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STUDIES ON THE EFFECTS OF THYROID HORMONE ON CHOLESTEROL AND LIPOPROTEIN METABOLISM

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ABSTRACT

Elevated plasma lipids, particularly cholesterol within low density lipoproteins, is an important risk factor for developing atherosclerosis which can cause angina pectoris, myocardial infarction, and stroke. Thyroid hormone (TH) has a strong influence on lipid metabolism and the aim of this thesis was to gain more insight into how TH modulates cholesterol and lipoprotein metabolism.

In contrast to normal rats, plasma cholesterol increases in hypophysectomized (Hx) rats upon cholesterol feeding. In paper I, it was found that the increased plasma cholesterol in Hx rats in response to cholesterol feeding is partly caused by an increased intestinal absorption of dietary cholesterol. TH was found to normalize the increased absorption of cholesterol in Hx rats. The changes in intestinal absorption of dietary cholesterol induced by hypophysectomy and TH could not be explained by changes in the intestinal gene expressions of sterol transporters ABCG5/G8 and NPC1L1. However, hepatic gene expressions of ABCG5/G8 were found to be diminished in Hx rats and strongly stimulated by TH, associated with a markedly reduced and stimulated biliary secretion of cholesterol, respectively, that may influence the absorption of dietary cholesterol.

The sterol transporter ABCG5/G8 is of major importance in the biliary secretion of cholesterol. However, alternative pathways that promote biliary cholesterol secretion have been proposed. TH stimulates both biliary cholesterol secretion and the hepatic gene expression of ABCG5/G8. In paper II, it was investigated if the TH-induced secretion of cholesterol into bile is mediated by ABCG5/G8, or if other pathways are involved. TH-induced secretion of cholesterol into bile was found to be largely dependent on the ABCG5/G8 transporter. It was also found that nuclear hormone receptor LXRα, reported to be positively regulated by TH and to have a stimulatory effect on ABCG5/G8 gene expression, is not critical for the TH-induced effect on ABCG5/G8 gene expression or on biliary cholesterol secretion.

In paper III, the responses to hyperthyroidism in humans were studied and compared to those of healthy subjects treated with the liver-specific thyromimetic eprotirome. Hyperthyroidism lowered VLDL-, LDL-, and HDL-cholesterol, apoB, Lp(a), and serum PCSK9. Bile acid synthesis was increased and serum FGF19 reduced. Cholesterol synthesis was unaltered while intestinal absorption of dietary cholesterol was reduced. Serum free fatty acids and glycerol were increased, while insulin, glucose, and FGF21 were unaltered. Eprotirome treatment resulted in similar reductions in lipoprotein cholesterol, apoB, Lp(a), and PCSK9. In contrast to hyperthyroidism, eprotirome reduced plasma triglycerides. There were no effects on bile acid synthesis, FGF19, or cholesterol absorption, in response to liver-selective stimulation of TH receptors by eprotirome.

LIST OF PUBLICATIONS

I. **Dramatically increased intestinal absorption of cholesterol following hypophysectomy is normalized by thyroid hormone**
Gälman C, Bonde Y, Matasconi M, Angelin B, and Rudling M
Gastroenterology. 134(4):1127-36 (2008)

II. **Stimulation of murine biliary cholesterol secretion by thyroid hormone is dependent on a functional ABCG5/G8 complex**
Bonde Y, Plösch T, Kuipers F, Angelin B, and Rudling M
Hepatology. 56(5):1828-37 (2012)

III. **Stimulation of human cholesterol and lipoprotein metabolism in hyperthyroidism: importance of liver-specific hormone actions**
Bonde Y, Breuer O, Lütjohann D, Sjöberg S, Angelin B, and Rudling M
Manuscript (2012)

CONTENTS

LIST OF ABBREVIATIONS

ABC	ATP-binding cassette
ABCG5	ATP-binding cassette subfamily G (WHITE) member 5
ABCG8	ATP-binding cassette subfamily G (WHITE) member 8
ACAT2	acyl coenzyme A:cholesterol acyltransferase 2
apo	apolipoprotein
BAAT	bile acid coenzyme A:amino acid N-acyltransferase
BSEP	bile salt export pump
CA	cholic acid
CDCA	chenodeoxycholic acid
CETP	cholesteryl ester transfer protein
CYP7A1	cholesterol 7α-hydroxylase
CYP8B1	sterol 12α-hydroxylase
DCA	deoxycholic acid
ER	endoplasmatic reticulum
FFA	free fatty acid
FGF19	fibroblast growth factor 19
FGF21	fibroblast growth factor 21
FGFR4	fibroblast growth factor receptor 4
FH	familial hypercholesterolemia
FPLC	fast protein liquid chromatography
FXR	farnesoid X receptor
GC-MS	gas chromatography-mass spectrometry
HDL	high density lipoprotein
HL	hepatic lipase
HMGCoA	3-hydroxy-3-methylglutaryl coenzyme A
HMGCoAR	3-hydroxy-3-methylglutaryl coenzyme A reductase
Hx	hypophysectomized
IBAT	ileal bile acid transporter
IDL	intermediate density lipoprotein
INSIG	insulin induced gene
LCA	lithocholic acid
LCAT	lecithin:cholesterol acyltransferase
LC-MS-MS	liquid chromatography-mass spectrometry-mass spectrometry
LDL	low density lipoprotein
LDLR	low density lipoprotein receptor
Lp(a)	lipoprotein(a)
LPL	lipoprotein lipase
LRH-1	liver receptor homolog-1
LRP	low density lipoprotein receptor-related protein
LXRα	liver X receptor α
NPC1L1	Niemann-Pick C1 Like 1
NTCP	sodium bile acid co-transporter
OATP	organic anion transporting polypeptide
OSTα	organic solute transporter α

OSTβ	organic solute transporter β
PAI-1	plasminogen-activator inhibitor type 1
PCR	polymerase chain reaction
PCSK9	proprotein subtilisin kexin type 9
RXR	retinoid X receptor
SHBG	sex hormone binding globulin
SHP	small heterodimer partner
SRBI	scavenger receptor class B type 1
SREBP2	sterol regulatory element binding protein 2
T3	triiodothyronine
T4	thyroxine
T2	diiodothyronine
TBG	thyroxine binding globulin
TBPA	thyroxine binding prealbumin
TH	thyroid hormone
TRα	thyroid hormone receptor α
TRβ	thyroid hormone receptor β
TRE	thyroid hormone response element
TRH	thyrotropin releasing hormone
TSH	thyroid stimulating hormone
VLDL	very low density lipoprotein

1 INTRODUCTION

1.1 CHOLESTEROL METABOLISM

Cholesterol synthesis

Cholesterol is a precursor for steroid hormones and bile acids, and also a crucial component of lipid membranes. Daily intake of cholesterol (~ 0.5 g) constitutes around 25% of total body cholesterol turnover, the rest is endogenously synthesized primarily by the liver and the intestine [1-3]. The rate-limiting enzyme in cholesterol synthesis is 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCoAR) that catalyzes the formation of mevalonate from 3-hydroxy-3-methylglutaryl coenzyme A [4, 5]. Cholesterol synthesis has a diurnal rhythm that peaks around midnight and reaches its nadir at noon in both humans and rodents [6]. Transcription of HMGCoAR is regulated by a feedback mechanism; when intracellular sterol levels are depleted, the sterol regulatory element binding protein 2 (SREBP2) is activated and translocated to the cell nucleus where it initiates transcription of HMGCoAR. When sterol levels are restored, SREBP2 is inactivated by insulin induced gene (INSIG) [7, 8] (Fig. 1). HMGCoAR is also regulated by hormones e.g. estrogen [9], glucagon, insulin [10], and thyroid hormone [11]. HMGCoAR inhibitors, statins, are the most widely used cholesterol-lowering drug today and reduce LDL-cholesterol levels by up to 60% [12].

Biliary secretion of cholesterol via ABCG5/G8

ABCG5 and ABCG8 are members of the ATP-binding cassette (ABC) transporter family that facilitates transport of substrates across cellular membranes [13]. ABC transporters function as full- or half-transporters of which the latter generally form homo- or heterodimers with other ABC family members [14]. ABCG5/G8 are highly expressed in hepatocytes and enterocytes [15, 16]. Their genes are located in a head to head position, separated by a short stretch of base pairs [17]. This orientation is typical for genes encoding subunits of functional complexes that share common regulatory sequences [18, 19]. ABCG5/G8 are co-immunoprecipitated when co-expressed in cultured cells [20]. If either one of the proteins is expressed alone, the protein stays in the endoplasmatic reticulum (ER) [20]. When both proteins are expressed, they are located to the apical plasma membrane [20, 21]. The mechanism for ER retention is not known. Some oligomeric membrane proteins contain ER retention motifs that become hidden when the proteins dimerize [22, 23]. Mutations in either of the genes encoding ABCG5/G8 cause identical clinical phenotypes characterized by the accumulation of cholesterol and plant sterols in plasma and tissues, a disorder referred to as sitosterolemia [15, 17]. *In vivo* studies show that disruption of either one [24, 25] or both [21, 26-28] genes reduces biliary cholesterol concentration and secretion. In contrast, induction of hepatic ABCG5/G8 gene expressions is associated with increased biliary cholesterol concentration and secretion [26, 28, 29], e.g. by administration of the LXR agonist T0901317 [26, 29, 30]. These data strongly suggest that ABCG5/G8 are half-transporters that heterodimerize with each other to form a functional complex expressed at the apical membrane of hepatocytes and enterocytes where it promotes sterol transport. However, hepatic gene expression of ABCG5/G8 is not always concurrent with biliary cholesterol secretion, suggesting that other pathways promote cholesterol transfer into bile as well [21, 31, 32]. Biliary cholesterol entering the intestine can be either reabsorbed or eliminated from the body by fecal excretion (Fig. 1).

Biliary secretion of cholesterol as bile acids

Hepatic bile acid synthesis

Bile acids are synthesized in the liver from cholesterol (Fig. 2) and facilitate the intestinal absorption of lipid soluble vitamins and lipids, including cholesterol, by acting as detergents and forming mixed micelles in which lipids are transported to the enterocytes. In addition, bile acids interact with nuclear receptors and function as signaling molecules in lipid and carbohydrate metabolism. Bile acid synthesis occurs through a classical pathway or an alternative pathway. In humans, the classical pathway accounts for approximately 90% of total synthesis, while in rodents the alternative pathway may contribute to more than 50% of total bile acid synthesis. Both pathways yield the end products cholic acid (CA) and chenodeoxycholic acid (CDCA), and the ratio between these is determined by the enzyme sterol 12α-hydroxylase (CYP8B1). In CYP8B1 knock-out mice, CA is absent. The first and rate-limiting step in the classical pathway is catalyzed by cholesterol 7α-hydroxylase (CYP7A1), and at least 16 other enzymes are involved in bile acid synthesis. An important terminal step is conjugation of the bile acid with either taurine or glycine, catalyzed by bile acid coenzyme A:amino acid N-acyltransferase (BAAT). Amino acid conjugation increases the solubility of bile acids in the aqueous milieu of the intestinal lumen, in which they exist as anionic salts. More than 98% of secreted bile acids are conjugated [33-35].

Intestinal bile acid synthesis and the enterohepatic circulation

Bile acids are secreted into bile canaliculi via the hepatic bile salt export pump (BSEP), and provide a strong osmotic force in the formation of bile [36], which other than bile acids mainly consists of water, cholesterol, phospholipids, and bilirubin. Bile is stored and concentrated in the gallbladder, that contracts after food intake and releases bile via the sphincter of Oddi into duodenum. In the intestine, the bacterial flora can modify bile acids in several ways. Dehydroxylation of bile acids is one of the most common modifications, and results in the formation of deoxycholic acid (DCA) and lithocholic acid (LCA) from CA and CDCA, respectively. In addition, bile acids are deconjugated by bacterial enzymes. Although unconjugated bile acids can be reabsorbed passively throughout the intestine, most bile acids are reabsorbed in the distal ileum by the ileal bile acid transporter (IBAT), located at the apical plasma membrane of enterocytes. Reabsorbed bile acids are then transported across the basolateral membrane by the organic solute transporters α and β (OSTα/β) to enter the circulation and return to the liver where they are taken up by the sodium bile acid co-transporter (NTCP) and organic anion transporting polypeptides (OATPs) (Fig. 2). The circulation of bile acids between the liver and intestine is referred to as the enterohepatic circulation, and the bile acid pool completes 8-10 cycles each day. Even though reabsorption of bile acids is approximately 95% efficient, some bile acids are lost with feces during each cycle and replaced by *de novo* synthesis. Fecal excretion of biliary cholesterol, as such or as bile acids, constitutes a major pathway by which the body eliminates cholesterol [37-39].

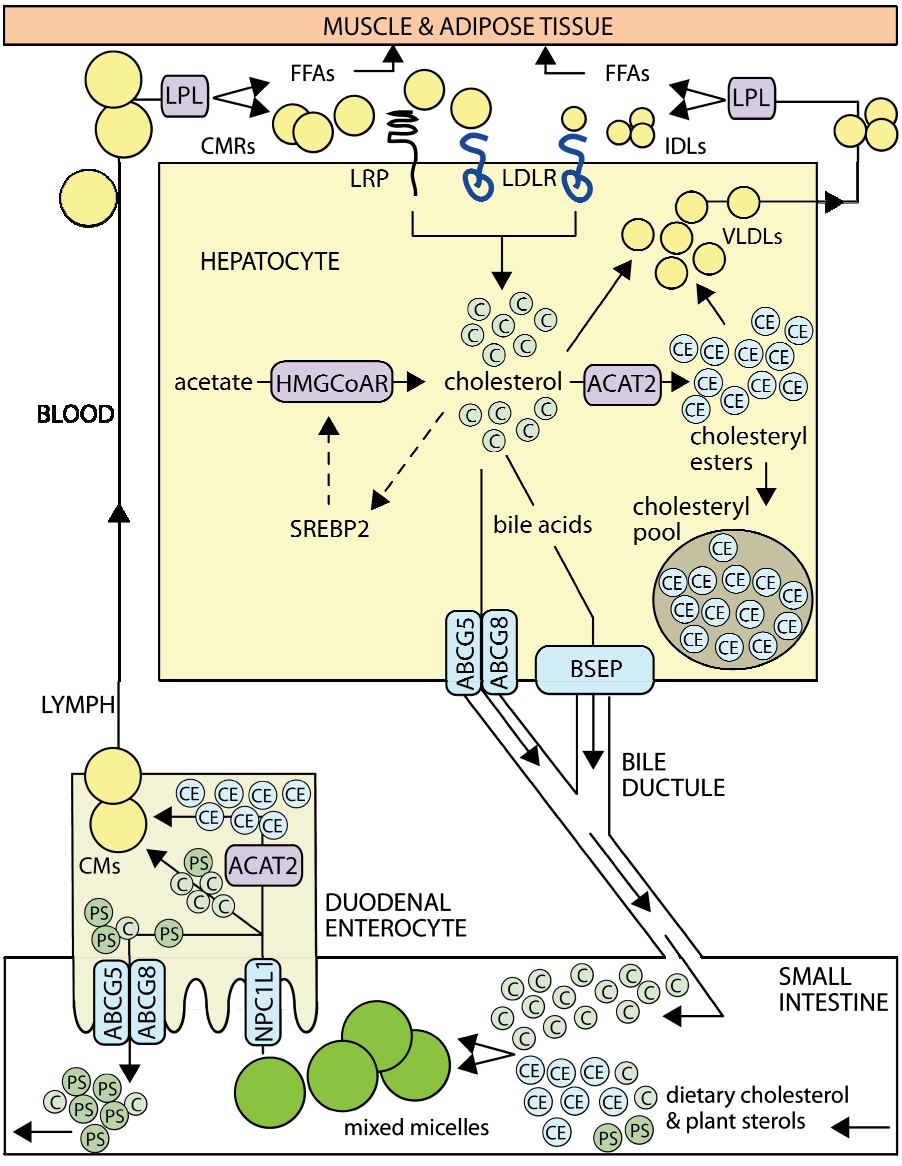


Fig. 1 Cholesterol metabolism. Hepatic cholesterol is stored as cholesteryl esters. Cholesterol and cholesteryl esters can be assembled into VLDLs and secreted into the blood. Cholesterol can also be secreted into bile, as such, or after the conversion to bile acids. Biliary and dietary cholesterol is absorbed in the proximal part of the small intestine and assembled into chylomicrons (CMs). After hydrolysis of the triglycerides within the chylomicron in peripheral tissues by LPL, the cholesterol content of the CM remnant (CMR) is taken up by the liver. High and low intracellular cholesterol levels exert negative and positive feedback, respectively, on cholesterol synthesis by modulating SREBP2.

Regulation of bile acid synthesis

Bile acids that return to the liver bind and activate the nuclear farnesoid X receptor (FXR) and activated FXR triggers the transcription of the nuclear receptor small heterodimer partner (SHP). SHP lacks a DNA-binding domain but represses transcription of CYP7A1 and CYP8B1 by inhibiting the transcriptional activities of other nuclear receptors that stimulate expression of these genes. E.g. by interacting with liver receptor homolog-1 (LRH-1), which normally stimulates transcription of CYP7A1, SHP decreases bile acid synthesis [40, 41]. Bile acid-activated FXR can also, independently of SHP, downregulate CYP7A1 by a mechanism involving fibroblast growth factor 19 (FGF19) and its receptor, fibroblast growth factor receptor 4 (FGFR4) [42].
In rodents, oxysterols can activate LXRα that binds to an element in the promoter of the CYP7A1 gene, thereby stimulating CYP7A1 transcription. This regulation is thought to be an important explanation to why plasma cholesterol levels in rodents fed cholesterol-enriched diets remain essentially unaltered. A corresponding regulation by LXRα of the human CYP7A1 gene has not been reported [43, 44] (Fig 2).

Cholesterol absorption

Cholesterol in the intestinal lumen originates from bile, diet, cell debris, and transintestinal efflux of cholesterol [3, 45]. Biliary cholesterol is mostly unesterified and more efficiently absorbed than dietary cholesterol, which is mostly esterified. There are large variations in the cholesterol absorption efficiency between individuals, but approximately 30-50% of the luminal cholesterol is absorbed. Absorption takes place in the proximal part of the small intestine where cholesterol, plant sterols, and other lipids form mixed micelles with bile acids [3]. When the micelles reach the brush border membranes of the enterocytes, uptake of cholesterol and plant sterols is mediated by the protein Niemann-Pick C1 Like 1 (NPC1L1) [46, 47]. Inside the cell, most of the cholesterol is esterified by acyl coenzyme A:cholesterol acyltransferase 2 (ACAT2) and together with triglycerides assembled in chylomicrons that transport the lipids to peripheral tissues and the liver [48, 49]. Some of the unesterified cholesterol and most of the plant sterols, which are poor substrates for ACAT2, are transported back into the intestinal lumen by ABCG5/G8 (Fig. 1). In mice, overexpression of ABCG5/G8 [50] or administration of LXR agonists that stimulate ABCG5/G8 gene expressions [30], results in a decreased absorption of cholesterol.

Ezetimibe is used as a monotherapy or in combination with statins to treat sitosterolemia and dyslipidemias of other origins. Ezetimibe inhibits NPC1L1-mediated uptake of sterols but the exact mechanism of action is not clear. NPC1L1 forms cholesterol-enriched microdomains with the proteins Flotillin-1 and 2 in the brush border membrane of enterocytes [51]. The microdomains are internalized by endocytosis [51, 52] and when the intracellular cholesterol level drops, NPC1L1 moves from the cytoplasm to the brush border membrane to mediate further uptake of cholesterol [53, 54]. Recent studies in mice show that treatment with ezetimibe prevents endocytosis of NPC1L1 and cholesterol, so that they are instead retained at the plasma membrane [55]. The importance of NPC1L1 is underscored by data showing that deletion of the gene for NPC1L1 in mice decreased cholesterol absorption by 70% [46].

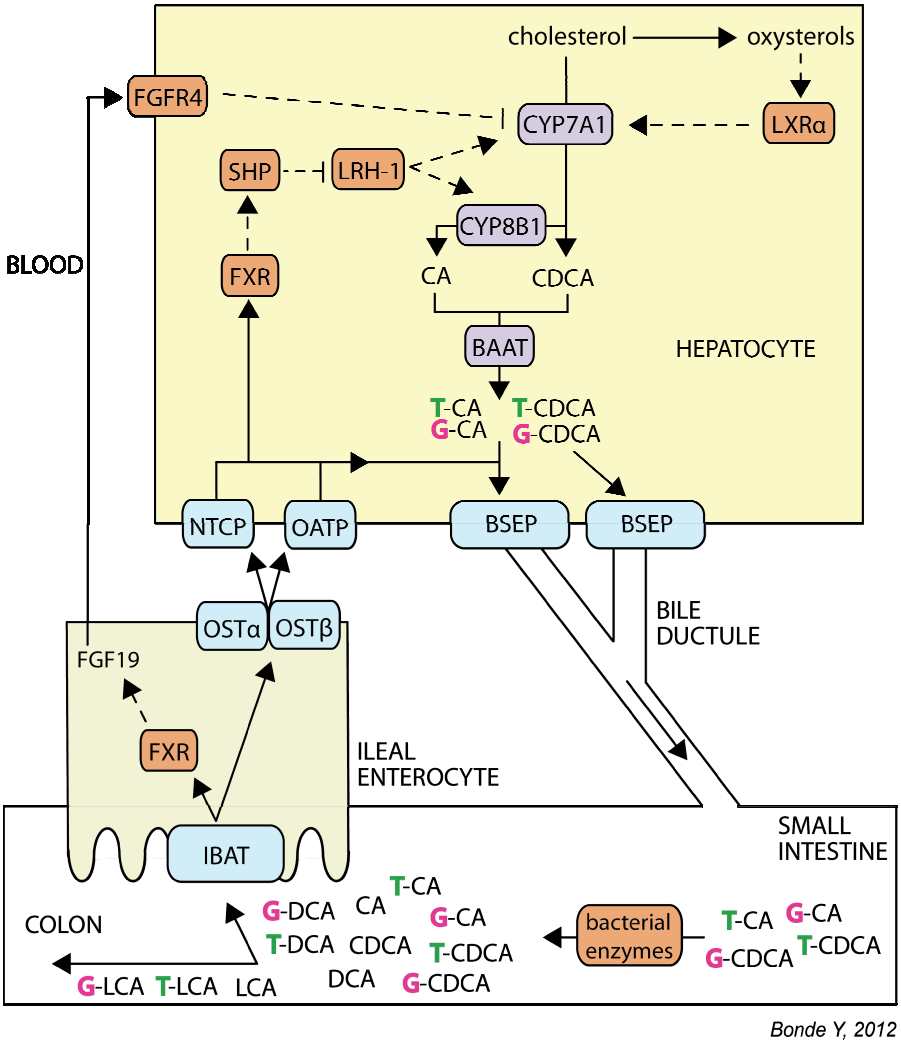


Fig. 2 Bile acid metabolism. Hepatic bile acids that enter the intestinal lumen in bile are reabsorbed in the distal ileum and return to the liver to be resecreted into bile. Bile acids inhibit their own synthesis by activating FXR in the liver and in the intestine. Activation of intestinal FXR stimulates secretion of FGF19 that interacts with its hepatic receptor, FGFR4, thereby inhibiting CYP7A1 activity.

1.2 LIPOPROTEIN METABOLISM

Due to its amphipathic structure, cholesterol is not soluble in blood, and has to be transported within lipoproteins. These are aggregates of lipids and proteins (apolipoproteins) and represent a continuous array of particles of varying size, composition, and density. They can be separated into chylomicrons, very low density lipoproteins (VLDLs), low density lipoproteins (LDLs), high density lipoproteins (HDLs), and lipoprotein(a) [Lp(a)]. Despite differences in size, composition, and density, lipoproteins share a common structure. The polar ends of apolipoproteins, cholesterol, and phospholipids face the blood while their nonpolar ends point inwards to the lipoprotein core, where nonpolar lipids such as cholesteryl esters and triglycerides reside. A schematic view of lipoprotein metabolism is shown in Fig 3.

Chylomicrons

Chylomicrons are formed in the enterocytes postprandially. They are the largest class of lipoproteins and approximately 90% of the lipid content is triglycerides, 5% cholesterol, and 5% phospholipids. After assembly, chylomicrons enter the lymphatic system and reach the circulation via the thoracic duct. Chylomicrons contain mostly apoB48 but may in addition contain apoAI, apoAII, apoAIV, apoCII and apoE. In peripheral tissues, apoCII and apoE activate lipoprotein lipase (LPL) that hydrolyses chylomicron triglycerides, resulting in free fatty acids (FFAs) to be used as energy supply in muscle and adipose tissue or to be stored. After hydrolysis, excess surface lipids and some of the apolipoproteins are transferred from the chylomicron remnant to HDLs, from which it acquires cholesterol esters. Still located on the chylomicron remnant, apoE interacts with hepatic LDL receptors (LDLRs) and LDLR-related proteins (LRPs) that clear the remnant particle from the circulation. The half-life of chylomicrons is approximately less than 15 min and chylomicrons are thus not normally present in blood samples from fasted subjects [3].

VLDLs

VLDLs are formed in the liver. The lipid content of VLDLs is approximately 70% triglycerides, 15% cholesterol, and 15% phospholipids. In humans, VLDLs contain apoB100 while in rodents, VLDLs may contain either apoB48 or apoB100. Although apoB is continuously synthesized, it is degraded unless sufficient lipids associate with it, and the secretion of VLDLs is thus dependent on the hepatic lipid level. Secreted VLDLs acquire additional apolipoproteins such as apoCI, apoCII, apoCIII, and apoE. LPL activated by apoCII and apoE hydrolyzes the triglycerides within VLDLs, and FFAs are released to be taken up by peripheral cells. The half-life of VLDLs is approximately 2 hours. The VLDL remnant, also referred to as intermediate density lipoprotein (IDL), can either be cleared from the circulation by interaction of the apoB100 and apoE with LDLRs, or be further hydrolyzed by hepatic lipase (HL) to form LDLs [3].

LDLs

LDLs are formed in the circulation by hydrolysis of triglycerides within IDLs. In humans, LDLs transport the major part, more than 70%, of the circulating cholesterol, while in rodents, HDL is the main cholesterol transporting particle. LDLs contain a single apoB100 that interacts with the LDLR resulting in the internalization of the LDL particle. Most LDLRs are located in the liver and 70-80% of the LDL-cholesterol is removed from the circulation by hepatic

LDLRs. There is also a LDLR-independent pathway for uptake of LDLs which appears to be nonsaturable and therefore strictly dependent on the LDL concentration. The half-life of LDLs is 2-3 days. Increased LDL-cholesterol levels is an independent risk factor for atherosclerosis [3].

Lp(a)

Lp(a) is thought to be formed at the hepatocyte surface or in plasma. It consists of an LDL particle with an apo(a) covalently bound to the apoB [56, 57]. The apo(a) is synthesized in the liver only [58] and shares homology with plasminogen. The function of Lp(a) is not fully understood although it is thought to enhance the activity of the plasminogen-activator inhibitor type 1 (PAI-1), thus acting on the balance between blood clotting and fibrinolysis. Circulating levels of Lp(a) vary 1000-fold between individuals and are regulated by Lp(a) synthesis rate which is dependent on polymorphisms in the apo(a) gene. Lp(a) has a longer half-life than the LDL and it is unclear how L(p) is removed from the circulation. *In vitro* and indirect *in vivo* data suggest that the LDLR is involved and that the kidneys may play an important role in Lp(a) catabolism. Increased levels of Lp(a) is an independent risk factor for cardiovascular disease [59].

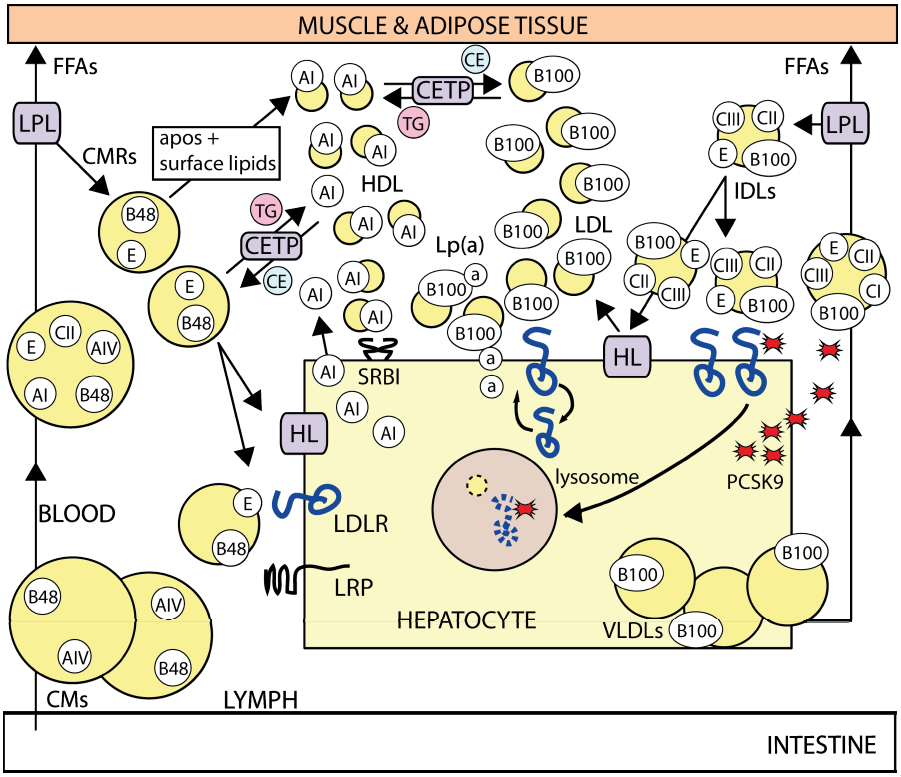
HDLs

HDLs are formed in the circulation after hepatic or intestinal secretion of free or partly lipidated apoAI, which is the major apolipoprotein of HDLs. ApoAI acts on peripheral cells in order for cholesterol to be transferred from these to the HDLs. This is the first step in a process called reverse cholesterol transport in which cholesterol is moved from peripheral tissues, unable to dispose excess cholesterol, to the liver where it can be eliminated from the body through biliary secretion. The cholesterol taken up by HDLs is esterified by the action of lecithin:cholesterol acyltransferase (LCAT) and moves to the core of HDL. ApoAI and apoAIV are thought to be activators of LCAT. The cholesteryl esters are either taken up by the hepatic scavenger receptor class B type I (SRBI), or transferred to apoB-containing lipoproteins in exchange for triglycerides by the action of cholesteryl ester transfer protein (CETP). HDLs can also be taken up by LDLRs and possibly by a separate apoE receptor present on hepatocytes. The half-life of HDLs is approximately 2-3 days. Other than apoAI, HDLs also contain apoAII, the second most abundant apo of HDL but of unclear physiological function, and apoAIV, apoCII, apoCIII and apoE. ApoCII is important in the activation of LPL, while apoCIII may inhibit LPL action. HDL is thought to protect against atherosclerosis [3].

The LDLR

LDLRs are present on all cells but more than 60% of the LDLRs in humans reside in the liver. LDLRs on the cell surface bind to the apoB100 on lipoproteins, and with even higher affinity, to apoE [60]. The LDLR-lipoprotein complex is internalized by endocytosis [61] and directed to lysosomes where the lipoprotein is degraded while the LDLR is released and returns to the cell surface. Even without ligand, surface LDLRs are internalized and recycled. Regulation of LDLR levels occurs both at the transcriptional and posttranslational level. When intracellular sterol levels are low, SREBP2 is activated and stimulates transcription of the LDLR gene as well as several other genes involved in cholesterol metabolism. LDLRs at the cell surface can be bound to proprotein convertase subtilisin kexin type 9 (PCSK9), a protein mainly expressed

in the liver, and the PCSK9-LDLR complex is internalized and directed to lysosomes for degradation. Since the LDLR may be recycled 150 times during its lifespan, minor changes in LDLR levels can result in large differences in circulating lipoprotein levels. The importance of the LDLR for clearance of lipoproteins is underscored by the characteristics of familial hypercholesterolemia (FH), a disease caused by mutations in the LDLR gene. In FH, homozygote individuals may have LDL-cholesterol levels of >16 mmol/L [62].



Bonde Y, 2012 (figure modified with permission from Persson L, Studies on PCSK9 in the regulation of cholesterol metabolism, 2011)

Fig. 3 Lipoprotein metabolism. Chylomicrons (CMs) and VLDLs are secreted from the intestine and the liver, respectively, and their triglyceride content is hydrolyzed in peripheral tissue by LPL. The remnant particles, CMRs and IDLs, can be taken up by the liver or further hydrolyzed by HL. Hydrolysis of IDLs yields LDLs that are taken up by the LDLR regulated by PCSK9. Lipids and apos are transferred between apoB-containing lipoproteins and HDL. HDL lipids are cleared by hepatic SRBI.

1.3 THYROID HORMONE

Thyroid hormone metabolism

The thyroid hormones (THs), triiodothyronine (T3) and thyroxine (T4), are synthesized in the thyroid gland. [63]. Thyrotropin releasing hormone (TRH), secreted from the hypothalamus, stimulates the release of thyroid stimulating hormone (TSH) from the pituitary. TSH in turn stimulates the secretion of TH from the thyroid gland. Low levels of TH stimulate TRH secretion, in opposite, *in vitro* and *in vivo* studies show that T3 directly inhibits transcription of the TRH gene in the hypothalamus. Genes encoding proteins involved in TSH synthesis and release are also transcriptionally regulated by TH and similar to the regulation of TRH, high levels of T3 and T4 inhibit synthesis and release of TSH. Most of the secreted TH is T4, which together with T3 is transported in the circulation bound to thyroxine binding globulin (TBG), thyroxine binding prealbumin (TBPA), and albumin [63, 64]. Intracellular levels of T3 and T4 are modulated by deiodinases that move iodine moieties from or onto the precursor molecule. Type I deiodinase converts T4 to T3 or to reverse T3 (rT3), an inactive metabolite. Type II deiodinase converts T4 to T3. Type III deiodinase converts T4 to rT3 and T3 to diiodothyronine (T2) [65, 66]. Extra-thyroidal pathways have been estimated to contribute to approximately 80% of the T3 produced daily in healthy subjects [67]. The regulation of TH synthesis and the deiodinase actions are shown in Fig. 4. Some TH is secreted from the liver into bile canaliculi to enter the intestine with bile. Most of the TH is reabsorbed why the fecal excretion of TH is very limited, the main route for TH excretion being with urine [64].

Thyroid hormone receptors

Within the cell, THs interact with nuclear TH receptors (TRs) and T3-binding to TRs is 10-fold higher in affinity compared to T4 [63]. TH-bound TRs bind as homodimers or heterodimers with retinoid X receptor (RXR) to TH response elements (TREs) in DNA, causing either initiation or repression of target gene transcription [68]. The TREs can vary considerably regarding the nucleotide sequences of half-sites and also the number, spacing, and orientation of half-sites [68], thus prediction of TREs from DNA nucleotide sequence-analysis is a difficult task. TRs may also bind to TREs in absence of TH, with the opposite result on target gene transcription as compared to when TH is present [68, 69]. Hence, in situations where TH levels are low, i.e. hypothyroidism, unliganded TRs may repress basal transcription of genes positively regulated by TH, rather than being inactive. Results of studies in which TH action is investigated in TR deficient models, may therefore differ from those of studies where the models have low or absent TH levels.

TRs are transcribed from two genes, *THRA* and *THRB* (rodents; *Thra* and *Thrb*), that encode TRα and TRβ, respectively. Alternative splicing and transcription start sites result in several isoforms that lack either the ligand-binding domain or the DNA-binding domain [68-70]. These isoforms may influence gene transcription by competing for binding of TH or to TREs. The highest expression levels of TRα is found in the heart, brain, and lungs whereas TRβ is predominantly expressed in the liver, kidneys, brain, heart, and thyroid. Gene-inactivation studies have provided *in vivo* data indicating that the TRα and TRβ have both isoform-specific and overlapping functions. In addition to TR-mediated effects of TH, data show that TH can bind to an integrin receptor at the plasma membrane and thereby activate signaling cascades [68-70].

Thyroid hormone and lipid metabolism

TH is a major regulator of lipid metabolism as evident from clinical findings and experimental animal models of hypo- and hyperthyroidism. The hypophysectomized (Hx) rat is a well-recognized model to study the effects of TH on lipid metabolism. Following hypophysectomy, plasma total triglyceride levels in rats are reduced by ~ 50% while plasma total cholesterol is slightly increased. Lipoprotein profiling reveals reduced HDL-cholesterol and markedly (~ 6-fold) increased LDL-cholesterol [71-73]. Hepatic cholesterol levels are increased, while the expression of HMGCoAR, LDLR, and CYP7A1 [74-76] are reduced, as is the hepatic fatty acid synthesis [77]. Treatment of Hx rats with TH increases the hepatic HMGCoAR, CYP7A1, and LDLR expressions [75, 76]. An additional feature of the Hx rat is a weaker resistance to cholesterol/fat-feeding [71]. In normal rats fed a cholesterol/fat-enriched diet, hepatic cholesterol content is 5-fold increased, but plasma cholesterol levels are essentially unaltered [71, 72]. In contrast, plasma cholesterol levels in Hx rats fed a cholesterol/fat-enriched diet are 6-fold increased, and the increase in hepatic cholesterol is significantly more pronounced. One of the main objectives of this thesis was to investigate the cause for this loss of resistance to dietary cholesterol.

Hypothyroidism in humans is associated with elevated plasma total and LDL-cholesterol levels, in part ascribed to impaired clearance of LDL via LDLRs. In contrast, hyperthyroidism is commonly associated with reduced plasma total and LDL-cholesterol levels [78, 79] explained by the reverse mechanism [80, 81]. Data on the effects of hypo- and hyperthyroidism on HDL-cholesterol and plasma total triglyceride levels have been less clear. HDL-cholesterol and plasma total triglyceride levels have been reported to be unaltered [82, 83] or increased [84] [85-87] in hypothyroidism. In hyperthyroidism, HDL-cholesterol levels have been reported as unaltered [88, 89] or decreased [82, 90, 91], while plasma total triglyceride levels have been reported to be decreased [82, 84], unaltered [90, 91], or increased [92]. Plasma triglyceride levels are partly determined by the activity of HL and LPL. In humans, HL activity is low in hypothyroidism [93, 94] and increased in hyperthyroidism [95]. LPL activity has been reported to be low in hypothyroidism [94, 96, 97] and to increase upon treatment.

Thyromimetics

The idea of using TH in order to lower plasma cholesterol levels has been investigated and discarded due to signs of overt hyperthyroidism, a potential life threatening condition, in treated subjects [98-100]. However, the discoveries of TRs, their differential tissue expression, and isomer-specific target genes, have promoted the development of compounds based on the structure of TH – thyromimetics – that are liver- and/or TRβ-selective, e.g. MB07811. MB07811 is metabolized to the TRβ agonist MB07344 after having entered the liver with high selectivity [101]. Other thyromimetics are GC-1, KB-141, T-0681, and eprotirome. Eprotirome, is a TRβ agonist that is absorbed within ~2h and rapidly disappears from plasma. It is highly liver-selective with a minimal uptake in extrahepatic tissues [102]. Eprotirome has prominent and beneficial effects on plasma lipid levels and importantly, the addition of eprotirome to individuals on statin therapy reduces LDL-cholesterol levels and total triglycerides by 32%, 33%, respectively [103].

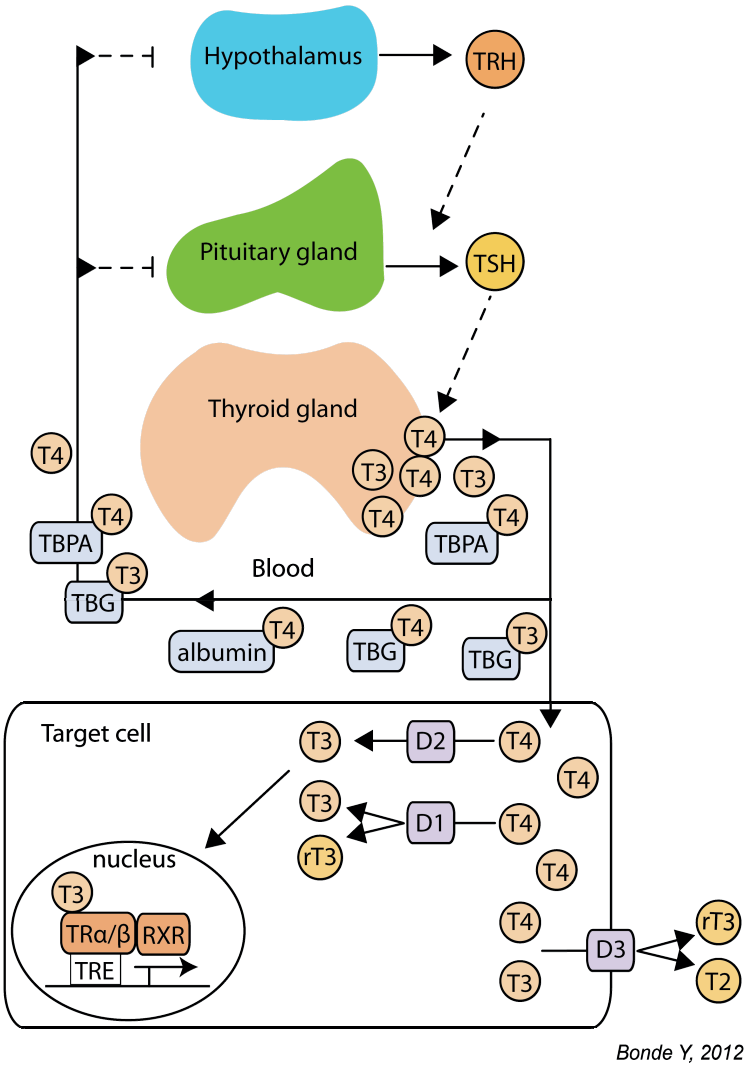


Fig. 4 Control of TH levels. TH secretion is under the control of TSH from the pituitary. Secretion of TSH in turn is controlled by TRH synthesized in the hypothalamus. TH exerts negative feedback on TRH and TSH synthesis and secretion. Intracellular TH binds to TRs and influences target gene expression. The intracellular levels of T4 and T3 are determined by deiodinases.

2 AIMS

The overall aim of this thesis was to study the effects of thyroid hormone on cholesterol and lipoprotein metabolism.

The specific aims of respective paper were:

I) To gain further insight into how hypophysectomy alters cholesterol and bile acid metabolism, and to understand why hypophysectomized rats, in contrast to normal animals, are sensitive to cholesterol feeding.

II) To clarify if the TH-induced stimulation of biliary cholesterol secretion is mediated by the ABCG5/G8 complex *in vivo*, and to evaluate if LXRα is involved in this stimulation.

III) To evaluate the effects of hyperthyroidism on cholesterol and lipoprotein metabolism in humans and to compare them to the effects of the liver-selective thyromimetic, eprotirome.

3 MATERIALS AND METHODS

3.1 ANIMALS AND HUMAN SUBJECTS

Paper I

All experimental procedures were approved by the Institutional Animal Care and Use Committee at Karolinska Institutet.
In total, 50 normal and 141 Hx male Sprague-Dawley rats (Taconic A&B, Ry, Denmark) were used in the experiments. Hypophysectomy was performed at Taconic Laboratories. Animals were kept under standardized conditions and had free access to water and chow, i.e. standard chow or chow enriched with 0.4% or 2% cholesterol/10% corn oil ± ezetimibe (3 mg/kg/day; Ezetrol MSD-SP Limited, Hoddesdon, UK). In hormonal substitution experiments, T4 (L-Thyroxine; Sigma-Aldrich, St Louis, MO) and cortisone (Solu-Cortef, Pfizer, Sollentuna, Sweden) were injected subcutaneously (42 μg/kg/day and 400 μg/kg/day, respectively), and bovine growth hormone (from Dr A.F. Parlow, National Hormone and Peptide Program, Harbor-UCLA Medical Center, Torrance, CA) was infused (1.5 mg/kg/day) by implanted pumps (Alzet model 2ML1; Palo Alto, CA). The time period of each experiment was 7 days.

Paper II

All experimental procedures were approved by the Ethics Committee for Animal Experiments of the University of Groningen.
Male mice homozygous for the disruption of the ABCG5 gene or the LXRα gene, and their respective wild-type counterparts, were used in the experiments. For detailed descriptions of how knockout mice were generated, see [25, 104]. Animals were kept under standardized conditions and had free access to drinking water and chow. Groups treated with T3 received drinking water supplemented with 0.5μg T3/mL (3,3',5-Triiodo-L-thyronine, Sigma, St Louis, MO) and 0.01% albumin (BSA, Sigma). After 14 days of treatment, mice were anesthetized and bile was collected for 30 min from cannulated gallbladders as described in [104].

Paper III

All subjects gave their written informed consent to participate in the studies which were approved by the Ethics Committee at the Karolinska Institute, Stockholm, Sweden, and the Capenhurst Independent Research Ethics Committee, Capenhurst, UK.
In the first study, twenty hyperthyroid patients (16 women and 4 men) were studied at two occasions; before initiation of treatment, and when serum free T3 was normalized (3.0-6.5 pmol/L). Blood samples were collected between 08:30 and 09:00 AM after an overnight fast.
In the second study, fourteen healthy volunteers (7 women and 7 men) that had been included in a study evaluating a potential drug interaction between eprotirome and warfarin using a randomized, double-blind cross-over design (KBT011; Eudra CT no. 2011-003029-92) were studied. Samples taken after 14 days of treatment with 100 μg/day of the thyromimetic compound eprotirome (Karo Bio AB, Sweden) were compared to samples obtained prior to treatment or after a wash-out period of 14 days after the last dose.

3.2 GENE EXPRESSION

Total RNA was extracted from individual or pooled samples of liver or intestinal tissue and cDNA was synthesized from 1 µg of RNA using reverse transcriptase enzymes. Quantitative real-time PCR was performed with primers and probes designed with Primer Express Software 2.0 (ABI). 18S, GAPDH and HPRT were used as endogenous controls and the comparative C_t method was used to quantify the results. For detailed descriptions see [105, 106]

3.3 SERUM AND PLASMA CHOLESTEROL, TRIGLYCERIDES, GLYCEROL, AND FFA

For analyses of plasma levels of total cholesterol and triglycerides, see respective paper. Serum levels of cholesterol and triglycerides within VLDL, LDL, and HDL particles as well as glycerol levels were measured by fast protein liquid chromatography (FPLC) [107]. Serum levels of FFAs were measured using kits from Kamiya Biomedical Company (Seattle, WA) using a Tecan Infinite M200 plate reader.

3.4 BILIARY CHOLESTEROL, PHOSPHOLIPIDS, AND BILE ACIDS

Cholesterol was extracted from 25µL of bile from each individual with and analyzed using GC-MS. For further details, see [106]. Phospholipids were extracted from individual bile samples as described in [108]. The concentration was subsequently determined as in Böttcher et al [109]. Bile acids were extracted from bile samples (2µL) from each individual and analyzed using GC-MS. For further details, see [106].

3.5 CHOLESTEROL SYNTHESIS

Assay of HMGCoAR activity

Enzymatic activity of microsomal HMGCoAR was assayed from the conversion of [^{14}C]HMGCoA to mevalonate in duplicate [110].

Assay of lathosterol

Lathosterol was extracted from duplicate samples of 25 µL of serum and analyzed with GC-MS. For assay of lathosterol in samples from subjects treated with eprotirome, see [111]. Serum levels of lathosterol were normalized for plasma cholesterol levels.

3.6 INTESTINAL CHOLESTEROL ABSORPTION

Assay of serum plant sterols

Campesterol and sitosterol were extracted from 25 μL (human) or 10 μL (rat) of serum in duplicate samples as described in Paper III and [105] and analyzed by GC-MS. For assay of plant sterols in samples from subjects treated with eprotirome, see [112]. Serum levels of plant sterols were normalized for plasma cholesterol levels.

The fecal dual-isotope method

This method is described in [105]. Animals received gastric gavage containing [^{14}C]-cholesterol and [5,6-^{3}H]-β-sitostanol and 24-hour stool was collected for each individual. The dosing mixture and fecal ^{14}C/^{3}H ratios were counted (disintegrations per min), and percent cholesterol absorbed was calculated per animal.

3.7 FECAL NEUTRAL STEROIDS

Neutral steroids were extracted from fecal homogenates and analyzed by GC-MS as described in [105].

3.8 BILE ACID SYNTHESIS

Assay of CYP7A1 protein expression

The methods used to assay protein expression of CYP7A1 and β-actin are described in [113] and [105], respectively. The ratio of CYP7A1/β-actin was calculated for each individual.

Assay of CYP7A1 activity

Enzymatic activity of microsomal CYP7A1 was assayed from the formation of 7α-hydroxycholesterol from endogenous microsomal cholesterol using isotope dilution-MS in duplicate [114].

Assay of 7α-hydroxy-4-cholestene-3-one (C4)

1 mL (human) or 200 μL (rat) of serum was used to assay C4. This method is described in in [115]. Serum levels of C4 were normalized for plasma cholesterol levels as outlined by Gälman et al [116].

Assay of 7α-hydroxycholesterol

The assay to measure serum levels 7α-hydroxycholesterol is described in [117]. Serum levels of 7α-hydroxycholesterol were normalized for plasma cholesterol levels

3.9 SERUM BILE ACIDS

Levels of CDCA, CA, DCA, and their respective glycine and taurine conjugates were assayed using 250 μL of serum in duplicate. Acetonitril was added to samples which were then vortexed and centrifuged at 13 000g for 15 min and before the upper phase was collected and dried under a stream of nitrogen gas. Samples were re-dissolved in methanol and analyzed by LC-MS-MS using D_4-bile acids as internal standards. For assay of bile acids in samples from subjects treated with eprotirome, see [118].

3.10 TH, TSH, INSULIN, AND GLUCOSE

Serum levels of free T3, free T4, TSH, insulin, and plasma glucose levels were measured using a *MODULAR ANALYTICS* P170/P800 (Roche/Hitachi) and kits from Roche Diagnostics GmbH (Mannheim, Germany).

3.11 LP(A)

Assay of Lp(a) levels in serum was carried out in duplicate using kits based on immunoturbidimetric principles from DiaSys Diagnostic Systems GmbH (Lp(a) 21 FS, Holzheim, Germany) and a Response® 910 analyzer.

3.12 APOLIPOPROTEINS

Kits from Kamiya Biomedical Company were used to determine serum levels of apoAI (KAI-002), AII (KAI-003), B (KAI-004), CII (KAI-005), and CIII (KAI-006) by immunoturbidimetric techniques. All analyses were carried out in duplicate and following the manufacturer's instructions.

3.13 ELISA ASSAYS

Kits for sandwich-based solid phase ELISAs were used to determine serum levels of the following; apoAIV (EZHAP0A4-73K, Millipore, Billerica, MA), FGF19 and 21 (FGF19; DF1900 and FGF21; DF2100, R&D Systems Europe Ltd., Abingdon, UK), PCSK9 (CY-8079, CycLex Co. Ldt., Nagano, Japan), and SHBG (MX52011, IBL International GmbH, Hamburg, Germany). All analyses were carried out in duplicate and following the manufacturers' instructions.

3.14 STATISTICS

Statistical analyses were performed using GraphPad Prism 5.0 Software, see respective paper for details of used methods.

4 RESULTS AND COMMENTS

4.1 DRAMATICALLY INCREASED INTESTINAL ABSORPTION OF CHOLESTEROL FOLLOWING HYPOPHYSECTOMY IS NORMALIZED BY THYROID HORMONE (PAPER I)

Intact CYP7A1 regulation in cholesterol/fat-fed Hx rats

Rats respond to cholesterol feeding by increasing the conversion of cholesterol to bile acids for subsequent fecal loss. This occurs through a LXR-mediated upregulation of the rate-limiting enzyme in bile acid synthesis, CYP7A1. In opposite to normal rats, plasma cholesterol levels increase in Hx rats upon cholesterol feeding. Whether the hypercholesterolemia in cholesterol-fed Hx rats is caused by a defective regulation of CYP7A1 was investigated by feeding normal and Hx rats a cholesterol/fat diet (chow ± 2% cholesterol/10% corn oil) and subsequently measuring CYP7A1 gene expression, protein mass, activity, and C4 (a serum marker reflecting CYP7A1 activity). The distribution of serum cholesterol in chow-fed Hx rats was shifted from HDL to LDL, while serum total cholesterol was only slightly increased. CYP7A1 protein mass and enzymatic activity, and C4 were reduced in Hx animals. These results are in line with previous data [71, 119, 120]. Cholesterol/fat feeding did not alter serum cholesterol in normal rats, whereas it caused a 4-fold increase in Hx rats. In addition, cholesterol/fat feeding increased hepatic cholesterol by 6-fold in Hx rats while in normal rats only a 3-fold increase was observed. In normal rats, CYP7A1 gene expression, protein mass, and C4 were increased (~25%), and CYP7A1 activity unaltered, by the cholesterol/fat diet. In Hx rats, CYP7A1 gene expression, protein mass, activity, and C4 were increased to levels seen in normal animals on this diet. Thus, the regulation of CYP7A1 in response to dietary cholesterol/fat appears to be fully functioning in Hx rats. This suggests that other mechanisms than reduced bile acid synthesis cause the increased sensitivity to dietary cholesterol. It was next investigated whether an increased level of intestinal absorption of cholesterol cause the increased sensitivity to dietary cholesterol observed in Hx rats.

Intestinal cholesterol absorption is doubled in Hx rats

The efficacy of intestinal absorption of dietary cholesterol in normal and Hx rats fed chow was measured using the fecal dual-isotope method. Serum cholesterol data were consistent with the first experiment, i.e. a slight increase in total cholesterol and a shift from HDL to LDL following hypophysectomy. Intestinal cholesterol absorption in Hx rats was doubled compared to normal rats (66% vs 32%, respectively). These data was supported by measurements of plant sterol levels in plasma (reflecting cholesterol absorption), which were increased in Hx rats. These results strongly suggest that intestinal cholesterol absorption is enhanced in Hx animals.
If the enhanced intestinal absorption of cholesterol is an important cause of the hypercholesterolemia in cholesterol/fat-fed Hx rats, blocking the absorption should prevent the hypercholesterolemia. To test this hypothesis, Hx rats were fed chow ± ezetimibe, or chow enriched with cholesterol (0.4% or 2%) ± ezetimibe. Ezetimibe is a drug that inhibits NPC1L1-mediated uptake of cholesterol from the intestinal lumen into enterocytes. Serum cholesterol doubled in groups given the cholesterol/fat diets. Ezetimibe reduced serum cholesterol in chow-fed Hx rats and partly normalized the characteristic lipoprotein profile (high LDL- and low HDL-cholesterol). In animals given the cholesterol/fat diets, ezetimibe eliminated the increase

in plasma cholesterol. These data suggest that enhanced intestinal absorption of cholesterol is of major importance for the hypercholesterolemia in Hx rats challenged with dietary cholesterol.

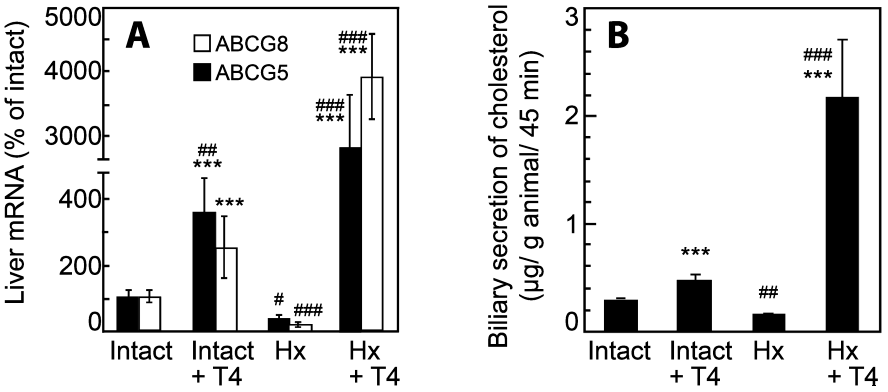


Fig. 5 Effects of T4-treatment on **A)** hepatic gene expression of ABCG5 (black bars) and ABCG8 (white bars) and **B)** biliary cholesterol secretion in intact and Hx rats. Data is shown as mean ± SEM. $^{###}$ = $p<0.001$, $^{##}$ = $p<0.01$, and $^{#}$ = $p<0.05$ vs intact, respectively. *** = $p<0.001$ vs Hx.

TH suppresses intestinal absorption of dietary cholesterol

Because the absorption of dietary cholesterol in Hx rats was increased, some pituitary factor(s) should have a suppressive effect on the absorption of cholesterol. To identify which pituitary hormone that might exert such an effect, Hx rats were substituted with growth hormone (GH), thyroid hormone (T4), or cortisone (C) alone and in all combinations (GH/C, GH/T4, C/T4, and GH/C/T4). Untreated Hx and normal rats served as control groups. Cholesterol absorption was estimated from serum levels of plant sterols. As expected, serum campesterol and sitosterol were increased, by 140% and 60%, respectively, in Hx rats. Substitution of Hx rats with only GH or cortisone had no effect on serum plant sterol levels. However, substitution with T4 significantly decreased both sterols. When the hormones were administered in combinations, T4 was obligate to significantly reduce plant sterols. This suggests that the suppressive effect on cholesterol absorption is exerted by thyroid hormone.

To confirm that T4 reduces cholesterol absorption in Hx rats, cholesterol absorption was assayed using the fecal dual-isotope method in normal rats, Hx rats, and Hx rats substituted with T4. It was confirmed that Hx rats have a significantly increased level of cholesterol absorption (29% vs 62%, compared with normal rats). Treatment of Hx rats with T4 strongly reduced cholesterol absorption (from 62% to 15%). To study whether these strong alterations in cholesterol absorption could be explained by changes in the intestinal cholesterol transporters ABCG5/G8 and NPC1L1, their intestinal gene expression levels were measured. There were no changes that could explain the increased cholesterol absorption. Because absorption of dietary cholesterol is also dependent on the amount of biliary cholesterol secreted into the intestine, hepatic ABCG5/G8 gene expressions were measured. In Hx rats, hepatic gene expressions of ABCG5/G8 were strongly suppressed (to 20% and 5% of normal rat levels, respectively), and

treatment with T4 increased their expressions by 60- and 190-fold, respectively. This suggests that Hx rats have a reduced biliary secretion of cholesterol into the intestine that is stimulated by T4. To test this possibility, the fecal excretion of cholesterol and neutral steroids was determined; fecal neutral steroids were reduced in Hx rats, and T4-treatment increased fecal neutral steroid output. Assay of hepatic ABCG5/G8 gene expressions in rats in the substitution experiment with GH, cortisone, and T4 showed that only T4 stimulates both ABCG5/G8 significantly.

In a final experiment, it was evaluated directly whether the hepatic secretion of cholesterol in T4-treated normal and Hx rats was in parity with the hepatic transcript levels of ABCG5/G8 by collecting biliary bile from normal and Hx rats after 7 days of treatment with T4 or saline. Assay of ABCG5/G8 transcripts were reduced by 60% and 80%, respectively, in Hx animals. In normal animals, T4 induced ABCG5 4-fold and ABCG8 2.5-fold, whereas, in Hx-animals, T4 induced ABCG5 74-fold and ABCG8 230-fold (Fig. 5A). Biliary cholesterol secretion was significantly reduced by 50% in Hx rats, and T4-treatment of Hx rats induced biliary cholesterol secretion 15-fold. Mean level of biliary cholesterol secretion in normal rats treated with T4 was increased by 65% compared to untreated normal rats, although it was not a statistically significant change. Thus, the hepatic secretion of cholesterol in normal rats, Hx rats, and Hx rats treated with T4 is in parity with the transcript levels of ABCG5/G8 (Fig. 5B).

4.2 STIMULATION OF MURINE BILIARY CHOLESTEROL SECRETION BY THYROID HORMONE IS DEPENDENT ON A FUNCTIONAL ABCG5/G8 COMPLEX (PAPER II)

By heterodimerization with each other, ABCG5 and ABCG8 form a complex that promotes transport of sterols from the liver into bile [15, 121, 122]. In paper I [105], it was found that TH-induced stimulation of biliary cholesterol secretion was associated with markedly increased hepatic ABCG5/G8 gene expressions. These data suggest that TH-induced stimulation of biliary cholesterol secretion is mediated by ABCG5/G8. Further, LXRα has been reported to be positively regulated by TRβ in the mouse [123] and the administration of the LXR agonist T0901317 to mice increases hepatic ABCG5/G8 gene expression and biliary cholesterol secretion [124-126]. To investigate if the TH-induced stimulation in biliary cholesterol secretion is mediated by the ABCG5/G8 complex, and if the effects of TH on biliary cholesterol secretion and hepatic ABCG5/G8 gene expressions are LXRα-dependent, mice homozygous for disruption of the genes encoding ABCG5 or LXRα, and their respective genetic wild-type counterparts, received drinking water ± T3.

TH-induced biliary cholesterol secretion depends on ABCG5/G8

As expected from paper I, hepatic ABCG5/G8 gene expressions were both increased in T3-treated *Abcg5*$^{+/+}$ mice (1.5-fold). ABCG8 gene expression was unaltered in *Abcg5*$^{-/-}$ control and in T3-treated *Abcg5*$^{-/-}$ mice. Opposite to what was expected [123], hepatic LXRα gene expression was unaltered in *Abcg5*$^{-/-}$ mice, while reduced in T3-treated *Abcg5*$^{+/+}$ and *Abcg5*$^{-/-}$ mice. Under basal conditions bile flow was the same in *Abcg5*$^{+/+}$ and *Abcg5*$^{-/-}$ mice. T3-treatment increased bile flow to similar extents in *Abcg5*$^{+/+}$ (1.9-fold) and in *Abcg5*$^{-/-}$ (1.8-fold) mice. Total bile acid secretion was unaltered in *Abcg5*$^{-/-}$ mice. T3-treatment of *Abcg5*$^{+/+}$ and of

Abcg5$^{-/-}$ mice tended to increase bile acid secretion but the differences did not reach statistical significance. Biliary cholesterol secretion was increased 3.1-fold in T3-treated *Abcg5*$^{+/+}$ mice. Basal secretion of biliary cholesterol in *Abcg5*$^{-/-}$ mice was only 28% of that seen in untreated *Abcg5*$^{+/+}$ mice. In T3-treated *Abcg5*$^{-/-}$ mice biliary cholesterol secretion was unaltered compared to *Abcg5*$^{-/-}$ mice, and did not differ from that of untreated *Abcg5*$^{+/+}$ mice. Biliary cholesterol secretion in T3-treated *Abcg5*$^{-/-}$ mice was 79% lower than in T3-treated *Abcg5*$^{+/+}$ mice (Fig. 6A). These results demonstrate that stimulation of biliary secretion of cholesterol by T3-treatment of mice is largely dependent on an intact ABCG5/G8 complex.

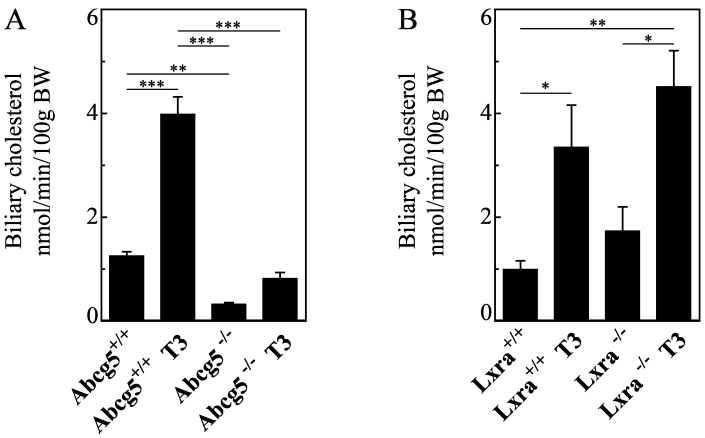


Fig. 6 Biliary cholesterol secretion in untreated and T3-treated **A)** *Abcg5*$^{+/+}$ and *Abcg5*$^{-/-}$ mice and **B)** *Lxra*$^{+/+}$ and *Lxra*$^{-/-}$ mice. Data is shown as mean ± SEM. *** = p<0.001, ** = p<0.01, and * = p<0.05, respectively.

No requirement for LXRα in TH-induced cholesterol secretion

LXRα gene expression was unaltered in T3-treated *Lxra*$^{+/+}$ mice, while ABCG5/G8 gene expression levels were both increased 2.1-fold and 1.5-fold, respectively. Gene expressions of ABCG5/G8 were unaltered in the *Lxra*$^{-/-}$ mice, while increased in T3-treated *Lxra*$^{-/-}$ mice (1.8-fold and 1.7-fold, respectively). Under basal conditions bile flow was the same in *Lxra*$^{+/+}$ and *Lxra*$^{-/-}$ mice. T3-treatment increased bile flow in *Lxra*$^{+/+}$ and in *Lxra*$^{-/-}$ mice (2.4-fold and 2.1-fold, respectively). Secretion of total bile acids was unaltered among the groups although there was a trend to an increased secretion in T3-treated mice. Biliary cholesterol secretion was similar in untreated *Lxra*$^{+/+}$ and *Lxra*$^{-/-}$ mice. In response to T3 treatment, it increased 3.5-fold in *Lxra*$^{+/+}$ and 2.6-fold in *Lxra*$^{-/-}$ mice, to similar levels (Fig. 6B). Thus, these results demonstrate that LXRα is not required for TH-induced stimulation of ABCG5/G8 gene expression or biliary secretion of cholesterol.

4.3 STIMULATION OF HUMAN CHOLESTEROL AND LIPOPROTEIN METABOLISM IN HYPERTHYROIDISM: IMPORTANCE OF LIVER-SPECIFIC HORMONE ACTIONS (PAPER III)

Effects of elevated endogenous TH levels in humans

Reduced lipoprotein cholesterol and serum PCSK9 levels

Compared to the euthyroid state, plasma total cholesterol was 28% lower in hyperthyroidism. The cholesterol content of VLDL, LDL, and HDL particles was 48%, 28%, and 15% lower, respectively. Compatible with the lower LDL- and HDL-cholesterol levels, serum apoB and apoAI levels were also lower, 27% and 14%, respectively. Serum levels of Lp(a) were 26% lower in hyperthyroidism. In the hyperthyroid state, there was a marked reduction in PCSK9 levels. Although clearly affected by TH, serum levels of PCSK9 did not correlate significantly with free TH levels. However, in agreement with the concept that the reduction in PCSK9 contributes to the TH-induced stimulation in clearance of LDL-cholesterol, there was a positive correlation between PCSK9 and plasma total and LDL-cholesterol levels in the hyperthyroid state. Notably, there was also a correlation between PCSK9 and LDL-cholesterol levels in the euthyroid state (Table 1).

Unaltered lipoprotein triglycerides but increased peripheral lipolysis

The plasma total triglyceride level was unaltered in hyperthyroidism, as was the triglyceride content of individual lipoprotein fractions. Thus, in the hyperthyroid state, circulating lipoproteins were depleted of cholesterol and therefore relatively enriched in triglycerides. The serum levels of FFAs and glycerol were both increased (20% and 37%, respectively), in the hyperthyroid state. ApoCII serum levels were unaltered whereas serum apoCIII levels were reduced 15%. Serum levels of apoAIV were 19% higher in hyperthyroidism, whereas apoAII, the second most abundant protein of the HDL particle, was 9% lower.

Unaltered serum FGF21, insulin, and glucose levels

FGF21 is a protein described as a novel metabolic regulator with positive impact on glucose and lipid homeostasis in experimental animal models [127]. Recently it was shown in mice that the administration of TH dose-dependently increased FGF21 hepatic expression and serum levels [128]. However, in the present study FGF21 serum levels were unaltered in the hyperthyroid state, as were the insulin and glucose levels.

Increased bile acid synthesis, reduced circulating FGF19, and reduced dietary cholesterol absorption

TH stimulates bile acid synthesis in animal models [75, 76], and attempts have been made to evaluate if this also applies to humans [82, 91, 129], but so far no clear stimulation has been reported. In this study, serum levels of C4, a metabolite that reflects bile acid synthetic rate [115, 130-132], were 43% higher in the hyperthyroid state, indicating that bile acid synthesis is stimulated by TH in humans. Of particular interest was that this increase in bile acid synthesis

was associated with 29% lower serum levels of FGF19, since FGF19 has been hypothesized to mediate inhibition of bile acid synthesis by suppressing the transcription of CYP7A1.
Serum levels of lathosterol that reflect cholesterol synthesis [133-136], were unaltered in hyperthyroidism. This indicates that, in contrast to animals [76, 137], cholesterol synthesis is not stimulated by TH in humans.

Reduced intestinal absorption of dietary cholesterol

Animal data indicate that the intestinal absorption of dietary cholesterol is reduced by TH [105], which likely contributes to the lowering of plasma cholesterol seen in response to this hormone. Since plant sterols and cholesterol share common transport pathways over the plasma membrane in enterocytes, serum levels of plant sterols can be used to indirectly estimate changes in the absorption of dietary cholesterol [136]. In the hyperthyroid state, serum levels of the plant sterols campesterol and sitosterol were lowered by 25% and 18%, respectively, suggesting that cholesterol absorption is impaired in hyperthyroidism.

Effects on serum bile acid composition and conjugation

The total amount of bile acids in serum was unaltered in the hyperthyroid state. However, there was a marked change in the proportions of individual bile acids: CDCA was 24% higher, and DCA acid 42% lower, in hyperthyroidism. CA was unaltered. As a result, the CDCA:(CA+DCA) ratio was higher in the hyperthyroid state. Further, in hyperthyroidism the relative amount of conjugated bile acids was 25% higher than in the euthyroid state, due to an increased amount of taurine conjugated bile acids.

Effects of hepatic stimulation of TRs by eprotirome in healthy subjects

Reduced lipoprotein cholesterol and PCSK9

Serum levels of sex hormone binding globulin (SHBG) were strikingly increased (80%) in response to eprotirome, indicating that there was a marked stimulation of hepatic TH receptors by this compound. In similarity with what was observed in the hyperthyroid state, plasma total cholesterol was 21% lower after treatment with eprotirome, and the cholesterol content of VLDL, LDL, and HDL particles was reduced by 20%, 29%, and 10% , respectively. In further agreement with the observations in hyperthyroidism, treatment with eprotirome reduced apoB and apoAI levels by 21% and 13%, respectively. Also consistent with the findings in hyperthyroid patients, treatment with eprotirome was associated with 25% and 17% lower Lp(a) and PCSK9 serum levels, respectively. Taken together, these findings clearly demonstrate that the liver is the main target for TH in the regulation of LDL-cholesterol, PCSK9 and Lp(a) in humans (Table 1).

Reduced lipoprotein triglycerides but not peripheral lipolysis

In contrast to the metabolic effects of hyperthyroidism in the patients, treatment with eprotirome reduced plasma triglycerides by 35%. The triglyceride content of VLDL, LDL, and HDL particles was reduced by 35%, 38%, and 46%, respectively. Further, also in contrast to the effects of hyperthyroidism, serum levels of FFAs and glycerol were unaltered in subjects treated with eprotirome. In concert with the observations in hyperthyroidism, serum levels of apoCII were unaltered and those of apoCIII reduced by 26%. In contrast to hyperthyroidism, but in agreement with the concept that apoAIV is mainly produced by the intestine [138, 139], serum levels of apoAIV were not altered by treatment with eprotirome. Similar to what was observed in hyperthyroidism, insulin, glucose, and FGF21 serum levels were not altered by eprotirome treatment.

Unaltered bile acid synthesis, FGF19, and dietary cholesterol absorption

The effect of eprotirome on bile acid synthesis was monitored from serum levels of the bile acid precursor 7α-hydroxycholesterol [133]. When eprotirome was given at a dose of 100 μg/day, the serum levels of 7α-hydroxycholesterol were unaltered, as were the levels of lathosterol. This indicates that, in contrast to hyperthyroidism, at this dose eprotirome does not increase bile acid synthesis. However, the fact that the ratio between 7α-hydroxycholesterol and lathosterol was increased (24%) following treatment may indicate that a larger proportion of newly synthesized cholesterol is converted into bile acids also in this situation. Again in contrast to what was observed in the hyperthyroid state, there was no change in the levels of the intestinally derived protein FGF19 following eprotirome treatment.

Total serum bile acids were 19% higher after treatment with eprotirome. The relative amounts of CA and DCA were unaltered, whereas CDCA was 20% higher, and thus the ratio CDCA:(CA+DCA) was increased by 30%, similar to what was seen in hyperthyroidism.

In contrast to what was observed in hyperthyroidism the levels of plant sterols, campesterol and sitosterol, were unaltered by treatment with eprotirome, in agreement with the contention that treatment with a liver-selective thyromimetic does not alter the absorption of dietary cholesterol from the intestine.

Table 1. Serum and plasma analyses

	Hyperthyroidism	Eprotirome
SHBG	↑	↑
Total cholesterol	↓	↓
VLDL-c	↓	↓
LDL-c	↓	↓
HDL-c	↓	↓
Total triglycerides	–	↓
VLDL-tg	–	↓
LDL-tg	–	↓
HDL-tg	–	↓
glycerol	↑	–
FFAs	↑	–
apoAI	↓	↓
apoAII	↓	
apoAIV	↑	–
apoB	↓	↓
apoCII	–	–
apoCIII	↓	↓
Lp(a)	↓	↓
PSCK9	↓	↓
FGF21	–	–
insulin	–	–
glucose	–	–
C4 (bile acid synthesis)	↑	–
FGF19	↓	–
lathosterol (cholesterol synthesis)	–	–
plant sterols (cholesterol absorption)	↓	–
total bile acids	–	↑
% CDCA	↑	↑
% CA	–	–
% DCA	↓	–
CDCA:(CA+DCA) ratio	↑	↑
% conjugated bile acids	↑	

↑ = increased ↓ = decreased – = unaltered

5 GENERAL DISCUSSION AND FUTURE PERSPECTIVES

Disorders of thyroid function are common, and the clinical abnormalities of hyper- and hypothyroidism are well established. A major aim of the present thesis was to investigate how elevated TH levels influence cholesterol and lipoprotein metabolism in humans. By paired comparisons of the hyperthyroid and the euthyroid state, the influence of interindividual genetic variation was minimized, and by means of eprotirome treatment, the liver-specific effects of TH in humans were evaluated. TH clearly exerts both hepatic and extrahepatic pleiotropic effects on cholesterol and lipoprotein metabolism that in general can be seen as positive as regards anti-atherogenic mechanisms (Table 1). We also explored some of these in more detail using animal models.

TH lowers plasma cholesterol levels in all lipoprotein fractions by its actions in the liver. LDL-cholesterol levels correlated with levels of PCSK9. From previous studies of lipoprotein kinetics in humans, it is clear that LDL is lowered by TH mainly through the stimulation of LDL clearance [140], presumed to reflect an increased number of hepatic LDLRs. The reduction in serum PCSK9 levels was similar in hyperthyroid patients and in eprotirome-treated subjects. Although it is not unlikely that hepatic LDLRs are also increased by TH via transcriptional activation, it is reasonable to assume that the reduced PCSK9 level contributes to an increased number of hepatic LDLRs in the hyperthyroid state. The potential role of induced changes in PCSK9 expression as a physiologic regulator of LDL-cholesterol levels is still not fully known. It is well established that treatment with statins increases both LDLRs and PCSK9 [141, 142]. Thus, the novel finding that TH clearly reduces PCSK9 levels probably explains the powerful LDL-lowering that is achieved when eprotirome is added to ongoing statin therapy [103]. Posttranslational regulation of hepatic LDLRs by modulation of PCSK9 seems to be a common feature of hormonal influence on lipoprotein metabolism in rodents [143], and lower circulating PCSK9 levels were recently demonstrated as an effect of endogenous estrogens in humans [144].

TH markedly reduced the levels of Lp(a) by its hepatic action. Despite much research it is still not completely understood how Lp(a) levels are regulated in an individual. It is generally believed that serum Lp(a) level is determined more by its synthesis in the liver and to a lesser extent by its clearance from the circulation [56]. The clear Lp(a) lowering effect observed in response to eprotirome supports the concept of the liver as the location of Lp(a) synthesis, and may be an additional advantage of such therapy. Thyromimetics should be useful to further explore the details of Lp(a) metabolism, and may also provide a possibility to evaluate the important question as to whether lowering of Lp(a) has positive clinical effects beyond lowering of LDL-cholesterol.

Previous data on the effects of TH on bile acid synthesis in humans have been unclear [82, 91, 129]. Bile acid synthesis, as measured by the serum marker C4, was increased in hyperthyroidism, in line with the effects of TH on CYP7A1 in rodents [75, 76, 145]. In contrast to animal data [76, 146], this occurred without any compensatory increase in cholesterol synthesis, assessed by measurements of serum lathosterol. In rodents, TH has a suppressive effect on the rate-limiting enzyme in cholic acid synthesis, CYP8B1, thereby increasing the synthesis of CDCA [147]. That the relative amount of CDCA in the serum bile acid pool was

increased, as observed in hyperthyroid as well as in eprotirome-treated subjects, suggests that such a response also exists in human liver. In further agreement with previous data, the conjugation of serum bile acids with taurine was increased in hyperthyroidism [148].

Circulating FGF19 levels were reduced in hyperthyroidism. FGF19, secreted from the intestine in response to transintestinal flux of reabsorbed bile acids, is believed to contribute to the feedback regulation of bile acid synthesis by suppression of CYP7A1 in the liver [127]. A reduced level of FGF19 in response to TH may thus either reflect a reduced flux of bile acids, or be a primary effect of TH in the gut. The fact that eprotirome did not result in a significant change in 7α-hydroxycholesterol (marker of bile acid synthesis rate) or FGF19 levels, suggests that at least part of the pronounced TH effect on bile acid synthesis may be related to a direct effect on the ileum, and not to a primary effect on the liver. The data on eprotirome treatment should be taken with some reservation, however, since it was previously shown that treatment with a higher dose (200 μg/day) was needed to establish a clear increase of C4 (marker of bile acid synthesis rate) in humans [102]. All the same, the fact that pronounced lowering of LDL occurred with eprotirome in the present study indicates that stimulation of LDL clearance by this drug is not heavily dependent on the conversion of cholesterol to bile acids.

Selective stimulation of TRs in the liver with eprotirome reduced circulating triglycerides in all lipoproteins, while in hyperthyroidism these were unaltered. In hyperthyroidism, but not during eprotirome treatment, serum FFAs and glycerol were increased, indicating augmented peripheral lipolysis. The levels of apolipoproteins produced mainly in the liver, such as apo B, apoAI, apoCII, and apoCIII, showed similar changes in the two models, whereas apoAIV which is mainly produced in the small intestine, was increased in hyperthyroidism but not during eprotirome treatment. In the present work, we did not evaluate the effects on lipoprotein lipase or hepatic lipase levels, and further studies will be important to make the picture complete as regards the pleiotropic effects of TH on lipoprotein metabolism. Another need for further work is exploration of how TH influences HDL metabolism, and whether the observed changes (with lowering of HDL-cholesterol and apoAI) reflect an increased flux of HDL-cholesterol through reverse cholesterol transport, or if they may indicate that the potentially anti-atherogenic reductions in LDL, VLDL and Lp(a) may be partly counteracted by a reduced protective effect in HDL.

Hyperthyroidism, but not eprotirome treatment, was also associated with reduced serum plant sterols which would indicate a reduced absorption of dietary cholesterol that may contribute to the cholesterol-lowering effects of TH. This difference between generalized and liver-selective hyperthyroidism suggests that the major influence on plant sterols in humans would be the result of intestinal effects by TH. Detailed studies on intestinal and biliary cholesterol fluxes were performed in rodents.

In contrast to normal rats, Hx rats display a pronounced increase in serum cholesterol levels in response to a cholesterol/fat diet, and studies have suggested that reduced hepatic LDLRs and CYP7A1 activity partly explain this [71, 149]. However, the fact that CYP7A1 activity was normalized upon cholesterol feeding suggests that the sensitivity in Hx rats to cholesterol-enriched diets is not due to an impaired regulation of CYP7A1. Instead, Hx rats were found to have a doubled rate of intestinal cholesterol absorption. NPC1L1 is the target of the cholesterol absorption inhibitor ezetimibe [46, 150], and the fact that ezetimibe prevented the increase in serum cholesterol in cholesterol/fat-fed Hx rats, suggests that the increased absorption of cholesterol in Hx animals is mediated by NPC1L1. Notably, ezetimibe also reduced LDL-

cholesterol in chow-fed Hx rats but not in normal rats. This further supports that increased cholesterol absorption is important for the dyslipidemia following hypophysectomy (increased LDL- and reduced HDL-cholesterol). The thyroid state may modify cholesterol absorption [151], and TH was obligate to normalize serum plant sterols (markers for intestinal cholesterol absorption) in substitution experiments in Hx rats, and by using the fecal dual-isotope method it was confirmed that TH reduces cholesterol absorption. The modulation of cholesterol absorption induced by hypophysectomy and TH were not explained by changes in the intestinal gene expressions of known sterol transporters NPC1L1 and ABCG5/G8. However, other mechanisms apart from the activity of these cholesterol transporters may also modulate absorption, such as intestinal permeability and intestinal transit time.

Apart from excreting sterols into the intestinal lumen, the ABCG5/G8 complex has been shown to be of major importance for biliary sterol secretion in mice. However, ABCG5/G8-independent mechanisms promoting cholesterol secretion have been suggested due to the following findings: 1) biliary cholesterol secretion/concentration is not completely abolished in single [24, 25] and double [122, 126, 152, 153] ABCG5/G8 knockout mice, 2) hepatic overexpression of SRBI in *Abcg5*$^{-/-}$ mice can restore their initially decreased biliary cholesterol secretion to wild-type levels [31], and 3) since transintestinal cholesterol efflux occurs in *Abcg5*$^{-/-}$ [154] and *Abcg8*$^{-/-}$ [45] mice via additional pathways not yet defined, such mechanisms may operate also in the liver. The hepatic ABCG5/G8 gene expressions and biliary cholesterol secretion were reduced in Hx rats and were strongly stimulated by TH-treatment. To test if the TH-induced stimulation of biliary cholesterol secretion is indeed mediated by the ABCG5/G8 complex, *Abcg5*$^{+/+}$ and *Abcg5*$^{-/-}$ mice were treated with TH. In line with the results in Hx rats, TH-treatment increased hepatic gene expression of ABCG5/G8 in *Abcg5*$^{+/+}$ mice but failed to increase ABCG8 gene expression in the *Abcg5*$^{-/-}$ mice. This lack of response may be due to a disruption in a regulatory region of *Abcg8* caused in the procedure of disrupting *Abcg5*. TH increased biliary cholesterol secretion 3.1-fold in *Abcg5*$^{+/+}$ mice whereas in *Abcg5*$^{-/-}$ mice this response was blunted. These results demonstrate that stimulation of biliary secretion of cholesterol by TH in mice is largely dependent on an intact ABCG5/G8 complex. However, TH-treatment restored the low biliary secretion of cholesterol in *Abcg5*$^{-/-}$ mice up to the basal rate observed in *Abcg5*$^{+/+}$ mice. This suggests that, although a functional ABCG5/G8 complex is required for the major stimulation of biliary cholesterol secretion by TH, there is also an additional, ABCG5/G8-independent, mechanism. The increased secretion in *Abcg5*$^{-/-}$ mice occurred simultaneously with a TH-induced doubled flow rate of bile, regardless of the genetic background of the animals. The increased bile flow could well facilitate the transport due to simple diffusion of cholesterol across the plasma membranes, and thus provide an explanation to the non-ABCG5/G8 driven cholesterol secretion.

Activation of LXR by selective agonists has similar effects on hepatic ABCG5/G8 gene expression levels and biliary cholesterol secretion as TH [124, 126]. It has been reported that LXRα is positively regulated at the transcriptional level by TRβ [123]. However, biliary cholesterol secretion rates did not differ between TH-treated *Lxra*$^{+/+}$ and *Lxra*$^{-/-}$ mice, clearly showing that stimulation of biliary cholesterol secretion in response to TH is independent of LXRα. It will be of great interest to study how TH – and eprotirome – influence biliary cholesterol secretion in humans, since such effects, if present, may contribute to lowering of lipoprotein cholesterol as well as reverse cholesterol transport.

In conclusion, TH exerts a number of pronounced effects on cholesterol and lipoprotein metabolism. Through further understanding of how these effects are mediated, it should be possible to develop new therapeutic strategies of clinical importance that positively modulate lipoprotein fluxes and cholesterol accumulation in the body. This would eventually be helpful in the treatment and prevention of major human disease entities such as dyslipidemia, atherosclerosis and biliary disease.

6 CONCLUSIONS

I) The loss of resistance to cholesterol/fat feeding in Hx rats is due to an increased intestinal absorption of dietary cholesterol, which is not explained by alterations in the expression of intestinal sterol transporters NPC1L1 and ABCG5/G8. TH impairs cholesterol absorption, but the mechanism does not involve altered intestinal expression of NPC1L1 or ABCG5/G8.

II) TH stimulates biliary cholesterol secretion. This is predominantly mediated by the ABCG5/G8 transporter complex. The TH-induced biliary cholesterol secretion is independent of LXRα.

III) TH exerts a number of important effects on cholesterol and lipoprotein metabolism in humans. Many of these originate from the effects of this hormone on the liver including lowering of PCSK9 and LDL-cholesterol levels, as well as reduction of apoB and the atherogenic Lp(a). Selective activation of hepatic TH receptors lowers plasma triglycerides, whereas the stimulation of peripheral lipolysis counteracts this action in hyperthyroidism. Bile acid synthesis is increased by TH, but this does not appear to be of critical importance for its lipid-lowering effects. The role of TH-stimulation of enterocytes for its effects on bile acid synthesis and cholesterol absorption may be of greater importance than previously recognized. The potential to use liver-selective TH analogs in the further exploration of how TH exerts its pleiotropic effects in humans should be great, whereas extended studies are needed to ascertain the possible clinical usefulness of therapy based on this concept.

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