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STUDIES ON CHOLESTEROL AND LIPOPROTEIN METABOLISM

– emphasis on diabetes and sugar

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THESIS FOR DOCTORAL DEGREE (Ph.D.)

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ABSTRACT

Cholesterol has important functions in the body; as a precursor in the synthesis of steroid hormones and bile acids (BAs), and as a component of cellular membranes. However, an elevated level of plasma cholesterol, transported in low density lipoprotein (LDL) particles, is one of the major risk factors and causes for cardiovascular disease. Therefore its metabolism is tightly regulated, from synthesis to excretion. Cholesterol can be excreted from the liver into the bile, directly or after conversion into BAs. By modulation of cholesterol and BA metabolism, carbohydrate and triglyceride (TG) metabolism can also be affected, and vice versa. The main focus of this thesis was to further characterize these relationships.

In **Paper I**, the effects of inhibiting the ileal bile acid transporter (IBAT; also known as apical sodium dependent bile acid transporter [ASBT]) on TG and glucose metabolism were studied. This was studied in IBAT-deficient mice fed a sucrose-enriched diet and in *ob/ob* mice treated with an IBAT inhibitor. Liver TG was reduced in the first model and plasma TG and blood glucose was reduced in the second. IBAT inhibition could therefore be a promising therapeutic agent. An unexpected finding was that BA synthesis was reduced by the sucrose-enriched diet.

This was further studied in **Paper II** in which rats were fed two different sucrose-enriched diets. The first one, with increased sucrose content and concomitantly reduced contents of fibers and fats, reduced BA synthesis. However, the second more controlled high-sucrose diet, in where the complex carbohydrates were replaced by sucrose, did not affect BA synthesis. It was therefore concluded that it was not sucrose *per se* in the first diet that reduced BA synthesis. Both high-sucrose diets induced a very strong reduction in the hepatic expression of the cholesterol transporters ATP-binding cassette sub-family G members 5 and 8 (Abcg5/8).

In **Paper III**, the effect of growth hormone (GH) on circulating levels of fibroblast growth factor 21 (FGF21) was investigated in three human studies with administration of different doses of GH, and for various durations. It was concluded that GH is not crucial for maintaining basal FGF21 levels and does not increase FGF21 levels acutely or after long-term administration of physiological doses. However, prolonged administration of supraphysiological doses increases FGF21.

In **Paper IV**, type 2 diabetic patients were shown to have lower levels of LDL cholesterol in interstitial fluid than healthy controls, when related to their serum levels. This was unexpected as it was hypothesized that these patients would have higher LDL levels in interstitial fluid and that this could explain their increased risk of cardiovascular disease. However, the reduced level may mirror an increased cellular uptake of apoB-containing lipoproteins from the interstitial fluid.

In conclusion, this thesis has further characterized the interactions between the metabolism of cholesterol and BAs, with that of TGs and glucose. It is shown that interruption of the enterohepatic circulation of BAs may be a promising drug target for improving glucose and TG metabolism. Furthermore, dietary sucrose may reduce the secretion of cholesterol into

bile, however, this needs to be confirmed. It is shown that the hormone-like protein FGF21 can be elevated by high GH levels in humans. Lastly, type 2 diabetic patients have unexpectedly low LDL cholesterol levels in interstitial fluid, presumably reflecting their increased propensity to develop atherosclerosis.

LIST OF SCIENTIFIC PAPERS

This thesis is based on the four papers listed below and they will be referred to throughout the text by their Roman numerals.

I. Lundåsen T, Andersson EM, Snaith M, Lindmark H, **Lundberg J**, Östlund-Lindqvist AM, Angelin B, Rudling M. Inhibition of intestinal bile acid transporter Slc10a2 improves triglyceride metabolism and normalizes elevated plasma glucose levels in mice. *PLoS One. 2012;7(5):e37787.*

II. **Apro J**, Beckman L, Angelin B, Rudling M. Influence of dietary sugar on cholesterol and bile acid metabolism in the rat: Marked reduction of hepatic Abcg5/8 expression following sucrose ingestion. *Accepted. Biochem Biophys Res Commun, April 2015.*

III. **Lundberg J**, Höybye C, Krusenstjerna-Hafstrøm T, Bina HA, Kharitonenkov A, Angelin B, Rudling M. Influence of growth hormone on circulating fibroblast growth factor 21 levels in humans. *J Intern Med. 2013;274:227–232.*

IV. **Apro J**, Parini P, Broijersén A, Angelin B, Rudling M. Levels of atherogenic lipoproteins are unexpectedly reduced in interstitial fluid from type 2 diabetes patients. *Submitted.*

R1 (Related publication)

Lundberg J, Rudling M, Angelin B. Interstitial fluid lipoproteins. *Curr Opin Lipidol 2013;24:327–331.*
Inserted after Paper IV.

CONTENTS

LIST OF ABBREVIATIONS

ABCA1	ATP-binding cassette sub-family A member 1
ABCG1	ATP-binding cassette sub-family G member 1
ABCG5	ATP-binding cassette sub-family G member 5
ABCG8	ATP-binding cassette sub-family G member 8
ACAT2	Acetyl-CoA acetyltransferase 2
AGEs	Advanced glycation end-products
AKT1	Protein kinase B alpha
ALT	Alanine aminotransferase
AMPK	AMP-activated protein kinase
Apo	Apolipoprotein
AST	Aspartate aminotransferase
BA	Bile acid
BSEP	Bile salt export pump
C4	7α-hydroxy-4-cholesten-3-one
cDNA	Complementary deoxyribonucleic acid
CETP	Cholesteryl ester transfer protein
ChREBP	Carbohydrate responsive-element binding protein
CYP7A1	Cholesterol 7α-hydroxylase
CYP8B1	Sterol 12α-hydroxylase
DMSO	Dimethyl sulfoxide
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
ERK1/2	Mitogen-activated protein kinase 3/1
FA	Fatty acid
FABP	Fatty acid-binding protein
FAT	Fatty acid translocase
FATPs	Fatty acid transport proteins
FFA	Free fatty acid
FGF15/19	Fibroblast growth factor 15/19
FGF21	Fibroblast growth factor 21

FGFR4	Fibroblast growth factor receptor 4
FOXO1	Forkhead box protein O1
FPLC	Fast protein liquid chromatography
GC-MS	Gas chromatography-mass spectrometry
GH	Growth hormone
GHRH	Growth hormone-releasing hormone
GLP-1	Glucagon-like peptide-1
HbA1c	Hemoglobin A1c
HDL	High density lipoprotein
HL	Hepatic lipase
HMG-CoA reductase	3-hydroxy-3-methylglutaryl-CoA reductase
HNF-4α	Hepatocyte nuclear factor 4α
HPLC	High-performance liquid chromatography
HSPGs	Heparan sulfate proteoglycans
IBAT/ ASBT	Ileal bile acid transporter/ Apical sodium dependent bile acid transporter
IBABP	Ileal bile acid-binding protein
IDL	Intermediate density lipoprotein
IF	Interstitial fluid
IGF-1	Insulin-like growth factor 1
IP	Intraperitoneal
IV	Intravenous
JNK	c-Jun N-terminal kinase
LCAT	Lecithin-cholesterol acyltransferase
LDL	Low density lipoprotein
LDLR	Low density lipoprotein receptor
Lp(a)	Lipoprotein(a)
LPL	Lipoprotein lipase
LRH-1	Liver receptor homolog 1
LRP	LDL receptor-related protein
LXR	Liver X receptor

MEK1/2	Mitogen-activated protein kinase kinase 1/2
MRP2	Multidrug resistance-associated protein 2
MTP	Microsomal triglyceride transport protein
NEFA	Non-esterified fatty acid
NF-κB	Nuclear factor-κB
NPC1L1	Niemann-Pick C1-Like 1
NTCP	Na+-dependent taurocholic cotransporting polypeptide
OATPs	Organic anion transporting polypeptides
OSTα/β	Organic solute transporter α/β
PCR	Polymerase chain reaction
PGC-1α	Peroxisome proliferator-activated receptor gamma coactivator-1-α
PL	Phospholipid
PLTP	Phospholipid transfer protein
PPARα	Peroxisome proliferator-activated receptor alpha
R_s	Spearman's rank correlation coefficient
RXR	Retinoid X receptor
SC	Subcutaneous
SD	Standard deviation
SE	Standard error
SHP	Small heterodimer partner
SR-B1	Scavenger receptor class B type 1
SREBP-1	Sterol regulatory element-binding protein 1
STAT5	Signal transducer and activator of transcription-5
T2D	Type 2 diabetes
TG	Triglyceride
TGR5 (Gpbar1)	G protein-coupled bile acid receptor 1
TICE	Transintestinal cholesterol efflux
VLDL	Very low density lipoprotein
WT	Wild type

1 INTRODUCTION

1.1 CARDIOVASCULAR DISEASE

Ischemic heart disease is still the leading cause of death worldwide, although death rates have declined in the Western world (1). Established risk factors for premature cardiovascular disease include elevated low density lipoproteins (LDL) and very low density lipoproteins (VLDL), low levels of high density lipoproteins (HDL), high lipoprotein(a) (Lp[a]), hypertension, diabetes mellitus, male gender, insulin resistance, obesity, family history, smoking, and lack of exercise (2).

1.1.1 Atherosclerosis

The process leading to atherosclerosis is thought to be initiated when LDL particles bind the proteoglycans in the vascular wall. These immobilized particles display an increased susceptibility to oxidation. The oxidized LDL, in itself, attracts monocytes and T lymphocytes, and it also stimulates the endothelial and smooth muscle cells to attract monocytes. Monocytes differentiate into macrophages that take up the modified LDL particles, leading to foam cell formation. The atherosclerotic process advances when smooth muscle cells migrate to the outer part of the vascular wall where they take up modified lipoproteins, which also contributes to foam cell formation. Furthermore, the smooth muscle cells synthesize extracellular matrix proteins that lead to the formation of a fibrous cap. At this stage, a chronic inflammation is ongoing, which contributes to atherosclerosis development. When the vessel lumen is narrowed as a consequence of the remodeling of the vessel wall, ischemic symptoms may occur. Furthermore, macrophages secrete matrix metalloproteinases that weaken the fibrous cap. This may ultimately lead to plaque rupture and initiation of a coagulation cascade that can result in thrombosis, ultimately resulting in acute events such as myocardial infarction or stroke (2; 3).

1.2 CHOLESTEROL AND LIPOPROTEIN METABOLISM

Cholesterol can be synthesized by all cells in the body, and the enzyme catalyzing the rate-limiting step is 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase. HMG-CoA reductase is inactivated when bound to statins and is transcriptionally reduced when intracellular cholesterol levels are high (4). Cholesterol is an important component in cell membranes and is a substrate for the synthesis of steroid hormones and bile acids (BAs) (4). Due to its hydrophobicity, cholesterol in blood plasma is transported in lipoproteins.

As mentioned above, elevated serum LDL cholesterol is one major risk factor and cause for cardiovascular disease. A recent meta-analysis of 27 statin trials has shown that with every mmol/L reduction of LDL cholesterol, the risk for major cardiovascular events is reduced by 21% (5). HDL cholesterol has an inverse relationship to coronary heart disease (6; 7), although it has never been shown that raising HDL lowers the risk for cardiovascular disease in humans (8; 9).

Cholesterol is transported in lipoproteins in plasma, together with triglycerides (TGs) and phospholipids (PLs). The core of the lipoprotein mainly consists of TGs and cholesteryl esters, whereas the surface is composed of PLs and unesterified cholesterol together with proteins. The categorization of lipoproteins is based on their density that is dependent of the lipid composition of the particle. The main lipoprotein classes are chylomicrons, VLDL, intermediate density lipoprotein (IDL), LDL and HDL; presented in the order of increasing density and decreasing diameter (Table 1). Additionally, the different subclasses of lipoproteins have different apolipoproteins bound to their surface, which determines their functions. Lipoprotein metabolism consists of the exogenous pathway, i.e. absorption of dietary fat in the intestine, the endogenous pathway, which is the transport of endogenous fat from the liver to peripheral tissues, and lastly, the reverse cholesterol transport, by which lipids are transported from peripheral tissues to the liver (Figure 1).

Table 1. *Characteristics of the major lipoprotein classes.*

Lipoprotein	Density (g/dL)	Diameter (nm)	TG (%)*	Cholesterol (%)*	PL (%)*
Chylomicron	0.95	75-1200	80-95	2-7	3-9
VLDL	0.95-1.006	30-80	55-80	5-15	10-20
IDL	1.006-1.019	25-35	20-50	20-40	15-25
LDL	1.019-1.063	18-25	5-15	40-50	20-25
HDL	1.063-1.21	5-12	5-10	15-25	20-30

** Percent composition of lipids, apolipoproteins make up the remaining part.*
Table modified from Ginsberg HN (10).

1.2.1 The exogenous pathway

Dietary fat in the intestine is hydrolyzed before absorption. More specifically, cholesteryl esters are hydrolyzed into unesterified cholesterol and free fatty acid (FFA) (11), TGs into FFA and monoacylglycerol (12), and PLs into FFA and lysophospholipids (13). The FFAs are either absorbed by the enterocyte via diffusion (14; 15) or by an active process (16) involving fatty acid transport proteins (FATPs) (17) or fatty acid translocase (FAT; also called CD36) (18). In the enterocyte, fatty acids (FAs) are transported by proteins in the fatty acid-binding protein (FABP) family (19). The FAs are transformed into TGs in the endoplasmic reticulum (ER) of the enterocyte (20) which enables chylomicron assembly. Cholesterol absorption is largely an active process, initiated by the Niemann-Pick C1-Like 1 (NPC1L1) protein located at the brush boarder membrane of the enterocyte (21). The absorption of cholesterol is also regulated by the heterodimer complex of ATP-binding cassette sub-family G members 5 (ABCG5) and 8 (ABCG8) which transport cholesterol from the enterocyte back into the intestinal lumen (22). In the ER of the enterocyte, acetyl-CoA acetyltransferase 2 (ACAT2) esterifies part of the absorbed free cholesterol (23). Chylomicrons are formed in a process located in the ER and Golgi. This process requires

apolipoprotein (apo)B-48 for activation of the lipidating function of microsomal triglyceride transport protein (MTP). Chylomicrons consist primarily of TGs, but also of PLs and cholesterol. In addition to apoB-48, chylomicrons carry apoAIV, apoAI, and apoCs. After assembly in the enterocyte, chylomicrons enter the blood via the lymph (11). In heart, skeletal muscle, macrophages, and adipose tissue, lipoprotein lipase (LPL) hydrolyzes TGs in chylomicrons, thereby generating chylomicron remnants (24). These are further depleted in TGs and PLs by hepatic lipase (HL) present in liver, endothelial cells, macrophages, and the blood stream (25). Finally, chylomicron remnants are cleared from the circulation by the liver, after binding to the LDL receptor (LDLR), the LDL receptor-related protein (LRP) or to heparan sulfate proteoglycans (HSPGs) (26).

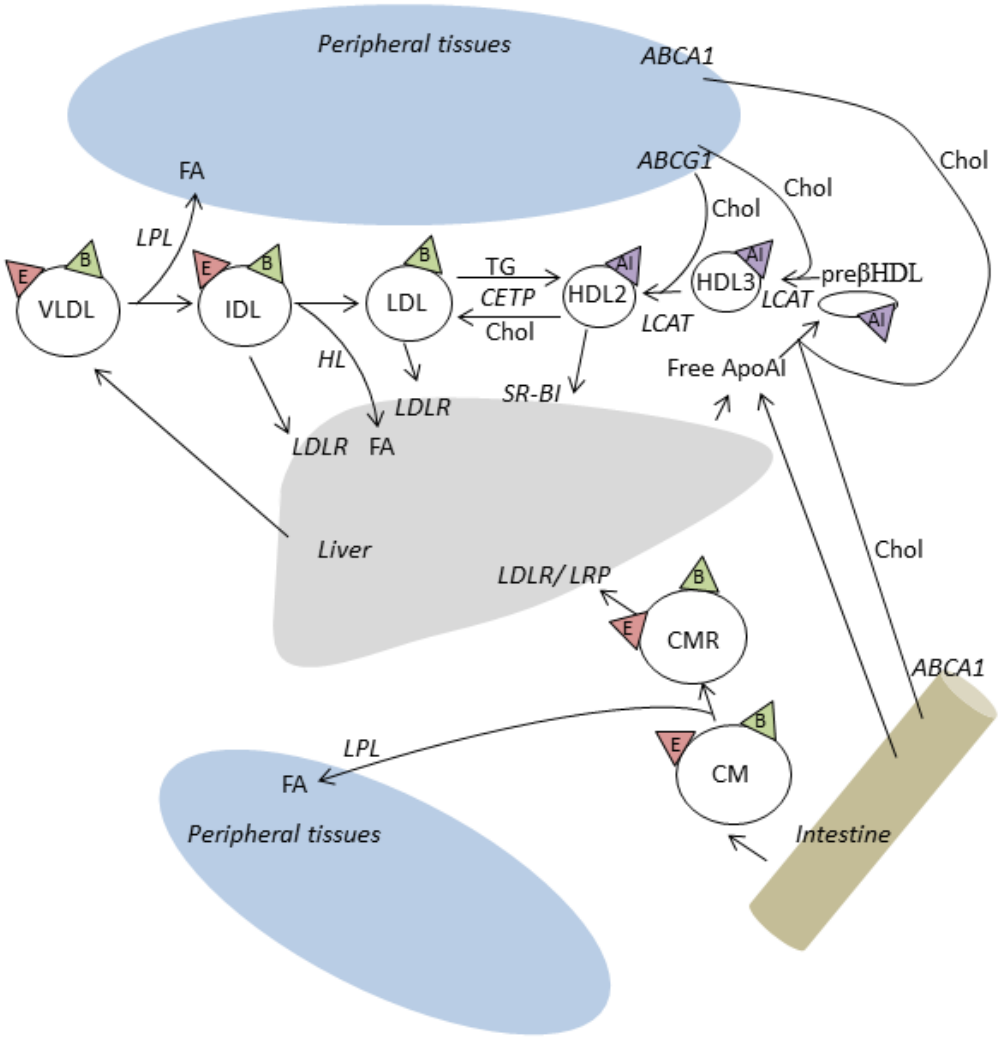

Figure 1. *Simplified schematic figure of lipoprotein metabolism.*
Chol = cholesterol, CM = chylomicron, CMR = chylomicron remnant.

1.2.2 The endogenous pathway

Liver FAs, that originate from adipocytes, chylomicron remnants, and the intestine via the portal vein, or are synthesized in liver, are synthesized into TGs in the ER (27; 28). VLDL formation requires MTP that lipidates apoB-100 (29) (apoB-48 and apoB-100 in rodents

(30)), after which these primordial VLDLs are further enriched in lipids. Thereafter the VLDL particles are transported to the Golgi (27) and are then secreted into the bloodstream. When in contact with LPL in peripheral tissues, VLDL TGs are hydrolyzed to FFAs and glycerol, and a more lipid-poor VLDL is created (24). In humans, the TG content of VLDL is also reduced by the action of cholesteryl ester transfer protein (CETP). CETP transfers TGs from VLDL to HDL in exchange for cholesteryl esters (31). When VLDLs are depleted of TGs, the particles are transformed into IDLs. IDLs are either taken up by the hepatic LDLR, LRP or HSPGs (26), or the TG and PL content is further reduced by HL which then leads to the formation of LDL (25). LDL is further enriched in cholesteryl esters in exchange for TGs through CETP action on HDL (31). LDL particles are predominantly cleared from the circulation by hepatic LDLRs (32).

1.2.3 Reverse cholesterol transport

Most cells cannot catabolize cholesterol, therefore reverse cholesterol transport is important in regulating cellular levels of cholesterol. Removal of cholesterol from macrophages is particularly important, as cholesterol-loaded macrophages lead to formation of foam cells. ApoAI is the main apolipoprotein in HDL and is synthesized by the liver and the intestine (33). HDL can acquire free cholesterol via ATP-binding cassette sub-family A member 1 (ABCA1), ATP-binding cassette sub-family G member 1 (ABCG1), and to a lesser extent by scavenger receptor class B type 1 (SR-BI) and aqueous diffusion (34). Whereas ABCA1 transfers cholesterol to lipid-free apoAI (35), ABCG1 requires apoAI to be partially lipidated to be able to transfer cholesterol (36). By the action of lecithin-cholesterol acyltransferase (LCAT), the cholesterol becomes esterified. The cholesteryl esters are translocated into the core of the HDL particles so that the originally discoidal particles become spherical (37). HDL particles acquire PLs from chylomicrons and LDLs by the action of phospholipid transfer protein (PLTP). Furthermore, PLTP can also fuse HDL particles that also generate pre-β HDL, the initial small HDL particle that is effective in acquiring cholesterol (38). HDL particles deliver cholesterol to the liver by the action of SR-BI (39). Larger HDL particles have higher affinity for the receptor, a selectivity that increases the removal of cholesteryl esters (40). Additionally, the action of CETP transfers cholesteryl esters to apoB-containing lipoproteins (31), which when taken up by the LDLR also clear the plasma from cholesterol. TGs and PLs in HDL particles are hydrolyzed by HL which also transforms HDL_2 into HDL_3, i.e. converts larger HDL particles into smaller ones (25).

1.2.4 Transintestinal cholesterol efflux

Another possible pathway for cholesterol excretion was recently proposed, termed transintestinal cholesterol efflux (TICE). The details of this pathway are not fully understood, but it is speculated that apoB-containing lipoproteins could deliver cholesterol to the intestine for excretion into the lumen (41).

1.2.5 Lipoprotein(a)

In addition to those mentioned above, Lp(a) is another lipoprotein present in humans and primates. Lp(a) is an LDL-like lipoprotein particle that is defined by the presence of apo(a) on its surface. The size of apo(a) depends on a copy variability of one of its domains, which makes Lp(a) size highly variable between subjects. The size of apo(a) is found to be inversely correlated with serum levels of Lp(a). Lp(a) is linked to atherosclerosis, presumably due to its participation in foam cell formation as well as by promoting oxidative and inflammatory processes in the vascular wall. Lp(a) assembly starts in the liver where apo(a) and apoB-100 are produced and becomes lipidated, followed by further lipidation through interactions with circulating LDL and IDL. Lp(a) is cleared from the circulation both through the kidneys and the liver, through the megalin and SR-BI receptor, respectively (42).

1.2.6 ABCG5/8

As mentioned earlier, the half-transporters ABCG5 and ABCG8 form an obligate heterodimer (43) that limits the intestinal absorption of cholesterol and plant sterols, by mediating efflux from the enterocyte back into the intestinal lumen (22). In liver, this heterodimer facilitates the biliary secretion of cholesterol and plant sterols (44). Thus, ABCG5/8 limits the accumulation of cholesterol in the body by two mechanisms (45). Both ABCG5 and ABCG8 are required for the efflux-function as both proteins are required for its transport from the ER to the cell surface (43). The main regulator of Abcg5/8 is the liver X receptor (LXR)α, which is activated by oxysterol metabolites, and positively regulates the transcription (46). Furthermore, hepatocyte nuclear factor 4α (HNF-4α) is also found to bind the promoter and upregulate ABCG5/8 transcription (47). The dietary regulation of Abcg5/8 is largely unknown. Cholesterol feeding of rats reduces Abcg5/8 expression whereas it increases the expression in mice (48). Recently it was shown that high-fructose feeding did not affect Abcg5/8 expression in mice (49).

1.3 BILE ACID METABOLISM

The liver plays an important role in maintaining the cholesterol homeostasis in the body. Hepatic cholesterol is either secreted directly into bile by ABCG5/8 (22), or after conversion into BAs that are actively secreted into bile via the ABC transporter bile salt export pump (BSEP) (50). Approximately 500 mg of cholesterol is converted into BAs each day in the human liver (51). The bile is stored and concentrated in the gallbladder and released into the small intestine after food intake to facilitate absorption of fat and fat-soluble vitamins (52). Most of the BAs (>95%) are absorbed in the ileum in the enterohepatic circulation of BAs (52). The BA pool size is 2-3 g and cycles approximately 10 times each day (53). See Figure 2 for a summary of the enterohepatic circulation of cholesterol and BAs.

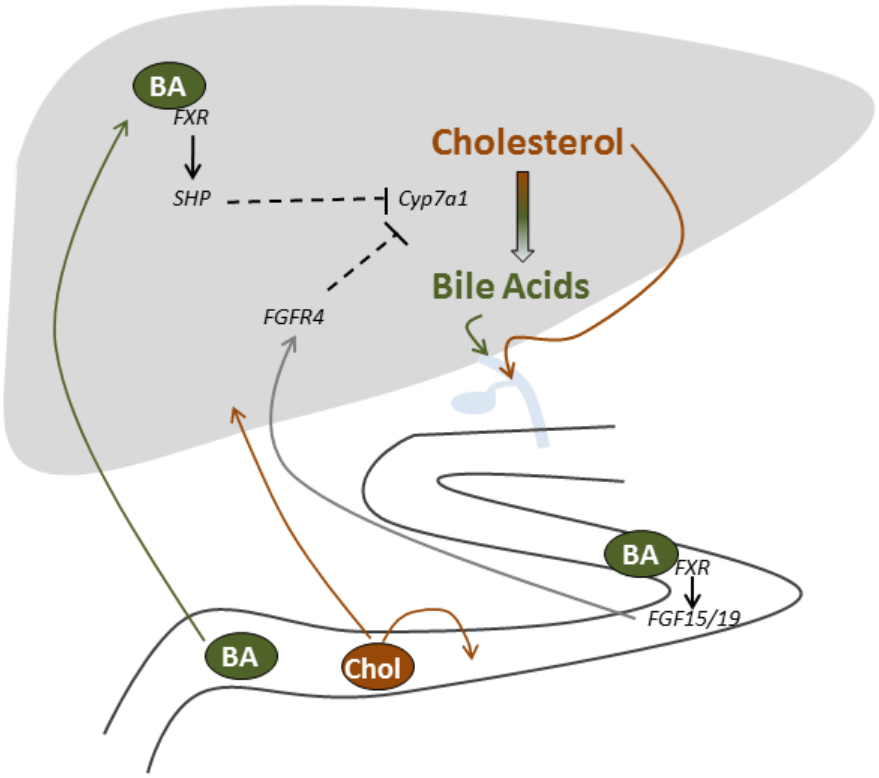

Figure 2. *Enterohepatic circulation of cholesterol and bile acids and the regulation of bile acid synthesis by the FXR-FGF15/19 pathway. Chol = cholesterol.*

1.3.1 Bile acid synthesis

Hepatic synthesis of primary BAs from cholesterol requires 17 different hepatic enzymes (51) and takes place in ER, cytoplasm, mitochondria and peroxisomes (52) (Figure 3). There are two main pathways for BA synthesis, the classic (neutral) and the alternative (acidic) pathway (54). Their relative contributions are difficult to estimate, however, the classic pathway is considered to be the main pathway in humans (54). The alternative pathway starts with 27-hydroxylation, mainly taking place in the liver and to a lesser extent in extra-hepatic tissues, creating intermediates that are transported to the liver for the latter steps. The product of the alternative pathway is chenodeoxycholic acid (53). The brain also contributes to BA synthesis with 24-hydroxylation (54). The first, and rate-limiting, enzyme in the classic pathway of BA synthesis is cholesterol 7α-hydroxylase (CYP7A1) (52). The product after the following enzymatic step in the BA synthetic pathway is 7α-hydroxy-4-cholesten-3-one (C4) which can be monitored in plasma and serve as a marker for BA synthesis (55). After this step, the classic biosynthetic pathway of BAs is divided into two sub-pathways resulting in the formation of chenodeoxycholic acid or cholic acid (52), the latter through the action of sterol 12α-hydroxylase (CYP8B1) (54). Rodents additionally produce α-muricholic acid and β-muricholic acid from chenodeoxycholic acid (53). Furthermore, before secretion into bile, almost all BAs are conjugated with glycine or taurine in humans and taurine in mice, which increases their solubility (51). When the BAs pass through the gastrointestinal tract, modifications by bacterial enzymes lead to the formation of secondary BAs. In humans,

deoxycholic acid is formed from cholic acid, and lithocholic acid and ursodeoxycholic acid are formed from chenodeoxycholic acid. The additional secondary BAs produced in rodents are hyocholic acid which is formed from α-muricholic acid, and ω-muricholic acid which is formed from β-muricholic acid (53; 56).

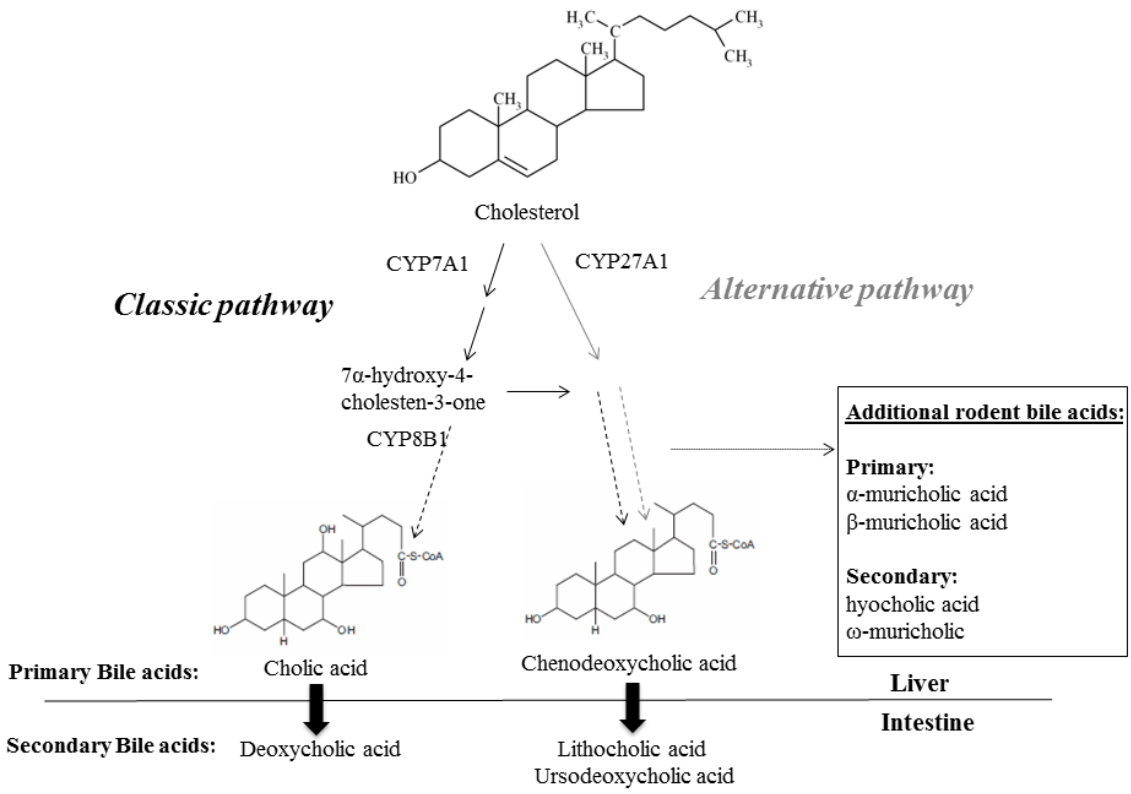

Figure 3. *Pathways for synthesis of primary and secondary bile acids.*
CYP27A1 = sterol 27-hydroxylase.

1.3.2 Regulation of bile acid synthesis

BA synthesis is largely determined by the levels and types of circulating BAs, which regulate CYP7A1 in at least two different ways (Figure 2). BAs in liver can activate the hepatic farnesoid X receptor (FXR), which forms a heterodimer with the retinoid X receptor (RXR). This heterodimer binds DNA response elements in target genes and upregulates their transcription. One of these is small heterodimer partner (SHP), which inhibits Cyp7a1 expression by reducing the activity of liver receptor homolog 1 (LRH-1) (57) and HNF-4α (58). Additionally, BAs in the intestine bind intestinal FXR (59) and upregulate the transcription of fibroblast growth factor 15 (Fgf15; human orthologue FGF19) (60). Presumably, FGF15/19 is subsequently transported to the liver where it represses the transcription of Cyp7a1 (60; 61). FGF15/19 exerts its repression of Cyp7a1 by binding FGF receptor 4 (FGFR4) (60; 62) together with βKlotho (63), which partly represses FGF15/19 by c-Jun N-terminal kinase (JNK) signaling (61).

While most BAs are FXR agonists, as outlined above, it has recently been shown that mouse muricholic acids and ursodeoxycholic acid are FXR antagonists, thereby stimulating BA synthesis when agonistic BAs such as chenodeoxycholic acid are present (64; 65).

In rodents, LXRα which is activated by oxysterol metabolites of cholesterol, forms a dimer with RXR which binds the promoter of Cyp7a1 and upregulates its expression (66). However, humans lack the LXR response element in the CYP7A1 promoter, and instead, LXRα induces SHP expression by binding its promoter, thereby repressing CYP7A1 (67).

Peroxisome proliferator-activated receptor alpha (PPARα) agonists such as fibrates decrease the expression of Cyp7a1 in rats and mice, possibly through antagonizing the effect of HNF4-α on the Cyp7a1 promoter (51). Furthermore, bezafibrate reduces the activity of CYP7A1 in humans (68).

Diet and nutritional status also regulates BA synthesis. Dietary fat increases BA synthesis in rats (69), although in humans, both extremely high and low intakes of fat reduces BA synthesis (70). Furthermore, glucose treatment of human hepatocytes stimulates CYP7A1 expression. This has been suggested to be mediated by glucose-mediated inhibition of AMP-activated protein kinase (AMPK) activity which increases HNF-4α transactivation of CYP7A1, and by epigenetic modifications of CYP7A1 by glucose (71). Insulin treatment of human and rat hepatocytes has been shown to either increase or reduce CYP7A1 expression (72-74). In human hepatocytes, insulin mediates phosphorylation and inhibition of forkhead box protein O1 (FOXO1). This leads to recruitment of HNF-4α to CYP7A1 and to increased HNF-4α/peroxisome proliferator-activated receptor gamma coactivator-1-α (PGC-1α) transactivation, both of which stimulate CYP7A1 expression (72). In contrast, in the rat, activated FOXO1 stimulates Cyp7a1 expression, through interaction with its binding site on the Cyp7a1 promoter (lacking in the human gene) (72). Prolonged insulin treatment of human hepatocytes increases the mature form of sterol regulatory element-binding protein 1 (SREBP-1)c, which blocks HNF-4α/PGC-1α interaction, thereby inhibiting CYP7A1 (72).

In mice, fasting stimulates Cyp7a1, through PPARα (75) and/or PGC-1α (76; 77). In humans (78) and rats (79) the opposite is found, fasting reduces BA synthesis.

1.3.3 Bile acid transporters in liver and intestine

Na+-dependent taurocholic cotransporting polypeptide (NTCP) is the main transporter responsible for the transport of BAs into the hepatocyte, however, organic anion transporting polypeptides (OATPs) also participate (50). Excretion of BAs from the hepatocyte is a crucial step in bile formation and is mediated by BSEP and multidrug resistance-associated protein 2 (MRP2) (50). The main protein responsible for absorption of luminal BAs in the terminal ileum is the intestinal bile acid transporter (IBAT), but OATPs may also contribute to some extent. Furthermore, deconjugated BAs are absorbed by passive absorption in the small and large intestine. When in the enterocyte, ileal bile acid-binding protein (IBABP) transports the BAs to the basolateral membrane where the dimeric organic solute transporter (OST)α/β transports the BAs out of the enterocyte (50). See Figure 4.

The level of BAs affects the expression of the BA transporters via FXR and SHP signaling. This leads to reduced cellular uptake and increased efflux, i.e. NTCP and IBAT are inhibited whereas BSEP, MRP2, OSTα/β and IBABP are stimulated (50).

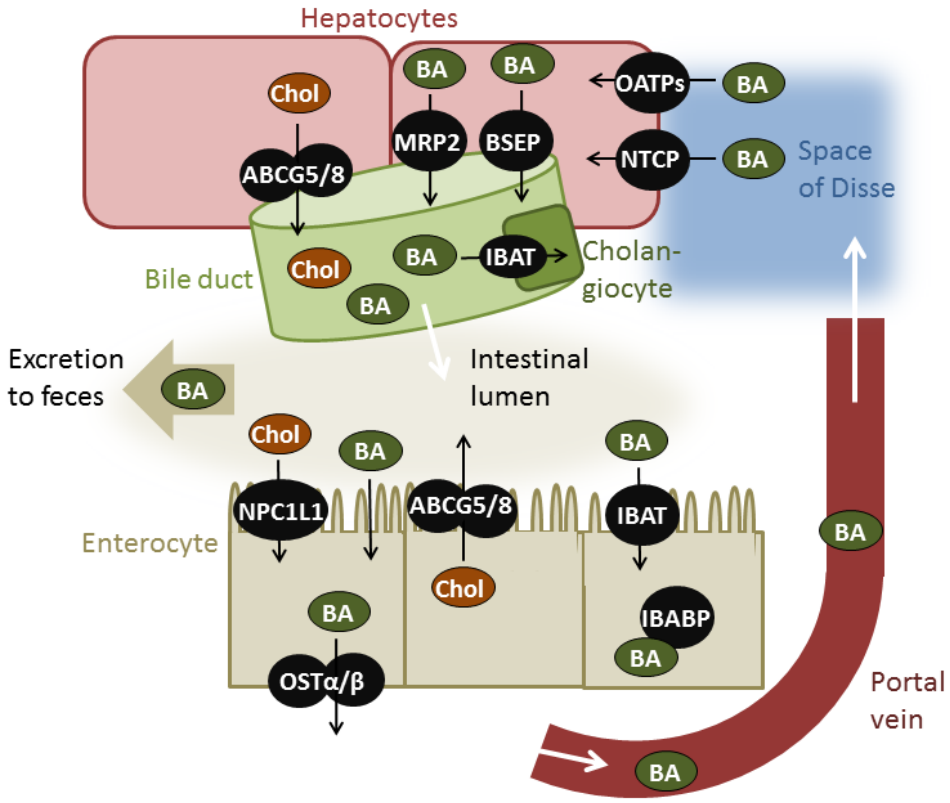

Figure 4. *Bile acid and cholesterol transporters in the enterohepatic circulation. Chol = cholesterol. Inspired by a figure by Alrefail and Gill (50).*

1.3.4 IBAT

As mentioned above, in mice, IBAT is the most important, and maybe the only, transporter in the absorption of BAs from the intestinal lumen (80). IBAT transports BAs across the apical membrane of the enterocyte which requires cotransport of sodium ions. IBAT is also known as Slc10a2 and apical sodium dependent bile acid transporter (ASBT) (81). Genetic removal of IBAT in mice dramatically increases fecal BA excretion. BA synthesis is strongly stimulated although this cannot compensate for the extensive fecal BA loss, as evident from a diminished BA pool. Furthermore, hepatic cholesterol is reduced (80). IBAT is also present in cholangiocytes and in proximal tubular cells in the kidney where it facilitates the absorption of BAs, together with OSTα/β. IBAT expression is reduced by both FXR and FGF15/19 signaling, while it is upregulated via the glucocorticoid receptor, the vitamin D receptor and PPARα (81).

1.4 INTERSTITIAL FLUID

Interstitial fluid (IF), together with the extracellular matrix, comprises the interstitial space, i.e. the space located outside the blood and lymphatic vessels and parenchymal cells (Figure 5). The volume of IF is about 20% of the body weight, which equals three times the blood

volume. The formation and composition of IF depends on the properties of the capillary wall, the hydrostatic pressure and the protein concentrations in plasma and IF (82). Lipoproteins (LDL and HDL) in serum diffuse passively to IF (83). The transport of molecules from IF to serum is either directly via the capillaries or via lymph. The extent of transport through each system is size dependent, where larger particles such as lipoproteins are expected to mainly be transported via lymph (84; 85).

Since IF is in close proximity to all cells in the body, the study of lipoproteins and other molecules in this fluid is highly relevant for understanding the physiology and pathophysiology of the human body. However, there are technical difficulties in collecting IF which makes it difficult to study. One option is to study prenodal lymph, which is relevant due to its close connection to IF. However, lymph can only be considered to be representative if it is collected directly from the organ of interest (82). The other relevant option for IF lipoprotein studies in humans is the study of suction-blister fluid, as in this thesis. A mild suction pressure is applied to the skin of the abdomen which separates epidermis from dermis and creates fluid filled blisters (82). This fluid has been found to be representative of whole body IF. However, the risk of hyperfiltration of proteins and local inflammation should be kept in mind (82).

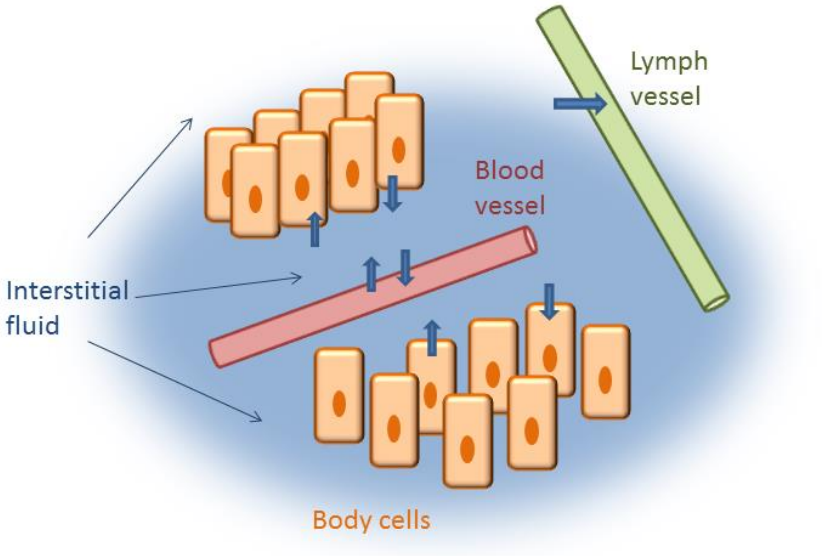

Figure 5. *Schematic figure of interstitial fluid and the flow (indicated by thick arrows) between interstitial fluid, blood, cells and lymph.*

1.4.1 IF lipoproteins

The current knowledge of IF lipoproteins has recently been reviewed (86) and is highlighted in this thesis as a related publication (R1). The most relevant aspects in relation to this thesis are also highlighted below.

IF lipoproteins are important to study since a considerable part of the lipoprotein metabolism takes place in IF, e.g. the interaction of LDL with the LDLR and the initiation of reverse

cholesterol transport. This fluid is also in immediate proximity to the atherosclerosis process. In peripheral lymph, the level of apoB is found to be 8-9% of the serum level, and apoAI is 12-21%. In abdominal suction-blister fluid, the corresponding values for apoB and apoAI are 16-20% and 23-28%, respectively. Cholesteryl ester and TG levels in suction-blister fluid is 21-24% of the serum level. Cholesterol levels in different lipoprotein fractions have also been studied and the levels in IF were found to be 18, 19 and 25% for VLDL, LDL and HDL cholesterol, respectively. This pattern is in agreement with other studies finding a relationship between percentage of serum level in IF and particle/molecular size of different solutes (86).

The role of IF in HDL metabolism has been more studied than the IF metabolism of apoB-containing lipoproteins. IF is suggested to play an important role in reverse cholesterol transport as small lipid-poor HDLs (pre-β) are generated by remodeling of αHDL in this fluid *in vitro*. This is explained by a low esterification rate and high PLTP activity (85). These particles are proposed to acquire cholesterol within the IF, after which they are transported via lymph to the bloodstream. In plasma, LCAT esterifies the cholesterol resulting in the formation of spherical αHDL (84; 85; 87; 88).

1.5 TYPE 2 DIABETES

Type 2 diabetes (T2D) is a state of insulin resistance and a relative insulin secretory defect leading to increased levels of blood glucose. Risk factors for T2D are age, obesity, family history, physical inactivity, consumption of red and processed meat, sugar-sweetened beverages, and reduced intake of fruit and vegetables. T2D often leads to microvascular and macrovascular complications (retinopathy, neuropathy and nephropathy; and ischemic heart disease, stroke and peripheral vascular disease; respectively). Furthermore, T2D increases premature mortality (89).

1.5.1 Lipoprotein metabolism in T2D

The lipid profile of T2D patients generally presents increased VLDL, reduced HDL cholesterol, and an increased number of small dense LDL particles. However, the LDL cholesterol concentration is less often increased (90; 91). VLDL is increased because of increased production by the liver. Possible mechanisms for this are that reduced insulin signaling increases apoB and MTP expression and increases hepatic FA and TG production (92). The small dense LDL present in T2D is a consequence of diminished lipolysis and long residence time of large VLDL in the circulation that allow for increased CETP action. This leads to TG-enriched but cholesterol-depleted LDL particles. By the action of HL, which is stimulated in T2D, small dense LDLs are formed (93). In T2D, increased CETP action also leads to TG-enriched and cholesterol-depleted HDL which are hydrolyzed by HL resulting in small HDLs and free apoAI (93). Also, the capacity for cholesterol efflux via HDL is reduced in T2D (94). This is partly dependent on reduced ABCA1 expression and efflux capacity in T2D patients (95). Reduced SR-BI may also contribute as it is suppressed by hyperglycemia *in vitro* (96). Glycation and oxidation of HDL also contributes to insufficient HDL function

(94). Moreover, the anti-inflammatory and anti-oxidative properties of HDL are reduced in T2D (94).

1.5.2 Cardiovascular disease in T2D

T2D patients display a 3-fold increased risk of developing cardiovascular disease (97). This is caused by several mechanisms, of which hyperglycemia, hyperlipidemia and inflammation are particularly important (98). The hyperglycemia affects several steps in atherosclerosis progression. Hyperglycemia increases the expression of inflammatory genes, which can, for example, lead to increased adhesion of monocytes to endothelial cells. Both hyperglycemia *per se* and the advanced glycation end-products (AGEs) formed as a consequence of the hyperglycemia, activate circulating monocytes. This increased inflammatory state also increases the oxidative stress. The increased oxidative stress and the increased AGE formation both contribute to the modifications of LDL. Thus the lipid accumulation in macrophages increases in T2D. Moreover, both hyperglycemia and AGEs stimulate the proliferation of vascular smooth muscle cells. The generation of proteoglycans and accumulation of collagen is also increased which contributes to increased LDL retention by the artery wall (98). The altered lipid profile in T2D with increased TG-rich lipoproteins and their remnants acts pro-inflammatory on endothelial cells and macrophages in addition to their contribution to increased lipid accumulation in macrophages. T2D patients also display increased levels of FFAs, which affect the smooth muscles cells to increase the retention of lipoproteins. As a consequence of their smaller LDL particle size, T2D patients have an increased number of LDL particles at a given LDL cholesterol concentration compared to non-diabetics. *In vitro*, smaller LDL particles more rapidly enter the vascular wall, cause greater production of pro-coagulant factors, are more easily oxidized, and are better retained by proteoglycans in the arterial wall. However, it is not certain if these smaller particles are also more atherogenic *in vivo,* or if the increased number is the important factor. The low HDL levels and an abnormal HDL composition in T2D causes a reduced reverse cholesterol transport and a reduced anti-inflammatory capacity of HDL (98). In addition to the increased inflammatory state in the vascular wall, the increased inflammation in adipose tissue also requires attention as it is also implicated in atherosclerosis. Inflammatory cytokines released from adipose tissue act on the liver to release circulating pro-inflammatory molecules. These may affect the vessel wall and the function of HDL (98).

1.6 FIBROBLAST GROWTH FACTOR 21 AND ITS METABOLIC EFFECTS

Fibroblast growth factor 21 (FGF21) is an atypical member of the FGF family since it, like FGF15/19, circulates and may function as a hormone. FGF21 is expressed in multiple tissues including liver, brown adipose tissue, white adipose tissue and pancreas, however, all circulating FGF21 originates from the liver. Hepatic FGF21 is induced by fasting via PPARα. FGF21 stimulates ketone production, hepatic FA oxidation and glucogenesis, and suppresses lipogenesis. The level of FGF21 is increased in obese rodents and humans which may reflect a state of FGF21 resistance (99). Administration of FGF21 to *ob/ob* mice or diabetic rhesus monkeys reduces plasma glucose and TGs (100; 101). The findings in monkeys also include

reduced LDL and increased HDL (101). Furthermore, FGF21 has been shown to reduce body weight as a result of increased energy expenditure and physical activity levels, to reduce hepatic TG levels due to reduced mature SREBP-1 protein and its target genes, and to improve insulin sensitivity (102). When a FGF21 analog was given to obese T2D patients, HDL cholesterol was increased, whereas LDL cholesterol and total TGs as well as body weight and fasting insulin were reduced (103).

1.7 GROWTH HORMONE AND ITS METABOLIC EFFECTS

The secretion of growth hormone (GH) from the anterior pituitary gland is stimulated by hypothalamic growth hormone-releasing hormone (GHRH) and ghrelin, and is inhibited by somatostatin. GH increases the level of insulin-like growth factor 1 (IGF-1) secreted from liver, and locally active IGF-1 in peripheral tissues. IGF-1 mediates negative feedback on GH secretion by modulating GHRH and somatostatin. The bioavailability of IGF-1 is regulated by its binding to several transport proteins in serum. GH is released in a pulsatile fashion, which bursts during night. GH secretion is increased by hypoglycemia and starvation, whereas it is reduced by hyperglycemia and FFAs. One important function of GH is to regulate linear growth together with IGF-1. GH also functions to increase resting energy expenditure and fat oxidation. Furthermore, GH increases insulin resistance and causes hyperglycemia (104). During fasting, GH increases lipolysis and lipid oxidation, and causes insulin resistance which reduces glucose oxidation, thus resulting in protein sparing as gluconeogenesis from amino acids is reduced (105).

GH deficiency leads to increased LDL cholesterol, and in women, also decreased HDL cholesterol. Furthermore, these patients often have impaired glucose tolerance due to increased adiposity. When substituted with GH, blood lipids are improved while there are mixed results on glucose tolerance (104).

1.8 DIETARY SUGARS AND ITS METABOLISM

Most dietary sugars are in the form of disaccharides, with lactose and sucrose (table sugar) being the most common. Sucrose is composed of equal numbers of glucose and fructose molecules, whereas lactose is composed of glucose and galactose. Glucose and fructose are also available as monosaccharides. After absorption, monosaccharides are transported in the portal vein to the liver, where much of the sugar is metabolized, and the remaining portion enters the circulation. In the postprandial state, most hepatic glucose is converted and stored as glycogen for later release as glucose, to supply peripheral tissues and to maintain blood glucose levels between meals, all under control of insulin and glucagon levels. The glucose which is transported to peripheral tissues is stored as glycogen (primarily in skeletal muscle) or is metabolized in the glycolytic pathway (106). In liver, fructose intermediates enter glycolysis at a later step than glucose intermediates, which is after the main rate-controlling step in glycolysis, catalyzed by phosphofructokinase. This is why fructose rapidly provides substrates for glycolysis, gluconeogenesis, glycogenesis and lipogenesis (107).

1.8.1 Metabolic effects of dietary sugars

The deleterious effects of dietary sugar on metabolic parameters, e.g. blood pressure and lipids, seen in animal studies have only partly been replicated in human studies (108). Epidemiological studies have found that sugar-sweetened beverages (fructose or sucrose), often consumed with an excess of calories, have negative effects on TGs, body weight, hepatic lipids, blood pressure, T2D risk, coronary heart disease and stroke (108; 109). The effects on body weight, TGs, cholesterol and liver TGs have been confirmed in clinical studies (108; 109). Importantly, the contributing effect of increased body weight has not totally been ruled out when it comes to the sugar-mediated effects. Whereas high fructose intake in humans (not given as sugar-sweetened beverages) is found to increase TG levels and liver lipids, body weight, glycemic control and cardiovascular disease risk is not affected (108). To summarize, dietary sugar have several deleterious metabolic effects if the intake is concomitant with excessive caloric intake or in the form of sugar-sweetened beverages.

2 AIMS

The main aim of this thesis was to investigate the interactions between the metabolism of cholesterol and BAs with that of glucose and TGs. The specific aims were as follows:

I. To investigate the effects of inhibiting the enterohepatic circulation of BAs on glucose and TG metabolism in mice.

II. To investigate the effects of dietary sugar on cholesterol and BA metabolism in rats.

III. To evaluate the effects of GH administration on circulating FGF21 levels in humans.

IV. To examine lipoproteins in IF and serum in patients with T2D and in healthy controls.

3 MATERIALS AND METHODS

A general description of the materials and methods used in this thesis follows below. For particular details regarding the methods used, see the respective papers.

3.1 ANIMALS

The studies were approved by the Ethics Committee of the University of Gothenburg (Paper I) or the Stockholm South Ethical Committee on Animal Research, Huddinge (Paper II). Rats and mice were kept under standardized conditions and the light cycle hours were between 6 a.m. and 6 p.m. Liver tissue and intestine was collected, frozen in liquid nitrogen and stored at -80°C for later analysis. Blood was collected and stored as serum or plasma.

3.1.1 Paper I, study design

In the first experiment, wild type (WT) mice, *Slc10a2+/-*, and *Slc10a2-/-* mice were fed standard chow (R3, Lactamin AB, Stockholm, Sweden). In the second experiment, WT mice and *Slc10a2-/-* mice were fed standard chow or a sucrose-rich diet (61% sucrose; D12329, Research Diets Inc, New Brunswick, NJ) together with drinking water supplemented with 10% fructose, for two weeks. In the third experiment, *ob/ob* mice were fed chow and received gavage with vehicle or a specific Slc10a2 inhibitor (AZD 7806, AstraZeneca R&D), for 11 days. Detailed compositions of the diets are given in Table 2.

3.1.2 Paper II, study design

In the first experiment, Sprague Dawley rats received either (a) a normal control diet (R36, Labfor, Lidköping, Sweden); (b) a high-sucrose diet i.e. control diet enriched with 60% sucrose, and drinking water supplemented with 10% fructose; (c) control diet supplemented with 2.5% cholestyramine (Questran Loc, Bristol-Myers Squibb, New York, NY); or (d) high-sucrose diet enriched with 2.5% cholestyramine, and drinking water supplemented with 10% fructose. At day 10, all animals were sacrificed by decapitation under Isoflurane anesthesia. In the second experiment, Sprague Dawley rats received either (a) a control diet (D11724, Research Diets Inc.) and were intraperitoneally (IP) injected with vehicle; (b) the same control diet and were given IP injections of a PPARα antagonist (GW 6471; Tocris Bioscience, Bristol, UK; 4 mg/kg body weight); (c) a controlled high-sucrose diet (65% sucrose; D11725, Research Diets Inc.) together with drinking water supplemented with 10% fructose, and were IP injected with vehicle; or (d) the same controlled high-sucrose diet and fructose water together with IP injections of GW 6471. The injections of vehicle (NaCl with 3.8% dimethyl sulfoxide [DMSO]) and GW 6471 were made in the morning on the last four days of the experiment. At day 10, all animals were sacrificed by decapitation while under Isoflurane anesthesia. In a supplemental experiment, WT mice and *ob/ob* mice were fed the same control diet or controlled high-sucrose diet and fructose-supplemented drinking water as in the second rat experiment. At day 10, all animals were anesthetized with Isoflurane, blood was collected by heart puncture and the animals were sacrificed by removal of the heart. Detailed compositions of the diets are given in Table 2.

Table 2. *Composition of the experimental diets used in Paper I and II.*

	Mouse experiment (Paper I)		**Rat experiment 1 (Paper II)**		**Rat experiment 2 (Paper II)**	
	Control diet (R3)	**Sucrose-rich diet** (D12329)	**Control diet** (R36)	**High-sucrose diet** (R36+60% sucrose)	**Control diet** (D11724)	**Controlled high-sucrose diet** (D11725)
Fat (g/kg)	50	48	40	16	50	50
Carbohydrate (g/kg)	515	743	557	823	660	660
Polysaccharide (g/kg)	496*	124	537	215	650	0
Mono-/Disaccharide (g/kg)	19*	611	20	608	0	650
Vitamin mix incl. sucrose (g/kg)		7			10	10
Protein (g/kg)	210	168	185	74	203	203
Fiber (g/kg)	35	0	35	14	50	50
Kcal/g	3.3	4.1	3.3	3.7	3.9	3.9

** Estimated amounts of polysaccharides and mono-/disaccharides, calculated from the amounts in the R36 diet (3.6% of carbohydrates are mono-/disaccharides).*

3.2 HUMANS

Written informed consent was obtained at inclusion in all studies. The studies were approved by the regional ethics committees at the Karolinska Institute (Paper III and IV) and the University of Aarhus (Paper III), and by the regional ethics review board in Stockholm (Paper IV). Studies were conducted in accordance with the Declaration of Helsinki.

3.2.1 Paper III, study design

Replacement therapy with GH to GH-deficient patients was studied in subjects with adult-onset GH deficiency, that were treated with conventional substitution with thyroxine, hydrocortisone, vasopressin or sex steroids for >2 years but not with GH. Subcutaneous (SC) injections of GH were made daily at bedtime for 1 month with 0.10-1.13 mg GH after which doses were titrated to reach normalized IGF-1 levels. Blood samples were collected in the morning after an over-night fast, at baseline and after 1, 3, 6 and 12 months of replacement therapy. The effects of acute GH administration were studied in healthy men in a randomized study (110). On separate days, subjects were given either (a) an intravenous (IV) injection of 0.5 mg GH; (b) an IV saline injection in combination with an oral glucose load (75 g); or (c) an IV injection of GH in combination with an oral glucose load. Blood was collected before and repeatedly after interventions, where samples collected before and 0.5, 1 and 3 hours after interventions were available for analysis in the present paper. The dose-response effects of GH were studied in young and old healthy men and in males with heterozygous familial hypercholesterolemia (111). Subjects were given daily SC injections of GH at bedtime for 3 weeks, with increasing doses every week (8.3, 17.0 and 33.0 µg/kg/d). Blood samples were collected each week after an overnight fast. The results from the three subject groups were pooled and presented together since there were no differences in FGF21 response between the groups.

3.2.2 Paper IV, study design

In a pilot study to evaluate the role of statin treatment on the transvascular gradient of LDL cholesterol, patients with peripheral vascular disease and hypercholesterolemia were studied. The patients were recruited from the Department of Surgery, Karolinska University Hospital Huddinge, where they were treated for intermittent claudication. The patients were randomized in a double-blind, cross-over fashion to receive either 320 mg of aspirin with placebo or 320 mg of aspirin with 80 mg of atorvastatin (Lipitor®, Pfizer), once daily. The treatments were given for four weeks each, separated by a four week wash-out period when only aspirin was administered. In this pilot study, IF and serum was collected in the fasting state in the same way as in the main diabetes study (see below).

Thirty-five T2D patients were recruited from the outpatient clinic of the Department of Endocrinology, Metabolism, and Diabetes, Karolinska University Hospital Huddinge. Thirty-five age- and gender-matched healthy controls were recruited via local advertisements, and their health was confirmed by a health check-up prior to inclusion in the study. Exclusion criteria for the healthy controls were abnormal fasting glucose, HbA1c, insulin, AST, ALT,

cystatin C, TGs, total cholesterol, HDL cholesterol, LDL cholesterol, hemoglobin and blood pressure, the presence of inflammatory or thyroid disease, or treatment with oral glucocorticoids. The exclusion criteria for the T2D patients were inflammatory disease or treatment with oral glucocorticoids. The number of subjects recruited was calculated to detect a 25% increase of the IF-to-serum gradient for LDL cholesterol in T2D patients with 95% power at a significance level of 0.05. IF was collected as described below and blood samples were taken 1 hour after the start of the suction-blister generation.

3.3 INTERSTITIAL FLUID COLLECTION

IF was collected by the suction-blister technique (112) after an overnight fast (Paper IV). Two plastic chambers (Ventipress Oy, Lappeenranta, Finland) were placed on the skin, five centimeters bilaterally of the umbilicus. A mild suction pressure (28-32 kPa) was applied for 1.5-2 h during which five fluid-filled blisters were formed underneath each chamber. The plastic chambers were removed and the IF was aspirated with a syringe. If there were signs of damage on the underlying tissue, i.e. reddish color of the fluid, the fluid was discarded. IF was stored at -80°C until later analysis.

3.4 SERUM AND IF ANALYSIS

Peripheral blood samples were stored as whole blood, serum, or plasma (as indicated in each paper) at -70°C or -80°C until analysis. All measurements in serum and IF were made in duplicates unless otherwise stated.

3.4.1 Apolipoprotein analysis

ApoAI and apoB in serum and IF were analyzed by enzyme-linked immunosorbent assays (ELISAs) from Mabtech (Nacka strand, Sweden) (Paper IV). A Lp(a) ELISA from Mercodia (Uppsala, Sweden) was used for Lp(a) analysis (Paper IV). For details regarding dilutions of serum and IF, see Paper IV.

3.4.2 Serum FGF21 assay

Serum FGF21 was analyzed using an in-house ELISA developed by Eli Lilly and Company (Indianapolis, IN) or a commercially available ELISA from R&D systems (Minneapolis, MN) (Paper III).

3.4.3 Serum/plasma lipid analysis

Non-esterified fatty acid (NEFA) levels were analyzed with an immunoturbidimetry reagent from DiaSys Diagnostic Systems (Holzheim, Germany) (Paper III) or by colorimetric reagents from Wako chemicals (Neuss, Germany and Richmond, VA) (Paper I and III). Total cholesterol and TG was determined using colorimetric reagents (Instrumentation Laboratory Scandinavia, Gothenburg, Sweden; and Roche Diagnostics, Mannheim, Germany), see Paper I and II for details.

3.4.4 Fast protein liquid chromatography (FPLC)

Concentrations of lipoprotein cholesterol, TGs (Paper I and IV) and free cholesterol (Paper IV) in serum, and in IF (Paper IV) were measured by FPLC. Lipoproteins were separated by size on a column. Reagents (cholesterol and TGs: Roche Diagnostics; and free cholesterol: Wako Diagnostics, Richmond, VA) were continuously added to the eluate on-line followed by measurements of absorbance. In Paper IV, the respective concentrations in the fractions were calculated from the area under the curve after integration of the individual chromatograms. For each analyte, serum samples from all study subjects were run in the same series, as were the IF samples. Samples were run once.

3.4.5 7α-hydroxy-4-cholesten-3-one (C4) assay

The marker for BA synthesis, C4 (55), was extracted from individual serum samples (Paper I and II) or pooled samples (Paper I) and analyzed by high-performance liquid chromatography (HPLC) (single samples). For details see Paper II.

3.4.6 Glucose and insulin analysis

Total plasma glucose was analyzed using the IL Test (Instrumentation Laboratory Scandinavia). Blood glucose was determined using a Bayer Elite glucometer (Bayer diagnostics, Germany). Plasma insulin levels were analyzed using a rodent insulin radioimmunoassay kit (Linco, St. Charles, MI). (Paper I).

3.5 TISSUE ANALYSIS

3.5.1 Quantitative real-time reverse transcriptase polymerase chain reaction (PCR) assay of mRNA

Total RNA was extracted from individual liver and intestinal samples using Trizol reagent (Invitrogen, Carlsbad, CA), and was transcribed to cDNA by random hexamer priming and Omniscript (Qiagen, Valencia, CA) (Paper I and II). Quantitative real-time PCR was run on cDNA in triplicates using an ABI prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA) following guidelines for SYBR Green assay (Paper II) or by TaqMan (Paper I). Data were normalized to expression of a housekeeping gene in the same preparations. The comparative Ct method was used to quantify the results.

3.5.2 Immunoblotting and ligand blot of liver proteins

In Paper I, a ligand blot using ^{125}I-labeled rabbit β-VLDL as the probe was used to detect LDLRs in liver membranes, as described previously (113). Western immunoblot technique was used to detect hepatic SR-BI, CYP7A1 and SREBP-1c, and phosphorylated and total levels of protein kinase B alpha (AKT1), mitogen-activated protein kinase 3/1 (ERK1/2) and mitogen-activated protein kinase kinase 1/2 (MEK1/2) (Paper I). SREBP1 was detected in cytoplasmic and nuclear protein preparations, prepared as described in Paper I. Total liver homogenates were prepared for analysis of phosphorylated proteins as described in Paper I. Microsomal samples were prepared for detection of CYP7A1 as previously described (114).

For SR-BI analysis, liver membranes were prepared as described (113). For a detailed protocol for SR-BI, see Galman C *et al.* (115); for CYP7A1, see Lundasen T *et al.* (116); and for SREBP-1c, and phosphorylated and total levels of AKT1, ERK1/2 and MEK1/2, see Paper I.

3.5.3 Liver lipid analysis

Liver lipids were extracted according to Folch *et al.* (117). In Paper II, liver cholesterol content was determined using gas chromatography-mass spectrometry (GC-MS) analysis, and expressed per mg liver protein. In Paper I, liver cholesterol and TG contents were determined with colorimetric reagents. The liver extractions were performed once per liver while the final analyzes were performed in duplicates. For detailed protocols see each respective study.

3.5.4 Enzymatic activities

Enzyme activities of HMG-CoA reductase and of CYP7A1 were assayed in hepatic microsomes from pooled samples, as described by others (114; 118) (Paper I).

3.6 STATISTICS

Data are given as means ± SE or means ± SD, as indicated. Graph Pad Prism software (GraphPad Software Inc., La Jolla, CA) was used for statistical analysis. See each paper for details on the used methods.

4 RESULTS AND COMMENTS

4.1 INHIBITION OF IBAT IMPROVES GLUCOSE AND TG METABOLISM IN MICE (PAPER I).

It was hypothesized that interruption of the enterohepatic circulation of BAs would induce BA synthesis and thereby increase cholesterol synthesis from acetate. A reduced acetate pool could improve TG metabolism by lowering of substrates needed for its formation. Plasma TGs were reduced by 35% in the *Slc10a2-/-* mice, whereas the levels of plasma glucose and liver TGs were unaltered. As expected, *Slc10a2-/-* mice had an increased BA synthesis, evident from increased Cyp7a1 mRNA, protein and enzymatic activity, and increased serum C4 levels. We found that serum C4 levels and enzymatic activity of CYP7A1 were not increased in parity with the mRNA levels of Cyp7a1. We therefore hypothesized that a state of substrate deficiency was present in these animals. Therefore, to provide more substrate, these mice were fed a sucrose-enriched diet. In contrast to our hypothesis, feeding *Slc10a2-/-* mice the high-sugar diet reduced hepatic Cyp7a1 mRNA and serum C4 levels (Figure 6), showing that this diet normalized the increased BA synthesis in these animals. Furthermore, *Slc10a2-/-* mice displayed a blunted increase in liver TG compared to WT mice. This was paralleled by lower levels of the mature form of SREBP-1c and mRNA levels of a set of lipogenic genes in *Slc10a2-/-* mice fed the sucrose diet compared to the WT mice fed the sucrose diet. However, there was no difference in plasma glucose or TG responses between WT and *Slc10a2-/-* mice.

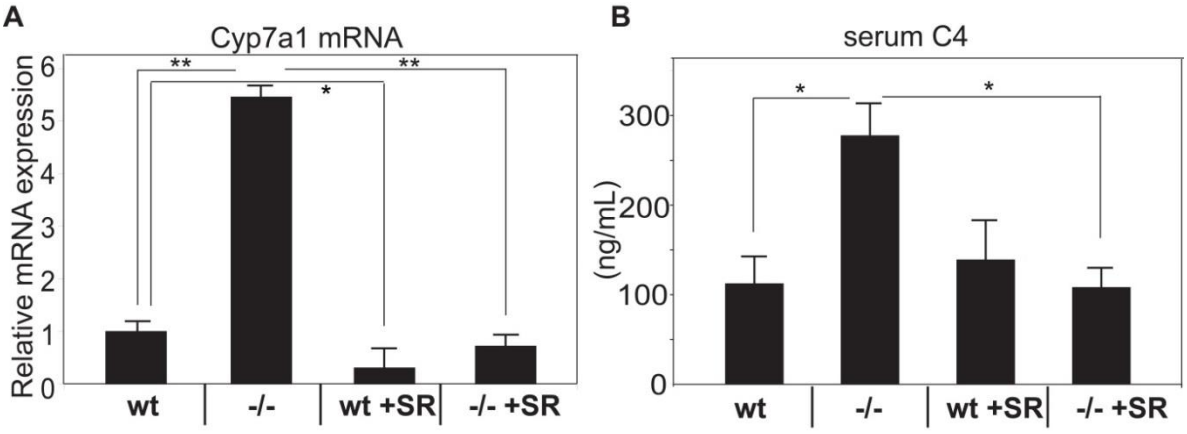

Figure 6. A) *Hepatic mRNA levels of Cyp7a1 and* ***B)*** *serum C4 levels, in WT and Slc10a2-/- mice fed regular chow or a sucrose-rich (SR) diet. Data are expressed as mean ± SE. mRNA expression was related to hypoxanthine guanine phosphoribosyl transferase and the data for the WT group on regular chow is normalized to 1. *, P<0.05; ** P<0.01 (Student's t test).*

To further evaluate the role of IBAT inhibition on glucose and TG metabolism, the effect of this inhibition was studied in a mouse model with elevated plasma glucose and TG levels. Therefore, *ob/ob* mice were treated with an inhibitor of IBAT. This treatment reduced blood glucose and plasma TG levels (Figure 7), whereas the hepatic TG levels were unaltered. At

the same time, the hepatic mRNA levels of Srebp-1c and a set of lipogenic genes were reduced. The results show that inhibition of IBAT is a promising drug target.

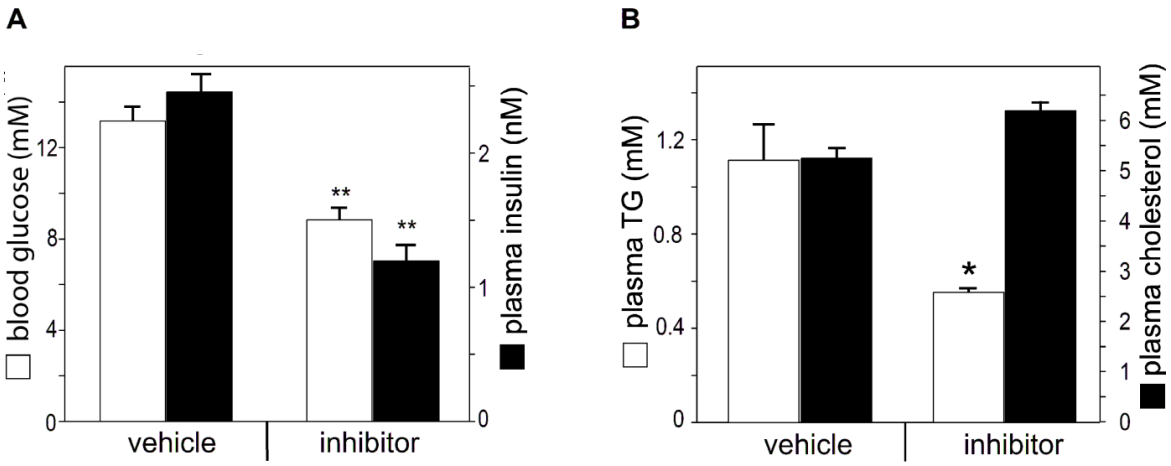

Figure 7. A) *Fasting levels of blood glucose and plasma insulin and* ***B)*** *plasma total TGs and cholesterol, in ob/ob mice treated with a specific Slc10a2 protein inhibitor, (AZD7806), or control vehicle. Data are represented as mean ± SE. *, P<0.05; ** P<0.01 (Student's t test).*

The mechanism behind the reduced BA synthesis by the sucrose-enriched diet was not fully explored in the paper. However, the concomitant increase in Fgf15 mRNA in the distal ileum by the high sugar diet suggests that this may be an important reason for the reduction of BA synthesis. Importantly, when WT mice were fed the high-sugar diet, Cyp7a1 mRNA was reduced while serum C4 was unaltered (Figure 6). This indicates that a high-sugar diet might only suppress BA synthesis when the basal synthesis is increased. Importantly, the high-sugar diet contained no fiber in contrast to the control diet with normal fiber content (Table 2). This difference may also contribute to the suppression of BA synthesis, as discussed in more detail in the sections about Paper II.

4.2 INFLUENCE OF DIETARY SUGAR ON CHOLESTEROL AND BILE ACID METABOLISM IN THE RAT (PAPER II)

Since Paper I showed that BA synthesis is clearly reduced by sugar only when given to mice with stimulated BA synthesis, the first experiment of this study was designed using normal rats and rats with stimulated BA synthesis (i.e. cholestyramine treated) fed a high-sucrose diet. This diet reduced BA synthesis, as shown by reduced levels of serum C4, in both normal rats and rats treated with cholestyramine (Figure 8A), however, Cyp7a1 mRNA was only reduced in the normal rats fed the high-sucrose diet (Figure 8B). However, when feeding normal rats a controlled high-sucrose diet in the second experiment of this study, BA synthesis was not affected (Figure 9). These results indicate that it was not the increased sucrose content *per se* in the first high-sucrose diet that reduced BA synthesis, rather some other change in the diet such as a reduction in fiber or fat (Table 2). Furthermore, in contrast to the results in Paper I (Figure 6), BA synthesis was more suppressed by the uncontrolled high-sucrose diet in the normal rats than in rats with stimulated basal BA synthesis (i.e. cholestyramine treated) (Figure 8).

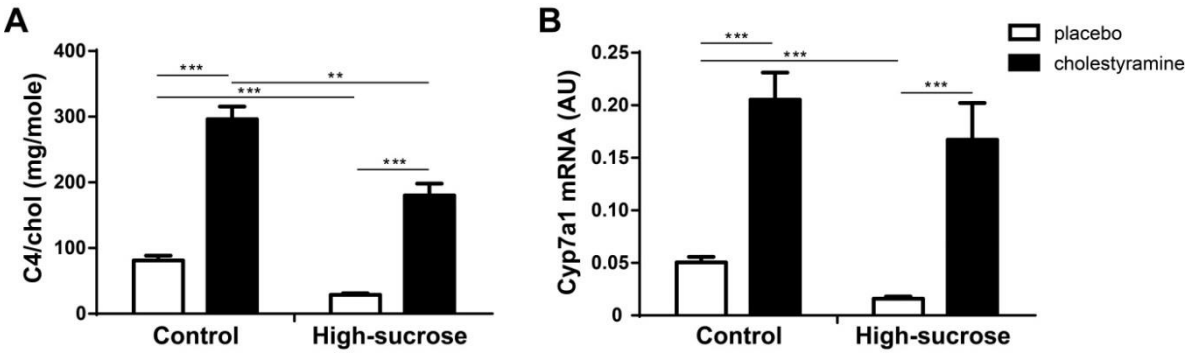

Figure 8. A) *Serum C4/cholesterol and* ***B)*** *liver Cyp7a1/β actin mRNA levels, after feeding rats a high-sucrose diet. Data are presented as means±SE. **, P<0.01; ***, P< 0.001 (Two-way ANOVA followed by Tukey's multiple comparison test).*

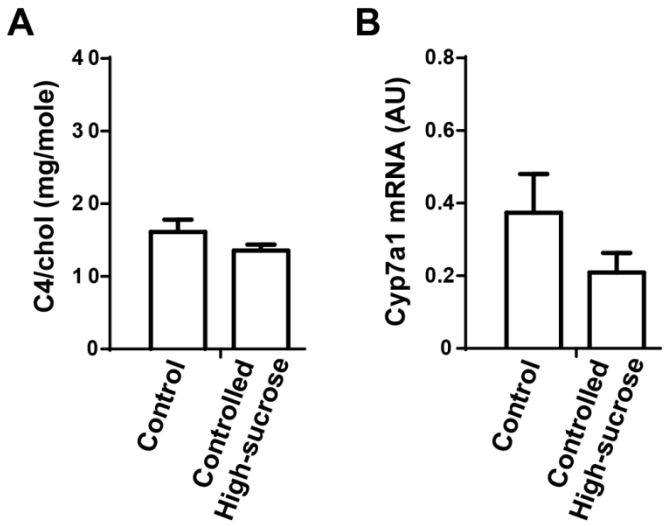

Figure 9. A) *Serum C4/cholesterol and* ***B)*** *liver Cyp7a1/β actin mRNA levels, after feeding rats a controlled high-sucrose diet. Data are presented as means±SE (Two-way ANOVA followed by Tukey's multiple comparison test).*

An important finding that was seen from both high-sucrose diets, was a very strong suppression of hepatic Abcg5/8 mRNA levels (Figure 10 and 11). In contrast, Abcg5 in proximal intestine was not affected by either of the high-sucrose diets, and Abcg8 was only modestly reduced by the high-sucrose diet in the first experiment while it was unaffected by the controlled high-sucrose diet in the second experiment. These results show that sucrose suppresses Abcg5/8 liver specifically in rats. The mechanism behind the suppression of Abcg5/8 could not be elucidated from the present data.

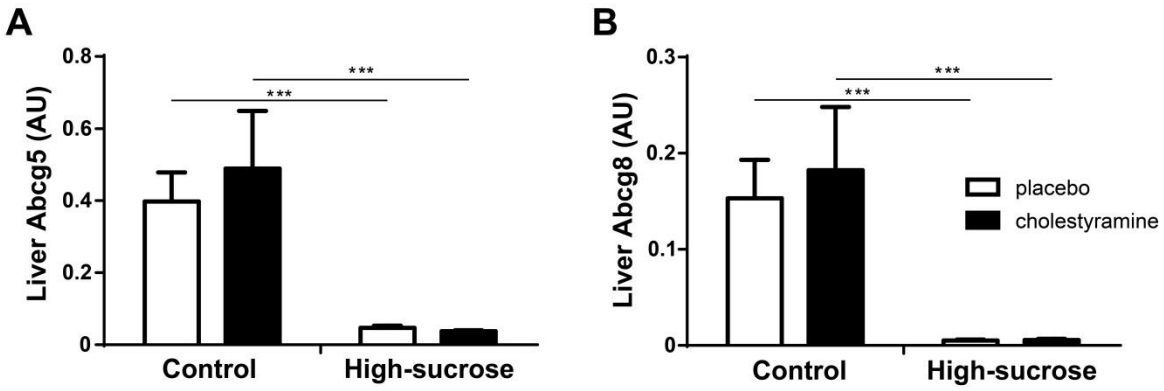

Figure 10. *Effects on **A)** Abcg5 and **B)** Abcg8 mRNA expression in liver by feeding rats a high-sucrose diet, mRNA expression was related to β actin mRNA. Data are presented as means±SE. ***, P< 0.001 (Two-way ANOVA followed by Tukey's multiple comparison test).*

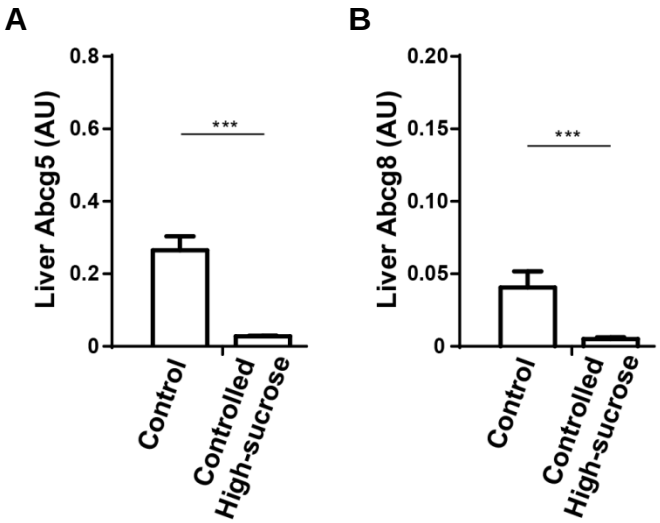

Figure 11. *Effects on **A)** Abcg5 and **B)** Abcg8 mRNA expression in liver by feeding rats a controlled high-sucrose diet, mRNA expression was related to β actin mRNA. Data are presented as means±SE. ***, P< 0.001 (Two-way ANOVA followed by Tukey's multiple comparison test).*

4.3 GH IS NOT AN IMPORTANT REGULATOR OF FGF21 IN HUMANS (PAPER III).

The GH-mediated regulation of FGF21 levels was investigated in humans, by analyzing three different studies in which GH was administered. Previous animal studies have shown that GH treatment increases hepatic mRNA expression and serum levels of FGF21 in mice (119) and hepatic mRNA expression in cattle (120). The first study in Paper III showed that GH deficiency was not associated with reduced circulating FGF21 levels (Figure 12A) in nine patients with adult-onset of GH deficiency. Furthermore, GH-replacement therapy for 12 months did not increase FGF21 levels in these patients (Figure 12A). From these results it was concluded that GH is not crucial for the maintenance of basal FGF21 levels. The acute effects of GH on FGF21 were evaluated in eight healthy men receiving a single bolus injection of GH. FGF21 levels did not increase up to 3 h after injection of GH (Figure 12B).

In addition, FGF21 levels were studied in a material where increasing doses of GH were given by daily injections. FGF21 was increased after one week's administration at the lowest dose and was progressively increased with increasing doses the two following weeks (Figure 12C). From these latter studies it was concluded that GH does not increase FGF21 levels acutely, however prolonged administration of supraphysiological doses to subjects with normal GH metabolism does increase FGF21.

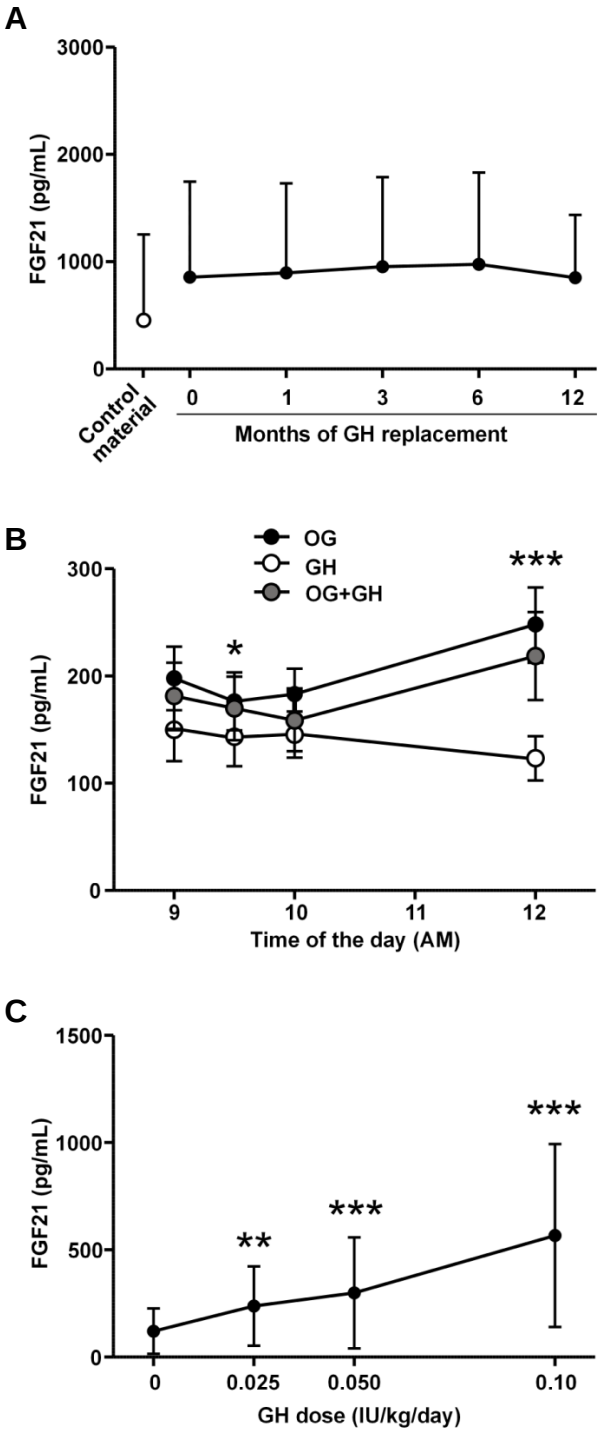

Figure 12.

A) *Serum FGF21 levels in growth hormone (GH)-deficient adult patients during GH replacement (n = 9; filled circles) and FGF21 levels in healthy control subjects (n = 76; open circle; previously reported (121)); data are means + SD.*

B) *Serum FGF21 levels in healthy volunteers (n = 8) who received an oral glucose load (OG; black circles), GH (white circles) or OG + GH (grey circles); data are means ± SE.*

C) *Serum FGF21 levels in healthy volunteers and patients with heterozygous familial hypercholesterolemia who received increasing doses of GH (n = 23), data are means ± SD.*

**P < 0.05; **P < 0.01; ***P < 0.001, compared with before GH and/or glucose administration (repeated measures ANOVA followed by Dunnett's multiple comparison test). Differences between the normal population and the baseline value in the GH-deficient patients (panel A) were tested by unpaired Student's t-test. In panel C, one value at the highest dose was excluded because it was 15 SDs higher than the mean.*

In mice, the GH-dependent stimulation of hepatic FGF21 synthesis is dependent on adipocyte lipolysis (119), whereas GH has a direct effect in the liver of cattle (120). Therefore, the circulating levels of NEFAs were analyzed in the above mentioned experiments. In the dose-response study, NEFA levels were increased, as were FGF21 levels. Consequently, a weak positive correlation between NEFA and FGF21 was found in this experiment (R_s = 0.26, P = 0.029). In the experiments where no effects were observed on FGF21 levels, there was no influence on NEFA levels. These data support the observation that the effect of supraphysiological doses of GH on FGF21 is at least in part dependent on NEFA levels.

4.3.1 Serum FGF21 is regulated by fructose and glucose (Paper III and unpublished results)

The acute effect of glucose on FGF21 was also studied in the acute GH experiment. In that experiment, FGF21 levels were increased 3 h after glucose intake (Figure 12B), which is in agreement with a previous human study (122). The levels of NEFAs were concomitantly reduced. In an unpublished study, mentioned in Paper III, it was shown that oral fructose administration similarly increases circulating FGF21 levels (Figure 13), a finding that was recently confirmed by Dushay *et al*. (123).

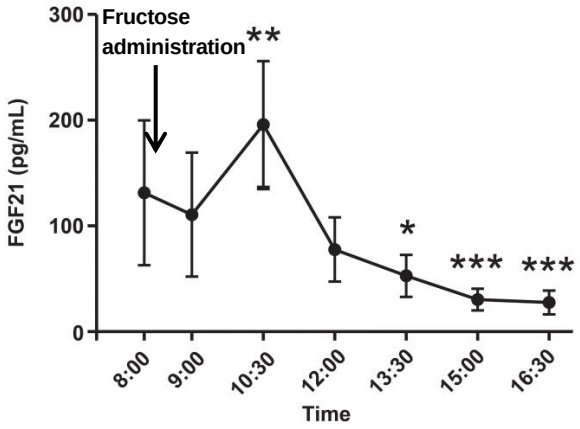

Figure 13. *Effects on circulating FGF21 by oral fructose administration in humans. Healthy subjects (n=6; age 29.1±1.3 years; BMI 20.5±0.5 kg/m^2) were studied during one day. They arrived fasted to the clinic in the morning and a baseline blood sample was taken at 8:00 a.m. Thereafter subjects were served a breakfast together with an oral fructose (75g) drink. Blood samples were taken at 9:00 a.m. and the following every 90th minutes until 4:30 p.m. Lunch was served immediately after the blood sampling at 12:00 noon. Data are presented as mean ± SE. *P < 0.05; **P < 0.01; ***P < 0.001 denotes differences from before fructose administration (Repeated measures ANOVA followed by Bonferroni's multiple comparison test, after natural log transformation of data).*

4.4 THE IF LEVELS OF APOB-CONTAINING LIPOPROTEIN PARTICLES ARE REDUCED IN T2D (PAPER IV)

Even though LDL cholesterol levels are not generally increased in T2D, it is a predictor for developing coronary artery disease and stroke in these patients (124; 125). As such, these patients undoubtedly benefit from lipid-lowering therapy (126-128). Moreover, the risk for cardiovascular events at a given concentration of LDL cholesterol is increased in T2D

compared to non-diabetics (97), for reasons still not fully understood. It has been shown that IV injected LDL particles disappear from the blood stream more rapidly in T2D patients than in healthy controls (129), presumably into the IF. Therefore, IF and peripheral serum samples were collected and analyzed in 35 T2D patients and 35 healthy controls. However, prior to the initiation of this study, the effects of statin treatment on the IF-to-serum gradient for LDL cholesterol were investigated in a crossover design. Statin treatment reduced the levels of LDL cholesterol in serum and IF by 50 and 54%, respectively. Consequently, statin treatment did not change the IF-to-serum gradients for cholesterol, indicating that the lipoprotein permeability of the vascular wall was unaltered during statin treatment.

Surprisingly, the levels of LDL and VLDL cholesterol in relation to the serum levels (IF-to-serum gradient) were reduced in T2D by 29 and 18%, respectively (Figure 14A), when compared to healthy controls. Similar reductions were shown for VLDL and LDL TGs and apoB (Figure 14B). Together with the results from Kornerup *et al.* (129), these data indicate that cholesterol may accumulate in peripheral tissues in T2D.

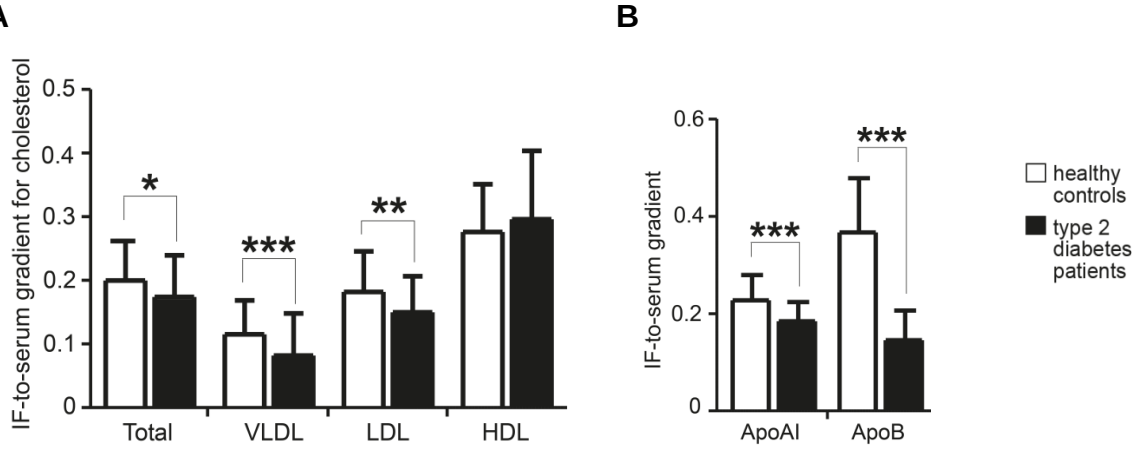

Figure 14. A) *IF-to-serum gradients for cholesterol in the different lipoproteins and* ***B)*** *IF-to-serum gradients for apoAI and apoB, in T2D patients and healthy controls. In the diabetes group, the IF-to-serum gradient for VLDL cholesterol is missing for one subject due to a zero concentration in serum which makes calculation of gradient impossible. One value for IF-to-serum gradient for apoAI in healthy controls is excluded because of being an outlier. One value for IF-to-serum gradient for apoB in healthy controls is missing. Data are presented as means±SD. *, P< 0.05; **, P<0.01; ***, P< 0.001 (Student's unpaired t-test).*

The IF-to-serum gradients for HDL cholesterol (Figure 14A) and TGs are similar in T2D patients and healthy controls, in contrast to the gradients for LDL and VLDL. However, apoAI was reduced in T2D patients when expressed in relation to their serum levels (Figure 14B). These data indicate that the accumulation of lipoproteins in peripheral tissues is specific to apoB-containing lipoprotein particles. The identification of the mechanisms behind these findings should likely increase our understanding for why atherosclerosis development is accelerated in T2D.

5 DISCUSSION

5.1 IBAT INHIBITION AS A DRUG TARGET

In Paper I it was evaluated whether interruption of the enterohepatic circulation of BAs alters glucose and TG metabolism in mice. First, it was confirmed that BA synthesis is stimulated in *Slc10a2-/-* mice, as previously shown (80). As this increased synthesis led to an increased demand for cholesterol, it was hypothesized that the increased synthesis of cholesterol should reduce the pool of acetate which in turn should reduce TGs. It was shown that plasma TGs were reduced in these animals. Surprisingly, when these mice were given a sucrose-enriched diet, in order to meet a putative deficiency of substrate for cholesterol and BA synthesis, BA synthesis was reduced. Moreover, *Slc10a2-/-* mice did not increase their liver TG levels as much as WT mice. As the sucrose-enriched diet normalized BA synthesis in these animals, an increased cholesterol and acetate consumption is unlikely to explain the reduced TG response. However, the gene expression of several lipogenic enzymes were lower in the *Slc10a2-/-* mice fed the sucrose-enriched diet, than in WT mice fed the same diet, which could explain the reduced lipogenic response in these animals. This reduced lipogenic response is opposite to what is found when administering a BA sequestrant, to diabetic mice, which also interrupts the enterohepatic circulation of BAs. Herrema *et al*. showed that liver TG levels and expression of lipogenic genes were increased in an FXR- and LXRα-dependent manner (130). FXR stimulation has been shown to reduce the expression of Srebp1c and other lipogenic genes via LXR inactivation (131). Consequently, the increased Srebp1c, shown by Herrema *et al*, in response to BA sequestrants, may be mediated by reduced FXR stimulation. However, others have shown improved hepatic lipids when treating diabetic mice with BA sequestrants (132). To further evaluate the potential of Slc10a2 inhibition in improving TG and glucose metabolism, *ob/ob* mice were treated with an inhibitor of Slc10a2. This treatment reduced serum TG and blood glucose levels. The lipogenic genes were reduced in *ob/ob* mice treated with the Slc10a2 inhibitor, as in the *Slc10a2-/-* mice fed the high-sugar diet, which could explain the reduced lipogenic response.

While editing Paper I, IBAT inhibition was reported to increase glucagon-like peptide-1 (GLP-1) and to attenuate elevated glucose and HbA1c levels in Zucker diabetic fatty rats (133). Therefore, elevated GLP-1 levels may explain the reduced blood glucose occurring during IBAT inhibition of *ob/ob* mice in Paper I. This is in line with the concept that GLP-1 improves insulin secretion, by which it may improve insulin sensitivity (134). Increased GLP-1 expression by IBAT inhibition is explained by the increased BA levels in the distal intestine (133), as delivery of bile salts to the distal intestine has been shown to stimulate GLP-1 secretion (135). The BAs activate G protein-coupled bile acid receptor 1 (Gpbar1, also known as TGR5) (136), which increases GLP-1 expression and secretion from intestinal cells (137). Moreover, as FAs in the intestine can also stimulate GLP-1 secretion (138), they are also likely mediators of the GLP-1-raising effect of IBAT inhibition, since such treatment increases the BA level in the intestine and therefore more FAs remain in the intestine. Moreover, a GLP receptor antagonist has been shown to reverse hepatic lipid accumulation in

ob/ob mice, and treatment of hepatocytes with GLP-1 reduces lipogenic gene expression (139). Thus, it is likely that GLP-1 is also a mediator of the lipid-improving effect of IBAT inhibition. In a previous study, treating diabetic rats with an IBAT inhibitor did not improve glucose handling (140). The different results between that study and Paper I may be related to the different diabetic animal models used and to the different structures and efficiencies of the IBAT inhibitors. Treatment of diabetic mice and rats with a BA sequestrant has also been shown to improve glucose handling (132; 140; 141) and GLP-1 was found to be a likely mediator of that effect (140; 141), while FXR and LXR were not (141). Studies using BA sequestrants and IBAT inhibitors are not fully comparable as they affect BA pool size and composition differently. While the BA pool size is not changed by BA sequestrants (130), IBAT inhibition reduces the BA pool size (80). This is likely to result in different metabolic responses.

Interestingly, feeding the sucrose-enriched diet to *Slc10a2-/-* mice normalized their increased BA synthesis. This was accompanied by increased Fgf15 expression in the distal intestine, potentially explaining the reduced synthesis. However, increased Fgf15 expression was seen also in the WT mice fed the sucrose-enriched diet, whereas the reduction of Cyp7a1 was much more pronounced in the *Slc10a2-/-* mice. Furthermore, C4 levels were unaffected in the WT mice. Taken together, these findings question the role of Fgf15 in regulating BA synthesis after sucrose feeding. The possible connection between dietary sucrose and Fgf15 was not further explored in this paper. As the reduced BA synthesis after sucrose feeding was much more pronounced in *Slc10a2-/-* mice, this could indicate that a stimulated basal BA synthesis is required for sucrose to repress BA synthesis. When looking closely into the high-sucrose diet it is obvious that it contains no fibers, which is in contrast to the control diet with normal fiber content. In light of the results in Paper II, it is questionable whether the sucrose content of the diet is indeed responsible for the reduction in BA synthesis.

5.2 EFFECTS OF DIETARY SUGAR ON BILE ACID AND CHOLESTEROL METABOLISM

In Paper II, the aim was to study the effect of dietary sucrose on BA synthesis as it was found in Paper I that feeding a sucrose-enriched diet to *Slc10a2-/-* mice reduced BA synthesis. Since feeding the same diet to WT mice did not affect BA synthesis to the same extent, the first study of Paper II was designed to include both normal rats and rats treated with cholestyramine as a mean of increasing their basal BA synthesis (similar to the phenotype of *Slc10a2-/-* mice). It was found that feeding rats a high-sucrose diet reduced BA synthesis, irrespective of normal or stimulated basal BA synthesis. The second experiment of Paper II was designed to test the hypothesis that PPARα mediated the effect of sucrose on BA synthesis, as the expression of the PPARα-regulated gene Fgf21 (99), together with others, was increased in the first experiment. Furthermore, PPARα agonists (fibrates) have been shown to suppress BA synthesis (51). To isolate the influence of sucrose, rats were fed a controlled high-sucrose diet, in which the complex carbohydrates in the control diet were replaced with sucrose in the controlled high-sucrose diet. In the diet used in the first

experiment, the fat, fiber and protein content were reduced in the high-sucrose diet compared to the control diet. This change in diet prevented us from finding any difference in BA synthesis between the study groups in the second experiment. This indicates that it was not the increased sucrose content of the high-sucrose diet that reduced BA synthesis in the first experiment, but rather the concomitant reduction of fat or fiber. As the controlled high-sucrose diet did not suppress BA synthesis, the PPARα hypothesis could not be tested. Therefore, the results from the study groups treated with the PPARα antagonist GW 6471 are not shown or discussed. Fat (69) and water-soluble fiber (142) has been found to stimulate BA synthesis. However, the main fibers of rodent diets are cellulose and other water-insoluble fibers that do not affect BA synthesis (142). Therefore, the BA-reducing effect of the high-sucrose diet used in the first experiment, is likely explained by the decreased fat content.

Although it was concluded that sucrose *per se* does not regulate BA synthesis in rats, insulin and glucose have been shown to regulate BA synthesis in a few different models. Insulin was shown early on to reduce Cyp7a1 expression in rat hepatocytes (74; 143), which was also later confirmed in human hepatocytes (144; 145). In primary human hepatocytes, a more detailed study of insulin regulation of CYP7A1 has shown that insulin stimulates CYP7A1 expression after short-term treatment, while long-term treatment suppresses its expression (72). In *in vitro* experiments, glucose has been shown to stimulate CYP7A1 expression in human hepatocytes (71). Furthermore, in mice expressing human CYP7A1, both glucose and insulin have been shown to stimulate CYP7A1 expression (146). In humans after an oral glucose tolerance test, BA synthesis is found to decline at 60 min and returning to baseline at 120 min (147). However, the lack of an untreated control group in that study makes it difficult to evaluate the difference from the normal diurnal variation, which displays decreasing values during night until 10:30 a.m. and peak values at 12:00 noon to 13:30 p.m. and at 9:00 to10:30 p.m. (148). Recently, feeding mice a high-fructose diet was found to reduce Cyp7a1 expression (49), while we here show that feeding mice a controlled high-sucrose diet does not affect Cyp7a1 expression.

A consistent finding in the two rat experiments was a dramatic reduction in hepatic Abcg5/8 mRNA expression by the sucrose-enriched diets. The dietary regulation of Abcg5/8 is largely unknown. Cholesterol feeding of rats reduces Abcg5/8 expression whereas it increases its expression in mice (48), however, LXR that is one likely mediator stimulates both rat and mice Abcg5/8 (46). Recently it was shown that high-fructose feeding did not affect Abcg5/8 expression in mice (49). Similar results were found in Paper II, when mice were fed a controlled high-sucrose diet. Insulin has been shown to reduce Abcg5/8 expression in rat hepatocytes (149), and insulin is thus a putative mediator of the sucrose-mediated effect on Abcg5/8. However, further studies are needed to clarify this.

Since LXR regulates both Cyp7a1 (150) and Abcg5/8 (46), it may be a mediator of the effects of the high-sucrose diet used in the first experiment. However, since liver cholesterol was increased by the high-sucrose diet in the first experiment, LXR is most likely activated

(66). This should lead to an upregulation of Cyp7a1 and Abcg5/8, which excludes LXR as a likely mediator. Furthermore, it could be concluded that the FXR-FGF15 pathway (60) is not a likely mediator of the effect of the high-sucrose diet on BA synthesis, since the mRNA expression of hepatic Fxr and Shp-1 and intestinal Fxr and Fgf15 was not affected. Another factor of potential relevance, which was not studied in the present work, is the possible influence of intestinal microflora in response to the different diets, which in turn could affect cholesterol and bile acid metabolism (151).

From Paper II it is evident that it is important to carefully design the composition of the experimental diets. However, it is not obvious which of the diets used in Paper II is the most interesting or relevant one. The controlled high-sucrose diet would be most relevant for evaluating the isolated effect of sucrose. However, the high-sucrose diet with concomitant reductions in protein, fiber and fat might more closely resemble a real dietary situation, as increased intake of refined sugar could be coupled to a reduced intake of a balanced diet.

5.3 REGULATION OF FGF21

FGF21 is known to be increased in association with obesity, insulin resistance, fatty liver disease and T2D in humans (152). However, the direct cause for the increase in FGF21, as well as the role of FGF21 in the development of these diseases, is still largely unknown.

In Paper III, the effects of GH and glucose on circulating FGF21 levels were studied in humans. It was shown that GH does not alter circulating FGF21 acutely or after long-term administration of physiological doses, whereas administration of supraphysiological GH doses increased FGF21. The effect of GH on FGF21 in humans has not previously been studied. However, in mice as well as in cattle, GH increases hepatic FGF21 expression (119; 120). It has been found in mice that the GH-dependent stimulation of hepatic FGF21 synthesis is dependent on adipocyte lipolysis (119). However, in cattle, GH has a direct effect on the liver by increasing signal transducer and activator of transcription-5 (STAT5) signaling (120). When FGF21 was increased by supraphysiological GH doses, a concomitant increase in circulating NEFA was found. This finding indicates that NEFA might, at least in part, mediate the GH-dependent effect on FGF21. In rodents (153; 154), fasting and a ketogenic diet increases circulating FGF21 levels. These states share increased NEFA levels as a common feature with GH administration at supraphysiological levels. Thus, NEFAs could be a mediator of the GH effect. However, since fasting and a ketogenic diet does not have an effect on FGF21 in humans (121), in contrast to rodents, even though NEFAs are increased, one may speculate that NEFAs are not such a strong regulator of FGF21 in humans as they might be in mice. We show that FGF21 is not affected by acute GH administration despite concomitant activation of STAT5 (110), which does not support STAT5 as an important GH-mediated regulator of FGF21 in humans. This is in contrast to what is found in cattle (120).

In mice, chronic elevation of FGF21 inhibits GH signaling and reduces IGF-1 levels in a similar way as fasting (155). Therefore, FGF21 has been suggested to be one mediator of GH

resistance during fasting. However, since FGF21 is not increased in humans by short-term fasting (121), while IGF-1 levels are reduced (156), one may speculate that there is a species difference in this regulation or that FGF21 mediates a later response in GH resistance during fasting. FGF21 is increased after prolonged fasting (starvation) in humans and may have effects on IGF-1 at that stage (121). Interestingly, FGF21 is increased in anorexia patients (157), together with increased GH levels and reduced IGF-1 levels. Thus, FGF21 may in part mediate GH resistance in this type of prolonged fasting (starvation) in humans (157).

In Paper III, it is shown that FGF21 displayed a nadir 30 min after glucose ingestion, followed by increasing levels, reaching statistical significance at 180 min. This is in agreement with a previous study by Lin *et al.* (122) and a recent study by Dushay *et al.* (123). In Paper III, as well as in the other two mentioned papers, there are no untreated control groups. This is of course a drawback, as it requires reliance on historic controls. In the study by Lin *et al*, it was shown that FGF21 levels after glucose administration negatively correlated with insulin. Therefore, and since a continuous insulin infusion has been found to suppress FGF21 in humans (158), the FGF21 serum pattern may be regulated by insulin. Dushay *et al.* showed the same insulin and FGF21 patterns and they discussed carbohydrate responsive-element binding protein (ChREBP) as a likely mediator of glucose stimulation of FGF21 (123). Additionally, Lin *et al.* discussed NEFAs as likely mediators of the glucose effect, as NEFAs are found to positively regulate FGF21 via PPARα (119). They refer to the NEFA data (110) in the same oral glucose tolerance test study material analyzed in Paper III, which shows that NEFA decreases early after glucose administration and thereafter increases. However, when FGF21 was analyzed in that same study in Paper III, it was found to increase above basal levels at 180 min, whereas the NEFA level at the same time were far from reaching basal levels, why it is doubtful that NEFAs are the main regulators of FGF21 after glucose ingestion. The insulin levels in this study material increased immediately after glucose ingestion and returned to baseline values at 180 min when FGF21 was high, in line with the study by Lin *et al*. This suggests that insulin may negatively regulate FGF21 after glucose intake.

This thesis presents observations (mentioned in Paper III) of a stimulatory effect of acute fructose ingestion on circulating FGF21 in humans. The lack of a control group in this study is a drawback, and we had to rely on historical controls regarding the normal diurnal rhythm of FGF21, which displays declining values during the morning, when our study was performed, and a main peak at 5:00 a.m. (159). The stimulatory effect of fructose on FGF21 was however recently confirmed in the paper by Dushay *et al.* (123). They showed a higher and earlier peak after fructose administration than after glucose administration. The same result can be seen when comparing the glucose and fructose studies in this thesis. This finding is not surprising as fructose and glucose are metabolized differently in liver, and have different physiological effects. ChREBP is suggested to be the link between fructose intake and FGF21 expression (123).

5.4 IF LIPOPROTEIN METABOLISM IN T2D

T2D patients display an increased risk of cardiovascular disease (97), of largely unknown reasons. Moreover, LDL cholesterol is more atherogenic per mole in these patients (5; 97; 125). Since it has been shown that IV injected LDL disappears more quickly from the blood stream in T2D than in non-diabetics (129), we hypothesized that the level of LDL cholesterol would be increased in IF of these patients. However, the opposite was found, i.e. a reduction in the IF-to-serum gradient. Reduced IF-to-serum gradients for apoB, VLDL cholesterol, and VLDL and LDL TGs were also found. However, HDL cholesterol was not affected, indicating specificity for apoB-containing lipoproteins. The lower IF-to-serum gradient for LDL cholesterol could be explained by an increased LDL cholesterol uptake by and/or adhesion to peripheral cells and/or macrophages. This explanation is supported by a report showing increased proteoglycan binding of LDL particles from T2D patients (160).

When designing this study, the aim was to recruit T2D patients with severe diabetes and it was considered to be an advantage if they also had cardiovascular disease. The rationale for this was that it might be easier to detect a difference between such patients and healthy controls. However, a drawback from these inclusion criteria was that many of the patients were treated with many different drugs that complicated the interpretation of the results. As statin treatment is powerful in reducing serum LDL cholesterol levels, we first evaluated if this treatment affected the IF-to-serum gradient of LDL cholesterol in non-diabetic subjects in a pilot study, and it was found not to be the case. This finding was also confirmed in the diabetic patients, and we can therefore rule out statin treatment as a reason for why the T2D patient group showed a lower IF-to-serum gradient for LDL cholesterol. The T2D patient group and the control group were matched for age and gender, but not for BMI. Because of this it cannot be excluded that the higher BMI of the T2D group explains the lower IF-to-serum gradient for LDL cholesterol. A weak negative correlation between IF-to-serum gradient for LDL cholesterol and BMI was also found (R_s -0.32, P 0.0062). It is possible that the higher abdominal mass increases the IF and therefore dilutes the lipoprotein particles. Therefore, it would be of importance to study healthy obese and normal weight patients to evaluate if BMI affects the IF-to-serum gradients.

The optimal location for collecting IF in order to best answer the hypothesis would be the vascular wall. However as this is not possible in humans *in vivo*, blister fluid was collected from the abdominal skin, using a mild suction (112). This fluid has been shown to represent skin IF (161). However it is important not to use too high vacuum pressure as this might lead to an increased movement of particles to the blister fluid. LDL has previously been analyzed in IF from aortic intima in necropsy samples, and the concentration was found to be twice the concentration in plasma (162). One could speculate that the intimal IF LDL level is somewhat lower in T2D than in non-diabetics, as show in skin IF, and that this would reflect an increased LDL retention to the intimal tissue cells.

5.5 FUTURE PERSPECTIVES

The results in this thesis have generated several new questions, which may be of interest for further study.

As it was shown that feeding rats a sucrose-enriched diet almost abolishes the hepatic Abcg5/8 mRNA expression, it would be of great interest to study the mechanisms behind this marked effect. One hypothesis is that insulin is mediating the sucrose effect. A feasible experiment to test this hypothesis would be to feed the same diet to insulin resistant or insulin deficient rats to see if the response is hampered. To evaluate the physiological importance of the Abcg5/8 lowering by sucrose, it would be of value to confirm the decreased mRNA levels with protein data, and to study if the cholesterol secretion into bile is altered. As the sucrose content of the experimental diet is as much as 65% it would also be interesting to study Abcg5/8 regulation by a diet with more moderate increase in sucrose content.

Following the unexpected finding of lower-than-expected levels of apoB-containing lipoproteins in IF in T2D patients, it is crucial to explore where these particles are located. One approach is to inject radiolabeled LDL particles and follow the transport of lipoproteins from the blood into the IF and map their distribution. If a rodent model with the same IF lipoprotein phenotype as in human T2D could be established, it would be possible to characterize this phenomenon in more detail. We have also begun to further characterize the properties of IF HDL particles. Since IF is the compartment where reverse cholesterol transport is initiated, a detailed study of functional properties of HDL in IF should provide important information on how dyslipidemia in T2D may promote the atherogenic process.

6 CONCLUSIONS

The main aim of this thesis was to investigate the interactions between the metabolism of cholesterol and BAs with that of glucose and TGs.

Paper I shows that inhibition of the enterohepatic circulation of BAs reduces blood glucose and plasma TGs in obese mice. Feeding a high-sugar diet lowers BA synthesis in mice with a high basal BA synthesis.

Paper II shows that feeding rats two different high-sucrose diets affects BA synthesis differently. A high-sucrose diet with concomitantly reduced fat and fiber content reduces BA synthesis, whereas a controlled high-sucrose diet does not affect BA synthesis in rats. Furthermore, both these high-sucrose diets markedly reduce Abcg5/8 gene expression in liver.

Paper III shows that GH is not a regulator of basal serum FGF21 levels in humans. However, prolonged administration of supraphysiological doses of GH increases serum FGF21. Furthermore, glucose administration to humans increases serum FGF21.

Paper IV shows that the levels of apoB-containing lipoprotein particles are reduced in relation to their serum levels in T2D patients compared to matched controls. This suggests that there is increased peripheral retention and/or catabolism of apoB-containing lipoproteins in this disease.

7 SVENSK POPULÄRVETENSKAPLIG SAMMANFATTNING

Hjärtinfarkt är en av de stora folksjukdomarna och är också en av de vanligaste dödsorsakerna. Hjärtinfarkt är en följd av en lång tids ansamling av kolesterol i kärlväggen tillsammans med inflammatoriska processer. Eftersom kolesterol har en central roll i utvecklingen av hjärt-kärlsjukdom är det viktigt att studera kolesterolmetabolismen. Samtidigt som kolesterol är skadligt i för höga koncentrationer, har det livsviktiga funktioner i kroppen t.ex. i bildandet av steroidhormoner och som byggsten i cellmembraner. Kolesterol i blodet transporteras i olika lipoproteiner. Kolesterolet i LDL-partiklarna (”low density lipoprotein”) ökar risken för hjärt-kärlsjukdom medan kolesterol i HDL (”high density lipoprotein”) i viss mån är skyddande. Kolesterol lämnar kroppen via levern på två olika sätt. Antingen utsöndras kolesterolet direkt till gallan, eller så omvandlas kolesterolet till gallsyror i levern som därefter utsöndras med gallan. Gallan töms sedan i tarmen när vi äter. De flesta gallsyrorna tas sedan upp från tarmen igen för att kunna återanvändas medan en del lämnar kroppen med avföringen. Det finns data som visar att metabolismen av blodsocker påverkas om man ändrar metabolismen av gallsyrorna. Detta förhållande har studerats i denna avhandling. Diabetiker har en ökad risk att drabbas av hjärt-kärlsjukdomar, men man vet inte riktigt varför. Den vanligaste typen av diabetes är åldersdiabetes, s.k. typ 2 diabetes. Hos dessa patienter är nivån av blodsocker förhöjd eftersom kroppens celler har svårt att ta upp socker från blodet. Den höga nivån av blodsocker är en orsak till att diabetiker har en ökad risk att drabbas av hjärt-kärlsjukdom eftersom blodets kolesteroltransportörer kan förändras av sockret och därför bli farligare än normalt. I denna avhandling har nivån av kolesterol i blod och den perifera vätskan, intercellulärvätskan, studerats hos diabetiker. Intercellulärvätskan är den vätska som närmast omger alla kroppens celler och dess volym är ca tre gånger så stor som hela blodvolymen.

Studierna i denna avhandling har visat följande:

- Genom att hindra gallsyror som utsöndrats till tarmen från att tas upp igen av kroppen kan man sänka blodsockernivån och fettnivån i blodet. Eftersom höga nivåer av blodsocker och blodfetter kan leda till och förvärra t.ex. diabetes och hjärt-kärlsjukdom vill man hitta sätt att sänka dessa nivåer. Genom att hindra att kroppen tar upp gallsyror från tarmen kan alltså det åstadkommas.
- En stor andel socker i maten kan minska bildningen av gallsyror i levern. Detta är negativt eftersom omvandlingen av kolesterol till gallsyror är ett sätt för kroppen att göra sig av med kolesterol. Resultaten visar att det troligtvis inte är sockret i sig som gör att bildandet av gallsyror minskar. Om man ökar intaget av socker äter man mindre av andra komponenter i maten, det är troligen minskningen av dem som leder till att det bildas mindre gallsyror. Vi kunde också visa att ett högt intag av socker minskade den transportör som transporterar kolesterol till gallan.
- Ett hormonliknande ämne i kroppen som heter FGF21 regleras av ett annat hormon som heter tillväxthormon. Eftersom djurstudier visat att FGF21 skulle kunna vara en

möjlig behandlingsmetod mot förhöjda nivåer av blodsocker och blodfetter är det viktigt att studera FGF21s reglering och funktioner.

- Diabetiker har lägre nivåer av kolesterol i intercellulärvätskan jämfört med friska. Vi tror att detta skulle kunna bero på att någon struktur i perifera vävnader tar upp kolesterolrika LDL-partiklar från intercellulärvätskan i ökad omfattning hos diabetiker. Detta ökade upptag skulle kunna vara en del i orsaken till varför diabetiker oftare får hjärt-kärlsjukdomar.

Resultaten från denna avhandling har ökat kunskapen kring hur metabolismen av gallsyror påverkar omsättningen av blodsocker och blodfetter. Avhandlingen har också fördjupat kunskaperna kring hur ett stort intag av socker påverkar metabolismen av kolesterol och gallsyror. De studierna gjordes i mus och råtta. Fler studier behövs för att studera förhållandena i människa. Avhandlingen har också ökat kunskapen kring hur det hormonlikande ämnet FGF21 regleras. Vidare visar avhandlingen att en viktig del av metabolismen av LDL hos typ 2 diabetiker sker i intercellulärvätskan, vilket bör leda till att kolesterol ansamlas i perifera vävnader hos typ 2 diabetiker.

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