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STUDIES ON THE REGULATION OF HEPATIC AND INTESTINAL LIPID METABOLISM

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ABSTRACT

Disturbances in the lipid homeostasis can result in a wide variety of different diseases and symptoms. This thesis is focused on the regulation of cholesterol metabolism and its relation to atherosclerosis development, and the effect of mitochondrial (mt)DNA depletion on hepatic β-oxidation of fatty acids.

ApoE deficient mice spontaneously develop atherosclerosis due to accumulation of chylomicron remnants in the circulation, a process highly dependent on intestinal cholesterol absorption. The lack of functional ApoE prevents receptor mediated uptake of lipoproteins, leading to very high levels of circulating cholesterol. We used two approaches to test the influence of plasma cholesterol levels on atherosclerosis development in ApoE deficient mice. In paper I we disrupted the *Cyp8b1* gene to disable the endogenous synthesis of cholic acid and thereby reduce the intestinal absorption of cholesterol. Cyp8b1/ApoE double knockout mice displayed reduced atherosclerotic lesions in aorta, reduced cholesterol levels in plasma and liver and reduced intestinal cholesterol absorption compared to ApoE knockout mice. In paper II we treated ApoE deficient mice with the thyroid receptor β modulator GC-1. Supplementation with thyroid hormone reduces plasma cholesterol in humans and rodents; this effect is mediated by the thyroid receptor isoform β (TRβ). We found that oral administration of GC-1 reduced atherosclerosis after 20 weeks of treatment. This reduction was accompanied by increased hepatic LDL-receptor expression, bile acid synthesis and fecal bile acid excretion, but serum cholesterol levels were not reduced. The onset and development of atherosclerosis was likely delayed by short-term (one to ten weeks) treatment effects, such as increased fecal excretion of neutral sterols, reduced intestinal cholesterol absorption and reduced plasma cholesterol levels, which were not significant after long-term (20 weeks) treatment.

Increased HDL-cholesterol and reduced intestinal cholesterol absorption may lead to improved anti-atherosclerotic effects on cholesterol metabolism, but the LXR-induced increase in HDL-cholesterol may depend on the intestinal cholesterol absorption. In paper III we investigated this by oral administration of the LXR agonist GW3965 and the cholesterol absorption inhibitor ezetimibe. The combined treatment effectively reduced the intestinal cholesterol absorption and increased serum HDL-cholesterol and ApoAI. Neither intestinal nor hepatic ABCA1 protein expression increased, but hepatic *ApoaI* expression was induced. The LXR-driven increase in HDL-cholesterol and ApoAI was thus independent of both intestinal cholesterol absorption and increased intestinal or hepatic ABCA1 protein. The combined treatment effectively increased fecal excretion of cholesterol.

Thymidine kinase (TK) 2 deficiency leads to mtDNA depletion. TK2 deficient mice have been developed and display symptoms of defect fat metabolism. In paper IV we studied the lipid phenotype of TK2 deficient mice in search for treatment options. TK2 deficient mice displayed elevated plasma levels of cholesterol, free fatty acids and long-chain acylcarnitines. In mice with hepatic mtDNA levels below 20%, the blood sugar and the ketone levels dropped. These mice also exhibited reduced hepatic mitochondrial β-oxidation and CPT activity, indicating reduced capacity to metabolize long-chain acylcarnitines. Hepatic ATP synthesis rates were unaltered in 14 days old TK2 deficient mice compared to controls.

LIST OF PUBLICATIONS

I. Slätis K., Gåfvels M., **Kannisto K**., Ovchinnikova O., Paulsson-Berne G., Parini P., Jiang Z-Y., Eggertsen G.
Abolished synthesis of cholic acid reduces atherosclerotic development in ApoE knockout mice.
Journal of Lipid Research 2010 51(11):3289-98

II. **Kannisto K**., Rehnmark S., Slätis K., Webb P., Larsson L., Eggertsen G., Gåfvels M. and Parini P.
The thyroid receptor β modulator GC-1 reduces atherosclerosis in ApoE deficient mice.
Manuscript

III. **Kannisto K**., Gåfvels M., Jiang Z-Y., Slätis K., Hu X., Steffensen KR. and Eggertsen G.
LXR driven induction of HDL-cholesterol is independent of intestinal cholesterol absorption and ABCA1 protein expression.
Manuscript

IV. Zhou X., **Kannisto K**.*, Curbo S., von Döbeln U., Hultenby K., Isetun S., Gåfvels M., Karlsson A.
Thymidine kinase 2 deficiency-induced mtDNA depletion in mouse liver leads to defect β-oxidation.
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* Shared first authorship with X.Z. and S.C.

CONTENTS

LIST OF ABBREVIATIONS

ABC	ATP-binding cassette
ACAT	Acyl-CoA: cholesterol acyltransferase
ACC	acetyl-CoA carboxylase
ADP	adenosine-5´-diphosphate
Apo	apolipoprotein
ATP	adenosine-5´-triphosphate
C4	7α-hydroxy-4-cholestene-3-one
CA	cholic acid
CD	cluster of differentiation
CDCA	chenodeoxy cholic acid
CETP	cholesteryl ester transfer protein
COX	cytochrome oxidase subunit
CPT	carnitine palmitoyltransferase
CVD	cardiovascular disease
CYP	cytochrome P450
DGUOK	deoxyguanosine kinase
DKO	double knockout
DNA	deoxyribonucleic acid
DNC	deoxyribonucleotide carrier
dNTP	deoxynucleotide triphosphate
dTMP	deoxynucleotide monophosphate
EL	endothelial lipase
FAS	fatty acid synthase
FGF	fibroblast growth factor
FXR	farnesoid x receptor
HDL	high-density lipoprotein
HL	hepatic lipase
Hmg-CoA	3-hydroxy-3-methyl-glutaryl-Coenzyme A
IDL	intermediate-density lipoprotein
LCAD	long-chain acyl-CoA dehydrogenase
LCAT	Lecithin-cholesterol acyltransferase
LDL	low-density lipoprotein
LPL	lipoprotein lipase
LRH	liver receptor homolog
LRP	LDL-receptor related protein
LXR	liver x receptor
MCA	muricholic acid
MCAD	medium-chain acyl-CoA dehydrogenase
MDS	mtDNA depletion syndrome
mtDNA	mitochondrial DNA
NAD(H)	nicotinamide adenine dinucleotide
NPC1L1	Niemann-Pick C1 Like 1
NR	nuclear receptor
POLG	polymerase gamma
RCT	reverse cholesterol transport

RNA	ribonucleic acid
SCAD	short-chain acyl-CoA dehydrogenase
SR	scavenger receptor
SREBP	sterol regulatory element binding protein
TAG	triacylglycerol
TH	thyroid hormone
TK	thymidine kinase
TR	thyroid hormone receptor
VLCAD	very long-chain acyl-CoA dehydrogenase
VLDL	very low-density lipoprotein

1 INTRODUCTION

1.1 LIPIDS

Lipids represent both polar and non-polar molecules that are involved in a multitude of functions in our bodies. This thesis is focused on the metabolism of two types of lipids; cholesterol and fatty acids.

1.1.1 Cholesterol

Cholesterol is a 27 carbon structure that functions as a component of cellular membranes; affecting membrane fluidity, and as a precursor for steroid hormones and bile acids (figure 1). Cholesterol is essential for mammalian life, but as for most things in life, excess levels can cause disease.

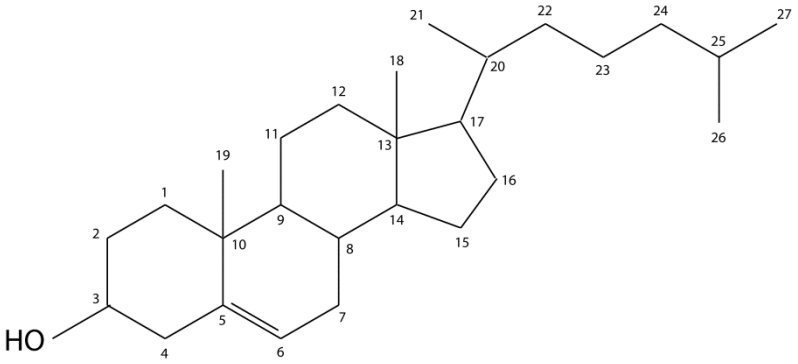

Figure 1. The chemical structure of cholesterol.

1.1.1.1 Hepatic cholesterol metabolism

The cells in our bodies can acquire cholesterol either by synthesis or by uptake of cholesterol from lipoproteins circulating in the blood stream (see section 1.1.3). The liver is a central organ for cholesterol metabolism where many of the plasma lipoproteins are formed and metabolized. The liver contributes 20-25 % of the total daily cholesterol synthesis. The synthesis starts with the condensation of acetyl-coenzyme A (Acetyl-CoA) to form mevalonate, a six carbon intermediate. This requires two important enzymes; 3-hydroxy-3-methyl-glutaryl-Coenzyme A (Hmg-CoA) reductase and –synthase, of which the former is the rate-limiting enzyme of cholesterol synthesis and the target of statins [1]. The endogenous cholesterol synthesis is regulated by the sterol regulatory element binding protein (SREBP) 2 which responds to intracellular cholesterol levels. When levels are high, SREBP2 is found in the endoplasmic reticulum (ER) as an inactive precursor, but as cholesterol levels decrease, SREBP-2 moves to the Golgi where it is proteolytically cleaved. The cytoplasmic portion of the peptide then moves into the nucleus where it binds to sterol response elements (SRE) in the promoter region and activates transcription of genes involved in cholesterol synthesis [2]. Within the cells, cholesterol is stored as cholesteryl esters (one cholesterol molecule bound to one fatty acid by an ester bond). Esterification is carried out by Acyl-CoA: cholesterol acyltransferase (ACAT) 1 and 2. ACAT 1 is ubiquitously expressed, while ACAT 2 is expressed only in enterocytes and

hepatocytes [3, 4]. Free cholesterol can be excreted from the liver into the bile via the ATP-binding cassette (ABC) G5/G8 heterodimer. Once formed, the cholesterol ring-structure is very stable and not easily metabolized. Therefore, formation of bile acids in the liver constitutes one of the major elimination routes of cholesterol from the body.

Bile acid synthesis requires an array of enzymes belonging to the Cytochrome P450 (CYP) superfamily. Formation of bile acids can occur through two pathways, often denoted as the classic and the alternative pathway. The classic pathway starts with hydroxylation of the 7α position in the cholesterol ring structure. This step is carried out by the enzyme encoded for by the cholesterol 7α-hydroxylase (*Cyp7a1*) gene and is the rate limiting step in bile acid synthesis. The classic pathway results in formation of either cholic acid (CA) or chenodeoxy cholic acid (CDCA) (figure 2), the two primary bile acids in humans. CA and CDCA have a common precursor; 7α-hydroxy-4-cholestene-3-one (C4), which can be hydroxylated in the C12 position by sterol 12α-hydroxylase (CYP8B1) and form CA. Alternatively, without 12α-hydroxylation, it is converted to CDCA. The alternative pathway is initiated by sterol 27-hydroxylase (CYP27A1) and results in formation of CDCA. Bile acid synthesis in mice differs slightly from that in humans as most of the synthesized CDCA is converted into muricholic acids (MCAs) [5] (figure 2 and 3).

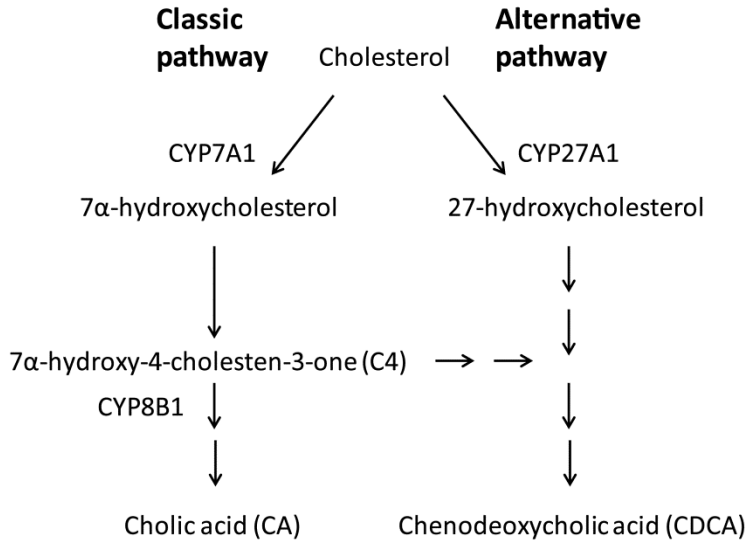

Figure 2. An overview of the main steps in bile acid synthesis.

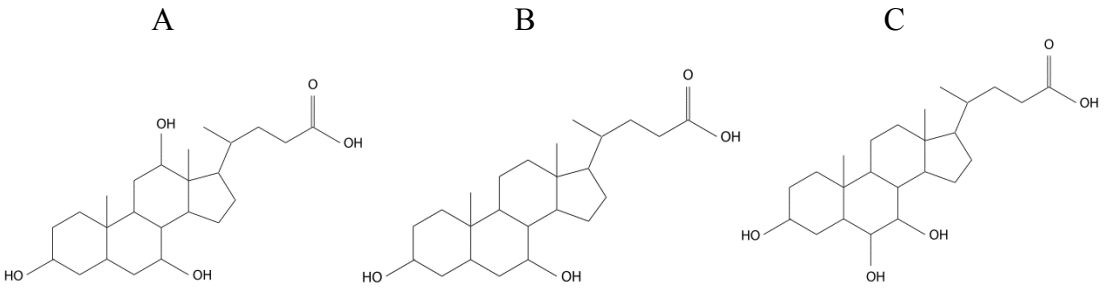

Figure 3. The chemical structures of the unconjugated primary bile acids in humans and mice: (A) cholic acid, (B) chenodeoxycholic acid and (C) muricholic acid. Bile acids are conjugated to taurine or glycine at the carboxylic acid side chain.

Because of this, the bile acid pool in mice is more hydrophilic than in humans. Bile acids are amphipathic molecules containing the same ring structure as cholesterol, but they are more hydrophilic than cholesterol and therefore have increased solubility in water. This property is important for transport of cholesterol in the bile and for solubilizing fats in the intestine. Primary bile acids are conjugated to either taurine or glycine at their carboxylic acid side chains, which lowers their pKa and optimizes their capabilities as emulsifiers. In mice, bile acids are mainly conjugated to taurine, while glycine-conjugates dominate in humans [6, 7]. During fasting periods, the bile acids are excreted to and collected in the gallbladder together with cholesterol and phospholipids, forming bile. The bile is released from the gallbladder into the proximal small intestine when food is ingested. Bile acids undergo enterohepatic circulation that preserves the bile acid pool as the major part of bile acids are re-absorbed in ileum and re-circulated to the liver. Bile acids and cholesterol exist in an intimate interplay, affecting synthesis rates, uptake and excretion of each other. The enterohepatic circulation (figure 4) facilitates this regulation by stimulating nuclear receptors such as the liver x receptor (LXR) and the farnesoid x receptor (FXR) (see section 1.3).

1.1.1.2 Intestinal cholesterol metabolism

The absorption of dietary cholesterol is believed to occur mainly in the jejunum of the small intestine and depends on bile composition and the amount of ingested cholesterol. Conjugated bile acids act as emulsifiers in the small intestine and form micelles together with cholesterol, phospholipids and other fats. In mice, the ratio of CA to β-MCA seems to be of importance for the emulsification of cholesterol [8]. CA-depletion in mice, due to disruption of *Cyp8b1*, leads to decreased intestinal cholesterol absorption. Contrary, dietary supplementation with CA increases intestinal cholesterol absorption [9, 10].

Several protein transporters are involved in the process of uptake and trafficking of cholesterol through the enterocyte. In 2004 the Niemann-Pick C1 Like 1 (NPC1L1) protein was shown to be critical for intestinal cholesterol absorption [11] and soon after it was also shown to be the target of the cholesterol absorption inhibitor ezetimibe [12]. NPC1L1 locates to the apical brush border membrane of intestinal enterocytes in mice and humans. Importantly, humans also express this protein in hepatocytes while the expression in mouse liver is very low [13]. Cholesterol binds to NPC1L1 and is subsequently incorporated into the enterocyte in clathrin-coated vesicles. Ezetimibe blocks the NPC1L1-mediated uptake of cholesterol by inhibiting the vesicular internalization of NPC1L1 [14]. Cholesteryl esters entering the small intestine are hydrolyzed by pancreatic enzymes before uptake and ezetimibe blocks the absorption of the free cholesterol formed after hydrolysis, but not the fatty acid moieties [15]. After uptake into the enterocyte by NPC1L1, the major part of cholesterol is esterified by ACAT2 and incorporated into chylomicrons. However, part of the internalized cholesterol can be transferred as free cholesterol to HDL via ABCA1. The small intestine may contribute up to 30 % of the total HDL-cholesterol in mice [16]. The ABCG5/G8 heterodimer balances the uptake of cholesterol via NPC1L1 by pumping free cholesterol back to the intestinal lumen (figure 4).

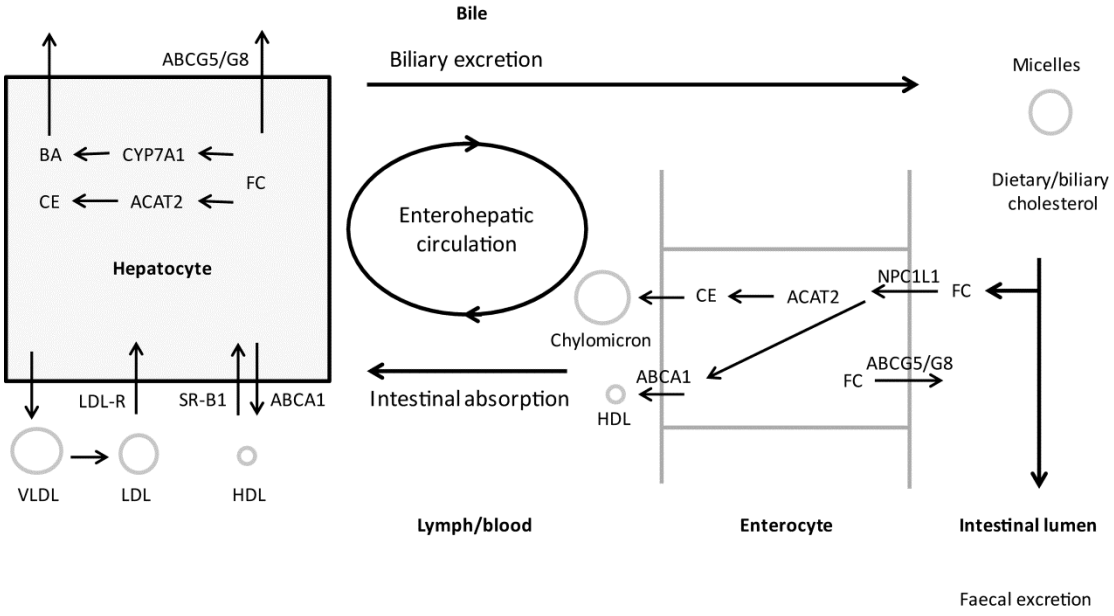

Figure 4. A schematic overview of the enterohepatic circulation of cholesterol. FC = free cholesterol, CE = cholesteryl esters, BA = bile acids.

1.1.2 Fatty acids

Fatty acids function as a source of energy; the break-down of fatty acids through β-oxidation is the first step in a process that yields a large quantity of adenosine triphosphate (ATP). They also function as cell membrane components and signaling molecules. Fatty acids are carbon-chain structures with a carboxylic acid at one end and they are classified by the length and number of double-bonds in the aliphatic tail. The hydrophobicity of the structure increases with increasing tail length. Most of the naturally occurring fatty acids have an even number of carbon atoms in their aliphatic tail. Fatty acids containing one or more double-bonds are classified as unsaturated fatty acids, while saturated fatty acids contain no double-bonds (figure 5).

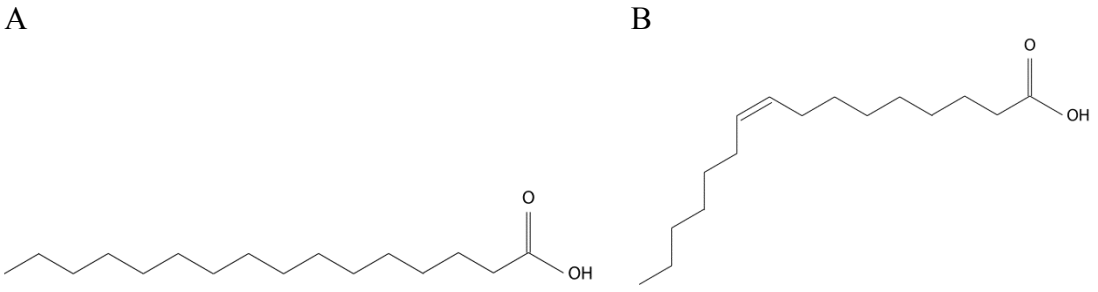

Figure 5. The chemical structures of two fatty acids.
(A) Palmitic acid (C16:0); a saturated fatty acid, (B) Palmitoleic acid (C16:1 n-7); an unsaturated fatty acid.

1.1.2.1 Hepatic fatty acid metabolism

The liver acquires fatty acids through synthesis within the cells (lipogenesis), absorption from dietary sources in the intestine or catabolism of stored triacylglycerol (TAG) in adipose tissue (lipolysis), which releases fatty acids into the blood stream.

The regulation of lipolysis in white adipose tissue is complex and many regulatory factors have been identified, but the hormonal regulation by insulin and catecholamines via hormone sensitive lipase (HSL) and the more recently identified adipose triglyceride lipase (ATGL) is well described [17, 18].

Lipogenesis takes place in the cytoplasm of the cells and requires malonyl-CoA, which is formed by carboxylation of acetyl-CoA through interaction with acetyl-CoA carboxylase (ACC). Malonyl-CoA and acetyl-CoA units are then assembled into fatty acids by the fatty acid synthase (FAS). The liver and the adipose tissue are the two major sites of fatty acid synthesis in the body. During lactation, the mammary glands also synthesize large amounts of fatty acids. Acetyl-CoA is derived from break-down of glucose; therefore lipogenesis is very sensitive to blood sugar and insulin levels. Lipogenesis is stimulated when food is ingested and blood sugar/insulin levels increase. The SREBP isoform 1c is a key transcriptional activator of genes involved in lipogenesis (*i.e*. ACC and FAS). SREBP1c is stimulated by insulin, but is also activated through LXR (section 1.2.1). High levels of free fatty acids are toxic to cells and the generated fatty acids are therefore rapidly esterified to glycerol, forming acylglycerols. The most common form is TAG which is either stored in lipid droplets within the cell or used for the assembly of very low-density lipoprotein (VLDL) particles that are released into the blood stream (see section 1.1.3). To utilize the energy bound within the fatty acid molecules, cells must first break down the molecules into acetyl-CoA. But, since acetyl-CoA is both substrate for lipogenesis and product of the oxidative break-down of fatty acids, these two reactions must be kept apart to ensure proper regulation. Therefore, β-oxidation of fatty acids takes place within the mitochondria.

Mitochondrial β-oxidation of fatty acids generates acetyl-CoA. To produce ATP, acetyl-CoA must enter the citric acid cycle where it is consumed together with water, resulting in reduction of NAD+ to NADH and production of CO_2. NADH is then used in the oxidative phosphorylation pathway (electron transfer chain) which ultimately results in the phosphorylation of ADP to ATP. All these steps occur inside the mitochondrion.

Long-chain fatty acids (fatty acids with aliphatic tail of 14 carbons or longer) are activated by acyl-CoA synthetase (ACS) forming acyl-CoA, which enter the mitochondrion in three steps; (1) carnitine palmitoyltransferase (CPT) I conjugates acyl-CoA to carnitine, (2) the acyl-carnitine conjugate is transferred into the mitochondrion by a translocase and (3) carnitine and acyl-CoA is released by CPT II (figure 6). Malonyl-CoA regulates this first step of β-oxidation by inhibiting CPT activity. The medium- and short-chain fatty acids are able to diffuse into the mitochondria directly and are activated by acyl-CoA synthetases in the mitochondrial matrix.

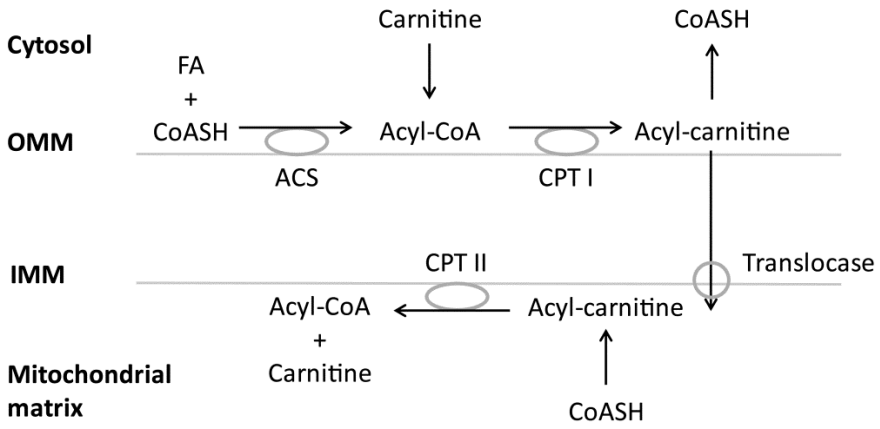

Figure 6. An overview of the transport of long-chain fatty acids into the mitochondrion. OMM = outer mitochondrial membrane, IMM = inner mitochondrial membrane, FA = fatty acid, ACS = acyl-CoA synthetase, CPT = carnitine palmitoyltransferase.

Inside the mitochondrion, acyl-CoA is repeatedly shortened by four reoccurring steps; (1) oxidation, (2) hydration, (3) oxidation and (4) thiolysis, yielding acetyl-CoA and acyl-CoA (two carbon atoms shorter in tail length compared to the initial acyl-CoA). This process continues until only acetyl-CoA remains (figure 7).

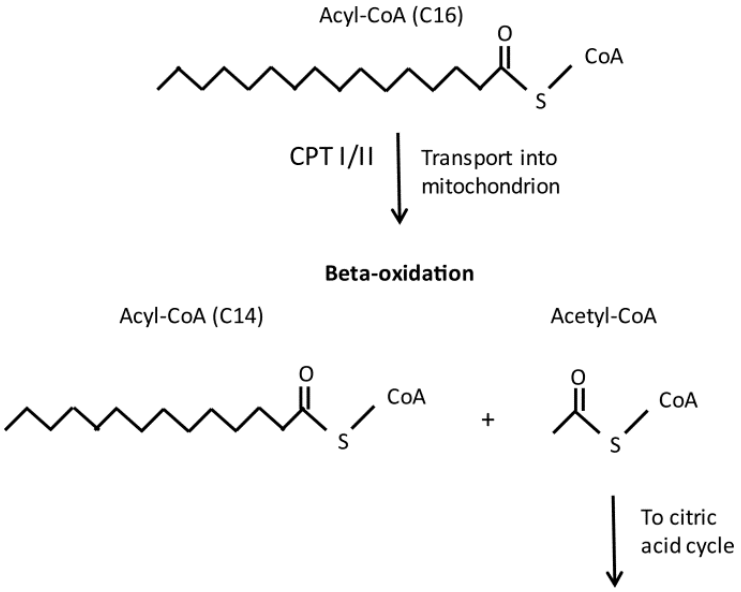

Figure 7. An overview of mitochondrial β-oxidation.

All of the enzymes involved in mitochondrial β-oxidation that are known today are products of genes encoded within the nuclear DNA. The mitochondrial genome, which is inherited maternally, consists of 37 genes which encode transfer (t)RNA, ribosomal (r)RNA and 13 peptides (proteins) involved in the oxidative phosphorylation system [19].

1.1.2.2 Intestinal fatty acid metabolism

TAGs are the major components of the fat that enters the small intestine from dietary sources. Acid lipase activity in the stomach (also in the saliva of rodents) initiates the

degradation of TAGs, resulting in fatty acids and diacylglycerols. In duodenum, pancreatic lipase continues the hydrolysis to release more free fatty acids and monoacylglycerides (predominantly 2-monoacylglycerols) that can be absorbed by enterocytes. Pancreatic lipase can only function in the interface between water and lipids; therefore efficient intestinal uptake of fatty acids is dependent of emulsification of fats by bile acids and formation of micelles. Fatty acids can enter the enterocytes through passive diffusion and possibly also through transporter mediated uptake. The specific proteins involved in this process are however not identified. One protein, cluster of differentiation 36 (CD36) interacts with fatty acids in enterocytes of the proximal intestine, but elimination of CD36 does not affect net absorption of fatty acids [20-22]. Inside the enterocyte, fatty acids are re-joined with monoacylglycerol to form TAGs that are transferred into chylomicrons and excreted in the lymph (see section 1.1.3) [23].

1.1.3 Transport of cholesterol and fatty acids by lipoproteins

Cholesterol and TAG have very low solubility in water. Therefore, to enable transport of these substances in the blood stream, they are packed in molecular complexes called lipoproteins. Lipoproteins consist of a hydrophilic surface, containing proteins, free cholesterol and phospholipids, and a hydrophobic core, containing TAG and cholesteryl esters. Lipoproteins are commonly classified according to their size or density as chylomicrons, very low-density lipoprotein (VLDL), intermediate-density lipoprotein (IDL), low-density lipoprotein (LDL) and high-density lipoprotein (HDL), starting with the largest particle. Each class also contains several subclasses, in which the lipoproteins are grouped according to their protein and lipid content, and sometimes their shape. The proteins attached to the lipoprotein surface are called apolipoproteins and determine the faith of the lipoprotein particle by binding to different receptors located on cell surfaces or by activation of lipolytic enzymes. These receptors facilitate uptake of lipids or entire lipoprotein particles into the cell, or can administer lipids to the lipoprotein particle. Transportation of cholesterol and fatty acids in the blood stream can be divided into three main routes: the exogenous pathway, the endogenous pathway and reverse cholesterol transport.

1.1.3.1 The exogenous pathway

The exogenous pathway constitutes the transport of cholesterol and fatty acids from the brush-border membrane of the small intestine and out to peripheral tissues, ending with the uptake of chylomicron remnants by the liver. It starts with the uptake of dietary cholesterol and fatty acids in the intestine. Most of the retrieved cholesterol and fatty acids are re-esterified into cholesteryl esters and TAG, and transferred into chylomicrons. Chylomicrons are the largest and most short-lived lipoprotein particles, consisting mostly of TAG, but also carry cholesterol and phospholipids. They are secreted by the enterocytes into lymph vessels, to be released into the blood stream via the thoracic duct. Their main purpose is to deliver fatty acids to the white adipose tissue, muscles and the liver. The nascent chylomicron contains the apolipoprotein ApoB48, an edited shorter form of ApoB which cannot bind efficiently to the LDL-receptor [24]. Upon release into the blood and interaction with HDL, chylomicrons acquire ApoCII and ApoE, necessary for efficient hydrolysis of triglycerides by lipoprotein lipase (LPL) and subsequent uptake of the free fatty acids into cells. After

delivering most of the triglycerides to the tissues, ApoCII is returned to HDL and the chylomicron remnants are taken up by the liver for breakdown. The uptake is dependent on functional ApoE for binding to the LDL-receptor and LDL-receptor related protein 1 (LRP1) [25-27]. In ApoE deficient mice, this last step fails and chylomicron remnants accumulate in the blood stream, making these genetically manipulated mice very sensitive to increased dietary cholesterol levels. Some of the cholesterol transported by chylomicrons can be taken up by peripheral tissues, but the major part is delivered to the liver.

1.1.3.2 The endogenous pathway

The endogenous pathway constitutes the transport of TAG and cholesterol from the liver in VLDL particles, for delivery to peripheral tissues, ending with the uptake of LDL by the liver. VLDL particles that are formed and secreted by the liver contain TAG, cholesterol, phospholipids and ApoB [28, 29]. ApoB secreted from human livers consists of the unedited full-length version; ApoB100, while mice secrete both ApoB100 and the edited form; ApoB48 [30-32]. Upon interaction with HDL, the VLDL particles acquire ApoCII and ApoE which facilitates the donation of fatty acids from VLDL to the tissues in the same way as for chylomicrons. In humans, VLDL donates TAG in exchange for cholesterol and phospholipids from HDL via the cholesteryl ester transfer protein (CETP). This step does not occur in mice, since mice are naturally devoid of CETP. As more and more TAGs are removed from the VLDL particles, they turn into IDL particles which still contain more than 50 % TAG, but no ApoCII. ApoCII is returned to HDL in circulation. IDL is either taken up by the liver by binding of ApoE to the LDL-receptor, or further relieved of TAG and ApoE turning into LDL. LDL particles contain low levels of TAG, high levels of cholesterol and phospholipids, and ApoB100. LDL delivers cholesterol to various organs by binding of ApoB to LDL-receptors on the cell surfaces. The complete LDL particle is endocytosed together with the LDL-receptor, of which the latter is re-circulated back to the cell surface [33, 34].

1.1.3.3 The reverse cholesterol transport

The reverse cholesterol transport constitutes the transport of cholesterol from peripheral tissues back to the liver for excretion in the bile, ending with the elimination of cholesterol from the body in the faeces. HDL particles are formed by secretion of ApoAI, mainly from the liver, which rapidly forms complexes with phospholipids and free cholesterol through interaction with the ATP-binding cassette (ABC) A1 protein expressed on cell surfaces. The liver is the major production site for HDL and hepatic ABCA1 is crucial for HDL synthesis and lipidation of ApoAI [35]. The HDL particle acquires more free cholesterol from peripheral tissues and cells, including macrophages, through interaction with ABCA1 and ABCG1 proteins expressed on cell surfaces. The acquired free cholesterol is esterified by Lecithin-cholesterol acyltransferase (LCAT) and stored in the HDL particle as cholesteryl esters. The accumulation of cholesteryl esters in HDL makes the particle increase in size and become spherical. HDL transports the acquired cholesterol to the liver where the scavenger receptor B1 (SR-B1) mediates uptake of cholesterol into the liver, but leaves the HDL particle in circulation. Excess ApoAI is metabolized in the kidneys. In the liver, cholesterol can be transferred to the bile as free cholesterol or bile acids for

subsequent elimination in the feces. HDL fractional catabolism and circulating levels are to some extent regulated by hepatic lipase (HL) and endothelial lipase (EL). Increased activity of HL or EL generally leads to reduced HDL levels and size, while defects in these lipases increase HDL [36]. Many apolipoproteins have been associated with HDL, but the major apolipoproteins in HDL are ApoAI and ApoAII [37, 38].

1.2 REGULATION OF LIPID HOMEOSTASIS BY NUCLEAR RECEPTORS

This chapter contains descriptions of the nuclear receptors studied in this thesis. Nuclear receptors are proteins which are activated by specific ligands (molecules) and thereby control the transcriptional expression of genes. Today there are 48 and 49 known nuclear receptors in humans and mice respectively, most of them are shared between these species [39]. We have studied the effects of oral administration of synthetic ligands for three of those receptors: the liver x receptor (LXR), the farnesoid x receptor (FXR) and the thyroid hormone receptor isoform β (TRβ). Upon activation, these receptors heterodimerize with the retinoid x receptor (RXR).

1.2.1 The Liver x receptor

The LXR subfamily consists of two members; LXRα (NR1H3) and LXRβ (NR1H2). LXRβ is ubiquitously expressed, while LXRα is mainly expressed in liver, adipose tissue, intestine, kidney and macrophages. These receptors regulate lipid metabolism and the importance of these receptors is evident in mice lacking LXR [40-42].

Both LXR isoforms are considered to be stimulated by oxysterols; 22(R)-hydroxycholesterol and 24(S), 25-epoxycholesterol [43, 44]. The relevance of these ligands for *in vivo* activation of LXR is however under debate since the ratio of oxysterols to cholesterol may be too low for LXR activation and direct proof of *in vivo* effects of increased oxysterol levels are missing [45].

Synthetic ligands for LXR have been developed and tested in animals. T0901317, the first synthetic non-steroidal ligand for LXR, opened the door for more extensive studies on LXR regulation *in vivo* [46]. Systemic stimulation of LXR was found not only to modulate cholesterol homeostasis, but also to induce SREBP1c and lipogenesis in the liver, leading to hepatic steatosis. Thereafter the development of synthetic LXR agonists was aimed at finding substances that could induce the beneficial effects on cholesterol homeostasis without affecting hepatic lipogenesis. One strategy was to develop tissue specific agonists that stimulated LXR in the intestine more efficiently than in liver. GW3965 is one such compound [47, 48] and in recent years several other compounds have been identified and tested in animal studies. Synthetic LXR agonists have been shown to be anti-atherogenic by oral administration in mice with ApoE or LDL-receptor deficiency [49, 50].

The anti-atherogenic properties of LXR agonists include increased HDL-cholesterol and RCT [51]. These effects are mediated through induction of genes involved in cholesterol transport; *Abca1*, *Abcg1* and *Abcg5*/*g8*. The activation of these genes is believed to be protective against accumulation of cholesterol within the cells. In mice, LXRα regulates bile acid synthesis by inducing *Cyp7a1*, but this regulation does not exist in humans due to lack of homology in the DR-4 promoter element [52].

Activation of LXR in CETP expressing animals (for example monkeys) is reported to increase LDL-cholesterol [53] and this poses an important obstacle to overcome for future development of LXR agonists intended for human use.

1.2.2 The Farnesoid x receptor

FXR (NR1H4) was originally identified as a receptor for farnesol, an intermediate in cholesterol synthesis, hence the name; farnesoid x receptor. FXR is a major regulator of bile acid synthesis in both humans and mice. Activation of FXR suppresses bile acid synthesis by reducing the expression of *Cyp7a1* and *Cyp8b1*. This is however an indirect action; FXR does not bind directly to these genes, but induces transcription of small heterodimer partner (*Shp*). SHP interacts with the liver receptor homolog-1 (LRH-1), and possibly also hepatocyte nuclear factor 4α (HNF4α), leading to inhibition of their transactivation of *Cyp7a1* [54, 55].

Bile acids are ligands to FXR and CDCA is considered to be the most potent [56-58]. However, CA seems to be an important mediator of FXR signaling in mice [9, 59]. The enterohepatic circulation of bile acids mediates FXR signaling between the intestine and liver; FXR is expressed in both organs. In the distal small intestine (ileum), uptake of bile acids activates FXR and induces expression of fibroblast growth factor 15 (Fgf15) in mice [60] or Fgf19 in humans [61], which is secreted into the blood. This peptide activates FGF receptor isotype 4 (FGFR4) in the hepatocytes, which in turn suppresses *Cyp7a1* and bile acid synthesis [60-62].

The development of a non-steroidal FXR agonist, GW4064 [63], was an important step in identifying FXR regulated pathways since bile acids also exert effects apart from the FXR regulation. The effects of FXR deficiency on atherosclerosis have been investigated in both $FXR^{-/-}/ApoE^{-/-}$ and $FXR^{-/-}/Ldlr^{-/-}$ mice, but results from these studies have not given conclusive answers. The $FXR^{-/-}/ApoE^{-/-}$ mice displayed increased or decreased atherosclerosis in males or females respectively, despite higher plasma levels of cholesterol in both genders after feeding with an atherogenic diet [64, 65]. Male, but not female, $FXR^{-/-}/Ldlr^{-/-}$ mice developed less atherosclerosis and had reduced plasma lipids compared to $Ldlr^{-/-}$ controls [66].

Interestingly, the *ApoaI* gene is negatively regulated by FXR in both humans and mice; FXR activation reduces *ApoaI* expression by binding to a response element in the gene [67]. Many studies in both mice and humans suggest a negative relationship between bile acids/FXR and HDL where increased bile acid concentration or FXR activation reduces HDL and ApoAI. Treatment with bile acid sequestrants in humans or FXR deficiency in mice typically raises HDL, but it is uncertain whether this is due to increased ApoAI secretion and synthesis of HDL, or if it is a result of reduced uptake of cholesterol via hepatic SR-B1 [68]. Inhibition of FXR could thus mediate anti-atherosclerotic actions through regulation of *ApoaI*.

FXR is also expressed in vascular smooth muscle cells and endothelial cells where it may induce macrophage recruitment if stimulated [69, 70].

1.2.3 Thyroid hormone and the thyroid hormone receptor

The lipid-lowering effect of thyroid hormone (TH) administration is well documented and described. The thyroid hormones T_4 and T_3 are synthesized and secreted from the thyroid gland. T_4 that has entered cells from the blood stream can be converted to T_3 by deiodinases. T_3 binds to the thyroid hormone receptors (TRs) in the nucleus of the cell and modulates the expression of genes involved in several metabolic pathways. The two TR isoforms α and β show different tissue specific expression and are encoded by different genes. TRα is involved in lipid metabolism in the heart, where stimulation increases the heart rate and cardiac output. TRβ mediates the effects of TH on hepatic cholesterol and bile acid synthesis by inducing bile acid synthesis and increasing LDL-receptor expression [71]. Contrary to LXR, TRβ regulates *Cyp7a1* expression in both humans and mice [72].

To specifically induce the hepatic metabolism of cholesterol and thereby reduce plasma cholesterol, TRβ specific modulators have been developed. One such modulator; GC-1, has been shown to effectively reduce plasma lipids without cardiac effects in animal studies. GC-1 has also been shown to increase bile acid synthesis and hepatic SR-B1 expression in normal and hypercholesterolemic mice [73]. Later, GC-1 was shown to reduce plasma cholesterol independently of the LDL-receptor by increasing hepatic bile acid synthesis and output in feces [74].

Several other TRβ specific modulators have also been developed and tested in humans and animals. For example, KB141 was shown to reduce plasma cholesterol and induce bile acid synthesis without affecting the heart in mice, rats and primates [75] and KB2115 had the same effect in humans [76, 77]. The reduction of atherosclerosis development in ApoE deficient mice by combined treatment with the TRβ specific modulator KB3495 and atorvastatin was however independent of plasma cholesterol [78]. There are so far no TRβ modulators approved for human medicine. The phase III program for eprotirome (KB2115) was discontinued after negative side-effects of cartilage damage seen in dogs exposed to the substance for 12 months (www.karobio.com).

1.3 LIPIDS IN DISEASE

The high diversity of lipids in our bodies makes lipid homeostasis a complex business, therefore imbalance in this homeostasis can result in a wide range of symptoms and diseases. This chapter describes the role of cholesterol in atherosclerosis and how mtDNA depletion might affect mitochondrial β-oxidation of fatty acids.

1.3.1 The role of cholesterol in atherosclerosis

In 1916 it was observed that feeding rabbits with cholesterol rich diets gave rise to aortic lesions much similar to those observed in humans [79]. Before that it had been observed that aortic lesions in men contained crystallized cholesterol, but this was the first evidence that cholesterol uptake from the food directly influenced the development of atherosclerotic lesions. High levels of ApoB-containing lipoproteins (VLDL/LDL) or low levels of ApoAI-containing lipoproteins (HDL) as independent, causative risk factors for cardiovascular disease [80, 81]. Today atherosclerosis is known to be a

disease with an important inflammatory component, often induced by hypercholesterolemia. The immune response elicits both pro-atherogenic and anti-atherogenic actions during the course of the disease [82, 83].

The atherosclerotic lesion is a slowly developing, degenerative process which is non-symptomatic until the size of the lesion or rupture blocks the artery and interrupts the flow of oxygenated blood, causing heart-attack or stroke. The retention and entrapment of LDL particles in the vascular wall is commonly accepted as the onset of atherosclerosis. High levels of plasma LDL mean more particles are available at the artery wall, increasing the risk of LDL entrapment. Studies have shown that the retention of LDL is dependent upon interaction of negatively charged proteoglycan sugars and certain positively charged domains of ApoB100 [84]. LDL particles trapped inside the vascular wall are more prone to oxidative damage [85]. Oxidized LDL stimulates the immune response and attracts monocytes that enter the artery wall. The monocytes differentiate into macrophages and when they become loaded with lipids, they turn into foam cells. The early stage atherosclerosis is defined as a fatty streak; the accumulation of lipids and foam cells in the artery wall. As the fatty streak develops into an advanced atherosclerotic lesion, a fibrous cap is formed over the plaque. Many different cell types are involved in the formation of an atherosclerotic plaque, but the most abundant ones are fibroblasts, smooth muscle cells, monocytes/macrophages and T-cells.

It is believed that the major anti-atherogenic property of HDL is the cholesterol uptake from macrophages through interaction with ABCA1 and ABCG1. The engulfment of oxidized LDL by macrophages thus makes cholesterol in the arterial wall accessible for removal through RCT. HDL and ApoAI also function as anti-oxidants, reducing oxidized LDL. In recent years, several studies have suggested that the functionality of the HDL particles is important for effective clearance of macrophage cholesterol; smaller particles and increased particle number may be more efficient than fewer and larger particles. Also, the pro- or anti-inflammatory properties of HDL can enhance or reverse the progress of atherosclerosis. Therefore, high levels of HDL may not always be anti-atherogenic [86, 87].

1.3.1.1 Atherosclerosis in ApoE deficient mice

Mice carry the major part of their plasma cholesterol in HDL, contrary to humans who carry it in LDL. Due to this, and their lower plasma levels of total cholesterol in general, mice are very resistant to atherosclerosis. Mice must therefore be genetically manipulated to gain a more human-like lipoprotein profile and develop human-like atherosclerotic plaques to be used for medical research. Several mouse models for atherosclerosis have been developed over the years, including LDL-receptor deficient mice ($Ldlr^{-/-}$), combined LDL-receptor and ApoE deficient mice ($ApoE^{-/-}/Ldlr^{-/-}$), combined ApoB100 transgenic and LDL-receptor deficient mice ($ApoB100/Ldlr^{-/-}$) and mice transgenic for the human ApoE3 mutation (ApoE*3-Leiden). But, the earliest and most commonly used model for atherosclerosis is the ApoE deficient mouse developed by gene targeted homologous recombination in pluripotent stem cells, in two separate laboratories in the 1990´s [88, 89]. The morphology of lesions in these mice is

comparable to human lesions and they develop from fatty streaks into complex lesions. The ApoE deficient mice develop atherosclerosis spontaneously on chow diet, but the process is accelerated by increased dietary cholesterol [90].

1.3.2 Defects in hepatic mitochondrial β-oxidation of fatty acids

A genetic classification of mitochondrial diseases of oxidative metabolism distinguishes two principal categories: disorders caused by mutations in the mtDNA and disorders caused by mutations in nuclear DNA. Defects in the nuclear genes encoding enzymes involved in mitochondrial β-oxidation have been identified and the abnormalities in plasma acylcarnitines that they result in have been described [91]. Hepatic disorders of fatty acid oxidation are severe, often lethal, diseases in infancy or the neonatal period, which result in hypoglycaemia and lipid accumulation in tissues that normally do not store large amounts of lipids (i.e. liver, heart and skeletal muscle). Symptoms do, however, vary greatly among individuals.

In humans, hepatic CPT deficiency is associated with delayed ketone body production (hypoketotic hypoglycemia) and dramatically increased long-chain acylcarnitines in serum after fasting. In mice, partial deficiency of the hepatic isoform CPT Ia (homozygous CPT $Ia^{-/-}$ is embryonic lethal) leads to reduced fasting serum glucose and increased free fatty acid levels [92]. The first oxidative step of long-chain fatty acid β-oxidation in mice is carried out by long-chain acyl-CoA dehydrogenase (LCAD) and disruption of this step causes hypoglycemia, increased free fatty acids in plasma and accumulation of fat in heart and liver. LCAD deficient mice also become hypothermic with abnormal brown adipose tissue. A dehydrogenase for very long-chain acyl-CoA has also been discovered (VLCAD). VLCAD deficiency in mice does not result in any definite clinical phenotype at rest, but stress, fasting or cold exposure results in impaired running speed and distance, hypoglycemia, hypothermia and lethargy. Contrary to mice, in humans, VLCAD covers a great part of palmitoyl-CoA oxidation and LCAD is not as highly expressed. The medium- and short-chain acyl-CoA´s are oxidized by MCAD and SCAD respectively [93]. The condition caused by long-chain fatty acid oxidation disorders can however be prevented by dietary interventions including fatty acid restriction and glucose supplementation. Therefore fatty acid oxidation defects, such as VLCAD, LCAD and CPT deficiencies, are included in the neonatal screening program (PKU-test in Sweden).

Mitochondrial (mt)DNA depletion syndromes (MDS) are severe autosomal recessive disorders that are characterized by quantitative reduction of mtDNA in affected tissues. Three classes of defective genes that cause MDS have been identified; (1) proteins that function directly at the mtDNA replication fork; *polymerase gamma* (POLG) and C10orf2/PEO1 (Twinkle), (2) enzymes involved in the salvage pathways of mitochondrial deoxynucleotides or in the control of the mitochondrial deoxynucleotide triphosphate pools; *thymidine kinase 2* (TK2), *deoxyguanosine kinase* (DGUOK) and *MpV17 mitochondrial inner membrane protein* (MPV17) and (3) proteins influencing several steps of mitochondrial protein replication; elongation factors, aminoacyl-tRNA synthetases or mtDNA encoded tRNA. These disorders

affect the function and replication of the mitochondrial genome itself. Insufficient ATP production for cellular requirements, due to the involvement of mitochondrially transcribed peptides in oxidative phosphorylation, is thought to be the biochemical basis of the disease. Children with MDS are often born after a normal pregnancy, but symptoms present soon after birth and are often fatal after months or a few years of age [94]. Whereas reduced activity of DGUOK, POLG or MPV17 leads to hepatic failure and encephalomyopathy, mutations in TK2 have mostly been associated with severe myopathy [95-97]. However, the symptoms of humans harboring TK2 mutations vary depending on the degree of functional reduction; partial impairment seems to cause mainly myopathy, while severe impairment may also cause liver failure and encephalomyopathy [98, 99].

Thymidine kinase isoforms 1 and 2 are enzymes encoded in the nuclear DNA. While TK1 is a cytosolic, cell-cycle dependent enzyme which is highly expressed in association with cell division, TK2 is found within the mitochondria and is cell-cycle independent. TK phosphorylates deoxythymidine to deoxythymidine monophosphate (dTMP), which provides dTMP for DNA replication; the process where DNA is copied. Down regulation of cytosolic TK1 and dNTP synthesis in non-dividing cells (tissues like muscle, liver and brain) forces mitochondrial TK2 (and DGUOK) to sustain the mitochondrial dNTP pool. In replicating tissues, the mitochondrial dNTP pool is maintained through cytosolic TK1 activity and influx of dNTP to the mitochondria via deoxyribonucleotide carrier (DNC) which bypasses the enzymatic step catalyzed by TK2. TK2 deficiency therefore leads to mitochondrial (mt)DNA depletion in non-replicating tissues [100] (figure 8).

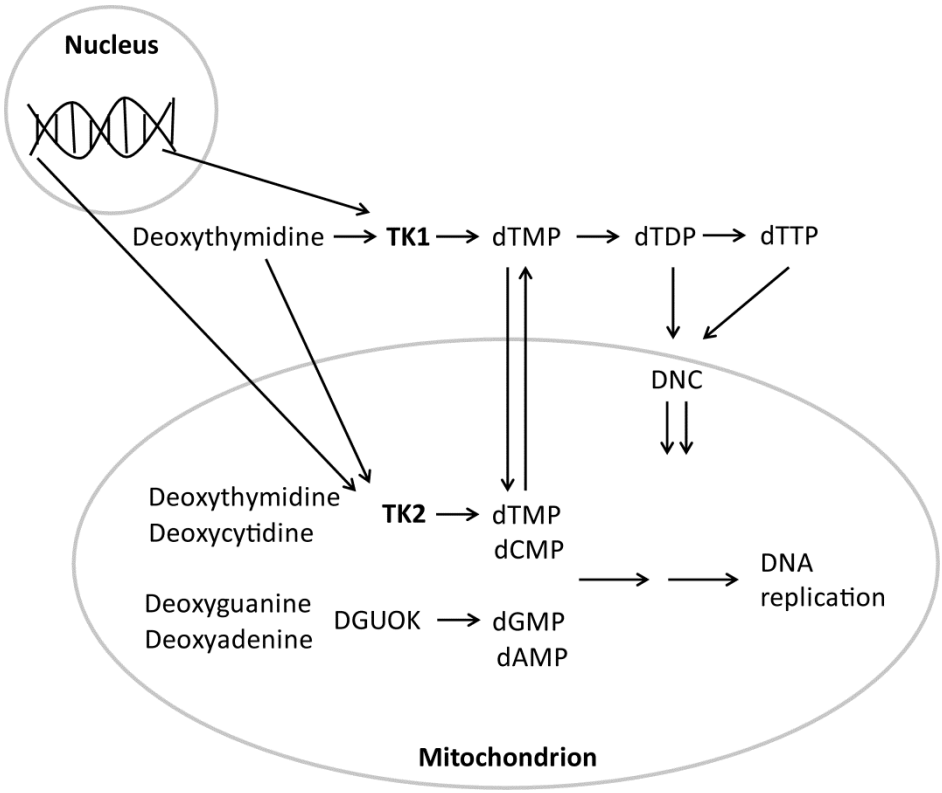

Figure 8. TK1 and TK2 are transcribed from genes in the cell nucleus. TK1 is cytosolic, while TK2 is located within the mitochondria. TK2 deficiency in non-dividing cells with low TK1 expression leads to dNTP depletion and impaired mitochondrial DNA replication.

1.3.2.1 The thymidine kinase deficient mouse model

The initial characterization of TK2 deficient mice revealed that they were born indistinguishable from their wild-type litter mates and grew normally during the first week of life. MtDNA levels in skeletal muscle, heart, spleen and liver were normal at birth, while adipose tissue and brain showed mtDNA depletion already at birth. Histological analysis of tissues revealed distortion of muscle fibres and mitochondrial christaes in the heart. The hypodermal fat layer and the brown adipose tissue of TK2 deficient mice did not develop normally and mice became increasingly hypothermic. Histological sections of adipose tissue showed heterogeneity in size and accumulation of lipid vesicles, and liver sections showed an increased number of lipid vesicles. The accumulated data suggested that the lipid metabolism was affected and perhaps involved in the disease progress [101]. The TK2 deficiency can be rescued by transgenic expression of drosophila melanogaster nucleoside kinase, which corrects the dTTP pool and mtDNA levels [102]. Some important species differences in TK2 activity between humans and rats have been described; humans have high activity in liver and lower activity in muscle, while rats display low activity levels in both muscle and liver [103]. The markedly lower TK2 activity in rodent liver has been proposed to make rodents more susceptible to TK2 deficiency-induced liver failure, compared to humans. The severity of TK2 deficiency for the mouse brain has been demonstrated by Hirano *et al* [104, 105]. They created a TK2 (H126N) knock in mouse that displayed very similar symptoms and survival rate as the TK2 knock out mouse. Mouse models

for TK2 deficiency may thus be more feasible as models for infantile fatal encephalomyopathy, than as comparable models for human partial TK2 deficiency.

2 AIMS

The aim of this thesis was to investigate and describe the regulation of hepatic and intestinal lipid metabolism, especially cholesterol metabolism and its relation to atherosclerotic development and the effect of mtDNA depletion on hepatic β-oxidation of fatty acids.

The following questions were asked and answered:

1. Does CA depletion reduce atherosclerosis development in ApoE deficient mice?

2. Does oral administration of GC-1 reduce atherosclerosis development in ApoE deficient mice?

3. Is the LXR driven increase in HDL-cholesterol dependent on intact intestinal cholesterol absorption?

4. Does reduced intestinal cholesterol absorption and simultaneous LXR stimulation lead to synergistic effects on cholesterol metabolism that may inhibit atherosclerosis development?

5. Does mtDNA depletion due to thymidine kinase 2 deficiency affect hepatic β-oxidation in mice?

3 MATERIALS AND METHODS

3.1 MICE

All animal experiments were conducted in accordance with accepted standards for animal care and approved by the institutional Animal Care and Use Committee at Karolinska Institutet.

In total 16 male ApoE(-/-)/Cyp8b1(-/-) (double knockout) and 24 male ApoE(-/-) mice, purchased through Taconic, Denmark, were used for paper I. 60 male ApoE -/- mice were used for paper II. 60 male C57Bl6 mice from our own in house breeding were used for paper III and 18 TK2(-/-) and 18 TK2(+/+) mice were used for paper IV.

For paper I mice of both genotypes were fed either chow diet or an atherogenic diet containing 0.2 % cholesterol and 10 % peanut oil. Mice received these diets from 1 week up to 5 months. Food and water was available *ad libitum*. Some mice received a single dose of radioactively labeled isotopes (^{14}C-cholesterol and ^{3}H-sitostanol) intragastrically and were housed individually in cages with grid floors for collection of feces during the last 24 hours of the experiment. All mice were fasted 4 hours prior to euthanisation with CO_2.

For paper II mice were fed a diet containing 0.2 % cholesterol and 10 % saturated fatty acids. 30 mice served as controls and the other 30 were treated with GC-1 (approximately 0.12 mg/kg/day mixed in the diet). Food and water was available *ad libitum*. Diets were given during 1, 10 and 20 weeks. 20 mice were killed at each time point (10 controls and 10 treated). All mice were fasted 4 hours prior to euthanisation with CO_2.

For paper III mice were fed a diet containing 0.2 % (w/w) cholesterol and 10 % (w/w) peanut oil, with or without supplementation of 0.012 % (w/w) ezetimibe (approximately 13 mg/kg/day mixed in the diet). All mice were gavaged once daily with 0.5 % methyl cellulose in water, with or without the LXR agonist GW3965 (40 mg/kg bw/day). The specified diets and treatments were given during 8 days. Food and water was available *ad libitum*. All mice received a single dose of radioactively labeled isotopes (^{14}C-cholesterol and ^{3}H-sitostanol) intragastrically and were housed individually in cages with grid floors for collection of feces during the last 24 hours of the experiment. All mice were fasted 4 hours prior to euthanisation with CO_2.

For paper IV suckling pups of seven to 14 days of age were used. The pups were housed with their mother until euthanisation with CO_2.

3.1.1 Genotyping of mice

For genotyping of mice with disrupted *Cyp8b1* or *ApoE* genes (and their wild-type counterparts) we collected 1-2 mm tail or ear biopsies. These were placed in direct PCR reagent (Viagen Biotech) supplemented with protein kinase K (Sigma-Aldrich) in room temperature or 37 °C water bath until complete lysis of the tissue. The reaction was stopped by incubation in 85 °C for 45 minutes, according to the manufacturer´s

protocol. PCR reactions were performed on crude lysates with primers for *ApoE* or *Cyp8b1* respectively.

Primers for mutant and wild-type ApoE alleles [106]
T1685E06: GCC TAG CCG AGG GAG AGC CG
T1685E07: TGT GAC TTG GGA GCT CTG CAG C
T1685E08: GCC GCC CCG ACT GCA TCT

Primers for mutant and wild-type Cyp8b1 alleles [107]
TCH-F2: AAA CCA CAC TGC TCG ACC TGC
CMN antisence: TGA GCT GAC CAC ATG TGT TCC
WTCH-F: CTT CCT CTA TCG CCT GAA GCC

Genomic DNA purification of ear biopsies from mice with disrupted *Tk2* gene (and their wild-type counterparts) was performed with the DNeasy Blood & Tissue kit (Qiagen) according to the manufacturer´s protocol.

Primers for the mutant Tk2 allele [101]
Tk2 F1: TAG TAG TTT AGA TGC CTG AGA CT
Tk2 R: GCC AGC TCA TTC CTC CCA CTC

3.2 QUANTIFICATION OF LIPIDS

3.2.1 Plasma lipids

For paper I-IV, size exclusion chromatography of plasma lipoproteins was performed as described earlier [108] and their lipid content was measured online with cholesterol CHOD-PAP and TG GPO-PAP reagents (Roche).

For paper IV, serum non-esterified fatty acid (NEFA) concentration was determined by an enzymatic assay and plasma acyl-carnitine metabolites were quantitated by an LC-MS/MS assay, as described in the article.

3.2.2 Extraction of lipids

For paper I-IV, total lipids were extracted with chloroform/methanol (2:1, by vol.) from serum and tissue samples using the method described by Folch *et al* [109]. Hydrolysis of sterol esters was performed by addition of KOH and ethanol to samples. Campesterol, free and total cholesterol, lathosterol and C4 were quantified by isotope dilution mass spectrometry [110-112]. Total cholesterol and triglycerides were also measured with the enzymatic cholesterol CHOD-PAP and triglycerides GPO-PAP reagents (Roche), using an Infinite F500 plate reader (Tecan) for detection. For this purpose, tritonX-100 was added to parts of extracts, the organic solvents were evaporated and lipids were solubilised in water. The remaining pieces of tissue after lipid extraction were dissolved in 1 M NaOH for protein determination by Bio-Rad DC assay (BioRad Laboratories). All lipids are related to the amount of protein in the extracted tissue.

3.2.3 Quantification of neutral sterols and bile acids in feces

For paper III, mice were placed in individual cages with grid floors for collection of feces during a 24-hour period. Fecal samples from each cage were analysed individually. For paper II, mice were housed together and fecal samples were collected cage-wise and analysed as pooled samples from the treatment groups. Feces were collected during two consecutive 24-hour periods. Homogenised and dried samples were weighed (~20 mg of pulverised faeces per sample) and hydrolysed by KOH in ethanol (120 °C) before extraction with hexane for neutral sterols. The extraction was repeated three times with 20 mL of hexane. Samples were then acidified with HCl and extracted for bile acids with 50 mL of dietylether. The extractions were performed in duplicate for all samples; d4-deoxycholic acid (DCA) and d4-lathosterol was added to one of the duplicates before hydrolysis, and the other was prepared without internal standards. All neutral sterol samples were trimethylsialylated prior to analysis and all bile acid samples were methylated with diazomethane and trimethylsialylated prior to analysis. Samples were quantitated in a similar manner as described in [113]. Amounts of DCA and lathosterol were quantified by isotope dilution mass spectrometry in samples containing internal standards. The other neutral sterols and bile acids were quantitated from the chromatogram (total ion current) of samples without added internal standard. Neutral sterols include: coprostanol, cholesterol, lathosterol, campesterol, stigmasterol, sitosterol and sitostanol and bile acids include: deoxycholic acid, chenodeoxycholic acid, cholic acid, α-, β- and ω-muricholic acid.

For paper III, total cholesterol content of selected fecal samples were also determined using the cholesterol CHOD-PAP reagent after extraction of total lipids as described in section 3.2.1.

For paper II neutral sterol content in the fecal samples was also determined by gas chromatography, using 5α-cholestane as an internal standard. Total bile acid concentration in the remaining water phase after hexane extraction was also determined with the enzymatic Total Bile Acids Assay (Diazyme Labs USA).

3.2.4 Fractional intestinal cholesterol absorption

In paper I and III we quantitated the fractional intestinal cholesterol absorption using the method described by Schwarz *et al* [114]. Mice were gavaged intragastrically with a single dose of 100 µL peanut oil containing 1 µCi ^{14}C-cholesterol and 2 µCi ^{3}H-sitostanol and were then immediately transferred to individual cages with grid floor. Feces were collected during 24 hours. The collected fecal samples were homogenized in water and parts of the homogenates were extracted for lipids in chloroform/methanol (2:1, by vol.) according to Folch *et al* [109]. The extracts were transferred to scintillation vials, dried down under N_2 and added 10 mL of Ultima Gold. Beta radiation was counted by a liquid scintillation counter. The per cent cholesterol absorption was calculated as:

Cholesterol abs (%) = **(**gavage (^{14}C/^{3}H) – fecal sample (^{14}C/^{3}H)**)**/ gavage (^{14}C/^{3}H).

Methodological limitations may give negative values after calculation; such values were set to 0 in all results presented within this thesis.

3.3 GENE EXPRESSION (REAL-TIME PCR)

For paper I-IV, total RNA was isolated from frozen liver tissue, scraped intestinal mucosa or cultured cells by homogenization in TRIzol (Life Technologies), subsequent steps were performed according to the manufacturer´s protocol. cDNA synthesis was carried out on 1 µg of total RNA using the High Capacity Reverse Transcriptase Kit (Applied Biosystems). In paper I and II cDNA samples from each treatment or genotype group were pooled by addition of equal amounts of cDNA from each individual. For measurements of mRNA levels, Power SYBR green PCR Master Mix (Applied Biosystems) was used with primers overlapping exon-exon boundaries. For detection Applied Biosystems PRISM 7500 Fast Real-time PCR System (Applied Biosystems) was used. The mRNA levels were related to the expression of mouse *hypoxanthine phosphoribosyl transferase* (*Hprt*) or *Cyclophilin*. All reactions were performed in triplicate and relative quantification was performed according to the comparative Ct-method ($2^{-\Delta\Delta Ct}$) [115].

For paper I, expressions of 45 selected genes involved in lipid metabolism were evaluated using a Low-Density TaqMan Array (LDA) (Applied Biosystems). Pooled cDNA samples were used for this purpose.

3.4 PROTEIN EXPRESSION

3.4.1 Immunohistochemistry

For paper I, the amount of T-cells and macrophages in atherosclerotic plaques were determined by immunohistochemistry. CD3+ and I-A+ (T-cells) cell numbers and CD68 and CD106 (macrophages) stained area/plaque area was determined as described in the article.

3.4.2 Sample preparation for western blot

For paper III, samples were homogenized in a buffer containing 20 mM Tris, 2 mM $MgCl_2$, 0.25 M sucrose and 1 % TritonX-100 for whole cell protein extraction. Samples were spun at 2000xg and the supernatant was collected.

For paper II and III, hepatic cell membrane fractions were prepared by tissue homogenization in a buffer containing 20 mM Tris, 2 mM $MgCl_2$, 0.25 M sucrose and protease inhibitor cocktail. After centrifugation 2000 xg, the supernatant was collected and centrifuged at 110,000 xg for 45 minutes. The supernatant was removed and the pellet was dissolved in 100 µL ice-cold buffer containing 80 mM NaCl, 50 mM Tris-HCl (pH 8), 2 mM $CaCl_2$, 1 % TritonX-100 and cOmplete Mini EDTA-free protease inhibitor cocktail (one tablet per 10 mL). A short centrifugation at 2000xg removed excess, undissolved material.

The total amount of protein in individual samples was determined by the Bio-rad DC assay (BioRad Laboratories) and equal amounts of total protein from each sample was added to make pooled samples.

For paper IV, total protein from liver tissue was prepared as described in the article.

3.4.3 Western blot

For paper III, analysis of ApoAI in individual serum samples was performed by mixing 1.5 µL of serum with NuPAGE LDS sample buffer (Life Technologies). Samples were then separated by SDS-PAGE.

Prepared samples from tissue (whole cell extracts or cell membrane fractions) were mixed with NuPAGE LDS sample buffer (Invitrogen) + 0. 1 M dithiotreitol (DTT) for separation on NuPAGE Bis-Tris gels (Invitrogen) or were mixed with Laemmli sample buffer (Bio-Rad Laboratories) + 0. 1 M dithiotreitol for separation on NuPAGE Tris-Acetate gels (Invitrogen). Proteins were transferred to nitrocellulose membranes and incubated with antibodies towards the LDL-receptor (Abcam), SR-B1 (Abcam), ABCA1 (Abcam), ABCG5 (a gift from Dr. Liqing Yu, Wake Forrest University [116]) or NPC1L1 (Novus Biologicals). All blots were incubated with respective secondary HRP-conjugated antibodies and after incubation with Super Signal West Dura Extended Duration Substrate (Pierce Biotechnology) the chemiluminescence intensity was detected in a Molecular Imager Gel Doc XR (Bio-Rad Laboratories). Densitometry analysis (rectangular volumes with global background subtraction) was performed with the Quantity One software (Bio-Rad Laboratories).

For paper III, the relative protein expression in whole cell homogenate was normalized against β-actin and related to the vehicle sample.

For paper II and III, the relative protein expressions in cell membrane fractions were determined by loading three amounts of each pool on the gel and calculating the ratio between $k_{treated}$ and $k_{vehicle/control}$ ($y=kx+m$; y = chemiluminescence intensity, x = amount of total protein (mg)) for each treated sample.

For paper IV, the relative protein expression was determined as described in the article.

3.5 HEPATIC ENZYME ACTIVITIES

3.5.1 Acyl-CoA:cholesterol acyltransferase (ACAT) 2 activity

For paper I, microsomes were prepared and ACAT activity was determined according to Parini *et al* [4]. Frozen liver tissue was homogenized in a solution containing 0.25 M sucrose, 0.1 M K_2HPO_4, 1 mM EDTA (pH 7.4) and protease inhibitor cocktail. After centrifugation 13,000 xg for 15 minutes, the supernatant was collected and centrifuged at 150,000 xg for 30 minutes. The pellet was dissolved in 300 µL 0.1 M K_2HPO_4 and the total protein concentration was determined by the Bio-rad DC assay (BioRad Laboratories). The microsomes were saturated with exogenous cholesterol complexed

to β-cyclodextrin and BSA for 15 minutes. The reaction was started by addition of ^{14}C-oleoyl-CoA and stopped after 20 minutes by addition of chloroform/methanol (2:1, by vol.). Samples were run in duplicates, where one duplicate contained pyripyropene A (PPPA), a specific ACAT2 inhibitor, to discriminate between ACAT1 and ACAT2 activity. ^{3}H-cholesteryl oleate was added as an internal control. The organic phase was evaporated and cholesteryl esters were separated from other lipids by thin layer chromatography (TLC) and the radioactivity was determined by mixing the scraped sample with Ultima Gold scintillation liquid and counting the activity in a beta-counter. ACAT2 activity was calculated by subtraction of ACAT1 activity from the total ACAT activity.

3.5.2 Mitochondrial β-oxidation

For paper IV, the mitochondrial β-oxidation of palmitic acid was determined in isolated hepatic mitochondria from TK2$^{-/-}$ and TK2$^{+/+}$ mice as described [117].

3.5.3 ATP production rate and citrate synthase activity

For paper IV, the mitochondrial ATP production rates and citrate synthase activities were determined in isolated hepatic mitochondria from TK2$^{-/-}$ and TK2$^{+/+}$ mice as described [118].

3.5.4 Carnitine palmitoyl transferase (CPT) activity

For paper IV, the mitochondrial CPT activity was determined in isolated hepatic mitochondria from TK2$^{-/-}$ and TK2$^{+/+}$ mice as described [119]. This method determines the total CPT activity, including CPT I and II.

3.6 CELL EXPERIMENTS

3.6.1 Primary mouse hepatocytes

Primary hepatocytes from two adult female C57Bl6 mice (Charles River) were isolated and seeded on 12-well plates coated with collagen (700,000-750,000 cells/well) according to [120] at the centre for transplantation surgery, CLINTEC.

3.6.2 HEPA1-6 cells

HEPA1-6 cells (ATCC, US) stored in liquid nitrogen were quickly defrosted in 37 °C water bath and centrifuged to remove the medium. Cells were then re-suspended in 37 °C DMEM High glucose medium (Life Technologies) with added heat-inactivated FBS (10 %) and Penicillin-Streptomycin (1 %). Cells were grown in cell culture flasks (75 cm^2) and were split twice (1/10) before experiments started, approximately one week and a half after thawing.

3.6.3 Protocol for treatment of cells with GW3965

Isolated primary hepatocytes were allowed to attach for 24 hours after seeding onto 12-well plates. Then, the medium was removed and 0.5 mL of medium containing either 0.1 % DMSO (control), 0.1 %DMSO + 1 μM GW3965 or 0.1 %DMSO + 10 μM GW3965 was added to four of the 12 wells on each plate. Plates were then left in the

incubator for 48 hours in 5 % CO_2, 37 °C. After 48 hours of treatment, the medium was removed and TRIzol (Life Technologies) was added to each well for RNA extraction. The TRIzol/cell mixture from each treatment group on one plate was pooled (four wells/pool) and RNA was isolated according to the manufacturer´s protocol.

HEPA1-6 cells were seeded on 6-well plates by diluting cells from a 75 cm^2 flask (approx. 90 % confluency) 1:3, adding 2 mL cell-suspension per well and allowing cells to attach for 24 hours before treatment. After 24 hours, three wells per plate were treated either with 0.1 % DMSO (control), 0.1 %DMSO + 1 μM GW3965 or 0.1 %DMSO + 10 μM GW3965 for 24, 48 or 96 hours. The medium was then removed and TRIzol (Life Technologies) was added to each well for RNA extraction. RNA was extracted separately from each well according to the manufacturer´s protocol.

3.7 STATISTICAL EVALUATION OF RESULTS

For paper I, III and IV parametric tests were performed using the STATISTICA software. Data is presented as mean ± SEM. Some data was log-transformed prior to statistical analysis to meet the criteria of homoscedasticity in parametric tests. For paper II non-parametric tests were performed using the PRISM software. Results are presented as individual data points with median. $P \leq 0.05$ was considered statistically significant in all tests.

Paper I: Figure 1 and table 1: the significance of difference was tested by student´s t-test. For the main experiment, where DKO and ApoE KO mice were fed chow or cholesterol diet for two weeks or cholesterol diet for five months, the significance of difference was tested by two-way ANOVA (factors: genotype (DKO or ApoE KO) and diet (chow diet 2W, cholesterol diet 2W or cholesterol diet 5M)) followed by post hoc comparison by the LSD test whenever significant main effects or interaction of factors were detected. For the additional experiment with GW4064 and CA supplementation, the significance of difference was tested by one-way ANOVA followed by post hoc comparison by the LSD test.

Paper II: Statistical significance of difference between groups for cholesterol levels in aortas was tested by one-tailed Mann-Whitney U-test (GC-1 treated expected to be lower than control). All other parameters were tested by two-tailed Mann-Whitney U-test for statistically significant differences between control and GC-1 treated samples. All tests were performed between GC-1 and control samples within the same treatment period. Statistical analysis was not performed on fecal excretion of sterols, hepatic protein expression or plasma C4 measurements, because measurements were performed on pooled material.

Paper III: The statistical significance of differences was tested by two-way ANOVA (factors: GW3965 and ezetimibe), followed by post-hoc comparison according to Dunnette´s test for comparison against the vehicle group. For figure 4c-d we used one-way ANOVA (factor: treatment), followed by post-hoc comparison according to Dunnette´s test for comparison against control treated cells. Post-hoc comparison was performed only when the ANOVA showed significant main effect(s) or interaction of factors ($p \leq 0.05$). For figure 1c, we used Spearman´s rank correlation to test for

correlation between the two parameters. For levels of ApoAI in serum, which were only comparable to vehicle samples on the same blot, we used two-tailed, unpaired t-test.

Paper IV: Two tailed, unpaired Student's t-test was used to test for statistical significance of difference between $TK2^{+/+}$ and $TK2^{-/-}$ mice of the same age.

4 RESULTS

4.1 PAPER I: CA DEPLETION REDUCES ATHEROSCLEROSIS IN APOE DEFICIENT MICE

In this paper we investigated the effect of CA depletion on atherosclerosis in ApoE deficient mice. Mice of ApoE$^{-/-}$/Cyp8b1$^{-/-}$ genotype (double knockout = DKO) were compared to ApoE$^{-/-}$/Cyp8b1$^{+/+}$ (ApoE KO) mice. Mice were fed chow diet or an atherogenic diet containing 0.2 % cholesterol and peanut oil for 2 weeks or 5 months. An additional experiment where mice on chow diet were treated with the FXR agonist GW4064 or CA supplementation was performed to test whether FXR stimulation in CA depleted mice could restore intestinal cholesterol absorption and to discriminate between effects of FXR stimulation and CA depletion on gene expression and bile composition.

CA depletion resulted in an approximate 50 % reduction of plaque area, as determined by oil Red-O staining, in aortas of the DKO mice after five months on the 0.2 % cholesterol diet. Mean plasma levels of cholesterol, mainly the cholesteryl esters, were lower in DKO mice compared to ApoE KO mice independently of the dietary regimen. The greatest difference occurred after feeding of the cholesterol diet for five months. The DKO mice fed either chow or 0.2 % cholesterol for two weeks displayed a 50-60 % reduction in the fractional intestinal cholesterol absorption compared to ApoE KO mice. The main fractions of bile acids in the ApoE KO mice on chow or cholesterol diet were CA and β-MCA. As expected, disruption of *Cyp8b1* resulted in complete abolishment of CA in the bile of the DKO mice. Increased levels of CDCA/α-MCA and β-MCA were detected in the bile of the DKO mice. The hepatic C4 content and Cyp7a1 mRNA levels were increased in DKO mice, indicating increased bile acid synthesis as a result of CA depletion and loss of FXR suppression. The hepatic cholesteryl ester content and ACAT2 activity was reduced, while the cholesterol synthesis was increased.

Oral administration of the synthetic FXR agonist GW4064 did not significantly affect bile composition or restore the cholesterol absorption in DKO mice, while oral supplementation with CA completely restored both bile composition and the cholesterol absorption. CA depletion or GW4064 treatment did not affect the intestinal gene expression for *Npc1l1*, *Abcg5* and *g8*, indicating that the effect of CA depletion on intestinal cholesterol absorption was mediated mainly by reduced micellar solubilisation of cholesterol and not by transcriptional effects on cholesterol transporters.

4.2 PAPER II: GC-1 REDUCES ATHEROSCLEROSIS IN APOE DEFICIENT MICE

In this paper, we investigated the effect of synthetic TRβ modulation on atherosclerosis in ApoE deficient mice. Mice were fed an atherogenic diet, containing 0.2 % cholesterol, supplemented with GC-1 for one, ten or 20 weeks. Control mice received the atherogenic diet without GC-1.

GC-1 reduced atherosclerosis in aortas of ApoE deficient mice after 20 weeks of treatment. Surprisingly, serum cholesterol levels were reduced by GC-1 after one or ten weeks treatment, but not after 20 weeks, despite a prominent induction of hepatic LDL-receptor protein expression. Hepatic SR-B1 expression was lower in GC-1 treated mice compared to control. Plasma levels of triglycerides or cholesterol in the HDL fraction could not be evaluated due to the high levels of ApoB-containing lipoproteins. GC-1 increased the C4 concentration in liver tissue and serum independently of treatment time and resulted in significantly increased *Cyp7a1* expression after ten weeks of treatment, indicating increased bile acid synthesis. Hepatic cholesterol synthesis was unaffected, but GC-1 treatment reduced hepatic cholesteryl esters after one week. Analysis of fecal samples revealed that GC-1 increased the neutral sterol content after one week treatment and the bile acid content after 20 weeks of treatment. The increased fecal neutral sterol content after one week treatment was paralleled by reduced serum levels of campesterol, indicating decreased intestinal cholesterol absorption.

The results from this study show that GC-1 reduced atherosclerosis in ApoE deficient mice by multiple effects on cholesterol metabolism. The lipid-lowering effect of oral treatment with GC-1 was however transient.

4.3 PAPER III: THE LXR DRIVEN INCREASE OF HDL-C IS INDEPENDENT OF INTESTINAL CHOLESTEROL ABSORPTION

In this paper, we investigated whether the LXR driven increase of HDL-cholesterol was dependent on intestinal cholesterol absorption and if combined LXR stimulation and blocked cholesterol absorption would lead to synergistic anti-atherogenic effects on cholesterol metabolism. We treated mice on a 0.2 % cholesterol diet with the LXR agonist GW3965, the cholesterol absorption inhibitor ezetimibe or a combination of both (GW3965+ezetimibe) for eight days.

The combined treatment with GW3965 and ezetimibe resulted in increased HDL-cholesterol, increased ApoAI levels in serum and reduced intestinal cholesterol absorption, but did not increase intestinal or hepatic ABCA1 protein expression. Hepatic SR-B1 expression in cellular membrane fractions were unaltered, indicating that the SR-B1 mediated uptake of HDL-cholesterol was not affected. Hepatic *ApoaI* mRNA levels were increased in mice treated with GW3965 or the combination of GW3965+ezetimibe.

An additional experiment was performed where primary hepatocytes from mice and the hepatoma cell line HEPA1-6 were treated with GW3965 to elucidate whether hepatic *ApoaI* expression was stimulated directly by the LXR agonist. Results from this experiment showed increased *ApoaI* expression in hepatocytes and HEPA1-6 cells after treatment with 10 μM GW3965 in the cell medium for 48 hours or longer, confirming that GW3965 could stimulate ApoAI production in hepatocytes. Thus, the LXR driven increase of HDL-cholesterol was found to be independent of the intestinal cholesterol absorption and did not require increased ABCA1 expression. Increased hepatic ApoAI secretion may represent an anti-atherogenic property of GW3965 which was independent of intestinal cholesterol absorption. We proposed a mechanism were "base-line" expression of ABCA1 is sufficient to lipidate increased ApoAI levels in plasma as part of the LXR driven increase of HDL-cholesterol.

We also compared the effect of ezetimibe or GW3965 treatment on intestinal cholesterol absorption, cholesterol content and transporters (*Npc1l1*, *Abcg5* and *Abca1*). We found that ezetimibe treatment lowered *Abcg5* and *Abca1* mRNA and protein, while *Npc1l1*was unaffected. GW3965 treatment increased *Abcg5* and *Abca1* mRNA and protein and lowered *Npc1l1* mRNA, as expected, but did not affect the intestinal cholesterol absorption or cholesterol content. However, neither ezetimibe nor GW3965 reduced NPC1L1 protein expression, despite the transcriptional down regulation by GW3965. Interestingly, in mice treated with the combination of GW3965+ezetimibe, the intestinal cholesterol absorption and cholesterol content was reduced (resembling ezetimibe treatment). Transcriptional regulation of *Npc1l1*, *Abcg5* and *Abca1* was comparable to the effect of GW3965 treatment, but ABCG5 and ABCA1 protein expressions were unaffected compared to vehicle treated mice. Unexpectedly, NPC1L1 protein expression was prominently reduced by the GW3965+ezetimibe treatment.

These results show that GW3965 did not reduce the intestinal cholesterol absorption, but also indicate that inhibited influx of cholesterol via NPC1L1 and/or low levels of intracellular cholesterol prevented post-transcriptional increase of intestinal ABCA1 and ABCG5, despite increased mRNA levels.

GW3965+ezetimibe treatment effectively increased fecal neutral sterol excretion and reduced hepatic cholesteryl esters; two anti-atherogenic properties of combined LXR activation and reduced intestinal cholesterol absorption.

4.4 PAPER IV: TK2 DEFICIENCY REDUCES HEPATIC MITOCHONDRIAL B-OXIDATION

In this paper we investigated whether the TK2 deficiency-induced mtDNA depletion in mice leads to defects in hepatic mitochondrial β-oxidation. We collected tissues from seven days (non-symptomatic), 12 and 14 days (symptomatic) old TK2 deficient mice. Their wild-type litter mates served as controls.

Transmission electron microscopy of liver sections from TK2 deficient mice showed increasingly hypertrophic mitochondria with increasing age. The hepatic protein expression of cytochrome oxidase subunit II (COXII), a mitochondrial protein involved in the electron transfer chain, was however unchanged. Hepatic adipophilin, a protein associated with lipid vesicles, was increased in 12 days old $TK2^{-/-}$ mice, but paradoxically, hepatic cholesteryl esters and triglycerides were increased in seven days old mice, but not in the older mice. Serum cholesterol and free fatty acids were increased in the 12 days old TK2 deficient mice, while triglycerides were unchanged. Analysis of plasma acylcarnitine metabolites revealed that long-chain acylcarnitines were elevated, indicating dysfunction in the primary steps of transfer or oxidation of long-chain fatty acids in mitochondria. Analysis of the palmitate oxidation rate and CPT activity in isolated hepatic mitochondria from 14 days old TK2 deficient mice revealed that both parameters were reduced. Despite mtDNA depletion in hepatic tissue at post-natal day 14, no significant differences were detected in the mitochondrial ATP production rates between TK2 deficient and control mice.

The results from this study show involvement of hepatic β-oxidation of long-chain fatty acids in the disease progress of TK2 deficient mice.

5 DISCUSSION AND COMMENTS

Paper I-III

Dyslipidemia is one major determinant of the pathogenesis of atherosclerosis, as well as overall mortality and morbidity in cardiovascular diseases (CVDs) [121, 122]. In paper I-III we tested different strategies to modulate plasma lipoproteins in ways that are considered to be anti-atherogenic. The ApoE deficient mouse model was used for paper I and II to elucidate whether the applied strategies would also reduce atherosclerosis. In both papers we employed strategies that were aimed at reducing ApoB-containing lipoproteins. In paper III we instead aimed at increasing ApoAI-containing lipoproteins in wild-type mice.

In paper I we showed that disruption of *Cyp8b1* had profound effect on plasma cholesterol levels and atherosclerosis development in ApoE deficient mice. The lipid-lowering effect of CA depletion was prominent and to this date no negative side-effects on survival, breeding or behavior have been observed in CA depleted mice. Development of inhibitors directed at CYP8B1 could therefore be a realistic alternative for future drug development. CA supplementation in humans has been reported to increase cholesterol absorption and bile content of CA [123], but the effect of CA depletion is not well documented in humans. So far, no polymorphism in the human Cyp8b1 gene has been associated with any disease [124]. The loss of FXR repression in CA depleted mice leads to increased bile acid synthesis and an expanded pool of bile, but the increased bile pool size did not compensate for the loss of CA in terms of intestinal cholesterol absorption. In fact, the reduced hydrophobicity of the bile acid pool in the DKO mice may also have contributed to the reduced absorption. We also showed that stimulation of FXR with a synthetic agonist did not restore the fractional intestinal cholesterol absorption in mice devoid of CA, meaning that the micellar composition and solubilization of cholesterol in the intestinal lumen is prior to FXR signaling for this parameter. Since CDCA is more potent as an FXR agonist in humans, and FXR does not regulate CYP7A1 and bile acid synthesis in humans in the same way as in mice, separation of the effect on intestinal cholesterol absorption from FXR signaling was important to demonstrate that the loss of CA is likely to reduce atherosclerosis and plasma lipids even when the FXR suppressive effect is lost.

In paper II we tested if oral administration of GC-1 would decrease atherosclerosis in ApoE deficient mice. Our results showed that the sustained, increased hepatic LDL-receptor expression observed after 20 weeks of oral GC-1 treatment was not enough to maintain reduced serum cholesterol levels in ApoE deficient mice, supporting the view of an LDL-receptor independent mechanism. Since LDL and VLDL levels are closely connected, it is not farfetched to think that hepatic VLDL secretion may be part of this mechanism. Secretion of TAG and cholesterol in VLDL is reduced in hyperthyroid rats [125], but the relationship between thyroid hormone and VLDL secretion is yet not clearly demonstrated since hypothyroidism may also decrease VLDL secretion [126].

An initial reduction in intestinal cholesterol absorption may also in part explain the reduced serum cholesterol levels after short-term treatment. We detected reduced levels of serum campesterol in mice treated with GC-1 for one week, but previous work by Davidson *et al* showed that synthesis rates of ApoB48 were increased in hyperthyroid rats [126], indicating that intestinal chylomicron production was increased. It should also be pointed out that observations on fecal excretion of neutral sterols and bile acids after GC-1 treatment have differed among labs. We detected increased fecal excretion of neutral sterols after one week treatment with GC-1 administered intraperitoneally or orally (see [73] and the present study), but others have instead detected a prominent increase in fecal bile acid content and no increase in fecal neutral sterols [74]. The definitive effect of GC-1 on hepatic VLDL secretion and intestinal cholesterol absorption thus remains to be clarified. Interestingly, GC-1 treatment for 20 weeks reduced atherosclerosis, but not serum cholesterol levels. These results demonstrate two things; (1) the reduction of serum cholesterol after treatment with GC-1 is transient and (2) the reduced atherosclerosis is either independent of serum cholesterol levels or represents a postponement of disease onset due to short-term treatment effects.

In paper III we tested whether increased HDL-cholesterol levels due to LXR stimulation were dependent on intestinal cholesterol absorption. Previous work by Tang *et al* suggested that the LXR driven increase in HDL-cholesterol might depend on intestinal cholesterol absorption [127]. However, our results clearly showed that the increase in HDL-cholesterol seen after LXR activation by GW3965 was not dependent on intestinal cholesterol absorption. In line with our results, it was recently reported that intestine-specific deletion of the *Microsomal triglyceride transfer protein* (*Mttp*) resulted in inhibited intestinal cholesterol absorption and down regulation of *Npc1l1*, but did not affect the ability of T0901317 to elevate serum HDL-cholesterol or the intestinal *Abca1* mRNA levels [128]. It is thus unlikely that the LXR driven increase in HDL-cholesterol is directly dependent on the uptake of cholesterol in the small intestine. We did however observe decreased cholesterol levels in the intestinal tissue after GW3965+ezetimibe treatment and it is possible that long-term treatment with this combination may result in further cholesterol depletion of intestinal cells, which in turn may affect the HDL-cholesterol levels in the long run. Ezetimibe treatment has, however, been shown to induce intestinal cholesterol synthesis and LDL-receptor expression [129], compensating for the loss of NPC1L1-mediated uptake.

The induction of hepatic *ApoaI* represents an interesting, anti-atherosclerotic effect that should be further investigated. The mechanism for this up regulation is unclear since *ApoaI* is not recognised as a direct LXR target gene. The opposing regulatory effects of LXR/FXR may hold the answer, as stimulation of FXR was reported to reduce *ApoaI* expression through a direct negative response element [67] and LXR activation was reported to reduce *Cyp8b1* and CA/βMCA ratio in mice [42], which could lead to reduced FXR suppression. The LXR induced increase in *ApoaI* may thus be indirectly mediated through loss of FXR suppression, but this mechanism needs further evaluation. It also remains to be shown whether the induced transcription also increases ApoAI secretion.

The suggestion of a mechanism where increased HDL-cholesterol levels are independent of increased ABCA1 expression is somewhat controversial, since functional ABCA1 is crucial for HDL-formation and overexpression of ABCA1 generally increases HDL-cholesterol in mice [130, 131]. The ABCA1 activity is however ATP dependent (ABCA1 is an ATPase) and regulated by phospholipid composition and cholesterol levels [132], meaning that the same amount of functional protein is likely to elicit different levels of activity depending on the cells lipid status.

Paper IV

MDS and mitochondrial fatty acid oxidation disorders are heterogeneous groups of disorders. Better understanding of the effects of mtDNA depletion in different organs and why symptoms differ is necessary for future development of optimal treatments. In paper IV we investigated the effect of mtDNA depletion on hepatic fatty acid metabolism. We aimed at describing the lipid phenotype of TK2 deficient mice in an attempt to find new possible treatment strategies and to better characterize this mouse model.

The progressive loss of mtDNA in livers of TK2 deficient mice was apparent after post-natal day seven, around the same time when the symptoms of TK2 deficiency became visible. The TK2 deficient mice displayed symptoms like those of long-chain acyl-CoA mitochondrial β-oxidation disorders. The accumulation of long-chain acylcarnitines and increased free fatty acid levels in plasma of TK2 deficient mice suggested disturbances in CPT or LCAD activities. The observation of symptoms common for CPT or LCAD deficiency and the partial reduction in CPT activity in TK2 deficient mice suggests involvement of several steps in the β-oxidation pathway of long-chain fatty acids. Since both *Lcad* and *CptIa-c and II* genes are located in the nuclear genome, the mtDNA depletion and transcriptional effects on the mitochondrial genome do not explain these defects, but it is likely that the hypertrophy of hepatic mitochondria leads to structural changes in the membranes that may reduce enzyme activity. We did not detect any significant reduction of the mitochondrial ATP production rates in 14 days old TK2 deficient mice and COXII protein expression was normal in 12 days old mice, indicating that the expression and function of mitochondrial proteins involved in the citric acid cycle or electron transfer chain were not primarily affected.

Dietary restriction of fatty acid content to medium-chain fatty acids could be beneficial for TK2 deficient mice. Glucose supplementation may not increase survival if the fatty acid oxidation defect has similar basis as LCAD or CPT deficiency, but may improve brain function. The effect on survival by dietary interventions may however be limited by the progressive loss of mtDNA and the multi-organ involvement in TK2 deficiency.

6 CONCLUDING REMARKS AND FUTURE PERSPECTIVES

This thesis describes some possible future approaches to modulate hepatic and intestinal lipid homeostasis with the aim of treating some specific lipid disorders; atherosclerosis and defect β-oxidation due to mtDNA depletion. Evaluation of the effects of total disruption of an enzyme may give important information on processes that are affected by the specific protein and its products, and this is one step in finding possible drug targets.

Paper I described the effect of CA depletion on atherosclerosis in male mice; the plasma lipid-lowering effect and atherosclerosis reduction was associated with reduced intestinal cholesterol absorption. CA depletion is thus anti-atherosclerotic and lipid-lowering in mice. Interestingly, the immunohistological examination of plaques from DKO mice showed a slight increase in CD68 stained area/plaque area and elevated mean numbers (although not statistically significant) of CD3+ and IA+ cells compared to ApoE mice, indicating that monocyte and T-cell infiltration may have been increased in DKO mice. Considering that CA depletion leads to increased bile acid pool and possibly increased levels of circulating bile acids in serum, the effect of CA depletion on the immune response and immune cell recruitment to the vascular wall should be more closely investigated. Furthermore, since FXR deficiency in mice has been reported to affect atherosclerosis development differently in male or female mice, studies on female CA depleted mice as well as studies of CA depletion in LDL-receptor deficient mice should be considered. In fact, preliminary data on female CYP8B1 deficient mice fed an atherogenic diet suggest that CA depletion has a pronounced anti-atherosclerotic effect on plasma lipoproteins that is gender-specific. The loss of hepatic FXR suppression in CA depleted mice may also increase hepatic *ApoaI* expression and secretion. Therefore, HDL metabolism should be more closely evaluated in these mice.

Paper II described the effect of TRβ-specific modulation by GC-1 on atherosclerosis. Atherosclerosis was reduced after 20 weeks of GC-1 treatment, despite a transient effect on serum cholesterol. The effect of GC-1 on hepatic VLDL secretion, intestinal cholesterol absorption and RCT remain to be determined, but measurements of these parameters will add greatly to the understanding of the GC-1 mediated effect on atherosclerosis. Furthermore, investigations of the immunological response to GC-1 treatment should be considered, since the TRβ-selective modulator KB3495 was recently observed to reduce macrophage content in aortic plaques and several inflammatory cytokines in serum ([78] and P. Parini personal communication). Also in this study, only male mice were used and GC-1 must be administered to females to complete the evaluation of this substance.

Paper III described the effect on HDL-cholesterol by combined treatment with GW3965 and ezetimibe. We concluded that HDL-cholesterol levels were not correlated with intestinal cholesterol absorption and that the LXR driven increase in HDL-

cholesterol was independent of both intestinal cholesterol absorption and ABCA1 protein expression. The proposed mechanism of induced hepatic *ApoaI* needs to be further evaluated and ApoAI secretion should be measured, preferably also the synthesis rate. Interestingly, T0901317 has been reported to increase *ApoaI* in intestinal cells and lymph [133], but since this LXR agonist is also a very potent PXR agonist [134], it is uncertain whether this effect is actually mediated directly through LXR. Also here, investigations of gender effects should be considered.

The use of LXR agonists in human medicine with the intention of increasing HDL-cholesterol is limited by the risk of increasing LDL-cholesterol. Combination of LXR stimulation with ezetimibe therapy, which lowers LDL-cholesterol may therefore compensate for this, but this must be tested in CETP expressing species. The anti-atherosclerotic effects of LXR are however not limited to cholesterol metabolism. Several studies have indicated inflammatory or immune regulatory roles for LXR [135]. The effects on HDL retention and functionality by the GW3965+ezetimibe treatment should be further investigated.

Paper IV described the effect of TK2 deficiency induced mtDNA depletion on hepatic β-oxidation. We concluded that TK2 deficiency in mice results in some symptoms that resemble mitochondrial long-chain fatty acid oxidation disorders. TK2 deficient mice could benefit from a fatty acid restricted diet, but this strategy is unlikely to save them from the continuous mtDNA depletion that will progress in resting cells, especially in the brain. To rescue the cells from mitochondrial dNTP depletion, induction of TK1 activity could be considered. However, stimulation of transcription factors that regulate TK1 expression is likely to induce cell-proliferation and increase the risk of tumor development. Therefore, it would be necessary to develop a technique which allowed for specific induction of TK1 or stabilization from proteasomal degradation.

Despite the apparent differences between species, mouse models have become valuable tools in medical research, especially because of the many possibilities of gene manipulation. Results from studies performed in species other than humans must always be valued with care, but as more and more knowledge about species differences are acquired, animal models can be increasingly refined.

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