;273:26755-26764.
44. Joyce C, Skinner K, Anderson RA, Rudel LL. Acyl-coenzyme A:cholesteryl acyltransferase 2. *Curr Opin Lipidol.* 1999;10:89-95.
45. Liang JJ, Oelkers P, Guo C, Chu PC, Dixon JL, Ginsberg HN, Sturley SL. Overexpression of human diacylglycerol acyltransferase 1, acyl-coa:cholesterol acyltransferase 1, or acyl-CoA:cholesterol acyltransferase 2 stimulates secretion of apolipoprotein B-containing lipoproteins in McA-RH7777 cells. *J Biol Chem.* 2004;279:44938-44944.
46. Temel RE, Hou L, Rudel LL, Shelness GS. ACAT2 stimulates cholesteryl ester secretion in apoB-containing lipoproteins. *J Lipid Res.* 2007;48:1618-1627.
47. Rudel LL, Haines J, Sawyer JK, Shah R, Wilson MS, Carr TP. Hepatic origin of cholesteryl oleate in coronary artery atherosclerosis in African green monkeys. Enrichment by dietary monounsaturated fat. *J Clin Invest.* 1997;100:74-83.
48. Araya J, Rodrigo R, Videla LA, Thielemann L, Orellana M, Pettinelli P, Poniachik J. Increase in long-chain polyunsaturated fatty acid n - 6/n - 3 ratio in relation to hepatic steatosis in patients with non-alcoholic fatty liver disease. *Clin Sci (Lond).* 2004;106:635-643.
49. Kirkeby K, Ingvaldsen P, Bjerkedal I. Fatty acid composition of serum lipids in men with myocardial infarction. *Acta Med Scand.* 1972;192:513-519.
50. Kingsbury KJ, Morgan DM, Stovold R, Brett CG, Anderson J. Polyunsaturated fatty acids and myocardial infarction. Follow-up of patients with aortoiliac and femoropopliteal atherosclerosis. *Lancet.* 1969;2:1325-1329.
51. Lawrie TD, McAlpine SG, Rifkind BM, Robinson JF. Serum fatty-acid patterns in coronary-artery disease. *Lancet.* 1961;1:421-424.
52. Warensjo E, Sundstrom J, Vessby B, Cederholm T, Riserus U. Markers of dietary fat quality and fatty acid desaturation as predictors of total and cardiovascular mortality: a population-based prospective study. *Am J Clin Nutr.* 2008;88:203-209.
53. Ma J, Folsom AR, Lewis L, Eckfeldt JH. Relation of plasma phospholipid and cholesterol ester fatty acid composition to carotid artery intima-media thickness: the Atherosclerosis Risk in Communities (ARIC) Study. *Am J Clin Nutr.* 1997;65:551-559.

54. Song BL, Qi W, Yang XY, Chang CC, Zhu JQ, Chang TY, Li BL. Organization of human ACAT-2 gene and its cell-type-specific promoter activity. *Biochem Biophys Res Commun.* 2001;282:580-588.
55. Li BL, Li XL, Duan ZJ, Lee O, Lin S, Ma ZM, Chang CC, Yang XY, Park JP, Mohandas TK, Noll W, Chan L, Chang TY. Human acyl-CoA:cholesterol acyltransferase-1 (ACAT-1) gene organization and evidence that the 4.3-kilobase ACAT-1 mRNA is produced from two different chromosomes. *J Biol Chem.* 1999;274:11060-11071.
56. Yang L, Lee O, Chen J, Chen J, Chang CC, Zhou P, Wang ZZ, Ma HH, Sha HF, Feng JX, Wang Y, Yang XY, Wang L, Dong R, Ornvold K, Li BL, Chang TY. Human acyl-coenzyme A:cholesterol acyltransferase 1 (acat1) sequences located in two different chromosomes (7 and 1) are required to produce a novel ACAT1 isoenzyme with additional sequence at the N terminus. *J Biol Chem.* 2004;279:46253-46262.
57. Chang TY, Li BLP, Chang CC, Urano Y. Acyl-Coenzyme a:Cholesterol Acyltransferases. *Am J Physiol Endocrinol Metab.* 2009.
58. Chang CC, Huh HY, Cadigan KM, Chang TY. Molecular cloning and functional expression of human acyl-coenzyme A:cholesterol acyltransferase cDNA in mutant Chinese hamster ovary cells. *J Biol Chem.* 1993;268:20747-20755.
59. Uelmen PJ, Oka K, Sullivan M, Chang CC, Chang TY, Chan L. Tissue-specific expression and cholesterol regulation of acylcoenzyme A:cholesterol acyltransferase (ACAT) in mice. Molecular cloning of mouse ACAT cDNA, chromosomal localization, and regulation of ACAT in vivo and in vitro. *J Biol Chem.* 1995;270:26192-26201.
60. Katsuren K, Tamura T, Arashiro R, Takata K, Matsuura T, Niikawa N, Ohta T. Structure of the human acyl-CoA:cholesterol acyltransferase-2 (ACAT-2) gene and its relation to dyslipidemia. *Biochim Biophys Acta.* 2001;1531:230-240.
61. Lei L, Xiong Y, Chen J, Yang JB, Wang Y, Yang XY, Chang CC, Song BL, Chang TY, Li BL. TNF-alpha stimulates the ACAT1 expression in differentiating monocytes to promote the CE-laden cell formation. *J Lipid Res.* 2009.
62. Song BL, Qi W, Wang CH, Yang JB, Yang XY, Lin ZX, Li BL. Preparation of an anti-Cdx-2 antibody for analysis of different species Cdx-2 binding to acat2 promoter. *Sheng Wu Hua Xue Yu Sheng Wu Wu Li Xue Bao (Shanghai).* 2003;35:6-12.
63. Song BL, Wang CH, Yao XM, Yang L, Zhang WJ, Wang ZZ, Zhao XN, Yang JB, Qi W, Yang XY, Inoue K, Lin ZX, Zhang HZ, Kodama T, Chang CC, Liu YK, Chang TY, Li BL. Human acyl-CoA:cholesterol acyltransferase 2 gene expression in intestinal Caco-2 cells and in hepatocellular carcinoma. *Biochem J.* 2006;394:617-626.
64. Plump AS, Smith JD, Hayek T, Aalto-Setala K, Walsh A, Verstuyft JG, Rubin EM, Breslow JL. Severe hypercholesterolemia and atherosclerosis in apolipoprotein E-deficient mice created by homologous recombination in ES cells. *Cell.* 1992;71:343-353.
65. Knowles JW, Maeda N. Genetic modifiers of atherosclerosis in mice. *Arterioscler Thromb Vasc Biol.* 2000;20:2336-2345.
66. Accad M, Smith SJ, Newland DL, Sanan DA, King LE, Jr., Linton MF, Fazio S, Farese RV, Jr. Massive xanthomatosis and altered composition of atherosclerotic lesions in hyperlipidemic mice lacking acyl CoA:cholesterol acyltransferase 1. *J Clin Invest.* 2000;105:711-719.
67. Fazio S, Major AS, Swift LL, Gleaves LA, Accad M, Linton MF, Farese RV, Jr. Increased atherosclerosis in LDL receptor-null mice lacking ACAT1 in macrophages. *J Clin Invest.* 2001;107:163-171.
68. Yagyu H, Kitamine T, Osuga J, Tozawa R, Chen Z, Kaji Y, Oka T, Perrey S, Tamura Y, Ohashi K, Okazaki H, Yahagi N, Shionoiri F, Iizuka Y, Harada K, Shimano H, Yamashita H, Gotoda T, Yamada N, Ishibashi S. Absence of ACAT-1 attenuates atherosclerosis but causes dry eye and cutaneous xanthomatosis in mice with congenital hyperlipidemia. *J Biol Chem.* 2000;275:21324-21330.

69. Su YR, Dove DE, Major AS, Hasty AH, Boone B, Linton MF, Fazio S. Reduced ABCA1-mediated cholesterol efflux and accelerated atherosclerosis in apolipoprotein E-deficient mice lacking macrophage-derived ACAT1. *Circulation.* 2005;111:2373-2381.
70. Willner EL, Tow B, Buhman KK, Wilson M, Sanan DA, Rudel LL, Farese RV, Jr. Deficiency of acyl CoA:cholesterol acyltransferase 2 prevents atherosclerosis in apolipoprotein E-deficient mice. *Proc Natl Acad Sci U S A.* 2003;100:1262-1267.
71. Bell TA, 3rd, Kelley K, Wilson MD, Sawyer JK, Rudel LL. Dietary fat-induced alterations in atherosclerosis are abolished by ACAT2-deficiency in ApoB100 only, LDLr-/- mice. *Arterioscler Thromb Vasc Biol.* 2007;27:1396-1402.
72. Lee RG, Kelley KL, Sawyer JK, Farese RV, Jr., Parks JS, Rudel LL. Plasma cholesteryl esters provided by lecithin:cholesterol acyltransferase and acyl-coenzyme a:cholesterol acyltransferase 2 have opposite atherosclerotic potential. *Circ Res.* 2004;95:998-1004.
73. Lambert G, Sakai N, Vaisman BL, Neufeld EB, Marteyn B, Chan CC, Paigen B, Lupia E, Thomas A, Striker LJ, Blanchette-Mackie J, Csako G, Brady JN, Costello R, Striker GE, Remaley AT, Brewer HB, Jr., Santamarina-Fojo S. Analysis of glomerulosclerosis and atherosclerosis in lecithin cholesterol acyltransferase-deficient mice. *J Biol Chem.* 2001;276:15090-15098.
74. Lee RG, Shah R, Sawyer JK, Hamilton RL, Parks JS, Rudel LL. ACAT2 contributes cholesteryl esters to newly secreted VLDL, whereas LCAT adds cholesteryl ester to LDL in mice. *J Lipid Res.* 2005;46:1205-1212.
75. Yu L, Hammer RE, Li-Hawkins J, Von Bergmann K, Lutjohann D, Cohen JC, Hobbs HH. Disruption of Abcg5 and Abcg8 in mice reveals their crucial role in biliary cholesterol secretion. *Proc Natl Acad Sci U S A.* 2002;99:16237-16242.
76. Rudel LL, Parks JS, Sawyer JK. Compared with dietary monounsaturated and saturated fat, polyunsaturated fat protects African green monkeys from coronary artery atherosclerosis. *Arterioscler Thromb Vasc Biol.* 1995;15:2101-2110.
77. Chang TY, Chang CC, Lu X, Lin S. Catalysis of ACAT may be completed within the plane of the membrane: a working hypothesis. *J Lipid Res.* 2001;42:1933-1938.
78. Chang CC, Lee CY, Chang ET, Cruz JC, Levesque MC, Chang TY. Recombinant acyl-CoA:cholesterol acyltransferase-1 (ACAT-1) purified to essential homogeneity utilizes cholesterol in mixed micelles or in vesicles in a highly cooperative manner. *J Biol Chem.* 1998;273:35132-35141.
79. Matsuda H, Hakamata H, Miyazaki A, Sakai M, Chang CC, Chang TY, Kobori S, Shichiri M, Horiuchi S. Activation of acyl-coenzyme A:cholesterol acyltransferase activity by cholesterol is not due to altered mRNA levels in HepG2 cells. *Biochim Biophys Acta.* 1996;1301:76-84.
80. Seo T, Oelkers PM, Giattina MR, Worgall TS, Sturley SL, Deckelbaum RJ. Differential modulation of ACAT1 and ACAT2 transcription and activity by long chain free fatty acids in cultured cells. *Biochemistry.* 2001;40:4756-4762.
81. Rudel LL, Davis M, Sawyer J, Shah R, Wallace J. Primates highly responsive to dietary cholesterol up-regulate hepatic ACAT2, and less responsive primates do not. *J Biol Chem.* 2002;277:31401-31406.
82. Roberts CK, Liang K, Barnard RJ, Kim CH, Vaziri ND. HMG-CoA reductase, cholesterol 7alpha-hydroxylase, LDL receptor, SR-B1, and ACAT in diet-induced syndrome X. *Kidney Int.* 2004;66:1503-1511.
83. Parini P, Gustafsson U, Davis MA, Larsson L, Einarsson C, Wilson M, Rudling M, Tomoda H, Omura S, Sahlin S, Angelin B, Rudel LL, Eriksson M. Cholesterol synthesis inhibition elicits an integrated molecular response in human livers including decreased ACAT2. *Arterioscler Thromb Vasc Biol.* 2008;28:1200-1206.
84. Wilson MD, Rudel LL. Review of cholesterol absorption with emphasis on dietary and biliary cholesterol. *J Lipid Res.* 1994;35:943-955.
85. Heinemann T, Axtmann G, von Bergmann K. Comparison of intestinal absorption of cholesterol with different plant sterols in man. *Eur J Clin Invest.* 1993;23:827-831.

86. Temel RE, Gebre AK, Parks JS, Rudel LL. Compared with Acyl-CoA:cholesterol O-acyltransferase (ACAT) 1 and lecithin:cholesterol acyltransferase, ACAT2 displays the greatest capacity to differentiate cholesterol from sitosterol. *J Biol Chem.* 2003;278:47594-47601.
87. Bose C, Bhuvaneswaran C, Udupa KB. Age-related alteration in hepatic acyl-CoA: cholesterol acyltransferase and its relation to LDL receptor and MAPK. *Mech Ageing Dev.* 2005;126:740-751.
88. Jiang ZY, Jiang CY, Wang L, Wang JC, Zhang SD, Einarsson C, Eriksson M, Han TQ, Parini P, Eggertsen G. Increased NPC1L1 and ACAT2 expression in the jejunal mucosa from Chinese gallstone patients. *Biochem Biophys Res Commun.* 2009;379:49-54.
89. Parini P, Jiang ZY, Einarsson C, Eggertsen G, Zhang SD, Rudel LL, Han TQ, Eriksson M. ACAT2 and human hepatic cholesterol metabolism: Identification of important gender-related differences in normolipidemic, non-obese Chinese patients. *Atherosclerosis.* 2009.
90. Tomoda H, Omura S. Potential therapeutics for obesity and atherosclerosis: inhibitors of neutral lipid metabolism from microorganisms. *Pharmacol Ther.* 2007;115:375-389.
91. Das A, Davis MA, Tomoda H, Omura S, Rudel LL. Identification of the interaction site within acyl-CoA:cholesterol acyltransferase 2 for the isoform-specific inhibitor pyripyropene A. *J Biol Chem.* 2008;283:10453-10460.
92. Lada AT, Davis M, Kent C, Chapman J, Tomoda H, Omura S, Rudel LL. Identification of ACAT1- and ACAT2-specific inhibitors using a novel, cell-based fluorescence assay: individual ACAT uniqueness. *J Lipid Res.* 2004;45:378-386.
93. Meuwese MC, de Groot E, Duivenvoorden R, Trip MD, Ose L, Maritz FJ, Basart DC, Kastelein JJ, Habib R, Davidson MH, Zwinderman AH, Schwocho LR, Stein EA. ACAT inhibition and progression of carotid atherosclerosis in patients with familial hypercholesterolemia: the CAPTIVATE randomized trial. *Jama.* 2009;301:1131-1139.
94. Nissen SE, Tuzcu EM, Brewer HB, Sipahi I, Nicholls SJ, Ganz P, Schoenhagen P, Waters DD, Pepine CJ, Crowe TD, Davidson MH, Deanfield JE, Wisniewski LM, Hanyok JJ, Kassalow LM. Effect of ACAT inhibition on the progression of coronary atherosclerosis. *N Engl J Med.* 2006;354:1253-1263.
95. Tardif JC, Gregoire J, L'Allier PL, Anderson TJ, Bertrand O, Reeves F, Title LM, Alfonso F, Schampaert E, Hassan A, McLain R, Pressler ML, Ibrahim R, Lesperance J, Blue J, Heinonen T, Rodes-Cabau J. Effects of the acyl coenzyme A:cholesterol acyltransferase inhibitor avasimibe on human atherosclerotic lesions. *Circulation.* 2004;110:3372-3377.
96. Rudel LL, Lee RG, Parini P. ACAT2 is a target for treatment of coronary heart disease associated with hypercholesterolemia. *Arterioscler Thromb Vasc Biol.* 2005;25:1112-1118.
97. Stein O, Stein Y. Lipid transfer proteins (LTP) and atherosclerosis. *Atherosclerosis.* 2005;178:217-230.
98. Gardner-Stephen DA, Gregory PA, Mackenzie PI. Identification and characterization of functional hepatocyte nuclear factor 1-binding sites in UDP-glucuronosyltransferase genes. *Methods Enzymol.* 2005;400:22-46.
99. Mendel DB, Hansen LP, Graves MK, Conley PB, Crabtree GR. HNF-1 alpha and HNF-1 beta (vHNF-1) share dimerization and homeo domains, but not activation domains, and form heterodimers in vitro. *Genes Dev.* 1991;5:1042-1056.
100. Ryffel GU. Mutations in the human genes encoding the transcription factors of the hepatocyte nuclear factor (HNF)1 and HNF4 families: functional and pathological consequences. *J Mol Endocrinol.* 2001;27:11-29.
101. Schrem H, Klempnauer J, Borlak J. Liver-enriched transcription factors in liver function and development. Part I: the hepatocyte nuclear factor network and liver-specific gene expression. *Pharmacol Rev.* 2002;54:129-158.
102. Pontoglio M. Hepatocyte nuclear factor 1, a transcription factor at the crossroads of glucose homeostasis. *J Am Soc Nephrol.* 2000;11 Suppl 16:S140-143.

103. Kuo CJ, Conley PB, Chen L, Sladek FM, Darnell JE, Jr., Crabtree GR. A transcriptional hierarchy involved in mammalian cell-type specification. *Nature.* 1992;355:457-461.
104. Power SC, Cereghini S. Positive regulation of the vHNF1 promoter by the orphan receptors COUP-TF1/Ear3 and COUP-TFII/Arp1. *Mol Cell Biol.* 1996;16:778-791.
105. Ktistaki E, Talianidis I. Modulation of hepatic gene expression by hepatocyte nuclear factor 1. *Science.* 1997;277:109-112.
106. Chiang JY, Kimmel R, Stroup D. Regulation of cholesterol 7alpha-hydroxylase gene (CYP7A1) transcription by the liver orphan receptor (LXRalpha). *Gene.* 2001;262:257-265.
107. Chen J, Cooper AD, Levy-Wilson B. Hepatocyte nuclear factor 1 binds to and transactivates the human but not the rat CYP7A1 promoter. *Biochem Biophys Res Commun.* 1999;260:829-834.
108. Mitchelmore C, Troelsen JT, Spodsberg N, Sjostrom H, Noren O. Interaction between the homeodomain proteins Cdx2 and HNF1alpha mediates expression of the lactase-phlorizin hydrolase gene. *Biochem J.* 2000;346 Pt 2:529-535.
109. Odom DT, Zizlsperger N, Gordon DB, Bell GW, Rinaldi NJ, Murray HL, Volkert TL, Schreiber J, Rolfe PA, Gifford DK, Fraenkel E, Bell GI, Young RA. Control of pancreas and liver gene expression by HNF transcription factors. *Science.* 2004;303:1378-1381.
110. Coffinier C, Thepot D, Babinet C, Yaniv M, Barra J. Essential role for the homeoprotein vHNF1/HNF1beta in visceral endoderm differentiation. *Development.* 1999;126:4785-4794.
111. Barbacci E, Reber M, Ott MO, Breillat C, Huetz F, Cereghini S. Variant hepatocyte nuclear factor 1 is required for visceral endoderm specification. *Development.* 1999;126:4795-4805.
112. Coffinier C, Gresh L, Fiette L, Tronche F, Schutz G, Babinet C, Pontoglio M, Yaniv M, Barra J. Bile system morphogenesis defects and liver dysfunction upon targeted deletion of HNF1beta. *Development.* 2002;129:1829-1838.
113. Shih DQ, Bussen M, Sehayek E, Ananthanarayanan M, Shneider BL, Suchy FJ, Shefer S, Bollileni JS, Gonzalez FJ, Breslow JL, Stoffel M. Hepatocyte nuclear factor-1alpha is an essential regulator of bile acid and plasma cholesterol metabolism. *Nat Genet.* 2001;27:375-382.
114. Pontoglio M, Sreenan S, Roe M, Pugh W, Ostrega D, Doyen A, Pick AJ, Baldwin A, Velho G, Froguel P, Levisetti M, Bonner-Weir S, Bell GI, Yaniv M, Polonsky KS. Defective insulin secretion in hepatocyte nuclear factor 1alpha-deficient mice. *J Clin Invest.* 1998;101:2215-2222.
115. Hertz R, Magenheim J, Berman I, Bar-Tana J. Fatty acyl-CoA thioesters are ligands of hepatic nuclear factor-4alpha. *Nature.* 1998;392:512-516.
116. Tanaka T, Jiang S, Hotta H, Takano K, Iwanari H, Sumi K, Daigo K, Ohashi R, Sugai M, Ikegame C, Umezu H, Hirayama Y, Midorikawa Y, Hippo Y, Watanabe A, Uchiyama Y, Hasegawa G, Reid P, Aburatani H, Hamakubo T, Sakai J, Naito M, Kodama T. Dysregulated expression of P1 and P2 promoter-driven hepatocyte nuclear factor-4alpha in the pathogenesis of human cancer. *J Pathol.* 2006;208:662-672.
117. Drewes T, Senkel S, Holewa B, Ryffel GU. Human hepatocyte nuclear factor 4 isoforms are encoded by distinct and differentially expressed genes. *Mol Cell Biol.* 1996;16:925-931.
118. Holewa B, Zapp D, Drewes T, Senkel S, Ryffel GU. HNF4beta, a new gene of the HNF4 family with distinct activation and expression profiles in oogenesis and embryogenesis of Xenopus laevis. *Mol Cell Biol.* 1997;17:687-694.
119. Eeckhoute J, Moerman E, Bouckenooghe T, Lukoviak B, Pattou F, Formstecher P, Kerr-Conte J, Vandewalle B, Laine B. Hepatocyte nuclear factor 4 alpha isoforms originated from the P1 promoter are expressed in human pancreatic beta-cells and exhibit stronger transcriptional potentials than P2 promoter-driven isoforms. *Endocrinology.* 2003;144:1686-1694.

120. Boj SF, Parrizas M, Maestro MA, Ferrer J. A transcription factor regulatory circuit in differentiated pancreatic cells. *Proc Natl Acad Sci U S A.* 2001;98:14481-14486.
121. Jiang S, Tanaka T, Iwanari H, Hotta H, Yamashita H, Kumakura J, Watanabe Y, Uchiyama Y, Aburatani H, Hamakubo T, Kodama T, Naito M. Expression and localization of P1 promoter-driven hepatocyte nuclear factor-4alpha (HNF4alpha) isoforms in human and rats. *Nucl Recept.* 2003;1:5.
122. Thomas H, Jaschkowitz K, Bulman M, Frayling TM, Mitchell SM, Roosen S, Lingott-Frieg A, Tack CJ, Ellard S, Ryffel GU, Hattersley AT. A distant upstream promoter of the HNF-4alpha gene connects the transcription factors involved in maturity-onset diabetes of the young. *Hum Mol Genet.* 2001;10:2089-2097.
123. Hirokane H, Nakahara M, Tachibana S, Shimizu M, Sato R. Bile acid reduces the secretion of very low density lipoprotein by repressing microsomal triglyceride transfer protein gene expression mediated by hepatocyte nuclear factor-4. *J Biol Chem.* 2004;279:45685-45692.
124. Misawa K, Horiba T, Arimura N, Hirano Y, Inoue J, Emoto N, Shimano H, Shimizu M, Sato R. Sterol regulatory element-binding protein-2 interacts with hepatocyte nuclear factor-4 to enhance sterol isomerase gene expression in hepatocytes. *J Biol Chem.* 2003;278:36176-36182.
125. Iwayanagi Y, Takada T, Suzuki H. HNF4alpha is a crucial modulator of the cholesterol-dependent regulation of NPC1L1. *Pharm Res.* 2008;25:1134-1141.
126. Chen WS, Manova K, Weinstein DC, Duncan SA, Plump AS, Prezioso VR, Bachvarova RF, Darnell JE, Jr. Disruption of the HNF-4 gene, expressed in visceral endoderm, leads to cell death in embryonic ectoderm and impaired gastrulation of mouse embryos. *Genes Dev.* 1994;8:2466-2477.
127. Hayhurst GP, Lee YH, Lambert G, Ward JM, Gonzalez FJ. Hepatocyte nuclear factor 4alpha (nuclear receptor 2A1) is essential for maintenance of hepatic gene expression and lipid homeostasis. *Mol Cell Biol.* 2001;21:1393-1403.
128. Stoffel M, Duncan SA. The maturity-onset diabetes of the young (MODY1) transcription factor HNF4alpha regulates expression of genes required for glucose transport and metabolism. *Proc Natl Acad Sci U S A.* 1997;94:13209-13214.
129. Fajans SS, Bell GI, Polonsky KS. Molecular mechanisms and clinical pathophysiology of maturity-onset diabetes of the young. *N Engl J Med.* 2001;345:971-980.
130. Yamagata K, Furuta H, Oda N, Kaisaki PJ, Menzel S, Cox NJ, Fajans SS, Signorini S, Stoffel M, Bell GI. Mutations in the hepatocyte nuclear factor-4alpha gene in maturity-onset diabetes of the young (MODY1). *Nature.* 1996;384:458-460.
131. Yamagata K, Oda N, Kaisaki PJ, Menzel S, Furuta H, Vaxillaire M, Southam L, Cox RD, Lathrop GM, Boriraj VV, Chen X, Cox NJ, Oda Y, Yano H, Le Beau MM, Yamada S, Nishigori H, Takeda J, Fajans SS, Hattersley AT, Iwasaki N, Hansen T, Pedersen O, Polonsky KS, Bell GI, et al. Mutations in the hepatocyte nuclear factor-1alpha gene in maturity-onset diabetes of the young (MODY3). *Nature.* 1996;384:455-458.
132. Karlsson E, Groop L. [MODY--an autosomal dominant type of diabetes. Diagnosis via genetic testing results in better treatment]. *Lakartidningen.* 2007;104:2600-2602.
133. Servitja JM, Ferrer J. Transcriptional networks controlling pancreatic development and beta cell function. *Diabetologia.* 2004;47:597-613.
134. Giuffrida FM, Reis AF. Genetic and clinical characteristics of maturity-onset diabetes of the young. *Diabetes Obes Metab.* 2005;7:318-326.
135. Johansen A, Ek J, Mortensen HB, Pedersen O, Hansen T. Half of clinically defined maturity-onset diabetes of the young patients in Denmark do not have mutations in HNF4A, GCK, and TCF1. *J Clin Endocrinol Metab.* 2005;90:4607-4614.
136. Pearson ER, Pruhova S, Tack CJ, Johansen A, Castleden HA, Lumb PJ, Wierzbicki AS, Clark PM, Lebl J, Pedersen O, Ellard S, Hansen T, Hattersley AT. Molecular genetics and phenotypic characteristics of MODY caused by

hepatocyte nuclear factor 4alpha mutations in a large European collection. *Diabetologia.* 2005;48:878-885.
137. Murphy R, Ellard S, Hattersley AT. Clinical implications of a molecular genetic classification of monogenic beta-cell diabetes. *Nat Clin Pract Endocrinol Metab.* 2008;4:200-213.
138. Lehto M, Bitzen PO, Isomaa B, Wipemo C, Wessman Y, Forsblom C, Tuomi T, Taskinen MR, Groop L. Mutation in the HNF-4alpha gene affects insulin secretion and triglyceride metabolism. *Diabetes.* 1999;48:423-425.
139. Lindner T, Gragnoli C, Furuta H, Cockburn BN, Petzold C, Rietzsch H, Weiss U, Schulze J, Bell GI. Hepatic function in a family with a nonsense mutation (R154X) in the hepatocyte nuclear factor-4alpha/MODY1 gene. *J Clin Invest.* 1997;100:1400-1405.
140. Eeckhoute J, Formstecher P, Laine B. Maturity-onset diabetes of the young Type 1 (MODY1)-associated mutations R154X and E276Q in hepatocyte nuclear factor 4alpha (HNF4alpha) gene impair recruitment of p300, a key transcriptional co-activator. *Mol Endocrinol.* 2001;15:1200-1210.
141. Shih DQ, Dansky HM, Fleisher M, Assmann G, Fajans SS, Stoffel M. Genotype/phenotype relationships in HNF-4alpha/MODY1: haploinsufficiency is associated with reduced apolipoprotein (AII), apolipoprotein (CIII), lipoprotein(a), and triglyceride levels. *Diabetes.* 2000;49:832-837.
142. Bellanne-Chantelot C, Carette C, Riveline JP, Valero R, Gautier JF, Larger E, Reznik Y, Ducluzeau PH, Sola A, Hartemann-Heurtier A, Lecomte P, Chaillous L, Laloi-Michelin M, Wilhem JM, Cuny P, Duron F, Guerci B, Jeandidier N, Mosnier-Pudar H, Assayag M, Dubois-Laforgue D, Velho G, Timsit J. The type and the position of HNF1A mutation modulate age at diagnosis of diabetes in patients with maturity-onset diabetes of the young (MODY)-3. *Diabetes.* 2008;57:503-508.
143. Richter S, Shih DQ, Pearson ER, Wolfrum C, Fajans SS, Hattersley AT, Stoffel M. Regulation of apolipoprotein M gene expression by MODY3 gene hepatocyte nuclear factor-1alpha: haploinsufficiency is associated with reduced serum apolipoprotein M levels. *Diabetes.* 2003;52:2989-2995.
144. Isomaa B, Henricsson M, Lehto M, Forsblom C, Karanko S, Sarelin L, Haggblom M, Groop L. Chronic diabetic complications in patients with MODY3 diabetes. *Diabetologia.* 1998;41:467-473.
145. Raile K, Klopocki E, Holder M, Wessel T, Galler A, Deiss D, Muller D, Riebel T, Horn D, Maringa M, Weber J, Ullmann R, Gruters A. Expanded clinical spectrum in hepatocyte nuclear factor 1b-maturity-onset diabetes of the young. *J Clin Endocrinol Metab.* 2009;94:2658-2664.
146. Scott C, Ioannou YA. The NPC1 protein: structure implies function. *Biochim Biophys Acta.* 2004;1685:8-13.
147. Vanier MT, Rodriguez-Lafrasse C, Rousson R, Gazzah N, Juge MC, Pentchev PG, Revol A, Louisot P. Type C Niemann-Pick disease: spectrum of phenotypic variation in disruption of intracellular LDL-derived cholesterol processing. *Biochim Biophys Acta.* 1991;1096:328-337.
148. Davies JP, Levy B, Ioannou YA. Evidence for a Niemann-pick C (NPC) gene family: identification and characterization of NPC1L1. *Genomics.* 2000;65:137-145.
149. Davies JP, Scott C, Oishi K, Liapis A, Ioannou YA. Inactivation of NPC1L1 causes multiple lipid transport defects and protects against diet-induced hypercholesterolemia. *J Biol Chem.* 2005;280:12710-12720.
150. Sane AT, Sinnett D, Delvin E, Bendayan M, Marcil V, Menard D, Beaulieu JF, Levy E. Localization and role of NPC1L1 in cholesterol absorption in human intestine. *J Lipid Res.* 2006;47:2112-2120.
151. Heimerl S, Langmann T, Moehle C, Mauerer R, Dean M, Beil FU, von Bergmann K, Schmitz G. Mutations in the human ATP-binding cassette transporters ABCG5 and ABCG8 in sitosterolemia. *Hum Mutat.* 2002;20:151.

152. Tang W, Ma Y, Jia L, Ioannou YA, Davies JP, Yu L. Genetic inactivation of NPC1L1 protects against sitosterolemia in mice lacking ABCG5/ABCG8. *J Lipid Res.* 2009;50:293-300.
153. Huff MW, Pollex RL, Hegele RA. NPC1L1: evolution from pharmacological target to physiological sterol transporter. *Arterioscler Thromb Vasc Biol.* 2006;26:2433-2438.
154. Altmann SW, Davis HR, Jr., Zhu LJ, Yao X, Hoos LM, Tetzloff G, Iyer SP, Maguire M, Golovko A, Zeng M, Wang L, Murgolo N, Graziano MP. Niemann-Pick C1 Like 1 protein is critical for intestinal cholesterol absorption. *Science.* 2004;303:1201-1204.
155. 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. *Arterioscler Thromb Vasc Biol.* 2007;27:841-849.
156. Telford DE, Sutherland BG, Edwards JY, Andrews JD, Barrett PH, Huff MW. The molecular mechanisms underlying the reduction of LDL apoB-100 by ezetimibe plus simvastatin. *J Lipid Res.* 2007;48:699-708.
157. Temel RE, Tang W, Ma Y, Rudel LL, Willingham MC, Ioannou YA, Davies JP, Nilsson LM, Yu L. Hepatic Niemann-Pick C1-like 1 regulates biliary cholesterol concentration and is a target of ezetimibe. *J Clin Invest.* 2007;117:1968-1978.
158. Yu L, Bharadwaj S, Brown JM, Ma Y, Du W, Davis MA, Michaely P, Liu P, Willingham MC, Rudel LL. Cholesterol-regulated translocation of NPC1L1 to the cell surface facilitates free cholesterol uptake. *J Biol Chem.* 2006;281:6616-6624.
159. Brown JM, Rudel LL, Yu L. NPC1L1 (Niemann-Pick C1-like 1) mediates sterol-specific unidirectional transport of non-esterified cholesterol in McArdle-RH7777 hepatoma cells. *Biochem J.* 2007;406:273-283.
160. Alrefai WA, Annaba F, Sarwar Z, Dwivedi A, Saksena S, Singla A, Dudeja PK, Gill RK. Modulation of human Niemann-Pick C1-like 1 gene expression by sterol: Role of sterol regulatory element binding protein 2. *Am J Physiol Gastrointest Liver Physiol.* 2007;292:G369-376.
161. Lally S, Tan CY, Owens D, Tomkin GH. Messenger RNA levels of genes involved in dysregulation of postprandial lipoproteins in type 2 diabetes: the role of Niemann-Pick C1-like 1, ATP-binding cassette, transporters G5 and G8, and of microsomal triglyceride transfer protein. *Diabetologia.* 2006;49:1008-1016.
162. Stamatatos L, Leventis R, Zuckermann MJ, Silvius JR. Interactions of cationic lipid vesicles with negatively charged phospholipid vesicles and biological membranes. *Biochemistry.* 1988;27:3917-3925.
163. Zabner J, Fasbender AJ, Moninger T, Poellinger KA, Welsh MJ. Cellular and molecular barriers to gene transfer by a cationic lipid. *J Biol Chem.* 1995;270:18997-19007.
164. Wrobel I, Collins D. Fusion of cationic liposomes with mammalian cells occurs after endocytosis. *Biochim Biophys Acta.* 1995;1235:296-304.
165. El Ouahabi A, Thiry M, Pector V, Fuks R, Ruysschaert JM, Vandenbranden M. The role of endosome destabilizing activity in the gene transfer process mediated by cationic lipids. *FEBS Lett.* 1997;414:187-192.
166. Wattiaux R, Jadot M, Warnier-Pirotte MT, Wattiaux-De Coninck S. Cationic lipids destabilize lysosomal membrane in vitro. *FEBS Lett.* 1997;417:199-202.
167. Hui SW, Langner M, Zhao YL, Ross P, Hurley E, Chan K. The role of helper lipids in cationic liposome-mediated gene transfer. *Biophys J.* 1996;71:590-599.
168. Zuidam NJ, Hirsch-Lerner D, Margulies S, Barenholz Y. Lamellarity of cationic liposomes and mode of preparation of lipoplexes affect transfection efficiency. *Biochim Biophys Acta.* 1999;1419:207-220.
169. Parini P, Johansson L, Broijersen A, Angelin B, Rudling M. Lipoprotein profiles in plasma and interstitial fluid analyzed with an automated gel-filtration system. *Eur J Clin Invest.* 2006;36:98-104.
170. Hengstler JG, Brulport M, Schormann W, Bauer A, Hermes M, Nussler AK, Fandrich F, Ruhnke M, Ungefroren H, Griffin L, Bockamp E, Oesch F, von Mach MA. Generation of human hepatocytes by stem cell technology: definition of the hepatocyte. *Expert Opin Drug Metab Toxicol.* 2005;1:61-74.

171. Novik EI, Maguire TJ, Orlova K, Schloss RS, Yarmush ML. Embryoid body-mediated differentiation of mouse embryonic stem cells along a hepatocyte lineage: insights from gene expression profiles. *Tissue Eng.* 2006;12:1515-1525.
172. Duffy AA, Martin MM, Elton TS. Transcriptional regulation of the AT1 receptor gene in immortalized human trophoblast cells. *Biochim Biophys Acta.* 2004;1680:158-170.
173. Auyeung DJ, Kessler FK, Ritter JK. Differential regulation of alternate UDP-glucuronosyltransferase 1A6 gene promoters by hepatic nuclear factor-1. *Toxicol Appl Pharmacol.* 2003;191:156-166.
174. Hatzis P, Talianidis I. Regulatory mechanisms controlling human hepatocyte nuclear factor 4alpha gene expression. *Mol Cell Biol.* 2001;21:7320-7330.
175. Bernard P, Goudonnet H, Artur Y, Desvergne B, Wahli W. Activation of the mouse TATA-less and human TATA-containing UDP-glucuronosyltransferase 1A1 promoters by hepatocyte nuclear factor 1. *Mol Pharmacol.* 1999;56:526-536.
176. Rowley CW, Staloch LJ, Divine JK, McCaul SP, Simon TC. Mechanisms of mutual functional interactions between HNF-4alpha and HNF-1alpha revealed by mutations that cause maturity onset diabetes of the young. *Am J Physiol Gastrointest Liver Physiol.* 2006;290:G466-475.
177. Pontoglio M, Barra J, Hadchouel M, Doyen A, Kress C, Bach JP, Babinet C, Yaniv M. Hepatocyte nuclear factor 1 inactivation results in hepatic dysfunction, phenylketonuria, and renal Fanconi syndrome. *Cell.* 1996;84:575-585.
178. Hani EH, Suaud L, Boutin P, Chevre JC, Durand E, Philippi A, Demenais F, Vionnet N, Furuta H, Velho G, Bell GI, Laine B, Froguel P. A missense mutation in hepatocyte nuclear factor-4 alpha, resulting in a reduced transactivation activity, in human late-onset non-insulin-dependent diabetes mellitus. *J Clin Invest.* 1998;101:521-526.
179. Navas MA, Munoz-Elias EJ, Kim J, Shih D, Stoffel M. Functional characterization of the MODY1 gene mutations HNF4(R127W), HNF4(V255M), and HNF4(E276Q). *Diabetes.* 1999;48:1459-1465.
180. Divine JK, McCaul SP, Simon TC. HNF-1alpha and endodermal transcription factors cooperatively activate Fabpl: MODY3 mutations abrogate cooperativity. *Am J Physiol Gastrointest Liver Physiol.* 2003;285:G62-72.
181. Eeckhoute J, Formstecher P, Laine B. Hepatocyte nuclear factor 4alpha enhances the hepatocyte nuclear factor 1alpha-mediated activation of transcription. *Nucleic Acids Res.* 2004;32:2586-2593.
182. Kritis AA, Ktistaki E, Barda D, Zannis VI, Talianidis I. An indirect negative autoregulatory mechanism involved in hepatocyte nuclear factor-1 gene expression. *Nucleic Acids Res.* 1993;21:5882-5889.
183. Glomset JA. The plasma lecithins:cholesterol acyltransferase reaction. *J Lipid Res.* 1968;9:155-167.
184. Kempen HJ, Glatz JF, Gevers Leuven JA, van der Voort HA, Katan MB. Serum lathosterol concentration is an indicator of whole-body cholesterol synthesis in humans. *J Lipid Res.* 1988;29:1149-1155.
185. Gylling H, Miettinen TA. Cholesterol absorption, synthesis, and LDL metabolism in NIDDM. *Diabetes Care.* 1997;20:90-95.
186. Galman C, Arvidsson I, Angelin B, Rudling M. Monitoring hepatic cholesterol 7alpha-hydroxylase activity by assay of the stable bile acid intermediate 7alpha-hydroxy-4-cholesten-3-one in peripheral blood. *J Lipid Res.* 2003;44:859-866.
187. Duran-Sandoval D, Mautino G, Martin G, Percevault F, Barbier O, Fruchart JC, Kuipers F, Staels B. Glucose regulates the expression of the farnesoid X receptor in liver. *Diabetes.* 2004;53:890-898.
188. Zhang Y, Lee FY, Barrera G, Lee H, Vales C, Gonzalez FJ, Willson TM, Edwards PA. Activation of the nuclear receptor FXR improves hyperglycemia and hyperlipidemia in diabetic mice. *Proc Natl Acad Sci U S A.* 2006;103:1006-1011.
189. Nagamine T, Hayakawa K, Kusakabe T, Takada H, Nakazato K, Hisanaga E, Iha M. Inhibitory effect of fucoidan on Huh7 hepatoma cells through downregulation of CXCL12. *Nutr Cancer.* 2009;61:340-347.

190. Pin RH, Reinblatt M, Fong Y. Utilizing alpha-fetoprotein expression to enhance oncolytic viral therapy in hepatocellular carcinoma. *Ann Surg.* 2004;240:659-665; discussion 665-656.
191. Sell S. Alpha-fetoprotein, stem cells and cancer: how study of the production of alpha-fetoprotein during chemical hepatocarcinogenesis led to reaffirmation of the stem cell theory of cancer. *Tumour Biol.* 2008;29:161-180.