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STUDIES ON CHOLESTEROL HOMEOSTASIS BY MODULATION OF LIPOPROTEINS AND BILE ACIDS

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ABSTRACT

Excess plasma cholesterol, in particular high levels of LDL cholesterol, is a major risk factor for the development of atherosclerosis. The liver has a central role in the regulation of cholesterol metabolism: i) a major part of the cholesterol present in the body is synthesized in the liver; ii) the level of plasma lipoproteins is regulated by liver uptake and secretion of these particles and iii) the liver is essential for elimination of cholesterol from the body as such or by its conversion into bile acids.
In this thesis we have investigated how different modifications of lipoproteins and bile acids affect cholesterol metabolism both *in vivo* and *in vitro*.

Stimulation and inhibition of bile acid synthesis was studied in patients treated with cholestyramine or chenodeoxycholic acid (CDCA). Following stimulation of bile acid synthesis with cholestyramine the mRNA levels for HNF4α were upregulated and positively correlated to the expression levels of CYP7A1, the rate limiting enzyme in bile acid synthesis. The results were further supported by the observation that CDCA treatment of primary human hepatocytes reduces the expression of HNF4α. From this study we can conclude that HNF4α appears to be a key factor in the regulation of bile acid formation in humans.

When bile acid synthesis is altered, the metabolism of cholesterol and lipoproteins is also affected. Following cholestyramine treatment the mRNA expression of LDLR and PCSK9 increased and in the opposite situation when patients were treated with CDCA, a reduction of LDLR and HMGCoAR expression was observed. The expression of these three genes was positively correlated to the expression of SREBP2 which indirectly suggests that SREBP2 regulates PCSK9 transcription in humans, *in vivo*.

It was previously reported that alcohol intake can reduce the risk for development of cardiovascular and gallstone disease, one possible explanation for the reduction could be an increased conversion of cholesterol into bile acids. In the third paper, primary human hepatocytes were used to investigate the effect of ethanol on the synthesis of bile acids. Following addition of ethanol to the cells the formation of bile acids was stimulated. In particular the synthesis of cholic acid increased, which suggests a stimulation of the neutral pathway for bile acid synthesis. The molecular mechanisms behind the effect remain to be elucidated.

Thyroid hormones (TH) regulate cholesterol metabolism but their use as lipid-lowering drugs are restricted because of negative cardiac effects. TH mimetic compounds modulating TH receptor β have been designed as potential drugs since they reduce serum cholesterol while avoiding apparent deleterious effects. In Paper IV the TH receptor modulator KB3495 was investigated for its potential to lower serum cholesterol and treat atherosclerosis.
In ApoE deficient mice KB3495, alone or in combination with atorvastatin, reduced atherosclerosis and lowered the cholesterol content in aorta, liver and skin. A reduced cholesterol synthesis, increased bile acid formation and induced fecal excretion of bile acids and neutral sterols was observed. Thus, the combined treatment of KB3495 and statins could provide a possible strategy to treat atherosclerosis.

LIST OF PUBLICATIONS

I. A. Abrahamsson, U. Gustafsson, E. Ellis, **L-M. Nilsson**, S. Sahlin, I. Björkhem, C. Einarsson, *Feedback regulation of bile acid synthesis in human liver: importance of HNF-4alpha for regulation of CYP7A1.* Biochem Biophys Res Commun 330 (2005) 395-399.

II. **L-M. Nilsson**, A. Abrahamsson, S. Sahlin, U. Gustafsson, B. Angelin, P. Parini, C. Einarsson, *Bile acids and lipoprotein metabolism: Effects of cholestyramine and chenodeoxycholic acid on human hepatic mRNA expression.* Biochem Biophys Res Commun 357 (2007) 707-711.

III. **L-M. Nilsson**, J. Sjövall, S. Strom, K. Bodin, G. Nowak, C. Einarsson, E. Ellis, *Ethanol stimulates bile acid formation in primary human hepatocytes.* Biochem Biophys Res Commun 364 (2007) 743-747.

IV. **L-M. Nilsson,** S. Rehnmark, P. Davoodpour, L. Larsson, J. Malm, P. Parini, *The Thyroid Receptor agonist beta modulator KB3495 reduces atherosclerosis independently of ApoB-cholesterol in ApoE depleted mice.* Manuscript

Other publications not included in this thesis:

- S. Sanyal, A. Båvner, A. Haroniti, **L-M. Nilsson**, T. Lundåsen, S. Rehnmark, M.R. Witt, C. Einarsson, I. Talianidis, J.A. Gustafsson, E. Treuter, *Involvement of corepressor complex subunit GPS2 in transcriptional pathways governing human bile acid biosynthesis.* Proc Natl Acad Sci U S A 104 (2007) 15665-15670.

- P. Kotokorpi, E. Ellis, P. Parini, **L-M. Nilsson**, S. Strom, K.R. Steffensen, J.A. Gustafsson, A. Mode, *Physiological differences between human and rat primary hepatocytes in response to liver X receptor activation by 3-[3-[N-(2-chloro-3-trifluoromethylbenzyl)-(2,2-diphenylethyl) amino] propyl oxy] phenylacetic acid hydrochloride (GW3965).* Mol Pharmacol 72 (2007) 947-955.

- R.E. Temel, W. Tang, Y. Ma, L.L. Rudel, M.C. Willingham, Y.A. Ioannou, J.P. Davies, **L-M. Nilsson**, L. Yu, *Hepatic Niemann-Pick C1-like 1 regulates biliary cholesterol concentration and is a target of ezetimibe.* J Clin Invest 117 (2007) 1968-1978.

- T. Lundåsen, M.C. Hunt, **L-M. Nilsson**, S. Sanyal, B. Angelin, S.E. Alexson, M. Rudling, *PPARalpha is a key regulator of hepatic FGF21.* Biochem Biophys Res Commun 360 (2007) 437-440.

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LIST OF ABBREVIATIONS

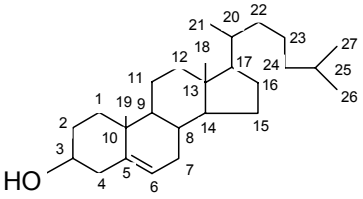
ABC	ATP-binding cassette transporter
ACAT	Acyl-coenzyme A:cholesterol O-acyltransferase
Apo	Apolipoprotein
ASBT	Apical sodium-dependent bile acid transporter
ASO	Antisense oligonucleotide
BA	Bile acid
BSEP	Bile salt export pump
C4	7α-hydroxy-4-cholesten-3-one
CA	Cholic acid
CDCA	Chenodeoxycholic acid
CE	Cholesteryl ester
CETP	Cholesteryl ester transfer protein
Coll	Collagen
CYP7A1	Cholesterol 7α-hydroxylase
CYP8B1	Sterol 12α-hydroxylase
CYP27A1	Sterol 27-hydroxylase
Dex	Dexamethasone
DCA	Deoxycholic acid
DIO	Iodothyronine deiodinase
EHS	Engelbreth-Holm-Swarm
ER	Endoplasmic reticulum
FC	Free cholesterol
FGF	Fibroblast growth factor
FXR	Farnesoid X receptor
HDL	High density lipoprotein
HMG-CoAR	3-hydroxy-3-methylglutaryl coenzyme A reductase
HNF4α	Hepatocyte nuclear factor 4α
IBABP	Ileal bile acid binding protein
IDL	Intermediate density lipoprotein
Ins	Insulin
INSIG	Insulin-induced gene
LCA	Lithocholic acid
LCAT	Lecitin:cholesterol acyltransferase
LDL	Low density lipoprotein
LDLR	Low density lipoprotein receptor
LPL	Lipoprotein lipase
LRH-1	Liver receptor homologue 1
LXR	Liver X receptor
NPC1L1	Niemann-Pick C1-like 1 protein
Ost	Organic solute transporter
PCSK9	Proprotein convertase subtilisin/kexin type 9
SCAP	SREBP cleavage-activating protein
SHP	Small heterodimer partner
SR-B1	Scavenger receptor B type I

SREBP	Sterol regulatory element binding protein
Std	Standard media
Swe	Swedish media
T3	Triiodothyronine
T4	Thyroxine
TG	Triglycerides
TH	Thyroid hormone
TICE	Trans intestinal cholesterol excretion
TR	Thyroid hormone receptor
UDCA	Ursodeoxycholic acid

1 INTRODUCTION

1.1 CHOLESTEROL METABOLISM

Cholesterol is the most common steroid found in animals and has an important role as a precursor of bile acids and sex hormones. It is also essential for maintaining the structure of plasma membranes since it affects their fluidity. Most cholesterol found in the body is synthesized *de novo* from acetate in a pathway regulated by the rate limiting enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCoAR). Cholesterol can be synthesized by most cells in the body but the liver and intestine accounts for major part of the synthesis (1-3).



Cholesterol

1.1.1 Lipoproteins

Lipids such as cholesterol and triglycerides (TG) are not freely soluble in the aqueous environment of plasma and therefore they are transported in the body as lipoprotein complexes. Lipoproteins have a hydrophobic core consisting of TGs and cholesteryl esters (CE), and a more hydrophilic surface monolayer consisting of phospholipids (PL) and unesterified cholesterol. The lipoprotein particles are associated with apolipoproteins (Apo) which have a structural function or confer specific properties to the lipoprotein. Lipoproteins vary in their composition and are classified into five main groups according to their density; chylomicrons, very low-density lipoproteins (VLDL), intermediate-density lipoproteins (IDL), low-density lipoproteins (LDL), and high-density lipoproteins (HDL) (4-8).

After a meal large TG rich chylomicrons are formed and secreted from the enterocytes in the intestine. Chylomicrons contain the apolipoprotein ApoB48 and can also contain ApoE, ApoAI, ApoAII and ApoAIV. The chylomicrons are transported in the lymph

and reach the circulation via the thoracic duct. In the circulation TGs are hydrolyzed by lipoprotein lipase (LPL) into free fatty acids (FFA) which are taken up by muscle and adipose tissues. The remnant particles are thereafter rapidly taken up by the liver, the half-life of chylomicrons is approximately 10 min.

VLDL particles are produced by the liver and as for chylomicrons the main function of VLDL is to transport TGs to peripheral tissue. VLDL is formed by a stepwise lipidation of ApoB100. First ApoB100 is lipidated and forms a pre-VLDL particle, this occurs in the endoplasmic reticulum (ER). Thereafter the particle can be further lipidated to form VLDL2 which is then transferred to the Golgi apparatus where it can be secreted or be further lipidated to form VLDL1 particles. The assembly of VLDL particles is dependant on fatty acids released from lipid droplets in the hepatocytes (9). In the circulation VLDL particles receive ApoCI, CII, CIII and E. Following hydrolysis of the TGs by LPL the particles are converted into IDL particles which are rapidly cleared from the plasma either by conversion to LDL particles or uptake by the liver. The half-life of circulating VLDL is approximately 2 hours.

LDL particles contain ApoB100 on their surface and are formed from VLDL and IDL. They have a longer half-life, 2-3 days, and in humans about 70% of plasma cholesterol is found in LDL particles. LDL delivers cholesterol to the liver and peripheral tissue where it is taken up by LDL receptors (LDLR).

HDL particles mainly consist of PLs, cholesterol and Apos. The major Apo is ApoAI but some HDL also contains ApoAII. During formation of HDL the ApoAI particles are secreted from liver and intestine. In plasma the particles acquire cholesterol and PLs via ATP-binding cassette transporter A1 (ABCA1) and discoidal pre-HDL particles are formed. Following esterification by lecitin:cholesterol acyltransferase (LCAT) CE-enriched spherical HDL particles are formed which then gain TGs from VLDLs, a step carried out by cholesteryl ester transfer protein (CETP) (10). HDL particles acquire cholesterol and PLs from peripheral tissues and transport it to the liver directly or via other lipoproteins. In the liver, scavenger receptor B1 (SR-B1) mediates the selective uptake of cholesterol from HDL. The various protein and lipid components in the HDL particle have different metabolic pathways. The half-life of ApoAI and ApoAII is believed to be 4-5 days, whereas the half-life of cholesterol esters may be 10 to 40 times shorter (4-8).

1.1.2 Hepatic cholesterol metabolism

The liver has a key role in the regulation of cholesterol homeostasis. As mentioned above, a major part of cholesterol synthesis takes place in the liver. The liver also regulates the levels of lipoproteins in plasma by their uptake and secretion. Chylomicrons, IDL, HDL and LDL are cleared from the plasma through the liver while VLDL and HDL are secreted from the liver into the circulation (4-7, 11).

Maintenance of stable cholesterol levels in the hepatocytes is also achieved by the formation of CEs from cholesterol and long-chain fatty acids, a reaction catalyzed by the enzyme acyl-CoA:cholesterol acyltransferase 2 (ACAT2) (12-13). Unlike free cholesterol CEs can be stored inside cells without being toxic.

A central function of the liver is the elimination of excess cholesterol from the body, either in bile through excretion of free cholesterol via the transporters ABCG5/ABCG8 (14-15), or through conversion into bile acids (BA) (16-18).

Animal studies have also suggested another pathway in which cholesterol excretion can take place independently of the biliary output. This trans intestinal cholesterol excretion (TICE) pathway describes the secretion of cholesterol into the feces directly by the enterocytes (19-20).

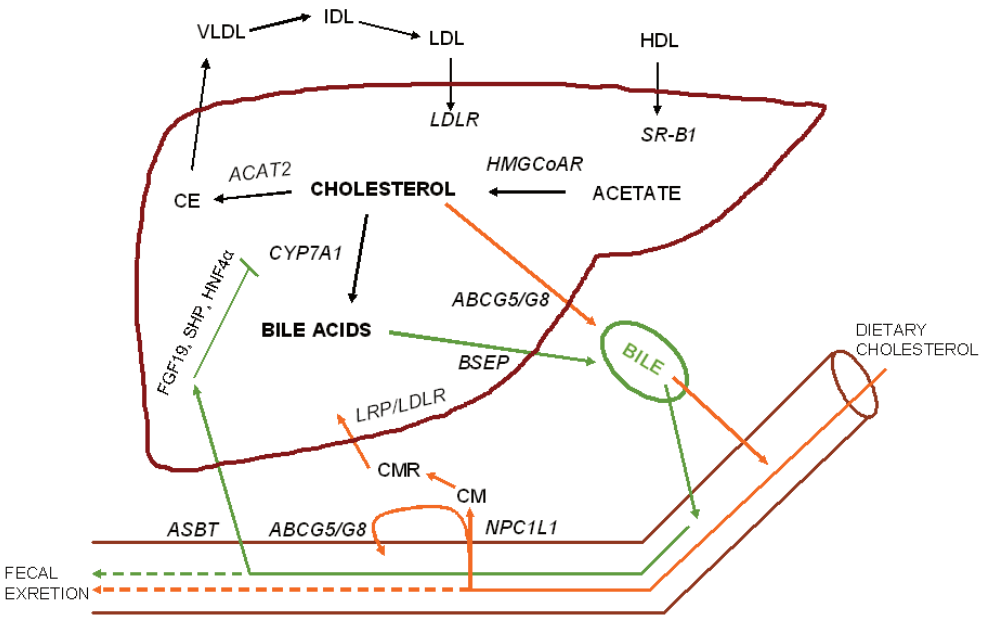


Figure 1. Schematic overview of cholesterol and bile acid transport in the human liver.

1.1.3 Regulation of the LDL receptor

The concentration of LDL particles in plasma is determined by their synthesis and by their removal by hepatic LDLRs. LDLRs bind and internalize lipoproteins containing ApoB100 and ApoE.

LDLR expression is regulated by sterol-responsive element binding protein-2 (SREBP2), a transcription factor that functions as a master switch for cholesterol metabolism. SREBP2 belongs to a SREBP-family which in humans and mice have three members; SREBP1a, SREBP1c and SREBP2. SREBPs are synthesized as precursors bound to the ER membrane and in order to be activated they require cleavage by the proprotein convertase SKI-1/S1P and metalloprotease S2P. The SREBP cleavage activating protein (SCAP) functions as a cholesterol sensor. When the sterol content in the liver is low, SCAP binds and escorts SREBPs to the Golgi apparatus where they are cleaved. Cleavage releases the amino-terminal domain which goes into the nucleus where it binds and activates its target genes, for example the LDLR. When cholesterol levels are restored another regulator named insulin-induced gene (INSIG) binds and retains the SREBP-SCAP complex at the ER, inhibiting cleavage and activation (21-26).

Recently a new pathway for LDLR regulation was discovered. It was found that a gain-of-function mutation in the gene coding for proprotein convertase subtilisin kexin type 9 (PCSK9) caused hypercholesterolemia (27). Since then a number of mutations have been found that both increase and inactivate the activity of PCSK9 (27-31) and it is now recognized that PCSK9 enhance the degradation of LDLR (32-34). PCSK9 is the ninth member of a family of proprotein convertase proteinases. Several of the members in the family are involved in sterol and lipid metabolism, for example SKI-1/S1P which cleaves SREBP precursors and is required for their activation (34). The molecular mechanism by which PCSK9 promotes degradation of LDLR is still not fully known but both intra- and extracellular PCSK9 may be involved. Presumably PCSK9 undergoes autocatalytic cleavage and then binds to the epidermal growth factor-like repeat A (EGF-A) domain of the LDLR thereby inducing degradation within intracellular acidic compartments (34-38).

1.2 BILE ACID METABOLISM

BAs are the major lipid components of bile and are synthesized from cholesterol in the liver. Their major function is to emulsify lipids to facilitate intestinal absorption. BAs are amphipathic compounds with one hydrophobic region and one hydrophilic region, a structure which allows them to interact with both lipids and water. The amphipathic structure of BAs results in formation of micelles which facilitate emulsion of lipids for transport to the intestinal epithelial cells where they are absorbed.

Most BAs are built of 24 carbon atoms, two or three OH-groups and a sidechain with a carboxyl group. In more than 98% of the BAs the carboxyl group is conjugated with glycine or taurine which increase the polarity. BAs synthesized in the liver are called primary BAs, in humans these are cholic acid (CA) and chenodeoxycholic acid (CDCA). In addition, rodents also form α- β- and γ-muricholic acids. Secondary BAs are formed from primary BAs by bacterial modifications during enterohepatic circulation; the major secondary BAs in humans are deoxycholic acid (DCA) and lithocholic acid (LCA). Bacterially modified BAs can be sent back to the liver for further modifications, thereby forming tertiary BAs. In humans the tertiary BA ursodeoxycholic acid (UDCA) is formed (16-18).

1.2.1 Enterohepatic circulation of bile acids

After synthesis BAs are stored in the gallbladder where the bile is concentrated. When food enters the gastrointestinal tract it stimulates gallbladder contraction and bile-flow to the intestine. In the intestine 95-99% of BAs are reabsorbed and returned to the liver. The majority of the BA reabsorption occurs in the distal part of the ileum where BAs are actively transported via the apical sodium-dependent bile acid transporter (ASBT or IBAT). ASBT is highly expressed at the apical brush border membranes of the enterocytes and transports both conjugated and unconjugated BAs with preference for conjugated BAs. Unconjugated BAs can also enter the enterocytes by passive diffusion throughout the intestine. Transport of BAs from the apical to the basolateral side of the enterocytes is mediated by the ileal bile acid binding protein (IBABP). The transportation of BAs from the enterocytes to the portal blood occurs through the heteromeric organic solute transporter (Ostα-Ostβ) (39-41).

BAs that are not taken up in the intestine are converted into secondary BAs by microbial enzymes. These secondary BAs can partly be taken up by passive diffusion in the large intestine or be excreted in feces. The loss of BAs in feces is compensated

for by *de novo* synthesis. The synthesis is highly regulated by the inflow of BAs by negative feedback regulation (16-18).

1.2.2 Bile acid synthesis

The human liver converts approximately 500 mg of cholesterol into BAs each day, a process involving at least 17 different enzymes. Primary BAs are synthesized via two major pathways: the classical (neutral) and the alternative (acidic) pathway. During normal conditions the classical pathway is the major route in humans and accounts for approximately 90% of total BA formation. In rodents the alternative pathway is believed to contribute to as much as 50% of the total BA synthesis (16-18).

The first step of the classical pathway is the hydroxylation at the 7α-position of cholesterol. This reaction is catalyzed by the rate limiting enzyme cholesterol 7α-hydroxylase (CYP7A1). CYP7A1 is a liver specific member of the cytochrome P450 superfamily. In the next step, 7α-hydroxycholesterol is converted to 7α-hydroxy-4-cholesten-3-one (C4). Thereafter, C4 may be hydroxylated by 12α-hydroxylase (CYP8B1), a pathway which eventually leads to formation of CA. If 12α-hydroxylation does not occur CDCA is formed. However, in the classical pathway CA is the predominant BA formed.

The alternative pathway starts with oxidation of the cholesterol side chain, producing oxysterols. The reaction is catalyzed by sterol 27-hydroxylase (CYP27A1) and contrary to 7α-hydroxylation this first reaction can also occur in extra-hepatic organs. In the next step 27-hydroxycholesterol is 7α-hydroxylated by CYP7B1, after several further reactions, CDCA or small amounts of CA are formed. Unlike the classical pathway no rate limiting steps can be clearly identified for the alternative pathway.

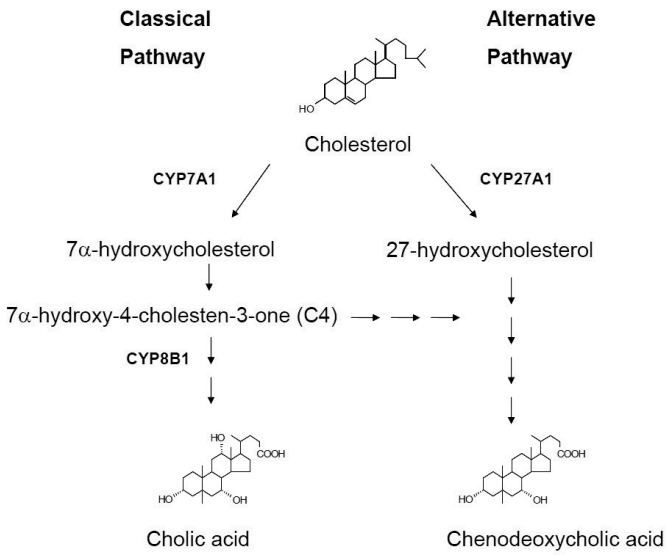


Figure 2. Overview of the main steps in bile acid synthesis.

1.2.3 Regulation of bile acid synthesis

Synthesis of BAs is regulated by a feedback mechanism through BAs returning to the liver from the intestine via the enterohepatic circulation.

BAs can bind to the nuclear hormone receptor farnesoid X receptor (FXR) which, in its activated form, initiates transcription of another orphan nuclear hormone receptor, small heterodimer partner (SHP). SHP binds the liver receptor homolog-1 (LRH-1) and possibly also the hepatocyte nuclear factor 4α (HNF4α), both positive regulators for CYP7A1. This protein to protein interaction causes a down regulation of CYP7A1 expression. Hydrophobic BAs induce a stronger induction of FXR and CDCA is the most potent inducer. In mice, CYP7A1 is also induced by the liver X receptor α (LXRα) but in contrast, the human CYP7A1 gene lacks a functional LXRα binding site (16-18, 42-43).

Mice deficient in SHP have increased synthesis of BAs, a finding consistent with a repressive role of SHP. However, when these mice are fed BAs the levels of CYP7A1 and CYP8B1 decrease, indicating that these enzymes also can be regulated through SHP-independent pathways. One suggested mechanism is by HNF4α inactivation through protein kinase C which acts via c-Jun kinase (16-18).

A more recently discovered pathway of bile acid regulation is through the intestinal fibroblast growth factor 19 (FGF19), in mice the orthologue is FGF15. In response to a

postprandial increase of BAs, FGF19 is secreted from the intestine to the liver via the portal blood. In the liver FGF19 interacts with FGF receptors (FGFR) and causes a down regulation of CYP7A1 expression (44-46). The binding of FGF19 to FGFRs is stabilized by the cofactor βKlotho (47-48). FGF19 expression is induced by BAs acting through FXR and can act independently to or in cooperation with SHP (42, 44-45, 49).
In addition to downregulating BA synthesis, FGF15 has also been shown to affect gallbladder filling. Following food intake the hormone cholecystokinin stimulates gallbladder contraction and emptying of bile into the intestine. In the distal part of the small intestine BAs induce FGF15 synthesis and secretion to the gallbladder where it stimulates gallbladder filling (50).

1.3 MODELS OF CHOLESTEROL AND BILE ACID METABOLISM

There are a number of different model systems in which the metabolism of cholesterol and BAs can be studied. Our ultimate objective is to elucidate the pathways relevant to humans or how our compound works in humans. However, studies in patients have many limitations; most importantly many studies are not possible from an ethical point of view and there are also practical problems. One possible disadvantage with clinical studies is the difficulty to recruit a large number of subjects. Another limitation of human studies is the large variation between patients. Because of these limitations various models of comparative physiology are used. Cell experiments are useful when a specific cell type should be studied and for analysis of molecular mechanisms. Another advantage is that the cell environment can be controlled and regulated. When complex or long-term diseases or whole body effects of a substance should be studied animal models are used. Because of its flexibility and the large number of genetically modified mouse models one of the most commonly utilized animal models is mice.
This thesis includes studies performed in humans, in primary human hepatocytes and in mice.

1.3.1 Primary cultures of human hepatocytes

In order to study human liver metabolism *in vitro,* primary hepatocytes and hepatoma cell lines can be used. Two frequently used liver cell lines are HepG2 and HuH7. Both HepG2 and HuH7 are suitable models for molecular studies but have lost many of their liver specific expression of P450 enzymes and phase II enzymes when compared to liver tissue or to human hepatocytes in primary cultures (51-53).

The currently used technique for isolation of primary human hepatocytes was first described in rat livers by Berry and Friend in 1969 (54). The method has thereafter been improved for isolation of human hepatocytes (55-57), see the method section for a detailed description.

Primary hepatocytes require attachment to extra cellular basement membranes to maintain their polarity and transcription of liver specific enzymes. Hepatocytes cultured on collagen or laminin preserve part of their hepatocyte specific proteins and the cells achieve a flattened polygonal shape (58-60), see figure 3. It has been shown that primary rat hepatocytes cultured on matrigel (a basement membrane preparation extracted from the Engelbreth-Holm-Swarm (EHS) mouse sarcoma) or in a sandwich system with matrigel and collagen maintain a more liver specific morphology and protein expression pattern. The hepatocytes have a cuboidal morphology with bile canaliculi, tight junctions and gap junctions and an increased expression of P450 enzymes compared to cells cultured on collagen alone and the liver specific enzymes are maintained (59-63). In human hepatocytes the effect of matrigel is more disputed. Although culturing the hepatocytes on matrigel or in a matrigel sandwich results in more adequate cell morphology, it has been reported that the expression of P450 enzymes is not changed (64) and that the expression may be dependent on plating density rather than on the type of extra cellular matrix used (59). On the other hand, others have shown that culturing the cells in matrigel sandwiches helps the maintenance and induction of P450 enzymes and other liver specific enzymes (65-66).
Under standard conditions primary hepatocytes can be kept in culture for approximately 10-14 days. For long-term studies, the culture media must be supplemented with e.g. hepatocyte growth factor and epidermal growth factor or alternatively the cells could be co-cultured with rat liver epithelial cells (67-69).

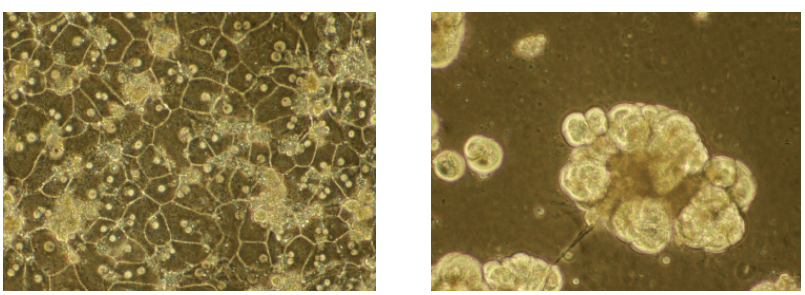


Figure 3. Primary human hepatocytes cultured on collagen (left panel) and EHS matrigel (right panel), 20X.

The majority of the *in vitro* studies on BA synthesis using liver cells of human origin are performed in HepG2 cells. However, studies on HepG2 cells are limited since they secrete BA precursors and unconjugated BAs not produced in healthy liver under standard conditions (70-73). One drawback with studies of BA metabolism in cell systems is the absence of enterohepatic circulation.

The first reports on cholesterol conversion to BAs in primary hepatocytes were done in rats in 1983 (74-75). Later studies in primary hepatocytes have shown that the capacity for BA formation and the hormonal regulation of their synthesis differs in human primary hepatocytes when compared to rat primary hepatocytes (76). Analysis of BA synthesis in primary human hepatocytes has shown that they form conjugated CA and CDCA (76-77).

1.3.2 Mouse models on atherosclerosis

Atherosclerosis is a complex disease with several factors influencing lesion development. Therefore, an animal model that resembles the human disease is needed to study atherosclerosis and investigate the effect of different therapeutical approaches. The mouse is a useful model; however, normal mice are relatively resistant to atherosclerosis. Therefore several genetically modified mouse models have been developed. Among the most commonly used mouse models for atherosclerosis are; $ApoE^{-/-}$ mice, $LDLR^{-/-}$ mice, ApoE*3Leiden transgenic mice and $LDLR^{-/-}ApoB^{100/100}$ mice (78-80).

In Paper IV we used $ApoE^{-/-}$ mice. This mouse model was simultaneously developed by two groups in 1992 (81-82) and is the most characterized model. $ApoE^{-/-}$ mice have been widely used because they develop advanced atherosclerotic lesions spontaneously and the lesions resemble those found in humans. A disadvantage with $ApoE^{-/-}$ mice is the nature and type of dyslipidemia present in these animals. In $ApoE^{-/-}$ mice the lipoproteins consist mainly of chylomicron remnants of intestinal origin which are distributed within the size/density range typical for VLDL and LDL particles (78, 80, 83-84).

1.4 MODULATION OF CHOLESTEROL AND BILE ACID METABOLISM

In this thesis modulation of cholesterol and BA metabolism has been studied. The effects of cholestyramine, statins, alcohol and thyroid hormones (TH) have been investigated.

1.4.1 Cholestyramine

Bile acid sequestrants such as cholestyramine was one of the first groups of substances used to lower serum cholesterol levels. Cholestyramine is a non-absorbed anion exchange resin that binds BAs in the small intestine and thereby increases fecal excretion of BAs. By preventing BAs from being reabsorbed, the enterohepatic circulation is interrupted. In order to compensate for the loss of BAs the liver increases the conversion of cholesterol into BAs and consequently serum cholesterol is lowered. Apart from working as a lipid lowering substance, cholestyramine may also be used to treat pruritus in patients with chronic liver disease or diarrhea caused by excess BAs (85-86).

1.4.2 Statins

The most common group of drugs used to decrease LDL cholesterol and reduce the risk for atherosclerosis are the statins. Statins are competitive inhibitors of HMGCoAR, the rate limiting enzyme in cholesterol synthesis. Following inhibition of HMGCoAR there is a depletion of cholesterol in the hepatocytes causing a compensatory upregulation of LDLR, which leads to reduced levels of plasma cholesterol by increased uptake of LDL particles (85, 87-88). There are several statins available on the market, the most commonly used in Sweden are: simvastatin, atorvastatin, pravastatin, and rosuvastatin (89).

1.4.3 Alcohol

A moderate daily intake of alcohol is associated with a reduction in total mortality and a reduced risk for development of coronary heart disease and arteriosclerosis (90-93). Regression analysis has indicated that approximately 50% of the protective effect of ethanol is associated with an increase in plasma HDL cholesterol (94). The lower risk for cardiovascular disease could also be a result of increased insulin sensitivity, anti-inflammatory effects or other changes involved in the lipoprotein metabolism, for example effects on apolipoproteins and lipases (93, 95-99). Experiments have shown that a moderate ethanol intake may decrease the risk of gallstone disease. One possible explanation for the reduction of gallstones may be an increased BA production (100-103).

There are a few studies which have investigated the effect of ethanol on BA synthesis but the results from these studies are conflicting. Crouse and Grundy showed that long-term intake of alcohol does not influence BA metabolism in humans (99). However,

another *in vivo* study reported that short-term intake of ethanol was associated with increased serum levels of the BA intermediates 7α -hydroxycholesterol and C4 (104), suggesting that the BA synthesis and CYP7A1 activity were increased (105-107). It has also been shown that alcohol intake increases BA excretion in feces in hyperlipidemic subjects, while this does not occur in normolipidemic subjects (108).

1.4.4 Thyroid hormones and thyroid hormone mimetics

THs are important for regulation of metabolism in various tissues and alterations in TH levels can lead to serious diseases. Hyperthyroidism is caused by excessive TH levels and is associated with an increased metabolic rate, tachycardia, muscle wasting, reduced body fat and reduced levels of serum LDL cholesterol. Hypothyroidism, deficiency of TH, have opposite symptoms, including weight gain, reduced body temperature, depression and high levels of serum LDL cholesterol (109-112).

The thyroid gland produces two hormones and the major one formed is thyroxine (T4) but the more active hormone triiodothyronine (T3) is also formed. However, most T3 is produced by deiodination of T4 in the periphery by deiodinases (iodothyronine deiodinase 1 and 2 (DIO1, DIO2)). Serum concentrations of THs are regulated by several mechanisms including negative feedback regulation by T3 and by regulation of DIO expression. The biological effects of THs are mediated by their binding and activation of TH receptors (TRs). TRs are encoded by two genes (α and β), both genes encode several alternative spliced mRNA isoforms. The tissue distribution and metabolic effects differ between TRα and TRβ where TRα has been shown to be abundantly expressed in heart and regulates the heart rate, while TRβ is highly expressed in the liver and plays a major role in regulating cholesterol metabolism (113-116).

Through the years several attempts have been made to separate the beneficial and the deleterious effects of TH by trying to find a substance that lowers cholesterol levels while avoiding side effects affecting the heart. In the 1960s one of the most extensive humans trials of a TH analogue was conducted. In the investigation, subjects were treated with dextrothyroxine (D-T4) which is the D-enantiomer of T4, but the study was terminated before completion because of an increased mortality (117-118). A subsequent investigation showed that contamination of the D-T4 with a stereoisomer of T4 may have contributed to the effect (119). Recently the knowledge about TRs has

improved and TH mimetic compounds which are selective for TRβ and the liver have been designed as potential drugs. The liver specificity of these substance is believed to be related to high rates of liver first-pass uptake and also differences in cellular uptake. There are a number of designed TH mimetics, for example GC-1, KB141, KB2115, MB07811 and T-0681 (120-123). In this thesis the TH mimetic compound KB3495 is investigated for its ability to reduce formation of atherosclerosis.

Like THs, many of the designed TH mimetics have been shown to reduce serum cholesterol (124-128) but, contrary to TH, the mimetics can reduce serum cholesterol at doses that that do not affect the heart rate (121). The cholesterol lowering effect have been suggested to be caused by increased levels of LDLR (125-126) but despite lowered cholesterol levels we failed to detect increased LDLR following GC-1 administration to mice in a previous study from our group (124), suggesting that other mechanisms may exist. Previous studies have also shown that TH and TH mimetics regulate the expression of CYP7A1 (124, 129-130), thus one pathway to lower cholesterol levels may be through an increased conversion into BAs.

2 AIMS

The aim of this project was to improve our understanding of how cholesterol homeostasis is regulated. We aimed to achieve this by studying modulation of lipoprotein and bile acid metabolism both *in vivo* and *in vitro*.
In more detail, the aims were:

- To characterize and optimize culture conditions for primary cultures of human hepatocytes with respect to bile acid synthesis.

- To determine the expression of key enzymes and nuclear factors regulating bile acid synthesis following inhibition or stimulation of bile acid synthesis.

- To study the expression of enzymes and nuclear factors involved in lipoprotein metabolism following inhibition or stimulation of bile acid synthesis.

- To investigate the effect of ethanol on the synthesis of bile acids using primary human hepatocytes.

- To examine the effect of a thyroid receptor beta modulator on the metabolism of cholesterol and bile acids.

3 METHODOLOGY

3.1 PATIENTS

In *Paper I* and *Paper II* patients (n=23) with uncomplicated gallstone disease who were referred to Danderyd´s Hospital for cholecystectomy gave their informed consent to participate in the study and were thus enrolled. One group of patients was treated with CDCA (15mg/kg/day) for three weeks prior to surgery; one group was treated with cholestyramine (8g twice a day) for three weeks prior to surgery. Finally, one group of patients received no treatment and served as controls.

The patients were all normolipidemic, of normal weight and otherwise healthy. They were all operated by laparoscopic technique in the morning between 8 and 10 a.m. During the operation a liver biopsy was taken and gallbladder bile collected for analysis of biliary lipids. The liver specimen was immediately frozen in liquid nitrogen for preparation of RNA, serum was obtained for analysis.

Studies were approved by the Ethical Committee at the Karolinska University Hospital Solna, Stockholm, Sweden.

3.2 ISOLATION AND CULTURE OF HUMAN HEPATOCYTES

In *Paper I*, *Paper III* and in *chapter 4.1* normal human liver tissue was obtained from patients (n=28) undergoing surgical liver resection due to cancer or alternatively from donor livers undergoing reduction prior to transplantation, or donor livers that could not be used for transplantation.

Hepatocytes were isolated by a two-step perfusion technique, utilizing EGTA and collagenase as previously described by Strom et al. (131). Briefly, 1-3 vessels were connected to a peristaltic pump and the tissue was perfused with 1 L Hank's Buffered Salt Solution (HBSS) with EGTA followed by HBSS without EGTA. The perfusion was continued with 1 L Eagle's minimum essential medium with Earle's salts (EMEM) containing 250 mg collagenase XI for approximately 20 min. The liver tissue was chopped with a pair of scissors, releasing the hepatocytes which were filtered through 2 layers of gauze, centrifuged at 50 g for 5 min and washed twice with EMEM. The hepatocytes were counted and cells were seeded in cell culture dishes precoated with matrigel, 3.5 million cells per P60 plate or 1.5 million cells per well in a 6-well plate. Hepatocytes were cultured under standard conditions in William’s E medium supplemented with glutamine, insulin (12 mM), penicillin G sodium (100 U/ml),

streptomycin sulphate (100 μg/ml) and gentamycin (85 μg/ml). The medium was changed one hour after plating and then daily until harvesting.

In *Paper I*, cells were treated with glycine conjugated BAs on day four. In *Paper III*, addition of ethanol or methanol was made on day four and the plates were covered with parafilm to prevent evaporation of alcohol. In both papers, the cells were harvested in Trizol on day 5 for quantification of specific mRNAs and cell culture medium was analyzed for BAs.

Approval to use parts of resected human liver specimens for research was given by the Ethics Committee at Karolinska Institute and from the Institutional Review Board at the University of Pittsburgh.

3.3 ANIMALS

In *Paper IV* ApoE-/- mice (n=80) were challenged with a western like diet containing 10% kcal from saturated fat and 0.2 % cholesterol (w/w). The animals had free access to food and water. The animals were divided into 4 groups, control group receiving diet without supplementation, KB3495 group receiving diet supplemented with 0.7 mg/kg of KB3495, atorvastatin group receiving diet supplemented with 110 mg/kg of atorvastatin and a combination group receiving diet supplemented with 0.7 mg/kg of KB3495 and 110 mg/kg of atorvastatin, corresponding to a daily dose of approximately 3.5 μg KB3495 and 550 μg atorvastatin. Half of the animals in each group were sacrificed after 10 weeks and the rest following 25 weeks of treatment. Mice were fasted for 4 hours prior sacrifice. Blood was drawn by cardiac puncture under carbon dioxide anesthesia. Livers and intestines were frozen in liquid nitrogen. Skin and aorta were frozen in liquid nitrogen or alternatively put into formalin. Feces was collected group-wise the last 48 h of the experiment in the 10 week experiment.

Studies were approved by the institutional Animal Care and Use Committee.

3.4 QUANTIFICATION OF BILE ACIDS

In *Paper III* and *chapter 4.1* BAs were analyzed in cell culture medium and in *Paper IV* BAs were analyzed in feces.

The feces was first homogenized in water and the mixture was hydrolyzed in 1 M KOH at 120^{o}C overnight. Samples were extracted with hexane, the water phase, together with 5-alfa-cholestane, was extracted again with diethyl ether. The samples were washed with water until neutral, evaporated and methylated with trimethylsilyldiazomethane and derivatized using hexamethyl-disilazane and trimethylchlorosilane in pyridine.

Samples were finally analyzed by gas chromatography as described by Grundy et al (132).

Cell medium were analyzed according to the same protocol with a few changes; D_5-CA and D_4-CDCA were used as internal standards and also in the first extraction diethyl ether was used, samples were finally analyzed by gas chromatography-mass spectrometry according to Björkhem et al. (133).

3.5 QUANTIFICATION OF LIPIDS

In *Paper IV* plasma lipoproteins from individual mice were separated by size exclusion chromatography (SEC) as previously described by Parini et al. (134). Plasma was carried over a Superose® 6 PC 3.2/30 column by an HPLC-pump. Separated lipoproteins were then mixed with cholesterol or TG reagents and absorption was monitored with an UV-VIS detector, data was collected every 20 seconds.

In *Paper IV* lipids from aorta, liver and skin from individual animals were extracted in chloroform/methanol according to Folch et al. (135). The lipid extracts from all three tissues were used to analyze total and free cholesterol levels, lipid extract from liver were also used for determination of TGs, lathosterols and C4. After lipid extraction, remaining tissue was dissolved in 1 N NaOH for protein determination using the method of Lowry (136).

Total and free cholesterol were determined by isotope dilution-mass spectrometry according to Björkhem et al. (137) with some modifications. Cholesterol esters were hydrolyzed in presence of KOH and ethanol and thereafter neutralized with H_3PO_4-Ortho-phosphoric acid prior to purification. D_6-labelled cholesterol was used as internal standard. Samples were purified using Isolute-MF C18 cartridges and derivatized with hexamethyl-disilazane and trimethylchlorosilane in pyridine prior to analysis by gas chromatography-mass spectrometry.

The TG content in the livers was analyzed using an enzymatic kit (Roche) and measuring the absorbance at 500 nm.

The liver concentration of lathosterol was analyzed by gas chromatography/mass spectrometry according to Lund et al. (138). Prior to analysis, D_4-lathosterol was added, the samples were purified using Isolute-MF C18 cartridges and derivatized with hexamethyl-disilazane and trimethylchlorosilane in pyridine.

C4 levels were analyzed according to Lövgren-Sandblom et al. (139). D_6-C4 was added, samples were purified using Isolute-SI cartridges and the C4 content was determined by liquid chromatography-mass spectrometry.

In *Paper IV* feces was analyzed for neutral sterols according to Miettinen et al. (140) with some modifications. Feces was first homogenized in water; the mixture was hydrolyzed in 1 M KOH at 120°C overnight. Samples were mixed with 5-alfa-cholestane and extracted twice with hexane. Thereafter samples were evaporated and derivatized using hexamethyl-disilazane and trimethyl-chlorosilane in pyridine. Samples were finally analyzed by gas chromatography.

3.6 QUANTIFICATION OF mRNA LEVELS

In all four papers, total RNA was isolated using Trizol reagent. RNA concentration was determined by spectrophotometry at 260 nm. The purity and condition of the RNA was determined by spectrophotometry at 280 nm and by agarose gel electrophoresis containing ethidium bromide. In *Paper I* and *Paper II*, RNA was first treated with RQ DNase, then transcribed into cDNA using Random Hexamer Primers, dNTP and superscript. mRNA expression was quantified using ABI taqman mastermix and probes. In *Paper III*, Omniscript and random hexamer primers were used to transcribe RNA into cDNA, in *Paper IV*, MultiScribe Reverse Transcriptase was used. In *Paper III* and *Paper IV*, mRNA was quantified by SYBR Green real time RT-PCR. In all four papers, each sample was analyzed in triplicate. Data were calculated by linearization of Ct values and corrected by the signal obtained in the same cDNA preparation for cyclophilin A or alternatively the mean value of cyclophilin A and GAPDH.

3.7 QUANTIFICATION OF PROTEIN EXPRESSION

Livers were homogenized in 20 M Tris-HCl, pH 7.5 / 2 mM $CaCl_2$ / 0.25 M sucrose. Homogenates were centrifuged at 4°C for 10 min at 2000g, supernatants were centrifuged at 110 000g for 45 min at 4°C. Pellets were suspended in 80 mM NaCl / 50 mM Tris-HCl, pH 8 / 2 mM $CaCl_2$ / 1% TritonX-100 and then centrifuged at 4°C for 10 min at 20 000g. The final supernatant was collected. After protein determination membranes were pooled groupwise. Pooled membranes (20, 30, 40 μg) were separated on 3-8% Tris-acetate gels (NuPAGE, Invitrogen). Proteins were transferred to nitrocellulose filter. ABCA1 protein was detected with mouse mAb (1:1000; Abcam) and as secondary antibody, a peroxidase-conjugated goat anti-mouse immunoglobulin

(1:20 000; Pierce). LDLR protein was detected with rabbit pAb (1:1000; Cayman) and a peroxidase-conjugated sheep anti-rabbit antibody (1:10 000; Immunokemi). SR-B1 was detected with rabbit antibody mAb (1:6000; Abcam) and a peroxidase-conjugated donkey anti-rabbit antibody (1:50 000; GE Healthcare). The specific bands were detected using a BioRad Universal Hood II (BioRad) and quantified by BioRad Quantity One software (BioRad). Signals were plotted by mg loaded protein and the slopes were calculated by method of least square. The slope of the control group was set to 100%.

3.8 QUANTIFICATION OF CHOLESTEROL 7A-HYDROXYLASE ACTIVITY

In *Paper III*, microsomes were obtained as described by Angelin at al. (141) and the enzyme activity of cholesterol 7α-hydroxylase was determined according to Einarsson et al. (142).

Frozen liver tissue was homogenized in 50 mM TRIS / 50 mM NaCl / 0.3 M sucrose / 10 mM EDTA / 10 mM DTT, pH 7.4. The homogenate was centrifuged at 20 000 g for 12 min at 6^{o}C. The supernatant was centrifuged at 100 000 g for 60 min at 6^{o}C. The pellet was homogenized in the same amount TRIS-buffer as previously but without DTT. Next the homogenate was centrifuged at 100 000 g for 60 min at 6^{o}C. Finally the pellet was homogenized in a 10% phosphate-buffer containing 100 mM PO_4 (KH_2PO_4 and K_2HPO_4) and 10 mM EDTA, pH 7.4.

Ethanol was added to the microsomes. The reaction was started with the addition of the cofactor NADPH and samples were incubated for 15 min at 37^{o}C. The incubation was terminated with Folch. D_6 7α-hydroxylase was added to all samples as an internal standard. The chloroform phase was evaporated and redissolved in Folch prior application to a TLC plate. Methanol was used to extract 7α-hydroxy-cholesterol from the silica gel. Samples were derivatized using hexamethyl-disilazane and trimethylchlorosilane in pyridine and analyzed by gas chromatography-mass spectrometry. Protein concentration was determined by using the method of Lowry (136).

3.9 QUANTIFICATION OF ACYL-COENZYME A:CHOLESTEROL O-ACYLTRANSFERASE ACTIVITY

In *Paper IV*, microsomes were prepared and ACAT activity analyzed according to Parini et al. (13).

Frozen liver tissue from individual animals was homogenized in 0.25 M sucrose / 0.1 M K_2HPO_4 / 1 mM EDTA, pH 7.4. Complete mini (Roche), a protease inhibitor, was added to the buffer before homogenization. Samples were centrifuged at 13 000 g for 15 min at 6^{o}C. The supernatant were centrifuged at 100 000 g for 35 min, the pellet was suspended in 0.1 M K_2HPO_4.

The microsomes were pre-incubated with a cholesterol-saturated solution of β-hydroxypropyl cyclodextrin and BSA for 30 min. Thereafter the reaction was started by the addition of C^{14}-oleoyl Co-A, after 20 min the reaction was terminated with Folch. In separate tubes, pyripyropene A, a specific ACAT2 inhibitor, was included in the preincubation and reaction mixture to separately identify ACAT1 and ACAT2 activities. H_3- cholesteryl oleate was added an internal control. The phases were separated by the addition of NaCl and the cholesteryl esters were isolated by TLC and the radioactivity was measured by scintillation spectrometry.

3.10 HISTOLOGICAL INVESTIGATION

In *Paper IV*, aortas were cryo-sectioned and stained for hematoxylin/eosin and oil red. Macrophages were detected with rat antibodies against Mac 1 and rabbit anti-rat secondary antibody. The samples were incubated with HRP labeled EnVision-rabbit before counterstaining with hematoxylin.

Skin biopsies were stained the same way as aortas. In the skin, thickness of dermis and hypodermis from the hypodermal muscle layer to the epidermis and thickness of the macrophage containing layer and thickness of the oil red stained layer was measured.

The histological investigation was done by MicroMorph Histology Services (Lund, Sweden).

3.11 STATISTICS

Statistical analyses were performed as described in the respective papers.

4 RESULTS AND DISCUSSION

4.1 INFLUENCE OF SUBSTRATE AND MEDIA SUPPLEMENTS ON BILE ACID PRODUCTION IN PRIMARY HUMAN HEPATOCYTES

Many investigators have studied the regulation of mRNA levels of enzymes important for BAs synthesis such as CYP7A1 and CYP8B1. However, in these different studies the culture conditions vary significantly (43-44, 143-145). In order to find the best way to culture primary human hepatocytes with respect to BA synthesis we investigated how different commonly used culture conditions alter the production of BAs.

Hepatocytes were isolated from normal human liver tissue obtained from patients (n=16) undergoing surgical liver resection due to cancer or from donor livers that could not be used for transplantation. Approval to use parts of resected human liver specimens for research was given by the Ethics Committee at Karolinska Institutet, alternatively from the Institutional Review Board at University of Pittsburgh. The hepatocytes were plated onto cell culture dishes pre-coated either with collagen or matrigel and cultured under standard conditions (described in the method section). In *Figure 4* cells were plated on collagen and covered with an EHS matrigel overlay on day two. Two different cell media were used; 'Standard media' which was supplemented with 120 nM insulin (ins) and 100 nM dexamethasone (dex) and 'Swedish media' which was supplemented with 12 nM ins, see Table 1. The medium was changed one hour after plating and then daily until harvesting. On day 5 the cells were harvested and cell culture medium was analyzed for BAs as described in the method section.

	Swe	**Swe + Dex**	**Std w/o Dex**	**Std**
Insulin	12 nM	12 nM	120 nM	120 nM
Dexamethasone	-	100 nM	-	100 nM

Table 1. Cell media composition.

In our experiments there were no difference of total BA production or BA composition when cells cultured on collagen was coated with an EHS matrigel overlay, see figure 4.

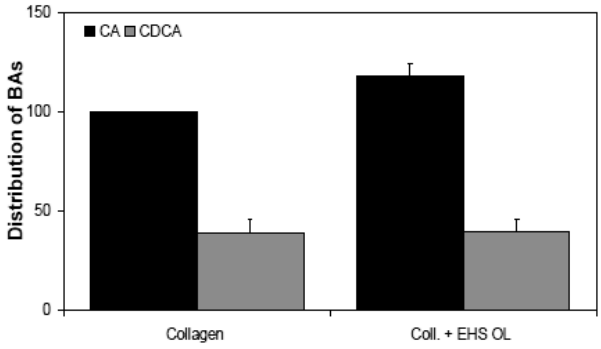


Figure 4. Bile acid levels in cell culture medium from primary human hepatocytes cultured on collagen or on collagen with an EHS matrigel overlay. Data are presented as relative values of means ± SEM, n=3 livers.

Next we measured BA synthesis in media from hepatocytes isolated from 6 different livers cultured in Standard (Std) or Swedish (Swe) media, on collagen or EHS matrigel. Cells cultured in either media produced more BAs when cultured on EHS, see figure 5. Cells cultured in Std media showed a markedly different composition to cells cultured in Swe media, with a higher proportion of CA leading to a much higher ratio of CA/CDCA, regardless of substrate.

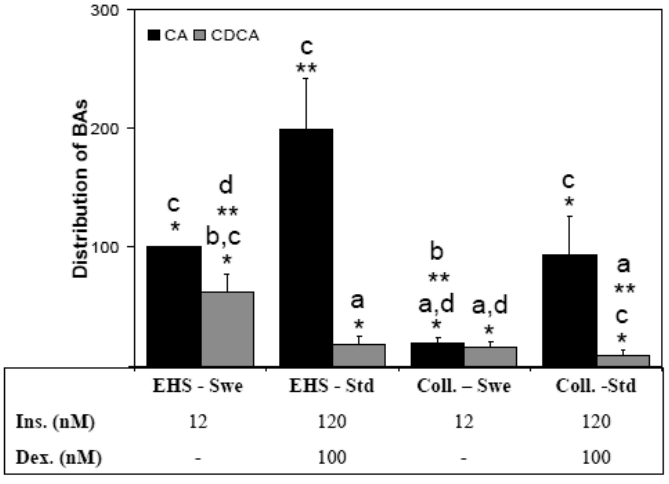


*Figure 5. Bile acid levels in cell culture medium from primary human hepatocytes cultured in different media and on different substrates. Data are presented as relative values of means ± SEM, * $p \leq 0.05$, ** $p \leq 0.01$. a = compared to EHS-Swe, b = compared to EHS-Std, c = compared to Coll.-Swe, d = compared to Coll.-Std, n = 6 livers.*

Since the difference between Std and Swe media is the concentration of both dex and ins we carried out a second set of experiments on EHS only where we included Swe media with dex and Std media without dex.

Analysis of BAs in media showed that ins at these concentrations does not affect BA synthesis whereas addition and removal of dex significantly altered the production of both CA and CDCA. Dex increased the total production of BAs, see figure 6.

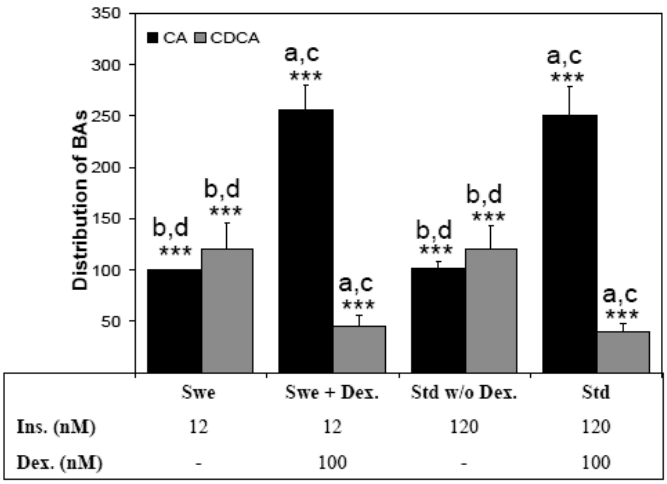


*Figure 6. Bile acid levels in cell culture medium from primary human hepatocytes cultured on EHS matrigel in different media. Data are presented as relative values of means ± SEM, *** p ≤ 0.001. a = compared to Swe, b = compared to Swe+Dex, c = compared to Std w/o Dex, d = compared to Std, n = 4 livers.*

In order to assay in what concentrations dex influences BA synthesis a dose experiment was performed. Primary human hepatocytes were cultured without dex (control) and with five doses of dex (0.1-200 nM), all cells were cultured on EHS and in low ins (12 nM). The lower dex concentration did not affect BA synthesis, but following treatment with 50 nM dex there is a significant increase of synthesized CA and a trend towards reduced levels of CDCA. This effect was sustained by the higher doses, see figure 7.

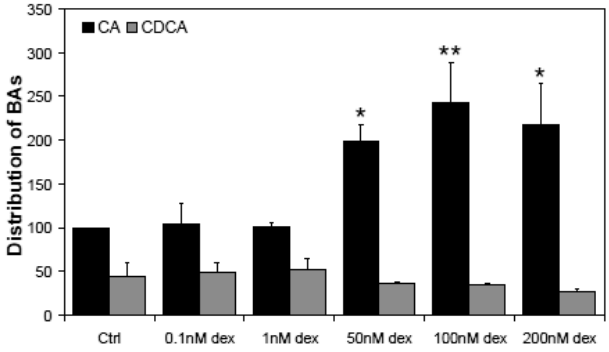


*Figure 7. Bile acid levels in cell culture medium from primary human hepatocytes cultured on EHS matrigel in Swedish media supplemented with dexamethasone. Data are presented as relative values of means ± SEM, * $p \leq 0.05$, ** $p \leq 0.01$ compared to controls, n = 3 livers.*

From our experiments it is clear that primary human hepatocytes synthesize more BAs when cultured on EHS compared to collagen. We also investigated the effect of dex and ins on BA formation and observed an increased BA formation and a changed BA composition following dex supplemention. The synthesis of CA was increased by dex and CDCA formation decreased.

4.2 PAPER I

In this project, the regulation of BA synthesis was studied in 23 patients with gallstone disease. The patients were either untreated and served as controls, or treated with cholestyramine or CDCA prior to surgery. In connection with the laparoscopic surgery a liver biopsy was taken and plasma and bile were collected.

Cholestyramine is a BA resin that acts by binding BAs, preventing them from uptake in the intestine and entering the enterohepatic circulation. Therefore CYP7A1 expression is increased and more BAs are formed. CDCA, on the other hand, increases the BA pool and also the negative feedback on CYP7A1 resulting in less synthesis of BAs. CYP7A1 expression is regulated by several pathways and in this study we addressed the question of which one is of the most importance during these conditions.

Following cholestyramine treatment the amount of CA in the bile increased and became the dominating BA, constituting about 76% of the BAs. In patients treated with CDCA, this BA became the major BA in the bile. The cholesterol saturation of the bile decreased to about 68%.

When we analyzed mRNA expression in the liver tissue we found that patients treated with cholestyramine had an almost 10-fold increase in mRNA expression of CYP7A1, whereas CDCA treatment decreased its expression by about 70% compared to controls. No significant differences were seen in CYP8B1, CYP7B1 or CYP27A1 levels, other enzymes important in BA synthesis.

We also investigated how the nuclear receptors and transcription factors involved in BA synthesis were affected by cholestyramine and CDCA treatment. Cholestyramine treated patients had a 60% increase in mRNA levels of HNF4α compared to controls, the expression of other analyzed nuclear factors, SHP, FXR, LRH-1, was not altered in the study. A significant correlation between CYP7A1 and HNF4α was observed.

In order to further investigate how the expression of HNF4α is regulated by changes in BA metabolism, mRNA from an earlier *in vitro* study using primary hepatocytes was analyzed. When RNA from primary human hepatocytes treated with glycine conjugated BAs was analyzed, a reduction of HNF4α by 25-30% was seen following CDCA treatment. The mRNA expression of CYP7A1 was decreased by 98%.

As described in the introduction BAs, especially CDCA, bind to and activate FXR which stimulates the expression of SHP. SHP causes an inhibition of CYP7A1 expression by interacting with LRH-1. In this study, neither FXR, SHP nor LRH-1 were significantly affected by CDCA or cholestyramine, suggesting that the SHP-dependent pathway of BA synthesis was not of major importance during these circumstances. This could be due to the fact that these nuclear factors are involved in many other regulatory functions in the cells and therefore their expression is under strict control *in vivo*. In addition, since we have not measured protein levels we can not exclude post-transcriptional regulations of the enzymes.

Following cholestyramine treatment, the expression of HNF4α increased and a positive correlation between HNF4α and CYP7A1 was observed, findings which suggest that HNF4α has an important role as a regulatory protein in the regulation of BA synthesis. The reduced level of HNF4α in primary human hepatocytes following CDCA treatment is in agreement with this concept. A recent study elucidating the effect of age on the regulation of BA synthesis in humans further supports the significance of HNF4α, but not SHP in BA regulation (146).

In 2006 it was shown that following CDCA treatment, the plasma levels of FGF19 were increased and after treatment with cholestyramine the FGF19 levels were reduced

(147). With this new knowledge it would be of great interest to investigate the plasma concentration of FGF19 in our patients. Unfortunately, this was not possible due to lack of material.

The results from this study suggest that during upregulation of BA synthesis with cholestyramine, the SHP-independent pathway, involving HNF4α, appears to be more important than the SHP-dependent pathway.

4.3 PAPER II

The study is an extension of the clinical study described in paper I. The same patient material was used. Here the effects of cholestyramine and CDCA on the metabolism of TGs, cholesterol and lipoproteins were investigated.
Modulation of BA synthesis by treatment with cholestyramine and CDCA influences the metabolism of lipoproteins, but the mechanisms behind the observed effects are not completely known in humans.

Following CDCA treatment, total plasma cholesterol levels increased from 5.5 to 6.0 in the patients, mainly due to an increase in LDL-cholesterol levels. Following cholestyramine treatment the opposite effect was observed. The total plasma cholesterol levels decreased from 5.4 to 4.5 mmol/L and LDL-cholesterol from 3.2 to 2.1 mmol/L.

To investigate the molecular mechanisms behind the changed lipoprotein profile, we analyzed mRNA expression in the liver biopsies. Following cholestyramine treatment mRNA expression of LDLR increased by 65% and that of PCSK9 increased by 70%. After CDCA treatment the levels of both LDLR and HMGCoAR mRNA decreased approximately by 50%. The expression of PCSK9 was not changed. The mRNA levels of LDLR, PCSK9 and HMGCoAR were significantly correlated to those of SREBP2.
There were no significant changes in mRNA levels for structures involved in HDL metabolism (ABCA1, APOA1, and CLA1), VLDL metabolism (APOA4, APOA5, APOCII, APOCIII, and APOE) or cellular lipid transport (ABCB4, ABCB11, ABCG5, ABCG8, and NPC1L1) in response to either treatment. Also, the mRNA levels of the genes controlling transcription (PGC1A, PGC1B, SREBP1, SREBP2, and HNF1A) were not affected.

A major finding is that following cholestyramine treatment the expression of PCSK9 is upregulated. PCSK9 expression was positively correlated to the expression of SREBP2, HMGCoAR and LDLR. The correlation between SREBP2 and PCSK9 indirectly suggests that PCSK9 expression is regulated by SREBP2, also in humans *in vivo*. It has previously been shown that PCSK9 is upregulated in transgenic mice overexpressing SREBP1 and SREBP2 (148). Another study has shown that insulin increases the expression of PCSK9 in primary rat hepatocytes and that the effect is mediated via SREBP1c (149). However, we did not obtain any correlation between SREBP1c and PCSK9, a finding in line with what has been observed in HepG2 cells and in primary human hepatocytes (150).

Our results suggest the following model; following cholestyramine treatment the negative feedback regulation of CYP7A1 decreases and the synthesis of BAs is increased. As a consequence, the sterol content in the liver is reduced and SREBP2 is activated. SREBP2 in turn, induces the expression of LDLR, which increases the cholesterol uptake from the plasma. The lowered plasma levels of LDL-cholesterol are compensated for by upregulation of HMGCoAR. At the same time the activation of SREBP2 also induces an upregulation of PCSK9 which degrades the LDLR. Thus, there is a dual regulation of the LDLR by SREBP2.
Unfortunately, in this study we did not have enough liver tissue to measure protein expression of LDLR, thus we do not know the net-effect on the LDLR protein expression. However, cholestyramine treatment caused a decrease in plasma levels of LDL indicating an increased protein expression of LDLR.

In the opposite situation, following CDCA treatment, CYP7A1 is inhibited causing increased sterol content in the liver which, in turn, reduces the expression of HMGCoAR and LDLR. In agreement with this, a trend towards increased LDL-cholesterol was observed following CDCA treatment.
However, *in vitro* studies have shown that the expression of LDLR is upregulated following CDCA treatment (151-153). The reason for the discrepancy between the *in vivo* and *in vitro* studies is unclear, probably *in vivo* treatment influences additional pathways and involves a crosstalk between liver, plasma and the intestine. In HepG2 cells the increased LDLR expression is suggested to be dependent on a mitogen-activated protein (MAP) kinase cascade since inhibition of MAP kinase activity blocks the upregulation of LDLR (152). More recently, a study using immortalized human

hepatocytes suggested that FXR signaling following CDCA treatment reduces PCSK9 expression and leads to increased LDLR expression (153). In the study, both treatment with CDCA and the synthetic FXR agonist GW4064 decreased PCSK9 expression while inducing LDLR expression. However, the effects were still present in FXR siRNA transfected cells, suggesting that other mechanisms may exist.

An improved knowledge about PCSK9 and the regulation of the protein is of special interest because of its therapeutic possibilities. PCSK9 has been shown to be upregulated by statin treatment (150, 154), thereby reducing the LDL-cholesterol lowering effect of statins. Therefore, PCSK9 is a promising target for new LDL lowering therapies, especially to be used in combination with statins. Recently, administration of an antisense oligo nucleotide (ASO) inhibitor targeting PCSK9 was shown to reduce plasma cholesterol in mice on a high fat diet (155).

From this study we can conclude that the increased BA synthesis during cholestyramine treatment leads to an upregulation of PCSK9 expression. The positive correlation between the expression of PCSK9 and SREBP2 indirectly suggests that SREBP2 regulates PCSK9 transcription in humans, *in vivo*.

4.4 PAPER III

Previous studies have reported that a moderate alcohol intake has positive effects on plasma levels of cholesterol (90, 97-99) as well as a reduced risk of gallstone disease (100-103). In this study we wanted to investigate whether the effects could be partly explained by an increased BA formation. Primary human hepatocytes were prepared from donor liver (n=11) and treated with ethanol.

Following addition of ethanol at a concentration of 7.7 mM (0.045%) to the cell culture medium, the amount of synthesized CA increased by approximately 20%. Treatment with 50 mM (0.29%) ethanol increased the production of CA by approximately 60%. In order to investigate if the stimulation of BA formation was specific for ethanol, the effect of 50 mM methanol on BA synthesis was investigated (n=6). Methanol addition did not affect BA synthesis. To examine if the increased BA synthesis was a direct effect of ethanol or if the effect was mediated by one of the products formed by ethanol oxidation, 4-methyl pyrazole (4-MP), which inhibits alcohol dehydrogenase was added

to the cells in combination with ethanol. When ethanol metabolism was inhibited by 4-MP, the stimulation of BA synthesis was abolished.
The increased BA synthesis was more pronounced for CA than for CDCA, hence the ratio between the BAs changed from 2.9 to 4.3, suggesting an increased bile acid synthesis via the classical pathway.

The mRNA expression of enzymes in the BA synthesis was studied using real time PCR. Large variations were seen in the expression of CYP7A1 between the livers. The expression of CYP27A1 and CYP8B1 was not changed following ethanol addition.
The time course of the ethanol effect was studied by analyzing BA content in the cell medium at different time points following ethanol addition. Increased production of BAs following addition of 50 mM ethanol was seen after 21 hours. The effect was even more apparent after 24 hours and remained stable until we stopped the experiment at 31 hours.
Although alcohol intake is very common, there are few studies of its effect on biliary lipids and BA formation in humans. In the present study we investigated if ethanol causes any changes in BA synthesis. It has previously been shown that alcohol intake increases the BA excretion in hyperlipidemic subjects, while the effect was not consistent in normolipidemic subject (108). It has also been shown that a single dose of ethanol increased the plasma concentrations of 7α-hydroxy-cholesterol and C4 in healthy subjects (104); both these intermediates reflect CYP7A1 activity and BA synthesis (105, 107). However, long-term intake of alcohol does not seem to influence the BA metabolism in humans (99).
There are difficulties, both ethical and practical, in recruiting healthy subjects to controlled long-term studies on the effects of ethanol and therefore there are only a few reports on how ethanol influences BAs. It should be noted that the above studies are rather small and involves a limited number of subjects and in addition some of the experiments were performed in subjects with a pre-existing alcohol abuse. Furthermore, the studies are quite old and the doses of ethanol were relatively high in some of the studies.

In this study treatment of primary human hepatocytes with ethanol increased the formation of BAs. The synthesis of CA was more increased than that of CDCA, indicating that the classical pathway of BA synthesis was upregulated upon ethanol addition to the cell medium. mRNA levels of CYP7A1, the rate limiting step in the

classical pathway, were measured in order to investigate how ethanol could stimulate BA formation. There was a great inter-individual variation in CYP7A1 expression and an increased CYP7A1 expression could not entirely explain the observed effects on BA synthesis. A possible explanation for the lack of mRNA induction in some livers could be that the transcription of CYP7A1 is already upregulated (de-repressed) in cultured hepatocytes. Next, we investigated if ethanol could have a direct stimulatory effect on the activity of CYP7A1. However, when ethanol was added to microsomes from human liver there was no effect on the cholesterol 7α-hydroxylase activity. Thus, the increased synthesis of BAs cannot fully be explained by increased mRNA levels of CYP7A1 or an increased direct activity of the enzyme.

A possible explanation for the increased synthesis of BAs following ethanol addition could be a change of redox levels ($NADH/NAD^+$) in the hepatocytes. Even small doses of ethanol cause an increase in the $NADH/NAD^+$ ratio which can influence BA formation (156-158). In line with this hypothesis, treatment with methanol, which does not change the redox levels, did not increase BA synthesis. However, the $NADH/NAD^+$ ratio is maximal when alcohol dehydrogenase is fully saturated at around 5-10 mM, and the greater increase with addition of 50 mM than with 7.7 mM ethanol argues against a direct effect of an ethanol-induced redox change. In order to investigate if an increased supply of substrate could cause the increased BA synthesis we added [2-$^2H_3$]-ethanol to the cells. But, the BAs formed did not seem to be a direct product of the labeled ethanol.

Thus, following treatment of primary human hepatocytes with ethanol, the formation of BAs is stimulated. An increased BA synthesis and thereby also an increased degradation of cholesterol could be a complementary explanation for the reduced risk of cardiovascular events and gallstone formation seen in subjects with a moderate alcohol intake compared to non-drinkers.

4.5 PAPER IV

In this study, we investigated the effects of KB3495 [Thyroid Receptor Agonists, PCT: WO/05092317A1] which is a selective TH mimetic compound designed to reduce the risk of developing atherosclerosis. The study describes the effect of KB3495 and the combined effect of KB3495 and atorvastatin on cholesterol metabolism.

ApoE deficient mice (n=80) were administered KB3495 and atorvastatin either alone or in combination for a period of 10 or 25 weeks. After 10 weeks of treatment the levels of CE in the aortas were approximately 60% lower in the group receiving KB3495 compared to the control group; there was also a tendency towards reduced CE levels in the combination group. These effects were seen despite clear differences in the levels of cholesterol in serum lipoprotein composition.

Also, in the liver the CE levels were reduced following treatment with KB3495, by 34% with KB3495 alone and by 59% with combination treatment. The reduced CE levels may be explained by a reduced cholesterol synthesis, supported by almost 60% lower lathosterol levels both in the KB3495 and combination group. However, lathosterol levels were not correlated to the mRNA expression of *Hmgcoar* indicating that post-transcriptional regulation may exist. The mRNA expression of *Ldlr* and *Pcsk9* were not affected by treatment with KB3495. Nevertheless, the protein expression for LDLR was decreased following KB3495.

Another possible explanation for the reduced cholesterol levels is an increased excretion of cholesterol. Fecal excretion of neutral sterols was increased in all treated groups compared to controls, the largest increase was observed in the combination group where the excretion was increased by 350%. Despite the increased excretion we did not observe any increase in the hepatic expression of *Abcg5* and *Abcg8* following KB3495 treatment suggesting that either *Abcg5* and *Abcg8* are regulated post-transcriptionally or that cholesterol is excreted through another pathway. A possible mechanism is via a direct excretion of plasma cholesterol from the enterocytes. Unfortunately, since no bile was collected the biliary lipid content could not be analyzed.

Cholesterol levels can also be lowered through an increased conversion into BAs and therefore BA synthesis was analysed. Fecal BA excretion increased by approximately 90% in the KB3495 group compared to controls, addition of atorvastatin partly reduced the effects of KB3495. Hepatic levels of C4 were not changed by KB3495 treatment alone but were increased in the combination group (157%). The same pattern was observed in the mRNA expression of *Cyp7a1* which was unchanged by KB3495 and increased by 204 % after combination treatment. The C4 and CYP7A1 results suggest that the neutral pathway may not be upregulated by KB3495 alone but with the combination treatment, however, BA analysis in the serum showed no major differences in BA composition between the groups. Intestinal mRNA expression of *Fgf15* was reduced to approximately 10% by KB3495 alone or in association with

atorvastatin. A reduced expression of Fgf15 suggests that BA synthesis is upregulated both by KB3495 alone and in combination with atorvastatin, a finding that could explain the observed increase in BA excretion. Hepatic expression of *Cyp8b1*, *Cyp27a1*, *Shp* and *Abcb11* was not changed by KB3495 treatment.

Next, we wanted to investigate the effects of maintaining the treatments for a period of 25 weeks.

After 25 weeks of a Western like diet the skin of the control group appeared thickened and the fur was sparse and in poor condition, therefore skin samples were collected and CE levels in the skin were analyzed. In animals receiving KB3495 CE levels were reduced by approximately 50%, in the combination group the levels were reduced by almost 95%. After histological staining the reduced lipid content was confirmed. The staining also showed an increase in the thickness of dermis and hypodermis in most of the animals which was due to a large number of foam cells/macrophages. This was most accentuated in the control animals, KB3495 treatment reduced the inflammation and swelling and in the combination group no macrophages were found at all.

Following 25 weeks CE levels in the aorta were reduced by 65% in the combination group and there was a clear trend towards reduced levels also in the group treated with KB3495. Aortas underwent histological examination to investigate if there also was a reduced plaque formation. The plaque area was 35% lower in KB3495 animals compared to controls (calculated by dividing the plaque area by the aorta circumference). The percentage of oil red staining in the plaques was 30% lower in KB3495 animals compared to controls (measured by dividing the area of oil red staining by the total plaque area). Thus, despite the fact that no significant changes of CE levels in aorta were observed following KB3495 alone the atherosclerosis was still reduced.

After 25 weeks of treatment, no major differences were observed in serum lipoprotein profile between the control animals and the KB3495 treated animals.

CE levels in the liver were not significantly lower in the KB3495 group, but a decrease was observed in the combination group (-37%). Lathosterol content in the liver showed the same pattern following 10 and 25 weeks of treatment. However, the expression of genes involved in hepatic cholesterol metabolism showed altered expression pattern. The mRNA levels of the *Ldlr* were increased to 142% in the KB3495 group whereas no change was observed in the atorvastatin and combination group. In contrast, *Pcsk9* expression was markedly upregulated following atorvastatin and combination

treatment. No significant changes were observed in the KB3495 group. Despite the high mRNA expression of *Pcsk9,* the protein level of LDLR was increased following combination treatment. Similar to 10 weeks of treatment, mRNA expression of *Hmgcoar* was not changed by KB3495 but increased following combination treatment (213%). Gene expression of *Abcg5* and *Abcg8* were not changed from 10 to 25 weeks. After 25 weeks hepatic C4 was increased following KB3495 and combination treatment (138%, 236% respectively). *Cyp7a1* expression was not changed in either group, whereas intestinal *Fgf15* expression was still reduced following KB3495 and combination treatment but not to the same extent. These results suggest that the BA synthesis is still upregulated following 25 weeks of treatment but unfortunately no feces were collected so the assumption could not be confirmed.

In this study we investigated the long term effects of KB3495 on the reverse cholesterol transport with special focus on the development of atherosclerosis. Using ApoE deficient animals we can for the first time show that atherosclerosis is reduced following treatment with KB3495. To our knowledge only one study on the combination of TH mimitics and statins is previously reported. In that study, the combination treatment caused a greater decrease on total plasma cholesterol than either drug administered as monotherapy (127). Unfortunately, only plasma cholesterol was analyzed and no mechanistic conclusions could be drawn.

Previous studies with selective TR mimetics have showed reduced levels of circulating cholesterol (124-128). A suggested mechanism has been an increased expression of LDLR. However, despite studies in rats where both T_3 and TR mimetics increase the expression of LDLR (125, 159-160) we could not detect any increase in LDLR expression, neither in this study nor in a previous study where mice were treated with GC-1 (124). To our knowledge only one study in mice has shown increased *Ldlr* expression following T3 and MB07811 administration and this effect was transient and only detected after 3 hours or 8 hours respectively (126). Thus, there might be a species difference in LDLR regulation in response to TR modulation. Also, the lack of response in LDLR expression observed in this study explains the unchanged levels of circulating cholesterol in our animals.

In the present study the formation of BAs is upregulated. This is in line with previous studies with TH and TH mimetics that have also shown that CYP7A1 expression is

regulated by TH in rodents (124, 129-130). As discussed in the introduction, CYP7A1 expression can be negatively regulated through a SHP dependent feedback inhibition, however in our results we can not detect any significant changes in SHP expression. Another pathway for CYP7A1 regulation is by FGF15 signalling from the intestine. Our results show that KB3495 strongly represses FGF15 expression in the intestine, thus suggesting that increased BA formation following TH treatment might be FGF15 dependent.

These results are restricted to mice and it is difficult to anticipate the effects of long term treatment in humans. Up till now only one study in humans using a compound related to KB3495 (KB2115) has been published (128). In the study, subjects with moderately elevated plasma cholesterol were treated with KB2115 for two weeks. Treatment decreased serum cholesterol, possibly through an increased conversion of cholesterol into BAs since C4 levels increased and lathosterol levels were not affected. These results are very interesting both from a therapeutic point of view but also since there are reported species differences in the regulation of BA formation by TH. Previous studies have shown that in contrast to rodents, mRNA levels of CYP7A1 are reduced by T_3 addition in human cells (161-162). Thus, these results emphasize the need for further investigations in this field.

The results from this study show that in ApoE deficient mice, the TRβ modulator KB3495 is effective in reducing atherosclerosis and that the reduction occurs independently of changes in the total cholesterol levels in ApoB-containing lipoproteins. Cholesterol levels are reduced by multiple ways; cholesterol synthesis is decreased, formation of BAs is induced, as well as the excretion of fecal BAs and neutral sterols. Thus, KB3495 seems to stimulate the reverse cholesterol transport in ApoE deficient mice leading to reduced atherosclerosis and CE deposition in the skin.

5 GENERAL DISCUSSION

Excess plasma cholesterol levels, in particular high levels of LDL cholesterol, is a major risk factor for the development of atherosclerosis. One major pathway for cholesterol elimination from the body is by its conversion into BAs. Hence, an increased understanding of this process is important for the development of new therapeutic strategies aimed to prevent and reduce atherosclerosis.
In this thesis we have investigated how different modifications of lipoproteins and bile acids affect cholesterol metabolism.

In *Paper I* and *Paper II,* CDCA was used to downregulate the synthesis of BAs. CDCA increases the enterohepatic circulation and thereby downregulates the synthesis of BAs. Of the BAs, CDCA is the strongest inhibitor of CYP7A1 expression and is therefore useful for studies on BA regulation. CDCA was previously used for treatment of gallstone disease, however, due to recurrence of gallstones and side effects, patients are currently only treated with CDCA in exceptional cases. Following a CDCA induced reduction of BA formation the sterol content in the liver increases and in order to compensate the liver reduces the expression of LDLR, as a consequence plasma LDL-cholesterol increases. The liver also downregulates the expression of HMGCoAR to reduce the synthesis of cholesterol.
In the opposite situation, when cholestyramine was used to upregulate BA synthesis an expected increase of CYP7A1 was observed. Increased levels of CYP7A1 were accompanied by an increased expression of HNF4α suggesting that this transcription factor is important for the regulation of BA synthesis in humans. Upregulation of BA synthesis causes a deprivation of cholesterol in the liver which is counteracted by an increased expression of LDLR. In our results there are also clear tendencies to increased levels of HMGCoAR. The induced levels of CYP7A1 and LDLR give lower LDL-cholesterol levels and therefore cholestyramine can be used as a lipid-lowering drug. Unfortunately, the SREBP2 activation caused by cholestyramine (and statins) also increases the expression of PCSK9 which may attenuate the effect of the therapy by inducing the degradation of LDLRs. Therefore a selective inhibition of PCSK9 may be a promising strategy for future drug development.
As mentioned in the introduction, cholestyramine was one of the first lipid-lowering drugs that were developed. Since then, statins have become the first choice of treatment

against atherosclerosis. Cholestyramine can still be an alternative treatment for those who do not respond to statin treatment but in that case cholesterol absorption inhibitors, like ezetimide, is more commonly used. These newer drugs are often more efficient and more palatable.

Also in *Paper III*, the upregulation of BA synthesis was studied. However, this time only BA formation and the expression of the BA synthesizing enzymes were studied. Following ethanol treatment of primary hepatocytes the BA formation was increased. Increased synthesis was observed in all studied livers. However, mRNA levels of CYP7A1 were only increased in some of the livers, suggesting that synthesis can be regulated also at another level, possibly through an increase in CYP7A1 protein expression. The increased BA synthesis could provide one explanation for the observed reduction of cardiovascular- and gallstone diseases following alcohol intake.

One possible weakness of the first studies in this thesis is that they are reliant on mRNA data. Since they are clinical studies we did not have enough material to analyze protein expression in the liver samples. Nevertheless, their strength and relevance is to elucidate the steady-state mRNA expression and offer the possibility to elaborate mechanistic models on the regulation of cholesterol metabolism in humans.

In *Paper IV* the effect of KB3495, a TR modulator, was investigated. The observed anti-atherosclerotic characteristic of KB3495 seems to by dependent on several mechanisms. KB3495, alone or in combination with statins, reduces the cholesterol content in aorta, liver and skin by an upregulation of the BA formation as well as a reduction of cholesterol synthesis and an increased fecal excretion of neutral sterols. Extremely interesting is that all the above effects take place independently of a reduction of cholesterol in ApoB-containing lipoproteins. Combined treatment of KB3495 and statins could provide a possible strategy to treat atherosclerosis.

In this thesis cholesterol homeostasis was investigated in different models. Although our main interest is how cholesterol metabolism is regulated in human, *in vivo*, studies of human liver metabolism can be complicated because of the difficulties to acquire healthy liver tissue. One possibility is to study patients that are scheduled for liver surgery where a small piece of liver tissue can be collected in connection to the surgery. This approach was used in *Paper I* and *Paper II* where patients with gallstone disease

were studied. The drawback of most clinical studies is that the number of subjects is limited, this is also the case in our studies. The small number of subjects may make it difficult to detect small differences between the groups, especially since the variation between patients is generally larger than variation between animals or cells. Another concern working with human samples is the type of patients to include. In our papers, patients with gallstone disease has been studied, even though the patients were otherwise healthy we can not exclude that their underlying disease may be the result of some variations in expression of genes involved in cholesterol or BA metabolism. However despite these shortcomings, studies in humans provide an invaluable opportunity to elucidate the mechanism present *in vivo*.

Studies on primary human hepatocytes have some of the same problems experienced with clinical studies. The studies are dependent on the availability of normal liver tissue. Most of the tissues used in these experiments are obtained from patients undergoing resections due to liver tumors and even though the 'normal' tissue surrounding the tumor is used, the general health status of the patients may vary. Since primary hepatocytes are well differentiated cells, the variation between cells from different patients can be rather large, therefore experiments may need to be repeated more times than when using carcinoma cells. The major advantages using primary human hepatocytes is the possibility to work *in vitro* with human cells that are more differentiated and maintain more liver specific functions compared to cell lines.

When the systemic effects of new, potential drugs are investigated the use of animal models are a necessary. Animal models are also very useful when long term and complex diseases are studied. In *Paper IV*, ApoE deficient mice were used because they spontaneously develop advanced atherosclerotic lesions that resemble those found in humans. Although this model is frequently used and well characterized it is not possible to necessarily assume that the same mechanisms are present in humans. Therefore, future clinical trials using TRβ modulators would be needed.

6 CONCLUSIONS

Cholesterol homeostasis is extremely important for several functions in the body and therefore the regulation of cholesterol metabolism is very complex and involves many mechanisms in multiple steps. In this thesis we attempted to elucidate some of the mechanism involved and to study the modulation of this process by selected substances.

From the studies the following conclusions can be drawn:

- Dexamethasone increases the synthesis of CA in primary cultures of human hepatocytes.

- HNF4α appears to be an important factor for the regulation of BA formation in humans.

- In humans SREBP2 regulates LDLR both by inhibition of LDLR expression and by upregulation of PCSK9 expression.

- Addition of ethanol to primary human hepatocytes stimulates the formation of BAs.

- KB3495 together with atorvastatin reduces atherosclerosis in mice independently of changes in plasma cholesterol levels.

7 SVENSK SAMMANFATTNING

Ateroskleros, eller åderförfettning, är sjukdom som på grund av vårt levnadssätt samt att vi lever allt längre, blivit vanligare de senaste åren. Vid ateroskleros bildas beläggningar i artärernas väggar vilka därmed blir tjockare och förlorar sin rörlighet, detta kan i sin tur leda till hjärtinfarkt, kärlkramp eller stroke. Idag finns läkemedel som minskar risken att utveckla ateroskleros men alla patienter uppnår inte sina behandlingsmål och somliga får biverkningar av behandlingen. Därför är det viktigt att få en ökad kunskap om de bakomliggande orsakerna till sjukdomen och fortsätta forskningen för att utveckla nya mediciner.

Det slutliga målet är att ta reda på hur kolesterolmetabolismen regleras i människa och hitta de läkemedel som fungerar för människor. Emellertid finns det många svårigheter med patientstudier; framförallt är många studier inte möjliga att genomföra på grund av etiska grunder och dessutom finns det flertalet praktiska problem. Därför kan kolesterol och gallsyraomsättningen även studeras i flera andra modellsystem. Den här avhandlingen innehåller studier utförda i människor, i leverceller isolerade från människor samt i en musmodell.

En av svårigheterna med studier på människor är att få tag i frisk levervävnad. I de två första studierna löstes detta problem genom att tillfråga patienter med gallsten om en liten bit levervävnad kunde erhållas i samband med deras gallstensoperation. Till följd av svårigheterna med att få patienter till studier är en vanlig nackdel med kliniska studier att antalet patienter är begränsat. Ett annat problem med humana studier är att variationen mellan människor ofta är större än variationen mellan djur eller celler.

I den tredje studien användes leverceller som vi först isolerat från levrar erhållna från patienter som opererats för cancer eller alternativt från levrar som tagits ut för organdonation men sedan inte kunnat transplanteras. I jämförelse med andra så kallade ”carcinoma cellinjer” behåller dessa nyisolerade leverceller många av sina leverspecifika egenskaper. Fördelen med cellförsök är att man kan studera en specifik celltyp samt att man har större kontroll på olika faktorer i sitt system och kan reglera dessa vilket underlättar cellmolekylära undersökningar. En annan fördel är att det inte finns något etiskt dilemma vilket medför att cellerna kan behandlas med vilket ämne som helst. Eftersom cellerna isoleras från levervävnad har de samma nackdelar som de kliniska studierna; det kan vara svårt att få tillräckligt många patienter till studierna och

variationen är stor mellan olika patienter. Att variationen är stor kan dock även vara en fördel då det kan spegla den naturliga variationen i populationen.
När nya, potentiella läkemedel undersöks är det nödvändigt att göra djurstudier. Studier på djur, vanligtvis möss, är även mycket användbara när man vill studera komplexa sjukdomar som tar flera år att utveckla eller när man vill analysera vilken effekt ett ämne har på alla organ i kroppen. Normala möss är relativt motståndskraftiga mot ateroskleros och därför har vi i detta arbete använt en genetiskt modifierad musstam som lätt utvecklar ateroskleros.

Kolesterol är inte vattenlösligt, därför transporteras det som lipoproteinkomplex i blodet. Lipoproteinet LDL transporterar kolesterol från levern ut i kroppen, lipoproteinet HDL transporterar kolesterol tillbaka till levern där kolesterol kan utsöndras från kroppen. Förenklat brukar LDL benämnas ”det onda kolesterolet” och HDL ”det goda kolesterolet” eftersom höga nivåer av LDL och låga nivåer av HDL ökar risken för ateroskleros.
Levern är ett viktigt organ för regleringen av kolesterolomsättningen. En stor del av kolesterolet som finns i kroppen bildas i levern och lipoproteinnivåerna i blodet regleras av upptag och utsöndring från levern. Levern är även nödvändig för nedbrytning och utsöndring av kolesterol från kroppen. Ett viktigt sätt att göra sig av med kolesterol är att omvandla det till gallsyror. Gallsyror är även nödvändiga för kolesterolabsorption i tarmen.
Framställning och nedbrytning av kolesterol och gallsyror är strikt reglerad av kroppen. Båda dessa ämnen kan reglera sin egen omsättning eller regleras av andra ämnen, exempelvis hormoner. Vid regleringen finns transkriptionsfaktorer vilka binder DNA och på så sätt styr genens aktivitet.

Syftet med denna avhandling är att förbättra vår kunskap om hur kolesterolnivåerna regleras och att studera hur modifiering av lipoproteiner och gallsyror påverkar kolesterolomsättningen.

I *Delarbete I* studerades hur bildandet av gallsyror regleras. Patienter var obehandlade eller behandlades antingen med ett läkemedel som ökar bildningen av gallsyror, alternativt med en gallsyra vilket minskar kroppens egen produktion av gallsyror. När patienterna gallstensopererades togs en leverbiopsi, blod och galla. Sammansättningen av lipider och gallsyror i gallan analyserades och i leverbiopsierna analyserades

uttrycket av gener som är viktiga för gallsyraproduktionen. I studien kom vi fram till att transkriptionsfaktorn HNF4α är av stor betydelse för regleringen av gallsyrasyntesen i människor.

Delarbete II är en utökning och fördjupning av den studie som gjordes i delarbete I. Samma patienter ingick i studien men i denna artikel undersöktes hur gallsyror påverkar lipoproteinomsättningen. Uttrycket av gener som styr nivåerna av lipoproteiner i blodet analyserades. Vi kunde visa att transkriptionsfaktorn SREBP2 reglerar uttrycket av den nyupptäckta genen PCSK9 hos människor.

I *Delarbete III* studerades om alkohol påverkar produktionen av gallsyror. Leverceller behandlades med etanol, metanol eller ett ämne som förhindrar nedbrytningen av alkohol. Cellernas gallsyrasyntes och uttrycket av gener involverade i bildningen av gallsyror bestämdes. Vid behandling såg vi en etanolspecifik ökning av gallsyraproduktionen. Det var framför allt produktionen av en gallsyra (cholsyra) som ökade vid alkoholbehandling vilket tyder på att den klassiska syntesvägen av gallsyror var uppreglerad. Den ökade syntesen av gallsyror kan vara en förklaring till att tidigare studier visat att ett ökat alkoholintag är associerat med en minskad risk för att utveckla hjärt-kärl- och gallstenssjukdomar.

I *Delarbete IV* undersöktes effekten av KB3495 vilket är en substans framställd för att minska ateroskleros. De möss som ätit KB3495 hade mindre ateroskleros samt lägre kolesterolnivåer i aorta, lever och hud än obehandlade möss. Ombildningen av kolesterol till gallsyror var ökad liksom utsöndringen av gallsyror och neutrala steroler i avföringen. Resultaten tyder på att KB3495 eventuellt skulle kunna användas som ett läkemedel för att motverka ateroskleros i framtiden.

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