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

STUDIES ON THE REGULATORY ROLES OF CHOLESTEROL AND BILE ACIDS

Charlotte Murphy

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The universe is full of magical things, patiently waiting for our wits to grow sharper.

Eden Phillpotts (1862 – 1960)

Dedicated to the memory of Anthony Pierse Murphy

ABSTRACT

Cholesterol is essential for normal growth and development in mammals. However, excess cholesterol can be harmful leading to diseases such as atherosclerosis and gallstones. Once formed the ring structure of cholesterol cannot be catabolised by the body and must therefore be converted to other compounds for excretion. In most vertebrates the faecal route of cholesterol excretion is through the formation of bile acids (BA) and the solubilisation of free cholesterol in bile. Cholesterol is converted into the primary bile acids cholic acid (CA) and chenodeoxycholic acid, the ratio of which is determined by the enzyme sterol 12α-hydroxylase (CYP8B1). Both cholesterol and BAs regulate their own synthesis, and other biological processes, through the actions of transcription factors such as the sterol regulatory element binding proteins (SREBP), the liver X receptor and the farnesoid X receptor.

A Cyp8b1 knockout mouse model has been created, which lacks the ability to synthesize CA. Using this model we investigated the regulatory effects of CA and cholesterol on metabolism and found that a reduction in CA resulted in decreased cholesterol absorption, increased cholesterol synthesis and was protective against large increases in hepatic cholesterol levels. Our results suggest an inhibitor of CYP8B1 may reduce cholesterol levels and thereby reduce the development of cardiovascular disease.

The gene involved in cholesterol synthesis most responsive to regulation by CA and cholesterol treatment was squalene epoxidase (Sqle). Therefore the promoter of this gene was investigated further. In analogy to the human SQLE promoter the murine promoter was activated by SREBPs. A 205bp region of the promoter, containing three novel sterol regulatory elements, was found to be responsible for SREBP-2 regulation.

While investigating the promoters of other genes involved in cholesterol synthesis mevalonate kinase was found to share a short common promoter with the cob(I)alamin adenotransferase gene (MMAB) catalysing the synthesis of AdoCbl, a cofactor for methylmalonyl CoA mutase. In mice the expression of Mmab was increased by statin treatment. Therefore, statins may represent a novel treatment for patients with methylmalonic aciduria type B, resulting from mutations in this gene.

Kommentar [SM1]: It's a common mechansims throughout the mammalia, the man and mouse stuff isn't wrong per se, but putting it in a broader contect is good wrt PhD stuff.

LIST OF PUBLICATIONS

I. Cholic acid as key regulator of cholesterol synthesis, intestinal absorption and hepatic storage in mice
Murphy C, Parini P, Wang J, Björkhem I, Eggertsen G, and Gåfvels M
Biochim Biophys Acta, 2005, 1735, 167-75

II. Studies on LXR- and FXR-mediated effects on cholesterol homeostasis in normal and cholic acid-depleted mice
Wang J, Einarsson C, **Murphy C**, Parini P, Björkhem I, Gåfvels M, and Eggertsen G
Journal of Lipid Research, 2006, 47, 421-30

III. Promoter analysis of the murine squalene epoxidase gene. Identification of a 205 bp homing region regulated by both SREBP'S and NF-Y
Murphy C, Ledmyr H, Ehrenborg E, and Gåfvels M
Biochim Biophys Acta, 2006, 1761, 1213-27

IV. Regulation by SREBP-2 defines a potential link between isoprenoid and adenosylcobalamin metabolism
Murphy C, Murray AM, Meaney S, and Gåfvels M
Biochem Biophys Res Commun. 2007 Apr 6;355(2):359-64

CONTENTS

LIST OF ABBREVIATIONS

ABC	ATP-binding cassette
ACAT	Acyl-CoenzymeA:cholesterol acyltransferase
AD	Alzheimer's disease
APP	Amyloid precursor protein
apo	Apolipoprotein
BA	Bile acids
CA	Cholic acid
CDCA	Chenodeoxycholic acid
CE	Cholesteryl ester
CSI	Cholesterol saturation index
CTX	Cerebrotendinous Xanthomatosis
CVD	Cardiovascular Disease
CYP7A1	Cholesterol 7α-hydroxylase
CYP27	Sterol 27-hydroxylase
CYP8B1	Sterol 12α-hydroxylase
ER	Endoplasmic Reticulum
FXR	Farnesoid X receptor
HDL	High density lipoprotein
HMG CoA	3-Hydroxy-3-methylglutaryl coenzyme A
HMGCR	3-Hydroxy-3-methylglutaryl coenzyme A reductase
HMGCS	3-Hydroxy-3-methylglutaryl coenzyme A synthase
LDL	Low density lipoprotein
LDL-R	Low density lipoprotein receptor
LRH-1	Liver receptor homolog-1
LRP	LDL-R related protein
NF-Y	Nuclear factor Y
NPC1L1	Niemann-Pick C1 Like 1
S1P	Site 1 protease
S2P	Site 2 protease
SCAP	SREBP cleavage activating protein
SHP	Short heterodimer partner
Sp1	Stimulating protein 1
SQLE	Squalene epoxidase
SR-B1	Scavenger receptor B-1
SRE	Sterol response element
SREBP	Sterol response element binding protein
TAG	Triacylglycerols
VLDL	Very low density lipoprotein

1 INTRODUCTION

1.1 CHOLESTEROL

Cholesterol is the most common steroid found in animals and is the precursor for most other animal steroids. As well as functioning as a precursor to steroid hormones and bile acids (BA), cholesterol is an essential structural component of animal cellular membranes, accounting for between 10% and 50% of the total lipid content. Cholesterol is a rigid structure and its presence stabilises the cell membrane, reducing its fluidity. Another important role for cholesterol is the lateral organisation of membrane associated proteins into functional clusters called lipid rafts[1]. These rafts may have a role in cholesterol efflux as the ATP-binding cassette (ABC) A1 transporter and scavenger receptor B-1 (SR-B1) have been localised to lipid rafts.

1.2 CHOLESTEROL SYNTHESIS

Most nucleated cells in mammals are capable of synthesizing cholesterol for their own use; however, the majority of cells also obtain cholesterol from lipoproteins synthesized by the liver and intestine. The brain is unique in that it does not obtain cholesterol from lipoproteins and transvascular diffusion, but synthesizes all of the cholesterol it requires. The brains ability to synthesize cholesterol is impressive, considering that it makes up only 2% of whole body mass and yet accounts for almost 25% of the cholesterol found in the body.

Isoprenoid biosynthesis is a complex procedure involving a large number of enzymes located both in the cytosol and the cytosolic surface of the endoplasmic reticulum (ER) as overviewed in Figure 1. Cholesterol is one important product of this pathway, however other products include geranyl-geranyl-pyrophosphate and solanesyl-pyrophosphate, involved in protein isoprenylation and ubiquination. The process begins with the conversion of acetyl-CoA by HMG CoA synthase (EC 2.3.3.10) to HMG CoA. HMG CoA is reduced by HMG CoA reductase (HMGCR) (EC 1.1.1.88), the rate-limiting step in isoprenoid biosynthesis, to CoA and mevalonate. The transcription and activity of HMGCR is regulated by cholesterol concentration via a negative feed back mechanism. When cellular cholesterol concentration is low HMGCR is actively

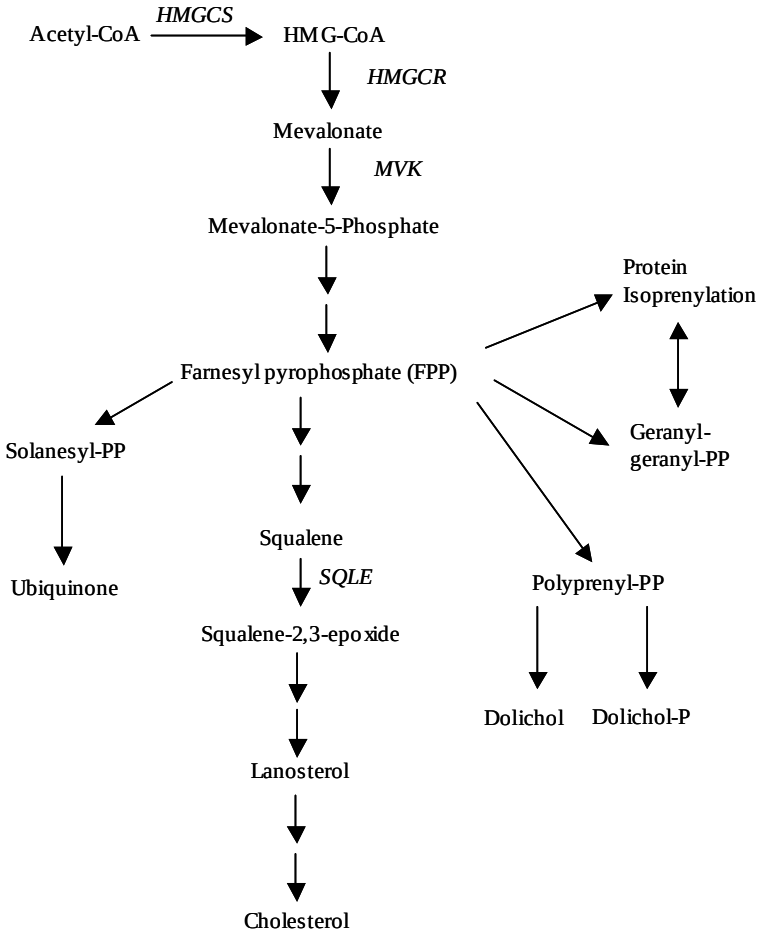


Figure 1 An overview of isoprenoid biosynthesis from acetyl-CoA to cholesterol

transcribed, but when cholesterol is abundant the protein Insig-1 binds to the sterol sensing domain of HMGCR leading to its ubiquitination and degradation[2]. HMGCR has a half-life of 2-3 hours that can be increased in the absence of exogenous cholesterol[3]. Mevalonate kinase (MVK) (EC 2.7.1.36) converts mevalonate to mevalonate-5-phosphate, which subsequently through a number of steps is converted to farnesyl-pyrophosphate. Farnesyl-pyrophosphate represents the substrate of squalene synthase (EC 2.5.1.21), and is converted to squalene. Squalestatins targeted against squalene synthase are available as anti-fungal treatments e.g. terbinafin (Lamisil®). In the only oxidation step in cholesterol biosynthesis, squalene is oxidised by squalene epoxidase (SQLE) (EC 1.14.99.7) forming 2,3-oxidosqualene. Having an extremely low specific activity compared to HMGCR or squalene synthase[4], SQLE is considered to be a secondary rate-limiting step in cholesterol biosynthesis and a potential target for

anti-hyperlipidemic drugs[5,6]. In contrast to HMGCR it is regulated by exogenous and endogenous sterols and not by nonsterol derivatives of mevalonate[7,8]. Many compounds have been found to inhibit SQLE including Green Tea[9], NB-598[10], FR194738[11,12] and selenium compounds[13]. The activity of SQLE is believed to be determined by changes in enzyme concentration resulting from an altered transcription level[5]. Although it is known that murine Sqle is regulated by cholesterol the precise mechanisms by which this strong regulation occurred were unclear and required further investigation. Lanosterol synthase (EC 5.4.99.7) catalyses the cyclization of squalene to lanosterol, which is in turn converted to cholesterol by further enzymatic steps.

1.3 INTESTINAL PROCESSING OF DIETARY CHOLESTEROL

The average Western diet contains 300-500 mg of cholesterol per day. This dietary cholesterol mixes with cholesterol released from the gallbladder, resulting in a total of 800-1200 mg of cholesterol presented intestinally, of which 30-50% is absorbed. Cholesterol is a hydrophobic compound requiring chemical detergents to aid its solubilisation and thereby its absorption in the gut. It has been demonstrated that the presence of BAs in the gut is a prerequisite for the absorption of lipids and lipid soluble vitamins from the diet[14]. In man and mouse models, an increase in CA has been shown to increase cholesterol absorption from the diet[15,16]. In the intestine the amphipathic BAs mix with cholesterol, lipids and fat-soluble vitamins from the diet to form mixed micelles, which are able to pass through the unstirred water layer to the intestinal enterocytes[17]. At the enterocytes the micelles disperse and their contents are available for absorption. The solubilisation of cholesterol by bile, its uptake and processing by enterocytes can be seen schematically in Figure 2.

1.3.1 Intestinal absorption of cholesterol

Cholesterol absorption is a selective, active protein-mediated process. Although the exact cholesterol transport protein remains elusive, a number of proteins are known to be involved in cholesterol absorption. Cholesterol, plant sterols and marine sterols are all absorbed from the diet. However, sterols other than cholesterol are selectively removed from enterocytes and transported back to the intestinal lumen by the ABC half transporters G5 and G8.

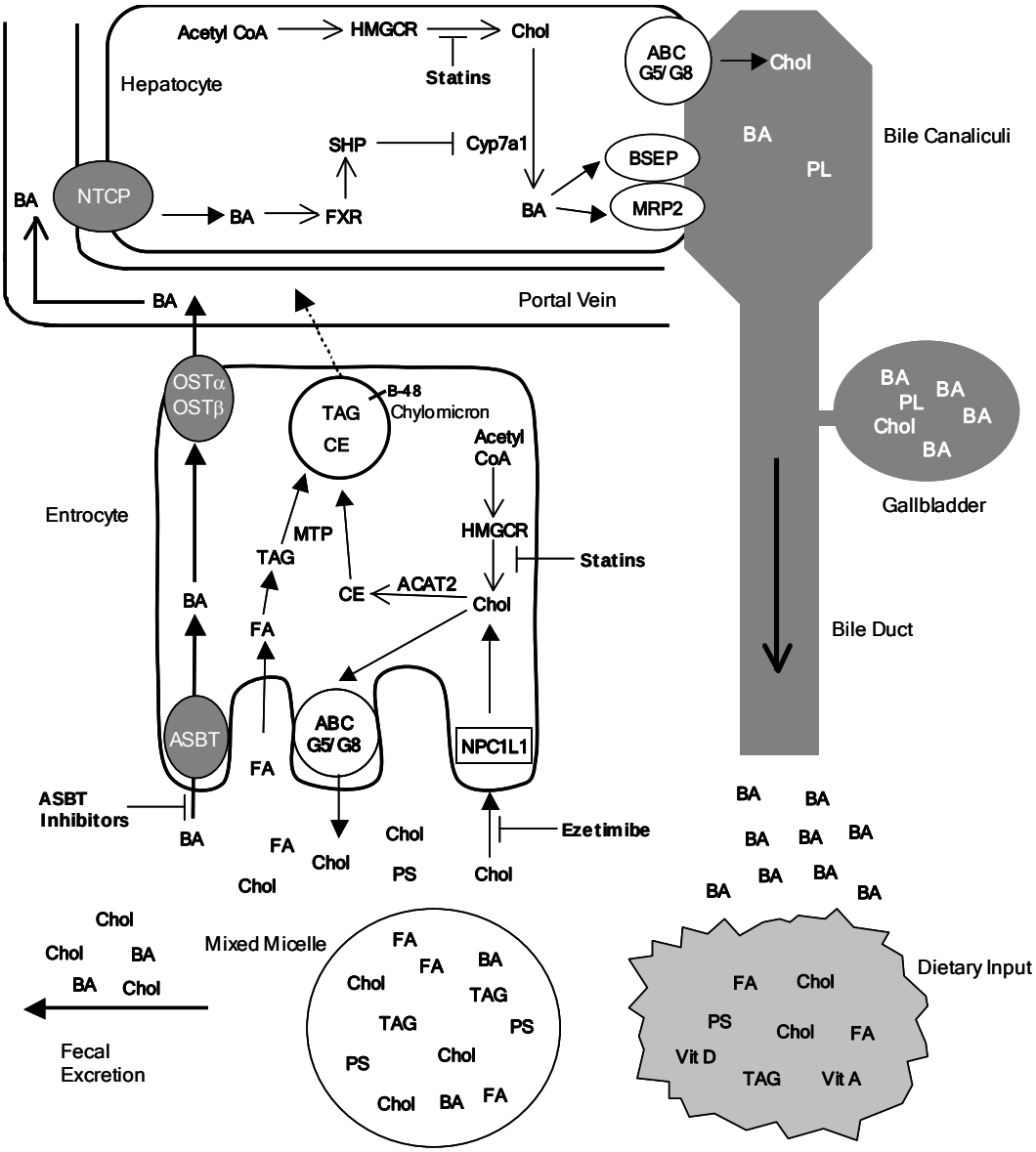


Figure 2 Enterohepatic regulation of BA and cholesterol metabolism. I. Intestinal lumen: Solubilisation of cholesterol (Chol), triacylglycerols (TAG), plant sterols (PS) and fatty acids (FA) by bile acids (BA). II. Enterocytes: Absorption, *de novo* synthesis, esterification and packaging of cholesterol into chylomicrons. III Hepatocyte: *De novo* cholesterol and BA synthesis regulated by the transcription factors, SREBP, FXR and SHP. IV. Integrated regulation: Enterohepatic recycling of BA and regulation of net faecal cholesterol and BA excretion.

Two newly discovered proteins have been suggested to be involved in the absorption of cholesterol: Mucin 1 (Muc 1) and Niemann-Pick C1 Like 1 protein (NPC1L1), as in murine knockout models the loss of either gene resulted in a decreased cholesterol absorption of 80% and 85%, respectively[18,19]. NPC1L1 is believed to be a good candidate for the cholesterol transporter protein. It is expressed at the brush border membrane of intestinal enterocytes, and cholesterol absorption in NPC1L1 knockout mice is unaffected by Ezetimibe, an inhibitor of intestinal cholesterol absorption.

1.3.2 Processing of cholesterol by enterocytes

As free cholesterol is toxic, once it is taken up by enterocytes cholesterol is rapidly esterified by acyl-CoenzymeA:cholesterol acyltransferase 2 (ACAT2) (Figure 2). The newly formed cholesteryl esters (CE) are then available for packaging into chylomicrons for transport around the body. Chylomicrons, the largest of the lipoproteins, are comprised mainly of triacylglycerols (TAG) (80-95%) with some CE. They express apoB-48, apoE and obtain apoC-II from circulating high density lipoproteins (HDL) in plasma. Striated muscle and adipose tissue use lipoprotein lipase to hydrolyse the TAG contained within the chylomicrons. With the progressive loss of TAG, the chylomicron shrinks and returns apoC-II to HDL, forming an enriched apoE chylomicron remnant that is rapidly taken up by the liver. In healthy individuals the half life of chylomicrons is only 10-15 minutes[20].

1.4 HEPATIC TURNOVER OF CHOLESTEROL AND LIPOPROTEIN SYNTHESIS

Chylomicron remnants, rich in cholesterol and apoE, are taken up by the liver via the low density lipoprotein receptor (LDL-R) and LDL receptor–related protein (LRP)[21]. The liver, capable of dealing with this large influx of cholesterol, handles incoming cholesterol in a number of ways. The first and most rapid response is the esterification of free cholesterol by ACAT2. This allows the storage of CEs in cytoplasmic droplets until it is required. The CEs can be used for the production of very low density lipoproteins (VLDL) or hydrolysed for the synthesis of BAs. Cholesterol can also be removed from the liver to the bile canaliculi by ABCG5/G8, where it is solubilised in the bile and either lost through feacal excretion or re-absorbed (Figure 2). In addition to dietary cholesterol the liver synthesizes a large amount of cholesterol each day, which

can be used for VLDL and BA synthesis. Although the liver can cope with a large influx of cholesterol, the storage of large amounts of cholesterol and other lipids may long-term lead to fatty liver and liver cirrhosis.

1.4.1 From very low density to low density lipoproteins

Lipids such as sterols and TAG are not soluble in the aqueous environment of plasma. Therefore they are packaged into lipoproteins for transport around the body. Lipoproteins are water-soluble particles with a lipid core (CE and TAG) surrounded by a protein and polar lipid bilayer (phospholipids, free cholesterol and apolipoproteins) and are classified according to their size and density.

The CE found in VLDL is derived from both *de novo* cholesterol biosynthesis and chylomicron remnants taken up by the liver. Nascent VLDL particles emerge from the liver in an assembly process[22,23], expressing apoB-100 on their surfaces and receive apoC-II and apoE from circulating HDL in analogy to chylomicrons. VLDL particles have a core rich in TAG (55-80%), which is hydrolyzed by peripheral tissues expressing lipoprotein lipase, causing the particle to shrink and become denser. The apoC-II and apoE are then recycled back to HDL. These modifications of the VLDL result in a particle called the intermediate density lipoprotein (IDL). If not taken up by the liver, the IDL looses more TAG, becoming LDL. VLDL has a half-life in circulation of about two hours; while the half-life of the cholesterol enriched LDL particles is 1-2 days.

LDL is the main source of cholesterol for peripheral tissue. Cells requiring cholesterol express LDL-R on their surfaces. After the LDL particle is absorbed by the cell the cholesterol is processed in the lysosome releasing free cholesterol, which is converted to CE by ACAT. LDL not taken up by other tissues is removed from the circulation by the liver.

1.5 HIGH DENSITY LIPOPROTEIN, MEDIATOR OF REVERSE CHOLESTEROL TRANSPORT

Lipid poor TAG-rich nascent HDL, secreted by the liver and intestine, expresses a number of apolipoproteins on its surface including apoA1, apoE and the C apoproteins.

As well as acting as a reservoir for apoC-II and apoE, HDL is involved in reverse cholesterol transport (RCT), acquiring excess cholesterol from peripheral tissues and macrophages[24,25]. In man HDL exchanges TAG for cholesterol from VLDL and LDL, through the action of the cholesterol ester transfer protein (CETP). Tissues expressing the SR-B1 receptor, such as the liver and steroid hormone synthesizing tissues, are able to take up cholesterol from HDL. Cholesterol is the precursor of steroid hormones such as testosterone and estrogen.

1.6 REGULATORY ROLES OF CHOLESTEROL

1.6.1 Sterol regulatory element binding proteins

Sterol regulatory element binding proteins (SREBPs) are a family of transcription factors that regulate cellular cholesterol and fatty acid homeostasis by responding to the cellular levels of free cholesterol[26,27]. There are three SREBP isoforms designated SREBP-1a, -1c and -2. SREBP-1a and -1c are encoded on the same gene, located on human chromosome 17. Through alternative transcription start sites SREBP-1a and –1c have alternative first exons, each of which is spliced to a common second exon[28]. SREBP-2 is derived from a second gene located on human chromosome 22. SREBPs bind as dimers to Sterol Regulatory Elements (SREs) in the promoters of a large number of genes, mostly activating their transcription. Sequences binding SREBPs appear to be rather heterogenous. Although there is some overlap in the genes regulated by SREBPs, SREBP-1c is thought to be the main regulator of the genes involved in fatty acid synthesis, whereas SREBP-2 regulates the genes involved in cholesterol biosynthesis. SREBP-1a is believed to activate genes involved in both processes[29]. As SREBP-1a is lowly expressed *in vivo* it was originally believed that SREBP-1c was the dominating SREBP1 isoform, although SREBP-1a is important in cell cultures. Subsequently it has been demonstrated that SREBP-1c homodimers are weak regulators of transcription and that heterodimers containing SREBP-1a are stronger[30]. Therefore the role that SREBP-1a plays in transcription may have been underestimated.

SREBP-2 activates its own transcription in combination with another transcription factor nuclear factor Y (NF-Y)[31]. Synthesized in the ER SREBP-2 remains bound to the membrane though an Insig-1:SCAP (SREBP cleavage activating protein) complex. Insig-1, a membrane bound protein, has a sterol sensing domain similar to HMGCR.

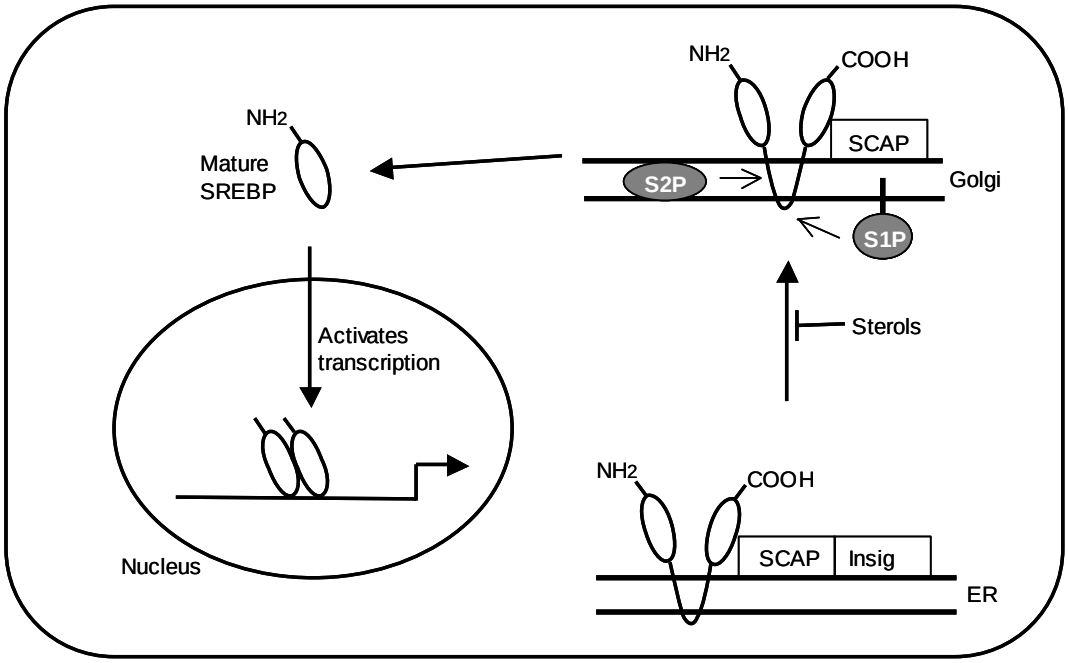


Figure 3 Overview of the processing of SREBP in the cell. In the presence of high concentrations of cholesterol SREBP remains bound to the ER. When cholesterol concentration drops SCAP releases Insig-1 allowing the SCAP:SREBP complex to move to the Golgi where the mature form of SREBP is released by a sequential two step proteolytic cleavage by S1P and S2P. This mature SREBP can then move to the nucleus where it dimerises and binds to promoters containing SREs.

When cholesterol levels are high Insig-1 remains bound to SCAP, which in turn is bound to SREBP-2, thus anchoring SREBP-2 to the membrane of the ER (Figure 3)[32]. When cholesterol concentration drops there is a conformational change in SCAP, releasing Insig-1 and allowing the SCAP:SREBP-2 complex to travel along the membrane to the Golgi where SREBP is enzymatically cleaved in a two-step process by two membrane bound proteases, site 1 protease (S1P) and site 2 protease (S2P). The released amino-terminal active form of SREBP-2 then moves to the nucleus where it dimerises and binds to promoters containing SREs. Mature SREBPs bind to DNA and are phosphorylated, increasing both their transcriptional activity and rate of degradation[33-35]. The mouse SREBP-2 knockout model is embryonically lethal at day 7–8, while SREBP-1 knockout mice are viable and healthy with an increased expression of SREBP-2[36]. This would suggest therefore that while SREBP-2 can

compensate for a loss or reduction of SREBP-1 the reverse is not true, implying that some genes are solely responsive to SREBP-2.

A number of genes involved in cholesterol and lipid metabolism have SREs in their promoters, including HMG CoA synthase[37], HMGCR[38], squalene synthase[39], fatty acid synthase[40] and LDL-R[41]. Putative SREs have also been identified in the promoter of NPC1L1[42]. In addition to their role in regulating cholesterol and lipid metabolism SREBPs have also been shown to regulate the expression of genes involved in processes other than cholesterol and lipid metabolism[43]. In yeast SREBPs have been shown to act as oxygen sensors[44], while in Drosophila melanogaster SREBP homologs are required for larval development[45]. Phagocytosis and bacterial pore-forming toxins induce the proteolytic activation of SREBPs and enhance lipid synthesis[46,47]. Due to the short shared promoter with MVK, the MMAB gene encoding Cob(I)alamin adenosyltransferase may be another target for SREBP regulation. Cob(I)alamin adenosyltransferase catalyses the conversion of cobalamin (vitamin B_{12}) to adenosylcobalamin, a cofactor required by methylmalonyl-CoA mutase. Regulation of this gene by SREBP-2, cholesterol and statins was investigated in this study.

1.6.1.1 Nulcear Factor-Y

Alone SREBPs are considered to be weak activators of gene expression and several *in vitro* studies indicate that they act coordinately with additional transcription factors, such as stimulating protein 1 (Sp1) and NF-Y to achieve full synergistic activation of their target genes[48,49]. NF-Y is a highly conserved transcription factor binding to CCAAT motifs in the promoters of a variety of genes including albumin, alpha-globin, collagen, and beta-actin. NF-Y is a trimeric protein that binds to DNA with a high specificity and affinity. The A subunit is the regulatory unit recognizing specific sequences[50]. The peptide sequences of subunits A and B are highly conserved during evolution as demonstrated by the fact that they are remarkably similar to the yeast transcription factors Hap2 and Hap3, both of which are required for specific binding to a CCAAT-like motif[51]. NF-Y has been shown to work in concert with SREBP-2 to activate the transcription of many genes involved in cholesterol biosynthesis including HMG CoA synthase, HMGCR, squalene synthase and Sqle[37,52-54]. However, binding of NF-Y to the murine promoter of Sqle, and this promoter's precise regulation by SREBP-2 and NF-Y had not been previously investigated.

1.6.1.2 Stimulating protein 1

Sp1 is a constitutive transcription factor interacting with a variety of gene promoters containing GC-box elements increasing or decreasing their transcription[55]. Sp1 is capable of synergistic transcriptional activity with promoters containing multiple Sp1 binding sites by direct promoter interaction that loops the intervening DNA[56,57]. Mutations that abolish Sp1 binding to the LDL-R promoter reduce its transcription[58]. In mice the loss of Sp1 is embryonically lethal at about day 11 of gestation[59]. As seen with the SREBP homolog, in Drosophila melanogaster the Sp1 homolog is required for the larval development[60]. Sp1, like SREBPs, can be phosphorylated altering its DNA binding and promoter activation[61].

1.6.2 Liver X receptor

The Liver X receptor (LXR), a former orphan nuclear receptor, interacts with oxysterols, the oxidised derivatives of cholesterol. It is theorised that LXR regulates cholesterol metabolism after binding oxysterols, thus activating the receptor and inducing its binding to specific DNA responsive elements. There are two isoforms of LXR, α and β. LXRα is expressed in liver, kidney, intestine, spleen and adrenals, while LXRβ has a ubiquitous expression[62]. LXR, like the Farnesoid X receptor (FXR), forms a heterodimer with the retinoid X receptor before binding to DNA. LXRα and β null mice have been created, and differences in their responses to a cholesterol rich diet have been observed[63,64].

LXR regulates cholesterol metabolism, storage and efflux by controlling the expression of a number of genes involved in cholesterol metabolism. Both LXRα and β regulate the transcription of SREBP-1c and indirectly, the expression of a number of genes involved in lipid metabolism[65], resulting in an increase in hepatic cholesterol ester formation. Activated LXR decreases hepatic cholesterol concentration in a number of ways. LXR activates the transcription of ABCG5/G8 decreasing the amount of cholesterol absorbed from the intestine and increasing the efflux of cholesterol from hepatocytes into bile. In mice, the expression of cholesterol 7α-hydroxylase (Cyp7a1), the rate limiting step in BA synthesis, is increased by LXRα thereby increasing the removal of cholesterol in the form of newly synthesized BAs[63,64]. LXR plays an important role in RCT by controling the expression of ABC transporters A1, G4, G1

and apoE in macrophages, enhancing the transport of cholesterol to HDL[66]. It is important to note that LXR is not the only protein able to bind oxysterols. The oxysterol binding protein (OSBP), and the family of OSBP related proteins, bind to oxysterols with a high affinity and appear to regulate a number of cellular processes[67]. Kandutsch first proposed the idea that cholesterol suppresses its own synthesis through oxygenated sterols[68]. The discovery that LXR, which regulates cholesterol metabolism, has oxysterols as specific ligands appears to fit this hypothesis. Kandutsch believed that 24S-25-epoxycholesterol was the most important regulatory sterol, and LXR shows the highest affinity for this oxysterol of all the sterols tested so far[69]. When cholesterol concentration increases the rate of cholesterol synthesis drops, possibly resulting in a shunting of 2,3-epoxide back to SQLE producing 2,3;22,23-dioxidosqualene, which is itself ultimately converted to 24S-25-epoxycholesterol[70]. However, there is some evidence to suggest that while LXR is required for normal cholesterol metabolism, oxysterols are not the natural ligand for this receptor. Firstly, it has been show in cell cultures that the addition of cholesterol, in the form of liposomes, decreases *de novo* cholesterol synthesis without the generation of oxysterols[71]. Also, *in vivo* studies where oxysterol levels have been perturbed show a minimal effect on cholesterol synthesis and homeostasis[72-74]. While it is clear that oxysterols bind to and activate LXR the possibility exists that another ligand is the true regulator of cholesterol metabolism.

1.7 BILE ACID SYNTHESIS

In humans the bile salt pool size is 3-4g, of which 0.5g is lost each day through faecal excretion. As well as being used to synthesize BAs, free cholesterol is also solubilised in the bile and lost through excretion. However, a paradox exists as the biliary cholesterol present in the bile is available for intestinal re-absorption and BAs themselves represent the facilitating agent.

BAs are synthesized by two pathways, the classic (neutral) pathway and the alternative (acidic) pathway, (Figure 4). Each pathway involves a number of different enzymes, of which several are cytochrome P450 enzymes, located in the smooth and rough endoplasmic reticulum, peroxisomes, mitochondria and cytosol[75]. The first step of the neutral pathway is the 7α-hydroxylation of cholesterol by the rate-limiting enzyme CYP7A1. The liver receptor homolog-1 (LRH-1) activates the transcription of the CYP7A1 gene[76-78]. In humans, expression of CYP7A1 is suppressed by cholesterol

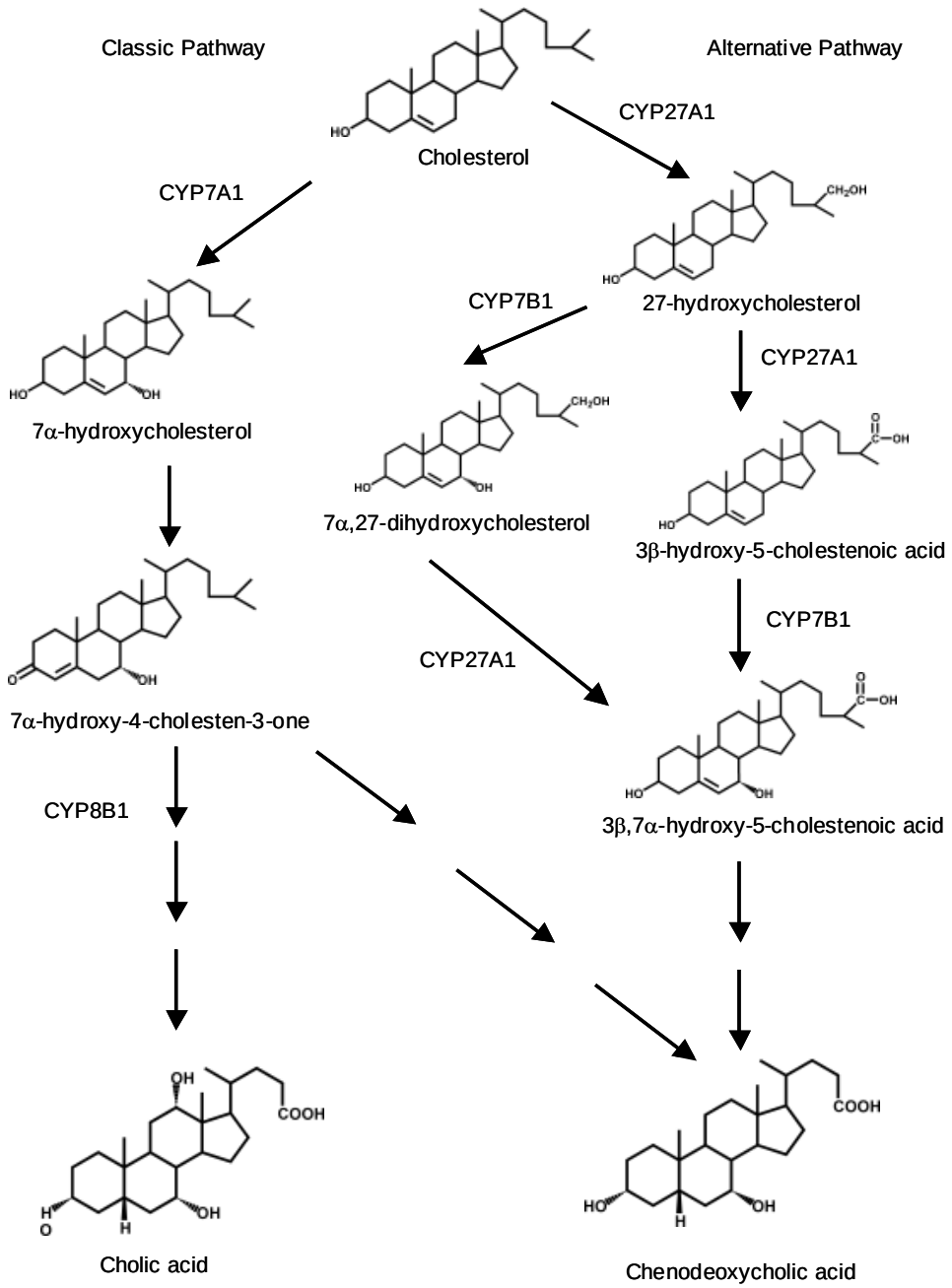


Figure 4 The major pathways of bile acid synthesis, the classical pathway initiated by CYP7A1 and the alternative pathway initiated by CYP27A1

treatment, while in mice it is increased through the action of the LXRα[79]. In both species FXR, a BA activated nuclear receptor, inhibits CYP7A1 expression through the action of the short heterodimer partner (SHP)[80], which seems to interfere with the transcriptional activation mediated by LXRα and LRH-1. Sterol 27-hydroxylase (CYP27A1) catalyses the first step in the alternative pathway of BA synthesis, the 27-hydroxylation of cholesterol (Figure 4), and also participates in the classical BA synthesis pathway.

In humans the BA pool is made up mainly of the primary BAs cholic acid (CA) and chenodeoxycholic acid (CDCA) and the secondary bile acids deoxycholic acid and lithocholic acid. CA and CDCA are the main products of the neutral pathway while CDCA is the main product of the acidic pathway. Sterol 12α-hydroxylase (CYP8B1) catalyses the hydroxylation of the steroid nucleus at the C12 α position required for CA synthesis[81]. The activity of CYP8B1 determines the overall ratio of CA to CDCA and thereby the hydrophobicity of the BA pool. As with CYP7A1, CYP8B1 expression is inhibited by FXR and SHP interfering with its activation by LRH-1[82].

1.8 REGULATORY ROLES OF BILE ACIDS

BAs appear to have a number of regulatory roles in addition to being biological detergents. As well as being natural ligands for FXRα, BAs are also ligands for G-protein coupled receptor (GPCR) TGR5 and activate mitogen activated protein kinase (MAPK) pathways[83]. In addition to regulating BA synthesis, FXR has been shown to be required for normal liver regeneration[84] and to control the growth of intestinal bacteria[85,86].

1.8.1 Farnesoid X receptor

FXR is a nuclear receptor that binds to specific DNA regions as a heterodimer with RXR. FXR is expressed in the liver, intestine, kidneys, adrenals and to a much lesser extent in white adipose tissue[87,88]. FXR was named due to the small activation by farnesoid, however, BAs were subsequently discovered to be more potent activators of FXR at concentrations similar to that found in the serum and tissues[80,89,90]. There are two FXR genes, α and β, although FXRβ is only functional in rodents, rabbits and dogs. FXRα has four isoforms due to alternative promoters and splicing.

An FXR knockout mouse has been created which is viable, fertile and there is no indication that the loss of FXR is prenatally lethal[91]. These mice do, however, develop liver tumors, have an increased serum cholesterol triglyceride concentration and a pro-atherogenic lipoprotein profile[91,92]. FXR/apoE double knockout mice show an increased atherosclerosic development[93], while conversely FXR/LDL-R double knockout mice show a reduced development[94]. The role FXR plays in the development of atherosclerosis, therefore, remains unclear.

1.8.2 Regulation of bile acid metabolism

As previously discussed BAs regulate their own biosynthesis in a FXRα:SHP dependent manner, inhibiting the expression of Cyp7a1 and Cyp8b1 genes. However, BAs regulate their own synthesis by SHP independent pathways as well. In the intestine FXR stimulates the synthesis of fibroblast growth factor 19 in man and the analog fibroblast growth factor 15 in mouse. This growth factor is released into the blood where it travels to the liver and binds to fibroblast growth factor receptor 4, activating the JNK pathway and thereby inhibiting CYP7A1 expression[95].

As well as regulating BA synthesis, FXR regulates BA transport. By suppressing Na+/taurocholate cotransporting polypeptide (NTCP) and inducing the bile salt export pump (BSEP) as well as the multidrug resistance associated protein 2 (MRP2), FXR increases the removal of BAs from hepatocytes while reducing their uptake. In the enterocytes FXR represses the apical sodium dependent bile acid transporter (ASBT), thereby reducing the reabsorption of BAs, and simultaneously inducing the basolateral organic solute transporters (OST) α and β increasing the transport of BAs into the circulation (Figure 2).

BAs are cytotoxic compounds and therefore their concentration must be kept low. Pregnane X receptor, a target for FXR activation[96], enhances the activation of genes involved in the breakdown of BAs, thereby protecting the liver. So far no direct link between FXR and human disease has been detected. However, mutations in genes regulated by FXR have been linked to inherited disorders such as the Dubin-Johnson syndrome and progressive familial intrahepatic cholestasis type 2 and 3[97].

1.8.3 Bile acid regulation of lipid metabolism

In humans, treatment with the BA sequestrant cholestyramine induced the production of VLDL, while CDCA treatment reduced hypertriglyceridemia[98]. In rodents FXRα interferes with the LXR activation of Cyp7a1, thereby reducing the consumption of cholesterol and increasing LDL cholesterol. FXR, through SHP, down regulates the expression of SREBP-1c[99], considered to be the global regulator of lipogensis[29]. In this way, FXR regulates hepatic fatty acid and triglyceride biosynthesis and VLDL production. FXR also binds to the promoter of murine fatty acid synthase activating its transcription[100]. In mice, activation of FXR is reported to prevent the formation of gallstones, while the loss of FXR increases susceptibility to gallstone formation[101]. FXR is expressed in both white adipose tissue in mice and differentiated 3T3-L1 cells, but not in undifferentiated preadipocytes[102]. Embryonic fibroblasts derived from FXR null mice have an impaired adipocyte differentiation[103], while treatment with an FXR agonist enhances the differentiation of induced 3T3-L1 cells[102]. FXR null mice do not show any regulation of adipocyte related genes when treated with an FXR agonist.

1.8.4 Bile acid regulation of glucose metabolism

Although cholestyramine treatment in type-II diabetic patients improved glycemic control[104], the precise mechanisms by which BA signalling via FXR regulates glucose metabolism remains unclear. In rodents, during fasting FXRα expression is increased by two transcription factors, peroxisome proliferator-activated receptor γ and hepatic nuclear factor 4α[105], implying an inhibitory role for insulin. However, in diabetic mice FXRα expression is decreased and insulin normalises expression[106]. Activation of FXR may increase glycogen levels and FXR null mice are glucose intolerant and insulin insensitive[107]. Obese leptin-deficient ob/ob knockout mice given an FXR agonist have lower concentrations of insulin than non-treated animals[103]. The current understanding of the role of FXR in glucose homeostasis is unclear and its significance as a target in the treatment of patients with type II diabetes awaits further studies.

1.8.5 Bile acids and energy homeostasis

BAs appear to play a role in energy homeostasis. In mice, treatment with BAs increases energy expenditure in brown adipose tissue, in a way that is dependent on cAMP-dependent thyroid hormone activating enzyme type 2[83]. Human skeletal myocytes treated with BAs also show increased oxygen consumption. These effects are FXR independent, mediated by the GPCR TGR5 receptor and induction of the MAPK pathway.

1.9 CHOLESTEROL AND BILE ACIDS IN HEALTH AND DISEASE

Alterations in normal cholesterol and BA synthesis result in a number of rare conditions affecting the overall health of the affected individuals. More common conditions such as obesity, type II diabetes and atherosclerosis are becoming serious global health issues, and cholesterol also plays a role in these diseases. Here I will briefly discuss some of the specific conditions in which cholesterol or BAs are involved.

1.9.1 Sitosterolemia

The loss of a functional ABCG5/G8 protein complex results in a condition called sitosterolemia[108]. Patients with this condition have an accumulation of plant sterols and an increased risk of atherosclerosis. In mouse models the loss of ABCG5/G8 has little effect on cholesterol absorption. However, over-expression of these transporters decreases cholesterol absorption, most likely due to the increased removal of cholesterol from the enterocytes[109]. Activation ABCG5/G8 transcription by LXR results in an increased cholesterol excretion[110,111].

1.9.2 Defects in cholesterol synthesis

Although very rare there are a number of conditions associated with a deficiency in one or more of the enzymatic steps involved in cholesterol biosynthesis. Cholesterol from the diet and compounds structurally related to cholesterol, such as desmosterol, are unable to compensate for a deficiency of cholesterol synthesis, especially in the brain

and nervous system. Table 1 introduces some of the syndromes resulting from impaired cholesterol synthesis. For a more thorough discussion of cholesterol synthesis disorders post squalene see review by Herman[112].

Table 1 Syndromes resulting from deficiencies in cholesterol biosynthesis

Disorder	**Affected gene**	**Clinical Symptoms**	**Animal Model**	**Phenotype**
Periodic Fever or Hyper IgD syndrome	Mevalonate Kinase	Reduced MVK activity, recurrent fever and other inflammatory symptoms	None	
Mevalonic Aciduria	Mevalonate Kinase	Loss of MVK acitvity, developmental delay, failure to thrive, hypotonia, ataxia, myopathy, and cataracts	None	
Smith-Lemli-Opitz syndrome (SLOS)	7-dehydrocholesterol reductase gene (DHCR7)	Mulitple malformations, distinctive facial features, small head size, mental retardation and behavioural problems	Dhcr7 -/-	Not viable after birth
Congenital Hemidysplasia with Ichthyosis Erythroderma & Limb Defects (CHILD)	NADH steroid dehydrogenase-like (NSDHL) encoding for 3β-hydroxysterol dehydrogenase	Ichthyosiform skin lesions with strict midline demarcation, shortened or absent limbs on affected side, malformations of the brain, lungs, kidney and inner ear	Bare patches (Bpa -/-) Striated (Str -/-)	Embryonic lethality in males, females small with skin and skeletal abnormalities
Lathosterolosis	Lathosterol 5-desaturase gene (SC5D)	Increased lathosterol, decreased cholesterol, similar phenotype to SLOS	Sc5d -/-	Stillborn
Desmosterolosis	DHCR24, demosterol reductase	Multiple malformations including dysmorphic facial features and limb abnormalities, accumulation of desmosterol	Dhcr24 -/-	Small infertile, with a shorter survival

1.9.3 Defects in bile acid synthesis

Although very rare, cases have been reported involving loss or reduction in one of the steps of BA synthesis, see Table 2. Of note is the fact that to date there are no reported cases of humans with a deficiency of CYP8B1, possibly due to a lack of any adverse symptoms.

Table 2 Deficiencies in bile acid biosynthesis

Affected Gene	Clinical symptoms	Phenotype of knockout mice
CYP7A1	Increased susceptibility of gallstones, increased LDL-C levels[113]	Decreased weight gain, BA pool size, cholesterol absorption, high mortality in pups, rescued by induction of Cyp7b1 after day 20-50.[114,115]
CYP7B1	Recurrent jaundice, acholic stools, hepatosplenomegaly, increased bleeding[116]	Increased levels of 25- and 27-hydroxycholesterol, increased cholesterol synthesis in kidneys of male mice[117]
CYP27A1	Accumulation of cholesterol in tendons and brain (CTX), early onset atherosclerosis, increased cholesterol synthesis[118]	Increased BA synthesis, no development of CTX[72]

*CTX – cerebrotendinous xanthomatosis

1.9.4 Atherosclerosis

Characterized by the formation of cholesterol-rich plaques on arterial walls, atherosclerosis is a progressive disease of unknown etiology showing a highly complex pathogenesis, which can begin at an early age and progress over decades. Fatty streaks, the first stage in atherosclerotic plaque formation, have be found in foetuses and new borns[119]. Over time these plaques grow, restricting the blood flow, or rupture, resulting in thombus and varying degrees of acute injury due to ischemia. Atherosclerosis is an inflammatory response to injury of the vessel wall[120,121]. Predisposing factors include hyperlipidemia, diabetes and the metabolic syndrome. Several dyslipidemias show Mendelian inheritance and result in monogenic alterations of the lipoprotein metabolism, such as familial hypercholesterolemia and Tangier Disease, as reviewed[122-124]. Dyslipidemias can result from a loss or gain of the lipoproteins in circulation, or

alterations in the apolipoproteins they express, and often lead to premature atherosclerosis. In contrast, the highly prevalent dyslipidemias may be of polygenic origin or related to life style components[125,126]. According to current concepts, endothelial dysfunction of the arterial vessel wall results in an influx of monocyte derived macrophages and smooth muscle cells. The macrophages, expressing scavenger receptors, accumulate cholesterol from modified LDL particles[127,128]. LDL can become modified in a number of ways including oxidation and, in diabetes, glycation. Unlike other cell types, macrophages do not down regulate their scavenger receptors when cellular cholesterol levels are high, thus transforming into foam cells. Therefore, the removal of cholesterol from macrophages by HDL is an obvious way of preventing atherosclerosis.

While inflammation appears to be a candidate etiologic factor in developing atherosclerosis, most primary and secondary preventative pharmaceutical measures are directed against hyperlipidemia. Statins, competitive inhibitors of HMGCR, reduce VLDL synthesis and up regulate the expression of LDL-R, thereby lowering the circulating levels of LDL cholesterol. Statins have also been proposed to treat CVD in a non-cholesterol related manner by stabilising plaques, preventing thrombus formation and acting as an anti-inflammatory agent that improves endothelial function. Ezetimibe, a cholesterol absorption inhibitor, has been shown to effectively reduce serum LDL cholesterol levels with no alteration in the absorption of fat-soluble vitamins and nutrients[129]. Understanding the role CA and FXR play in cholesterol metabolism may uncover novel ways of treating atherosclerosis. By using a CA-free mouse model we have a unique opportunity to discover the regulatory role of CA on cholesterol synthesis, absorption, storage and transport.

1.9.4.1 LDL:HDL and atherosclerosis

Extrahepatic tissues are incapable of handling excess cholesterol; therefore it must be transported to the liver. A high LDL:HDL ratio is a known independent risk factor for the development of atherosclerosis and CVD, while high HDL levels are a negative risk factor. Studies have shown that for every 1mg/dL increase in HDL-C the risk of CVD is reduced by 2-3%[130,131]. The removal of cholesterol from macrophages is especially important to prevent the formation of foam cells. As well as its role in RCT, HDL is believed to have other protective roles including enhanced vasorelaxation, suppressed

induction of cell-adhesion molecules by tumor-necrosis factor, anti-inflammatory and antioxidative effects[132]. Therefore, HDL is an attractive potential target for the treatment of CVD.

1.9.5 Obesity and type II diabetes

Obesity is becoming a very serious global health problem for industrialised nations. It has been proposed that due to the increase in childhood obesity life expectancy in the United States may actually fall for the first time in recent history[133]. The American Heart Associations statistical sourcebook, “A Nation at Risk: Obesity in the United States” classifies obesity as a major, modifiable risk factor for CVD. Obesity also limits the beneficial effects of statin treatment[134]. However, reducing waist to hip ratio and moderate exercise for at least 30 minutes per day are effective in reducing the risk of CVD[135].

Along with the rise in obesity there is also an increase in non-insulin dependent type-II diabetes. These patients are able to produce insulin, however, they are resistant to its effects. Type-II diabetes usually affects overweight middle-aged people. Many of the mechanisms linking obesity and CVD are believed to be related to insulin resistance. Both obesity and type-II diabetes result in an altered distribution of lipoproteins with an increase in VLDL and small dense LDL particles and decreased HDL levels. In diabetes there is also a delayed removal of chylomicron remnants. In alloxan-induced diabetic mice the loss of CA was protective against large increases in VLDL and LDL with cholesterol treatment[136].

1.9.6 Alzheimer’s disease

Alzheimer’s disease (AD) is the most common cause of dementia in the elderly. It is defined histopathologically by the presence of extracellular neuritic plaques and intracellular neurofibrillary tangles in the brain. The deposition of plaque gradually disturbs the neural activity resulting in disturbed awareness and memory loss and eventually death. Cholesterol is believed to play a role in AD. The apoE4 isoform of apoE, the major cholesterol transporter in the brain, is a known risk factor for the development of AD[137]. The plaques found in the brains of AD patients are rich in β-amyloid protein, produced by abnormal cleavage of the amyloid precursor protein

(APP). There is some evidence from cell culture experiments that cholesterol may regulate the processing of ubiquitously expressed APP in favour of plaque generating β-amyloid formation over α-amyloid[138]. A C → A polymorphism at position -911 in the promoter of HMGCR has been identified as a risk factor for the development of AD in an Italian population[139].

2 AIMS OF THE STUDY

Paper I & II

To investigate the regulatory roles of BAs and cholesterol in cholesterol metabolism using a CA free mouse model

Paper III

To investigate the cholesterol regulation of the mouse Sqle gene by cholesterol and the transcription factors: SREBP-2 and NF-Y.

Paper IV

To investigate the shared promoter of MVK and MMAB and determine their regulation by cholesterol, SREBP-2 and statins.

3 MATERIALS AND METHODS

The following is a brief summary of the materials and methods used. Standard molecular techniques were used for RNA purification, PCR, Q-PCR, cloning, mutagensis, transcription start site mapping, gel shift assay, transfection and short interfering RNA (siRNA). A more detailed account is provided in the respective papers.

3.1 EXPERIMENTAL ANIMALS

A Cyp8b1 knockout mouse model was created on a mixed C57/Bl6;129 background[140]. While grossly phenotypically normal these mice are unable to synthesise CA and have an enlarged bile acid pool mainly containing muricholates and some CDCA. In this study male mice, 4-5 months old and 25-32g body weight were housed at 24°C with the light period over 0800-2000h. Male Cyp8b1 +/+ and -/- mice on a mixed C57/Bl6;129 background and inbred C57/Bl6 and SV40 mice were used in our studies.

3.2 DIETS AND TREATMENTS

The mice were fed either a control diet of powdered food with peanut oil (10 or 20%) or the same diet supplemented with 0.1% or 0.5% (w/w) sodium cholate, or 0.5% or 1% (w/w) cholesterol or 0.5% cholesterol and 0.25% sodium cholate for 6-7 days. Mice were given 1% methylcellulose containing, TO-901317 (LXR agonist) or GW4064 (FXR agonist) by gavage for 5 days, 10 mg/kg/day or 50 mg/kg/day, respectively.

3.3 CHOLESTEROL ABSORPTION

Cholesterol absorption was measured for individual mice using the dual faecal isotope method as described by Schwarz et al[141].

3.4 MEASUREMENT OF CHOLESTEROL AND LATHOSTEROL

Lipid extracts were prepared from pieces of approximately 100mg frozen liver tissue in 2ml Folch by stirring overnight at room temperature under argon gas. Concentrations of total and free cholesterol and lathosterol were assayed by isotope dilution-mass spectrometry with the use of deuterium-labeled cholesterol and lathosterol as internal

standards [142,143]. To obtain a relative index of hepatic *de novo* cholesterologenesis in the liver the ratio of lathosterol to cholesterol concentration was calculated.

3.5 IN VITRO MODELS

HEK293 cells were used to analyse the promoter activity of murine Sqle and human HMGCR luciferase promoter constructs. 3T3-L1 cells were use to analyse intact murine chromatin response to SREBP-2 siRNA or over expression of SREBP-1c or –2.

3.6 GENOTYPING OF ALZHEIMER'S DISEASE PATIENTS AND CONTROLS

Aged matched AD patients and controls were genotyped for the C → A mutation at position –911 in the promoter of HMGCR by allelic discrimination with the Applied Biosystems SNP genotyping assay, assay number, C__27476930_10, as of manufacturers protocol, using the ABI Prism 7000 Sequence Detection System.

4 RESULTS

4.1 REGULATORY IMPACT OF BILE ACIDS AND CHOLESTEROL ON CHOLESTEROL METABOLISM AS STUDIED IN THE CHOLIC ACID FREE MOUSE, PAPERS I AND II

4.1.1 Cholesterol absorption

As seen in Papers I and II, Cyp8b1 -/- mice, lacking CA, have a decreased absorption of cholesterol from the diet (Paper I Figure 5, Paper II Figure 3b). Treatment with CA to either Cyp8b1 +/+ or -/- mice increases the hydrophobic quality of the bile and cholesterol absorption. This shows that the loss of CA per se does not alter the ability to absorb cholesterol from the diet. This is analogous to Cyp7a1 and Cyp27 null mice, whose reduced cholesterol absorption is also restored with CA feeding[144,145].

While cholesterol feeding did not effect the BA composition of Cyp8b1 -/- mice, wildtype mice had a reduction in CA and an increase in muricholates. In mice cholesterol is known to inhibit the expression of Cyp8b1 and increase the expression of Cyp7a1, thereby driving BA synthesis towards the production of muricholates and a less efficient BA pool. While the percentage of cholesterol absorbed decreased with cholesterol treatment the overall amount of cholesterol absorbed most likely increased. When given CA or cholesterol alone, the CA-free mice consistently absorbed less cholesterol compared to the intact animals.

Through binding to FXR, CA inhibits the expression of both Cyp7a1 and Cyp8b1 genes in mice. Combining CA with cholesterol feeding resulted in an increase of CA and a decrease in muricholates in the bile of Cyp8b1 +/+ and -/- mice and further increased cholesterol absorption compared to cholesterol treatment alone (Paper II Figure 3b). Cyp8b1 -/- mice actively absorbed slightly more cholesterol then their Cyp8b1 +/+ littermates when given both CA and cholesterol.

4.1.2 Hepatic storage and cholesterol secretion into bile

In Papers I and II we show that CA treatment increases the hepatic levels of free and esterified cholesterol in Cyp8b1 intact mice (Cyp8b1+/+ and C57/Bl6 mice), while Cyp8b1 -/- mice showed an increase in free cholesterol only when given the 0.5% CA diet. It is only when CA is given in conjuncture with cholesterol that Cyp8b1 -/- mice show similar increases in hepatic free and esterified cholesterol as intact mice. Lack of CA therefore appears to protect the Cyp8b1 -/- mice from the large increases in free and esterified cholesterol seen in intact animals after cholesterol feeding.

When fed the control diet the cholesterol saturation index (CSI) did not differ significantly between the Cyp8b1 +/+ and -/- mice (Paper II, Table 2). However, when fed cholesterol the Cyp8b1 +/+ mice had a CSI 2.5-fold greater than that of Cyp8b1-/- mice. Also cholesterol crystals were only found in the bile of Cyp8b1+/+ mice. When given CA in combination with cholesterol the CSI again did not differ significantly between the Cyp8b1 +/+ and -/- mice.

4.1.3 Cholesterol synthesis

Cyp8b1 -/- mice have a decreased absorption of cholesterol from the intestine. However, they have a compensatory increased expression of the genes involved in cholesterol synthesis and a higher rate of hepatic cholesterol synthesis, thereby maintaining overall cholesterol balance (Figure 5). The greatest alteration in expression was seen with the Sqle gene. Both CA and cholesterol treatment reduced the expression of cholesterol synthesizing genes and the overall rate of cholesterol synthesis, as determined by the lathosterol:cholesterol ratio.

4.1.4 Bile acid synthesis

As shown in Paper II, Cyp8b1 -/- mice have an increased BA synthesis compared to Cyp8b1 +/+ mice, as demonstrated from an increased Cyp7a1 expression and fecal excretion of BAs (Paper II Figure 2a and b). As previously shown this leads to an expansion of the BA pool[140]. While cholesterol treatment increased the rate of BA synthesis in both genotypes, the Cyp8b1 -/- mice always maintained a higher rate of synthesis than their intact littermates. This increased removal of cholesterol in the form

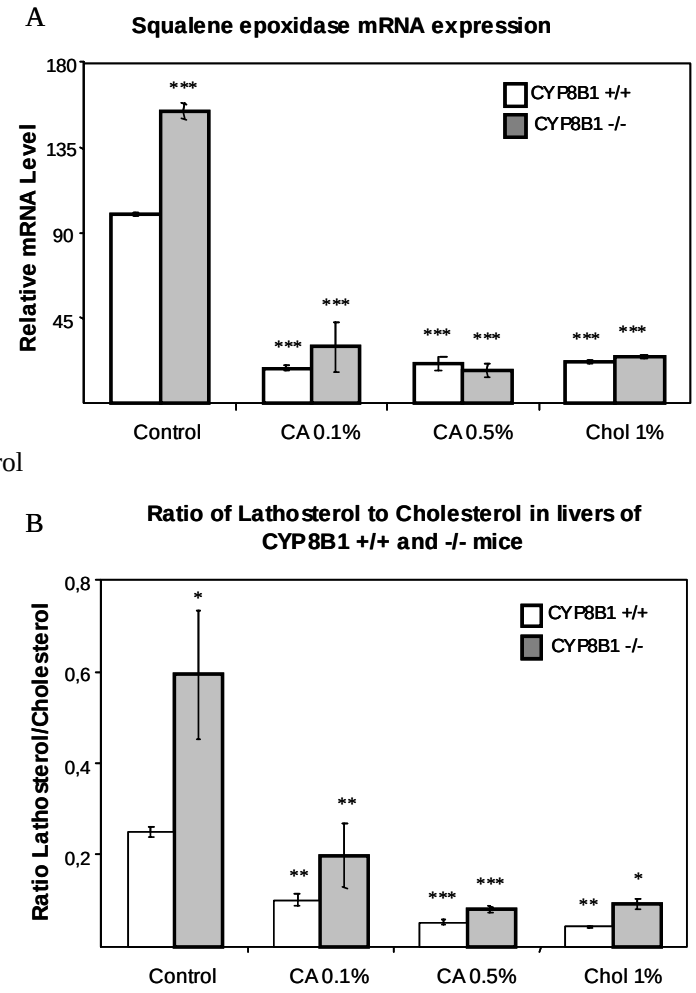


Figure 5 Increased cholesterol synthesis A. Hepatic mRNA expression of Sqle in Cyp8b1 +/+ and -/- mice fed either a control diet, 0.1% CA, 0.5% CA or 1% cholesterol B. Lathosterol:cholesterol ratio (ng/µg) was used as an indication of the relative level of *de novo* cholesterol synthesis in the livers of CYP8B1 +/+ and -/- mice fed control diet, 0.1% CA, 0.5% CA or 1% cholesterol. *P<0.05, **P<0.001, ***P<0.0001

of BAs could provide one explanation as to why Cyp8b1 -/- mice accumulate much less hepatic cholesterol than intact mice. As Cyp8b1 +/+ and -/- mice have similar levels of Cyp27a1 expression, the increased rate of BA synthesis appears to be mainly via the classical pathway and activation of Cyp7a1.

In mice, cholesterol and its derivatives such as oxysterols, promote cholesterol removal via the synthesis of new BAs through the LXR-mediated activation of Cyp7a1. Treatment with an LXR agonist resulted in the same increase in Cyp7a1 expression as seen with cholesterol treatment, and either treatment resulted in a greater increase of Cyp7a1 expression in the Cyp8b1 -/- mice (Paper II Figure 2b and 6a).

In mice, CA is a natural ligand for FXR, which is known to regulate the expression of genes involved in BA synthesis through interaction with SHP. By interfering with LXR

activation, SHP downregulates the expression of Cyp7a1 and Cyp8b1 thereby decreasing BA synthesis. Logically, as removal of CA increased expression of Cyp7a1 and the rate of BA synthesis, addition of CA resulted in a decreased expression of Cyp7a1 and a decreased rate of BA synthesis. We also show that in Cyp8b1 +/+ and -/- mice an FXR agonist mediated the same effects on Cyp7a1 expression and BA synthesis as CA treatment. It is therefore apparent that CA, through its interaction with FXR, is a key regulator of its own synthesis.

4.1.5 Lipid profiles in serum

Under control dietary conditions the lipid profile of Cyp8b1 +/+ and -/- mice is very similar, with the majority of cholesterol being located in the HDL fraction (Paper II Figure 4b). Plasma cholesterol levels also remained very similar under all test conditions for both Cyp8b1 +/+ and -/- mice. However in man, the development of atherosclerosis is mainly dependent on whether cholesterol is confined to the LDL or HDL faction, rather than the overall cholesterol concentration[130,131]. In the Cyp8b1 -/- mice cholesterol treatment resulted in an increased expression of ABCA1 and higher levels of HDL cholesterol. However, the addition of CA to the cholesterol diet resulted in a shift towards VLDL and LDL particles. It would therefore appear that loss of CA in mice would generate increased HDL levels under certain circumstances, an effect that can be lost when the CA is replaced.

4.2 MOLECULAR REGULATION OF CHOLESTEROL SYNTHESIS BY CHOLESTEROL, PAPERS III AND IV

4.2.1 Regulation of cholesterol synthesis: SREBP-2

SREBP-2 has been shown to regulate the expression of a number of genes involved in cholesterol biosynthesis including MVK and SQLE[43]. As shown in Papers III and IV, in mice the promoters of both these genes, as defined by transcription start site mapping, contain potential responsive elements for NF-Y and SREBP-2. Both genes were regulated by cholesterol feeding in mice, and by overexpression or removal of SREBP-2 by siRNA in the murine cell line 3T3-L1 (Figure 6). SREBP-2 appears to be the major regulator of murine Mvk expression in the whole animal[43]. However, the

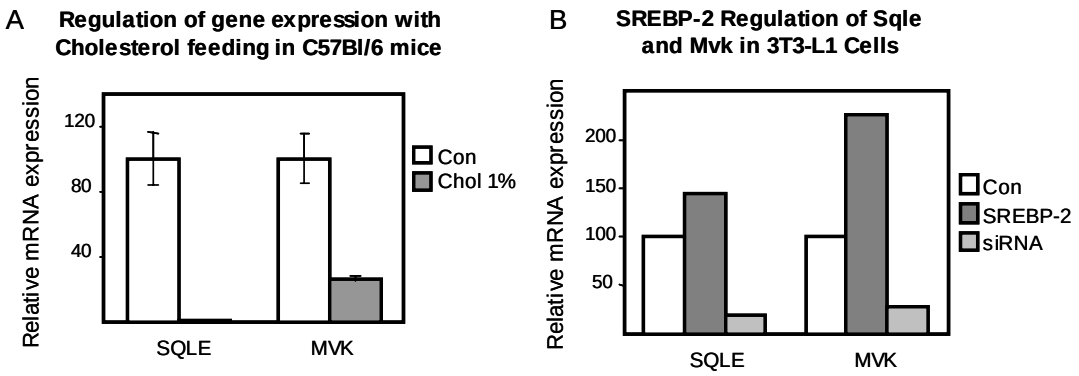


Figure 6 A. Hepatic mRNA expression of Sqle and Mvk in C57/Bl6 mice fed either a control diet or 1% cholesterol. B. Expression of Sqle and Mvk in the murine cell line 3T3-L1 either under control conditions or co-transfected with either SREBP-2 overexpression plasmid or SREBP-2 siRNA.

precise element(s) responsible for this regulation may lie outside of the promoter region as defined by our studies, as the Mvk luciferase promoter construct showed the greatest response to SREBP-1c (Paper IV Figure 3c).

The promoter of the human SQLE gene had previously been shown to respond to SREBP-2[54]. However, the precise elements to which SREBP-2 was binding had not been demonstrated. We created a murine Sqle luciferase promoter construct corresponding to -1944 to +215, relative to the major transcription site that we identified. This construct was activated by all three SREBPs, but was most highly responsive to SREBP-2. Truncation of this construct in both the 5' and 3' directions identified a 205 bp minimal responsive region responsible for regulation by SREBPs. This region did not contain the conserved SRE element believed to be responsible for SREBP regulation in the human SQLE promoter. In the first such demonstration, by gel shift assay we showed that SREBP-2 bound to three novel SREs in the promoter of Sqle (Figure 7). However, mutation of all three SREs did not abolish SREBP-2 activation (Paper III Figure 4b). Therefore, it is likely that one or more SREs remain to be identified in this 205 bp region.

LDL-R	+	+	+										
SRE Repeat 1				+	+								
SRE Repeat 2						+	+						
SRE/A								+	+				
SRE/B										+	+		
SRE/C												+	+
Cold LDL-R			+		+		+		+		+		+
Lane	1	2	3	4	5	6	7	8	9	10	11	12	13

SREBP-2 →

Figure 7 Competition gel shift assay of SRE elements using programmed rabbit reticulocyte lysate. ^{32}P-labelled LDL-R SRE oligo was used as positive control and to compete for SREBP-2 binding cold LDL-R SRE oligo was used at 2000-fold excess. Programmed lysate is present in all lanes except lane 1. Competition of specific SREBP-2 bands is visible in lanes 3, 5, 9, 11 and 13. No SREBP-2 binding is visible in lane 6.

4.2.2 Regulation of cholesterol synthesis: NF-Y and Sp1

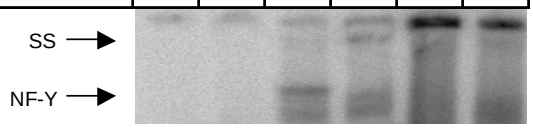
The promoter of human MVK contains four potential NF-Y elements, while the mouse promoter contains five, three of which are conserved with the human promoter (Paper IV Figure 1b). These sites remain to be tested. Two potential NF-Y sites were identified in the promoter of the murine Sqle gene (Paper III Figure 1d), although only the proximal site was able to bind to NF-Y (Figure 8) and mutational loss of this site resulted in an abrogated response to SREBP-2 (Paper III Figure 5b). Intriguingly it was the most distal site in the human SQLE promoter that was believed to be responsible for NF-Y regulation, although binding to NF-Y was never demonstrated[54]. Co-transfection with dominant negative NF-Y A subunit unable to bind DNA, resulted in an abrogated response to SREBP-2, demonstrating the requirement of SREBP-2 for functional NF-Y to activate the murine Sqle promoter (Paper III, Figure 5c).

Sp1 elements are often found in the promoters of SREBP sensitive genes. The rat, mouse and human SQLE promoters contain numerous potential Sp1 sites, some of which were conserved between the species. The Mvk promoter has two potential Sp1 sites, one of which is conserved between mouse and human. NF-Y is known to enhance

and stabilise the binding of SREBP-2 to DNA; however, the precise manner in which NF-Y and Sp1 enhance SREBP-2 regulation remains unclear.

Figure 8 Gel shift assay to assess the binding of NF-Y to the proximal and distal NF-Y sites in the murine Sqle promoter. Addition of NF-Y antibody produced a super shifted (SS) band visible in lane 4

Extract			+	+	+	+
Proximal NF-Y	+	+	+	+		
Distal NF-Y					+	+
NF-Y Antibody		+		+		+
Lane	1	2	3	4	5	6



4.2.3 Regulation of cholesterol synthesis: FXR

Our analysis of the murine Sqle promoter identified a potiental FXR regulatory element at position –1611 in the promoter (Paper III Figure 1c). This site found in the mouse Sqle promoter is highly homologous to the conserved FXR binding site in the rat and human SHP-1 promoter described by Goodwin et al[146]. While binding of FXR to this potential regulatory element remains to be tested, treatment with the FXR agonist GW4064 caused an increased expression of Sqle, Hmgcs and Hmgcr in Cyp8b1 +/+ mice, that appears to be SREBP-2 independent (Figure 9). Cyp8b1-/- mice given GW4064 had a reduced expression of these genes, approaching the levels seen in Cyp8b1+/+ control mice. While further analysis is required this opens up the possibility that BAs can directly regulate cholesterol biosynthesis through FXR.

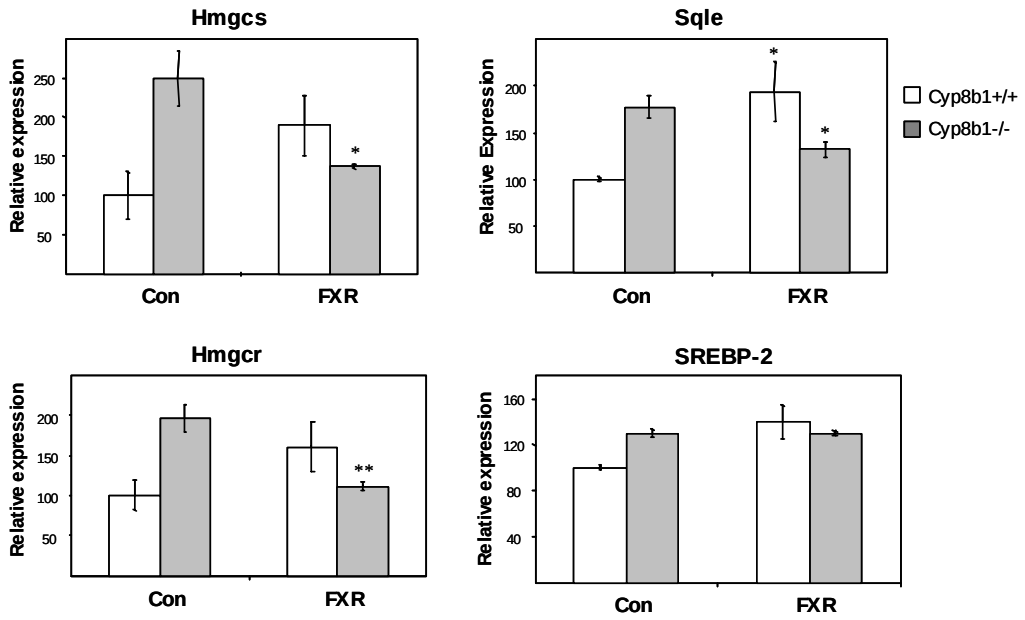


Figure 9 Hepatic mRNA expression of HMG CoA synthease (HMGCS), Squalene epoxidase (Sqle), HMG CoA reductase (HMGCR) and SREBP-2 in Cyp8b1 +/+ and -/- mice treated with GW4064, a FXR agonist for one week. *P<0.05, **P<0.001

4.3 REGULATION OF THE MMAB GENE, PAPER IV

C57/Bl6 mice given 1% cholesterol showed a significant decrease in the mRNA levels of the Mmab gene, while SV40 mice treated with atorvastatin showed a significant increase in Mmab mRNA levels (Figure 10a). Murine 3T3-L1 cells were used to determine the effects of SREBP-2 on Mmab expression with intact chromatin. Increased concentrations of SREBP-2 activated the transcription of Mmab in these cells, while the reduction of SREBP-2 by siRNA resulted in a decreased expression (Figure 10b). This demonstrates that the murine Mmab gene is under the control of SREBP-2. A luciferase promoter construct was created for the shared promoter of murine Mvk and Mmab. In either orientation the constructs showed a similar basal expression in 3T3-L1 cells, and similar activation by SREBP-1c and –2 (Paper IV Figure 3c).

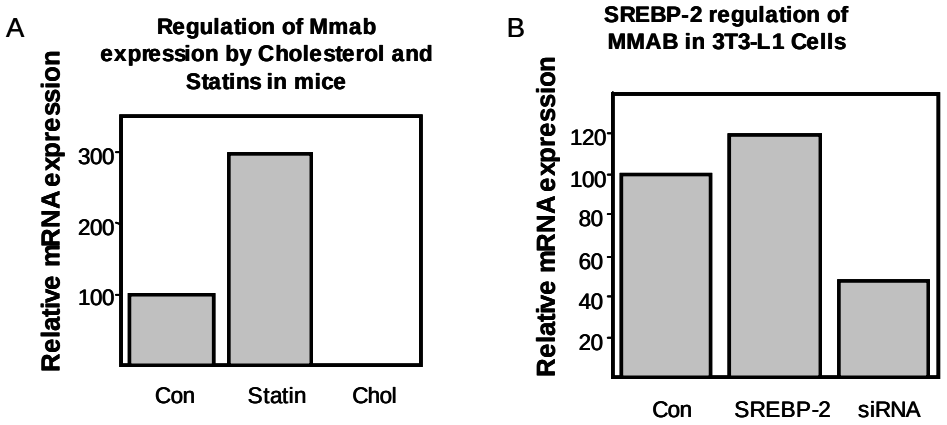


Figure 10 A. Hepatic mRNA expression of Mmab in the livers of SV40 mice treated with atorvastatin or C57/Bl6 mice treated with 1% cholesterol for one week. B. Expression of Mmab mRNA in the murine cell line 3T3-L1 with transfection of either SREBP-2 overexpression plasmid or siRNA for SREBP-2.

4.4 ALZHEIMER'S DISEASE AND CHOLESTEROL SYNTHESIS IN BRAIN

Over 1 Kb of the human HMGCR promoter was cloned into the expression vector pGL-3 Basic, with or without the C → A polymorphism at –911 in the promoter, previously identified as an AD risk factor in an Italian population[139]. In HEK293 cells the luciferase expression was 50% less for the HMGCR promoter construct containing the polymorphism, however, this altered promoter was more responsive to activation by SREBP-2 (Figure 11).

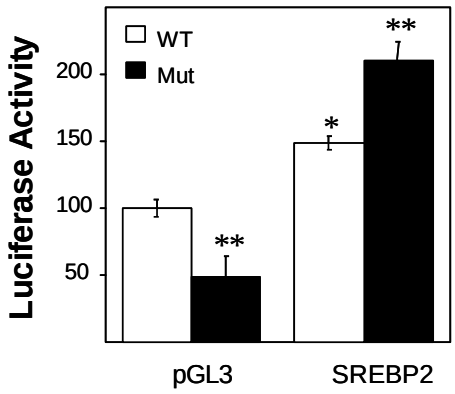


Figure 11 Expression of HMG CoA reductase promoter luciferase constructs without the C → A polymorphism (WT) or with the polymorphism (Mut), co-transfected with either empty pGL-3 Basic vector or SREBP-2 over expression plasmid. *P<0.05, **P<0.001

This is a substantial reduction in expression with only a single base change. A similar construct expressed in other cell lines showed no alteration in expression[147] possibly resulting from differences between HEK293 cells, an embryonic cell line, and the cells used by other investigators. Screening of a Swedish population of age matched healthy controls and AD patients showed no significant increase of the C → A polymorphism in AD patients compared to healthy controls, Table 3.

Table 3 Genotyping of a Swedish population for the HMGCR promoter C-911A polymorphism

Genotype				
Status	AA	AC	CC	Total
Controls	4	67	276	347
AD cases	4	73	290	367
Total	8	140	566	714

5 GENERAL CONCLUSIONS AND FUTURE PERSPECTIVES

5.1 CHOLIC ACID FORMATION AND CHOLESTEROL ABSORPTION

Cholesterol absorption is a multifactorial process involving a number of proteins, any of which is a potential target for reducing cholesterol absorption. A number of strategies are currently used to treat hypercholesterolemia by reducing the absorption of dietary cholesterol. ACAT2 has been proposed as one possible drug target for hypercholesterolemia and atherosclerosis treatment[148]. A group of African Green monkeys hyper-responsive to atherogenic diets showed an increased hepatic expression of ACAT2[149], while in apoE -/- mice loss of ACAT2 protected against diet-induced hypercholesterolemia, through a decreased cholesterol absorption[150]. Another key factor is the potential cholesterol transporter NPC1L1. Ezetimibe, a cholesterol absorption inhibitor, binds to NPC1L1 and increases its expression in porcine liver and intestine[151]. Polymorphisms in NCP1L1 have been identified in people with low LDL levels and cholesterol absorption[152]. In mice NPC1L1 expression may be inhibited by cholesterol[153], and in a human cell line by LXR[152]. In cell culture studies, NPC1L1 has been shown to be present in the cell membranes and intracellular compartments. When cholesterol levels drop, however, it is translocated to the cell surface in the apical domain[154].

The inactivation of the Cyp8b1 gene in mice results in a lack of CA, an altered BA pool, decreased cholesterol absorption, increased cholesterol and BA synthesis and a decreased storage of cholesterol in the liver. In mice the ability of cholesterol to drive the expression of Cyp7a1 offers these animals another possibility to actively remove excess cholesterol. Taken together this results in a decrease of the levels of atherogenic VLDL and LDL. Humans lack this LXR mediated regulation of CYP7A1 expression. Therefore there are some species differences between rodents and man that directly affect cholesterol and BA metabolism. A pharmaceutical that inhibits the activity of CYP8B1 may provide a novel way to treat hypercholesterolemia and reduce atherosclerosis by inhibiting the absorption of cholesterol from the diet. The combination with ezetimibe and BA sequesterants may further increase the efficiency of such a strategy.

Similar to rodents the addition of CA to the diet is reported to cause an enhanced cholesterol absorption in man[15]. Loss or reduction in CA content in human bile would probably result in a BA pool less efficient for cholesterol absorption. Whether or not this will also increase BA synthesis cannot yet be stated, as the percentage of CDCA may increase. In man CDCA is considered to be the natural ligand for FXR[155,156], an inhibitor of BA synthesis.

Understanding the precise mechanisms controlling the genes involved in BA and cholesterol metabolism in mouse models aids our understanding of the possible effects of drug treatment in man. An important focus of future studies is to delineate effects of CA mediated via micellar solubilisation on the one hand and FXR activation on the other.

5.2 REGULATION OF CHOLESTEROL SYNTHESIS BY FXR

FXR is known to regulate BA synthesis, however, the possibility that it can also regulate a previous step, namely *de novo* cholesterol biosynthesis, is a more novel concept. Cyp8b1 -/- mice treated with FXR agonist showed a decreased expression of the genes involved in cholesterol synthesis to a level approaching that of Cyp8b1 +/+ mice. This may indicate that under control conditions FXR, through BAs or other ligands, reduces cholesterol synthesis. However, intact mice given FXR agonist showed an increased expression of genes involved in cholesterol synthesis. Direct binding of FXR to the potential FXR element at position –1611 in the murine Sqle promoter remains to be proven. If FXR does bind to the Sqle promoter perhaps more potential FXR elements in other genes involved in cholesterol synthesis may be identified and tested. In the near future the possible effects of FXR on cholesterol synthesis, absorption and lipid profile will be examined using the unique CA-free mouse model.

5.3 COMPLEX REGULATION OF SREBPS

SREBPs have a complex and finely tuned regulation[157]. Much work has been done to understand the post-transcriptional regulation of SREBPs, although more is waiting to be uncovered. The complex interaction between SREBPs and the MED15 subunit of the mediator complex are beginning to come to light[158]. The important role of SREBP-

1c and NF-Y in regulating the cell cycle demonstrates the importance of these transcription factors[159-162]. However, the precise mechanisms by which SREBP interact with other transcription factors such as NF-Y and Sp1 are still unclear. Once SREBP is cleaved, becoming mature SREBP protein, it binds to DNA with the aid of other transcription factors such as NF-Y and Sp1. It is only after binding to DNA that SREBPs are phosphorylated increasing both their activity and degradation[33-35]. Therefore the roles that NF-Y and Sp1 play in aiding SREBP binding to DNA are very important and require further investigation.

5.4 ROLE OF HMG COA REDUCTASE IN ALZHEIMER'S DISEASE

HMGCR is a highly regulated protein, with the majority of its regulation being post-translational. Nevertheless, it is important to investigate whether this C $\rightarrow$ A polymorphism results in an altered rate of cholesterol synthesis. One simple way to test this is to measure the cholesterol:lathosterol ratio in the plasma of people with one or both alleles containing the polymorphism. If these patients show no alterations in their rate of cholesterol synthesis this polymorphism does not exert any overall effects on the body. However, this technique is not a direct indicator of the rate of cholesterol synthesis in the brain. Why such an alteration would predispose an Italian population and not a Swedish one would perhaps be multifactorial involving multiple unknown genes and lifestyle differences.

5.5 POTENTIAL NEW TREATMENTS FOR METHYLMALONIC ACIDURIA TYPE B

The discovery that Mmab expression, a gene encoding for Cob(I)alamin adenosyltransferase, was increased with statin treatment in mice was surprising. Cob(I)alamin adenosyltransferase catalyses the conversion of cobalamin to adenosylcobalamin, a cofactor required by methylmalonyl-CoA mutase for the synthesis of succinyl-CoA. However, very little is currently known about Cob(I)alamin adenosyltransferase and its regulation. Treatment with statins will increase the expression of genes involved in cholesterol biosynthesis while the overall rate of biosynthesis decreases. It has previously been proposed by other researchers that Periodic Fever or Hyper IgD syndrome, resulting from a decreased MVK activity, be treated with statins[163]. The possibility that statin treatment may help patients with

methylmalonic aciduria type B demonstrating a residual adenosylating activity should be fully explored. The first way to do this is to investigate if statin treatment increases the activity of this enzyme in humans. Currently we are planning to perform experiments on isolated fibroblasts from healthy controls and patients with methylmalonic aciduria type B, with and without some residual Cob(I)alamin adenosyltransferase activity. If statin treatment can increase Cob(I)alamin adenosyltransferase activity in cell culture then the possibility of treating patients with statins becomes more favourable.

6 HISTORICAL ASPECTS

The study of bile and its relationship to health is far older than that of cholesterol. The ancient Greeks and Romans believed that to be healthy a person had to have a balance between their four humors, blood, phlegm, black bile and yellow bile. This belief was still held true in medieval times when an imbalance in the humors was believed to cause personality changes. For example too much black bile would make you melancholy. As bile accounted for half of their understood humors it is easy to assume that it was a large component in their medicine. In 1943 Konrad Bloch first demonstrated that CA is synthesized from cholesterol[164], thus cementing the relationship between cholesterol and bile. While bile acids have remained a focus of study since these ancient times, their central role in medicine has been lost. However, the importance of bile is making a come back and it has been shown that BAs, as natural ligands for FXR, are required for the normal regeneration of liver tissue[84]. Through a deeper understanding of nuclear receptors, such as FXR, the important roles BA play in health and disease, in addition to their detergent properties, is coming to light.

In 1775 Conradi isolated cholesterol from gallstones and made observations about its solubility. The term cholesterine was first proposed by Chevreul in 1816, from the Greek *chole* meaning bile and *steros* meaning solid, while the name cholesterol was introduced into English and French literature around the 20th century. A connection between cholesterol and bile was established early in cholesterol research, with Chevreul identifying cholesterol in human and animal blood and bile in 1824. In 1843 Vogel identified cholesterol in atherosclerotic plaques of arteries, thus establishing a link between CVD and cholesterol. In 1906 Pribram observed that there was an increase in blood cholesterol upon feeding of cholesterol, thus demonstrating that cholesterol can be absorbed from the diet. From his work with dogs Mueller showed that cholesterol was transported via the thoracic duct[165] and that bile and pancreatic juice aided in the absorption of cholesterol[166]. The first demonstration that cholesterol could be synthesised *in vivo* was in 1914 and these findings were confirmed in human adults in 1921[167]. Therefore in 1915 J Howard Mueller was wrong to state that[165]:

“it is now a generally accepted fact that the cholesterol of the body has its origin in the food, and not, as has been held by some investigators, in an actual synthesis within the body”

Windaus first proposed the structural formula of cholesterol in 1919, which was modified in 1932 to the current accepted formula. During the 1930’s Schoenheimer developed new a technique involving radio labeled molecules as a way of tracking them through the body. This technique is still used today. In 1937 along with Rittenburg, Schoenheimer used deuterium oxide to study cholesterol formation and suggested that in mammals cholesterol resulted from the coupling of a large number of smaller molecules[168]. After Schoenheimers death in 1941 his work was continued by Rittenburg and Bloch, who went on to study cholesterol synthesis using deuterium labeled acetic acid[169].

In the beginning of the 1970’s a group of Japanese researchers began looking for fungal metabolites that inhibited cholesterol metabolism by competitively binding with HMGCR, the rate limiting enzymatic step in cholesterol biosynthesis, and in 1976 they were successful[170]. This was the discovery of statins, one of the most common treatments for hypercholesterolemia used today. Continuing experiments from this group, and parallel experiments at Merck and Company, led to the discovery of lovastatin, the first commercially marketed statin, which was isolated from *Aspergillus terrus*[171].

Since these early studies into cholesterol and BA metabolism much progress has been made into understanding the synthesis, absorption, transport, functions, regulation of their metabolism and their involvement in health as well as disease. However, there is still much left to discover about these very important compounds.

7 POPULAR SCIENCE SUMMARY

Most people are aware that too much cholesterol can be bad for your health, increasing the risk of developing such conditions as heart disease and stroke. Many pharmaceuticals are currently available to reduce cholesterol levels. One popular treatment is with a class of drugs called statins, which reduce cholesterol levels by inhibiting the body's synthesis of cholesterol. Cholesterol, however, is a fundamental requirement for normal growth and development. While the body is capable of synthesizing all of the cholesterol it requires, it also absorbs cholesterol from the diet. Before cholesterol, a water insoluble compound, can be absorbed it must first be solubilised in the gut by detergents called bile acids (BA).

Once made or absorbed by the body cholesterol cannot be broken down. Instead cholesterol is used to synthesize BAs, some of which are lost each day in the faeces. This represents the major way that the body removes excess cholesterol. As with cholesterol, BAs are required for health and a lack reduces the body's ability to absorb cholesterol, and also fat-soluble vitamins and other lipids from the diet.

The synthesis and metabolism of both cholesterol and BAs is tightly controlled. Both of these compounds can regulate their own synthesis and metabolism through the action of proteins called transcription factors. DNA contains genes, the blueprints for all of the proteins synthesized in the body. By controlling the activity of a gene the concentration of the protein it encodes for can also be controlled. Transcription factors bind to DNA and regulate the activity of the gene, and thereby the level of protein produced. Transcription factors are themselves regulated. BAs and cholesterol can regulate transcription factors involved in both their own metabolism and other metabolic processes. For example the transcription factor activated by BAs has been shown to have an important role in liver regeneration and controlling bacterial growth in the gut.

By understanding precisely how BAs and cholesterol interact with different transcription factors, and how these transcription factors interact with other proteins and DNA, can allow for the discovery of novel drug targets. In addition, by understanding the complex interactions within the body the possible effects of new drugs can be better predicted.

During the course of this study we uncovered a gene involved in vitamin B metabolism that, in mice, was regulated by cholesterol and statin treatment. When given statins the activity of this gene increased, indicating a possible increase in the protein for which it encodes. People with mutations or alterations in this gene may produce dysfunctional protein and develop a condition called methylmalonic aciduria type B. This is a very rare yet serious condition with a high incidence of mortality in infants in the first few weeks of life. Some patients still have residual protein activity. Increasing the amount of protein may alleviate some of the symptoms of this condition in these patients. Treatment with statins, which increases the activity of this genes in mice, may be one way to do this.

As time goes on we learn more and more about cholesterol and BAs and their importance in both health and disease. As the incidence of obesity increases in the industrialised world there is an increase in related health problems. By understanding the delicate balance within the body and how different processes affect one another more elegant and refined drugs to control disease may be developed.

Populärvetenskaplig sammanfattning

De flesta är medvetna om att för mycket kolesterol kan var skadligt för hälsan och öka risken för exempelvis hjärtsjukdomar och stroke. Det finns idag många läkemedel på marknaden som kan sänka kolesterolvärdet. En populär behandlingsmetod är en grupp av läkemedel som kallas statiner, vilka förhindrar kroppens förmåga att framställa kolesterol. Kolesterol spelar dock en viktig roll i kroppens utveckling och samtidigt som kroppen själv kan framställa allt kolesterol den behöver, kan den även ta upp kolesterol från kosten. Innan kolesterol, som är ett vattenolösligt änne, kan tas upp av kroppen måste det först lösas upp i tarmarna av ett ämne (lösningsmedel) som kallas gallsyra.

När kolesterol väl har framställts eller tagits upp av kroppen kan det inte brytas ned. Istället används det till att framställa gallsyra, vilken delvis försvinner med avföringen. Detta utgör kroppens huvudsakliga sätt att göra sig av med överflödigt kolesterol. Gallsyror är på samma sätt som kolesterol nödvändiga för kroppen och ett underskott

reducerar förmågan att ta upp kolestorol samt fettlösliga vitaminer och andra lipider från dieten.

Framställning och avsättning av kolesterol och gallsyra är hårt reglerad av kroppen. Båda dessa ämnen kan reglera sin egen framställning och omsättning genom proteiner (transkriptionsfaktorer) som binder till DNA. DNA innehåller gener vilka utgör mallen för alla protein som framställs av kroppen. Genom att kontrollera aktiviteten hos en gen kan även dess protein kontrolleras. Transkriptionsfaktorer fäster vid DNA och reglerar genens aktivitet vilket styr mängden protein som produceras. Även transkriptionsfaktorerna själva är reglerade av kroppen. Gallsyra och kolesterol kan genom transkriptionsfaktorer reglera såväl sin egen omsättning som andra processer i kroppen. Till exempel så har transkriptionsfaktorn som aktiveras av gallsyra visat sig spela en viktig roll i leverns återbildning och för att kontrollera bakterietillväxten i tarmsystemet.

Genom att förstå hur gallsyra och kolesterol interagerar med olika transkriptionsfaktorer och hur dessa i sin tur interagerar med andra proteiner och DNA, kan möjliga effekter av nya läkemedel bättre förutsägas.

Under studien upptäckte vi att kolesterol-och statinbehandling påverkar omsättingen av vitamin B. Vid behandling med statin ökar aktiviteten hos genen som aktiverar vitamin B vilket även indikerar en möjlig ökning av detta protein. Hos människor med mutationer eller förändringar i denna gen kan ett defekt protein produceras och ett tillstånd som kallas methylmalonsyra aciduri typ B utvecklas. Detta tillstånd är väldigt ovanligt men allvarligt då det medför en hög dödlighet hos spädbarn under deras första levnadsveckor. En del patienter har dock fortfarande en liten aktivitet i detta protein och hos dessa skulle en ökning av proteinet kunna avhjälpa några av symptomen för detta tillstånd. Behandling med statiner ökar denna gens aktivitet hos möss. Statin skulle därför kunna vara en möjlig behandlingsform för detta tillstånd.

Vad tiden lider lär vi oss mer om kolesterol och gallsyror och deras betydelse för vår hälsa. När andelen överviktiga ökar i västvärlden ökar även hälsoproblemen relaterade till detta. Genom att förstå balansen mellan olika processer i kroppen och hur dessa påverkar varandra kan träffsäkrare läkemedel framställas för att behandla dessa hälsoproblem.

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