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STUDIES ON PCSK9 IN THE REGULATION OF CHOLESTEROL METABOLISM

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ABSTRACT

Elevated levels of plasma cholesterol, mainly in low density lipoproteins (LDL), are a major risk factor for coronary heart disease. The level of plasma LDL cholesterol (LDL-C) is largely dependent on the number of hepatic LDL receptors (LDLRs). Increased numbers of LDLRs lead to higher uptake of LDL particles and lower concentration of plasma LDL-C. Proprotein convertase subtilisin Kexin Type 9 (PCSK9) is a novel key regulator in cholesterol metabolism. PCSK9 reduces the number of available hepatic LDLRs leading to increased plasma levels of LDL-C. Inhibition of PCSK9 has a great potential as a cholesterol-lowering agent. However, the physiological role of PCSK9 is largely unknown.

In this study we investigated the regulation of PCSK9 in rats and humans, by hormones and diets and during the diurnal phases. We can show that:

I) Reduced bile acid synthesis, but not changes in PCSK9, likely contributes to the age-dependent hypercholesterolemia in rats. Treatment with growth hormone (GH) restores bile acid synthesis to juvenile levels and upregulates the gene transcription of PCSK9

II) Hormonal and dietary regulation of hepatic LDLRs in the rat is frequently mediated by PCSK9. Treatment with estrogen, glucagon and a cholesterol-enriched diet reduces PCSK9 expression, while it is increased by insulin. The transcription factor SREBP-2 is partly involved in the hormonal and dietary regulation of PCSK9, although our results suggest that the glucagon-mediated suppression of PCSK9 may involve other mechanisms.

III) Circulating PCSK9 has a pronounced diurnal variation and is strongly reduced during fasting in humans. These changes are presumably related to oscillations in hepatic intracellular cholesterol levels mediated by SREBP-2. GH treatment reduces circulating PCSK9 in men, whereas a ketogenic diet does not alter circulating PCSK9 levels.

IV) Endogenous estrogen exerts rapid and distinct effects on cholesterol metabolism in females, with reduced levels of circulating PCSK9, plasma total and LDL-C, whereas the levels of HDL- and LDL-triglycerides (TGs) and apoAI increase. Some of the effects of estrogen on cholesterol metabolism may be mediated by a rapid induction of GH secretion.

The hormonal regulation of PCSK9 can partly explain the cholesterol-lowering effects of GH, estrogen, glucagon and thyroid hormone. Our results further indicate that such hormonal regulation may involve SREBP-2 independent mechanisms. The regulation of PCSK9 during the diurnal phases and fasting may explain why plasma LDL-C levels remain stable during these situations. We have also shown that PCSK9 can be dietary regulated, partly explaining the pronounced resistance to development of hypercholesterolemia following a cholesterol-enriched diet in the rat. Neither a ketogenic nor a vegan diet alters circulating PCSK9 in humans. Hormonal, dietary and diurnal regulation of PCSK9 may influence serum LDL-C levels, a fact that should be considered in the use of anti-PCSK9 agents.

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CONTENTS

LIST OF ABBREVIATIONS

ABC	ATP-binding cassette transporter
ACAT	Acyl-coenzyme A:cholesterol O-acyltransferase
ACTH	Adenocorticotrophic hormone
Apo	Apolipoprotein
ARH	Autosomal recessive hypercholesterolemia
ASBT	Apical sodium-dependent bile acid transporter
BSEP	Bile salt export pump
C4	7α-hydroxy-4-cholesten-3-one
CDCA	Chenodeoxycholic acid
CE	Cholesteryl esters
CETP	Cholesteryl esters transfer protein
CHD	Coronary heart disease
CYP7A1	Cholesterol 7α-hydroxylase
EGF	Epidermal growth factor
ER	Endoplasmatic reticulum
FC	Free cholesterol
FGF	Fibroblast growth factor
FH	Familial hypercholesterolemia
FPLC	Fast performance liquid chromatography
FSH	Follicle stimulating hormone
FXR	Farnesoid X receptor
GH	Growth hormone
GOF	Gain of function
HDL	High density lipoprotein
HMG-CoA	3-hydroxy-3-methylglutaryl coenzyme A
HNF	Hepatocyte nuclear factor
IDL	Intermediate density lipoprotein
Idol	Inducible degrader of LDL receptor
IGF1	Insulin growth factor 1
INSIG	Insulin-induced gene
IVF	In vitro fertilization
LCAT	Lecitin:cholesterol acyltransferase
LDL	Low density lipoprotein
LDL-C	Low density lipoprotein cholesterol
LDLR	Low density lipoprotein receptor
LOF	Loss of function
Lp(a)	Lipoprotein(a)
LPL	Lipoprotein lipase
LRH-1	Liver receptor homologue 1
LRP	Low density lipoprotein receptor related protein
LXR	Liver X receptor
MTP	Microsomal triglyceride transfer protein
NPC1L1	Neimann-Pick C1 Like 1
PCSK9	Proprotein Convertase Subtilisin Kexin Type 9

PL	Phospholipids
PPAR	Peroxisome proliferator-activated receptor
SCAP	SREBP cleavage-activating protein
SHP	Small heterodimer
SR-BI	Scavenger receptor B1
SRE	Sterol regulatory element
SREBP	Sterol regulatory element binding protein
TG	Triglycerides
TH	Thyroid hormone
VLDL	Very low density lipoprotein
VLDLR	Very low density lipoprotein receptor

1 INTRODUCTION

Cardiovascular disease, e.g. coronary heart disease (CHD) and stroke, is the most common cause of death in the western world today. CHD is the result of atherosclerosis, a condition characterized by the progressive thickening of the arteries, due to entry of lipids and inflammation. Epidemiological studies have shown that age, male sex, plasma cholesterol, smoking, hypertension, obesity and diabetes are important risk factors linked to the progression of atherosclerosis. An elevated level of plasma cholesterol, mainly in low density lipoprotein (LDL), increases the likelihood for the onset and progression of atherosclerosis (1, 2), and most important, the reduction of LDL cholesterol (LDL-C) reduces the risk for CHD (3).

Plasma LDL-C is influenced by several different physiological and pharmacological factors such as genes, hormones, diets and drugs. In recent years, the protein PCSK9 has evolved as a key modulator of plasma LDL-C levels. Different gene variations of PCSK9 can result in both hyper- and hypocholesterolemia and drugs such as the cholesterol lowering statins also alter PCSK9. Regulation of PCSK9 might actually explain some of the physiological and pharmacological responses on plasma LDL-C, and the focus of this thesis has been to study the regulation of PCSK9 *in vivo*.

1.1 CHOLESTEROL

Cholesterol is a hydrophobic molecule ($C_{27}H_{45}OH$) with several important biological functions; as a component of cellular membranes; a precursor of bile acids and steroid hormones. Excessive amounts of free cholesterol can be toxic and therefore cholesterol homeostasis is tightly regulated by a balance between endogenous synthesis, dietary uptake, and biliary excretion. The liver has a central role in the overall body cholesterol homeostasis and, together with the intestine, it accounts for the major part of cholesterol´s endogenous synthesis (4). In the liver cholesterol is also degraded to bile acids which are secreted into bile together with free cholesterol, and fecal excretion of bile is the main way for the body to eliminate cholesterol.

1.2 LIPOPROTEINS

Cholesterol is a water-insoluble molecule packed within lipoproteins to enable transport in the blood stream. Lipoproteins have a hydrophobic core of cholesteryl esters (CE) and triglycerides (TGs) surrounded by a monolayer of more polar lipids such as

phospholipids (PL), and unesterified (free) cholesterol (FC) together with specific apolipoproteins (apos). Apos give the lipoprotein specific structure and ability to interact with specific receptors and enzymes. Lipoproteins are often classified from their densities into five major classes; chylomicrons, very low density lipoproteins (VLDL), intermediate density lipoproteins (IDL), low density lipoproteins (LDL) and high density lipoproteins (HDL).

1.2.1 Chylomicrons

The largest lipoprotein particles are chylomicrons, being produced postprandially in the intestine containing 85-95% of TGs and apoB48. Chylomicrons also contain other apos such as apoAI and apoAII, and after transportation via the lymph to the blood these particles acquire apoC and apoE. Chylomicrons are transported to peripheral tissues where lipoprotein lipase (LPL) hydrolyses TGs into free fatty acids (FFA) for energy supply to muscles and adipose tissue. The size of the chylomicrons is then reduced and the remaining remnant particles are efficiently removed from the circulation by the liver. Chylomicrons have a short half life of approximately 10 min, and are not normally present in fasting blood samples.

1.2.2 VLDL

VLDL particles also supply the peripheral tissues with TGs, they are more dense then chylomicrons and contain 50-80% TGs and an apoB100. VLDLs are produced in the liver, by a cascade of lipidation steps of apoB100 where the availability of microsomal triglyceride transfer protein (MTP) and lipids are required (5, 6). The amount of secreted VLDL particles is dependent upon how many particles escape hepatic degradation, ~55% of the newly synthesized VLDL-particles are rapidly taken up by hepatic LDL-receptors (LDLRs) (7), and people with LDLR defects have increased secretion of VLDL (8). When VLDL is secreted into the circulation it can receive apoCI, apoCII, apoCIII and apoE. In peripheral tissues, TGs are hydrolyzed by LPL making the VLDL particle denser thereby converting them into intermediate lipoprotein (IDL) particles. The half-life of circulating VLDLs is around 2 hours, whereas the IDL particles are rapidly removed either by hepatic LDL receptors (LDLR) or by conversion into more dense LDL particles.

1.2.3 LDL

In human plasma about 70% of the cholesterol is present in LDL particles. The LDL particle consists of almost 50% cholesterol and has one apoB100 molecule on the surface. LDL particles are largely cleared from plasma through hepatic LDLRs and the number of LDLRs thereby regulate the level of plasma LDL-C (9), LDL also deliver cholesterol to extrahepatic tissues such as the adrenals and gonads. The half-life of circulating LDL is 2-3 days, and during that time LDL particles can infiltrate the endothelial barrier in the arterial wall where they are susceptible to modifications such as oxidation and aggregation (10). Modified LDL particles are recognized and internalized by macrophages that accumulate large amounts of cholesterol which transform them into foam cells, starting the development of inflammation and atherosclerosis (10). Increased levels of LDL-C is strongly correlated to the risk to develop atherosclerosis (3). The concentration of LDL-C is of relevance in clinical work and is generally calculated using Friedwald's formula (11), where measurements of total cholesterol, HDL-cholesterol (HDL-C) and triglycerides are used. In today's clinical work, measurements of apoB, apoA1 and the calculation of their ratio (apoB/apoAI) are frequently used as a complement to LDL-C. ApoB reflects the number of LDL and VLDL particles and apoB/apoAI has proven to be a strong predictor for the risk of CHD than LDL cholesterol.

1.2.4 HDL

HDL particles are small, dense and consist of apos (55%), PL (25-30%) and cholesterol (15-20%). They transport cholesterol from peripheral tissues back to the liver, and this reverse cholesterol transport is considered as an important anti-atherogenic pathway. ApoAI is the dominant apo in HDL and is synthesized in liver and intestine. HDL also contains apoAII, apoAIV, apoC and apoE. Secreted apoAI acquires PL and FC through interaction with ATP binding cassette transporter A1 (ABCA1) leading to the formation of pre-HDL particles. FC, taken up by the pre-HDL in the peripheral tissues, is then esterified by lecithin: cholesterol acyltransferase (LCAT), thereby generating more spherical particles. Through cholesteryl ester transfer protein (CETP) HDL can acquire TGs from VLDLs and LDLs in exchange for CE. HDL can also interact with the hepatic scavenger receptor BI (SR-BI) which mediates the selective uptake of HDL-cholesterol for degradation into bile acids or for the secretion of cholesterol into bile.(12, 13) In rodents, cholesterol is mainly transported in HDL and the levels of LDL-C are very low.

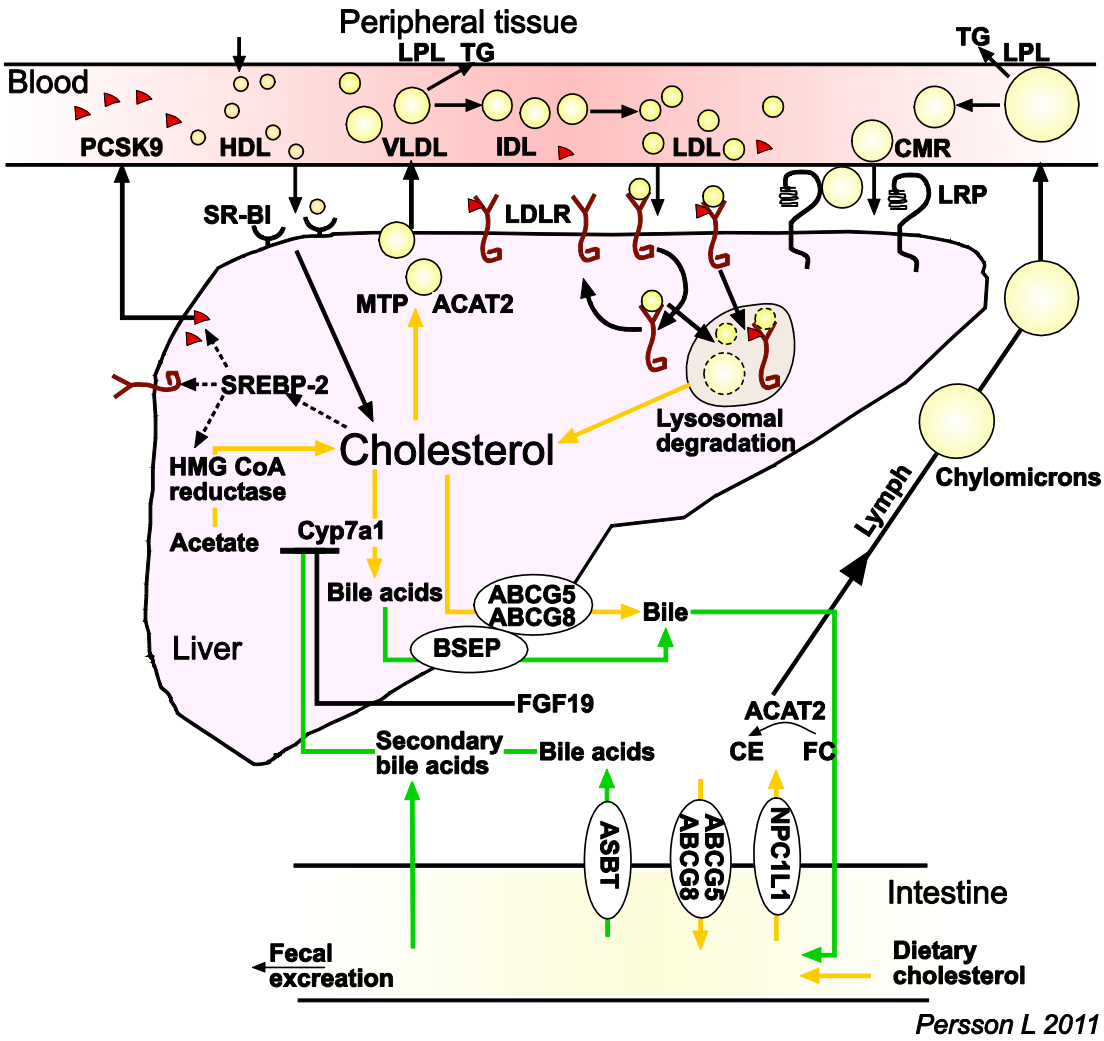

Fig.1 Schematic picture of the liver and cholesterol and lipoprotein metabolism, yellow lines represent cholesterol flux whereas green lines show bile acids flux.

1.3 LDL RECEPTOR

Plasma LDL-C levels are mainly determined by the number of hepatic LDLRs (14), the more LDLRs, the higher the uptake of apoB particles as LDL (9). LDLRs are normally present on all mammalian cells but the liver harbors the largest number of LDLRs although the adrenals and corpora lutea have the highest LDLR densities (14, 15).

The LDLR is a membrane bound glycoprotein, synthesized in the endoplasmatic reticulum (ER), processed in the Golgi complex and transported to the cell surface. It consists of five domains, 1) the N-terminus with cysteine-rich type A repeats which serve as the ligand binding domain, 2) the domain homologous to the epidermal growth factor (EGF)-precursor, important in the lysosomal dissociation of LDLR and its ligand, 3) the O-linked sugar domain, 4) the transmembrane domain, 5) and the C-terminal, a short cytoplasmic region containing signals for internalization.(16, 17)

ApoB100 and apoE serve as ligands for the LDLR and apoE containing particles bind with higher affinity to the LDLR as compared to apoB100 particles (18). On the cell surface LDLRs concentrate to clathrin-coated pits and are internalized, with or without its ligand, into the cell by endocytosis (19) where the adaptor protein ARH (autosomal recessive hypercholesterolemia) is required (20, 21). The endosome is directed to the more acidic lysosome where the receptor-complex is separated, allowing the receptor to return to the surface and be reutilized (22). The lipoprotein is then degraded and the liberated cholesterol can be stored in the cell, used for VLDL production, secreted into bile as such or after the degradation into bile acids. Each such LDLR-cycle takes about 12 min (23). As the LDLR can have a lifespan of about 30 hours it may recycle around 150 times (22). Therefore, minor changes of the number of LDLRs can result in major differences of plasma LDL-C levels. Mutations in the LDLR gene cause defective LDL catabolism and is one important cause for the relatively frequent disease Familial Hypercholesterolemia (FH) (24).

The gene encoding the LDLR is located on chromosome 19, and is transcriptionally regulated by sterol regulator element binding protein-2 (SREBP-2) (25). When intracellular cholesterol decreases, SREBP-2 is activated (see below), as e.g. during statin treatment, leading to a transcriptional up regulation of the LDLR gene, increased LDLR levels, and increased plasma clearance of LDL-C. The regulation of LDLR has generally been considered to occur at a transcriptional level. However, recent results have shown that LDLRs are frequently regulated, in a posttranscriptionally manner, by the protein PCSK9 (as described below). *In vitro* studies have shown that the protein inducible degrader of LDLR (Idol) may also regulate LDLRs at an posttranscriptional level (26). Idol is activated by the nuclear liver X receptor (LXR) and triggers ubiquitination of the LDLR on its cytoplasmic domain, thereby targeting the LDLR for degradation. Thus, changes of intracellular cholesterol levels can regulate LDLR at the transcriptional level by SREBP-2, as well as at a posttranscriptional level by PCSK9 and possibly also through LXR-Idol involvement (27). The influence of Idol on the LDLR *in vivo* is, however, largely unknown. The LDLR is transcriptionally and posttranscriptionally regulated by hormonal, dietary and pharmacological agents, but the exact mechanisms involved are less clear.

The LDLR is a member of the LDLR gene family, a group of receptors with a high degree of structural similarities that is engaged in receptor-mediated endocytosis of different ligands. The most prominent receptors are LDLR, LDLR related protein (LRP)-1, gp330/megalin, VLDL receptor (VLDLR) and apoE receptor type 2 (apoER2). LRP1 is a multiligand receptor mainly expressed in the liver, and is involved in the uptake of chylomicron remnants through apoE, whereas the VLDLR is expressed mainly in the heart and can internalize apoE-lipoproteins and other ligands. The role of VLDLR, gp330 and apoER2 in lipoprotein metabolism is still not understood.(28, 29)

1.4 FAMILIAL HYPERCHOLESTEROLEMIA

FH is an autosomal disease, with a prevalence of 1/500, causing hypercholesterolemia, tendinous xanthomas, premature atherosclerosis and CHD (24, 30). The clinical disorder is manifested with a gene dosage effect where heterozygous carriers have approximately two-fold higher plasma LDL-C levels, and homozygous carriers with two defective alleles have five-fold higher plasma LDL-C levels as compared to the normal population. FH patients and especially homozygotes are therefore, at an early age, in need of lipid lowering treatment to prevent CHD. Mutations in the gene for LDLR are the most common reason for FH, and over 1000 separate LDLR mutations have been described leading to defective LDL catabolism and hypercholesterolemia. Also other mutations in the LDLR-ligand complex lead to FH. Thus, studies in France (31) and England (32) have reported that around 5-7% of FH patients carry mutations in the apoB100 gene leading to impaired LDLR-ligand interaction, and that 0.7-1.5% of FH patients carry mutations in the gene for PCSK9. Though the prevalence of LDLR mutations in FH patients is 74-79%, approximately 10% of FH patients have other still unknown causes for their disease. ARH is a recessive inherited disease also leading to hypercholesterolemia due to defective LDLR endocytosis, although this disease has a very low prevalence.

1.5 REGULATION OF CHOLESTEROL METABOLISM

1.5.1 The SREBP pathway

The SREBP regulatory system, located in the ER membrane, is important for maintaining cholesterol homeostasis by increasing cholesterol synthesis and uptake when intracellular cholesterol levels drops, and down regulating these processes when cholesterol is in excess (33). SREBP-1a and -1c are produced from the same gene and

are mainly involved in the regulation of fatty acid metabolism and lipogenesis. SREBP-2 is produced by another gene and functions as a master switch for cholesterol metabolism by binding to sterol regulatory elements (SRE) in the promoters of a large number of genes. The cholesterol sensitive membrane protein SCAP (SREBP cleavage-activating protein) forms a complex with the SREBPs, and during excess of cholesterol in the ER-membrane, SCAP binds to Insig, anchoring the SCAP/SREBP-2 complex to the ER-membrane. When cholesterol levels drop, SCAP is released from Insig, allowing the SCAP/SREBP-2 complex to travel to the Golgi complex where two proteases cleave SREBP-2, so that the active transcription factor domain can enter the nucleus and activate its target genes(33-35). SREBP-2 activates the transcription of e.g. PCSK9, LDLR and 3-hydroxy-3-methylglutaryl-Coenxyme A (HMG-CoA) reductase (rate limiting enzyme in cholesterol synthesis) (36, 37) and also its own transcription (38). The SCAP/SREBP complex responds with great precision to small changes in the ER-membranes, with a threshold effect (39). The SCAP/Insig ratio is important for the cholesterol sensitivity in the ER-membrane as is also the transport of cholesterol between different cellular organelles (39). How the SCAP/Insig ratio and cholesterol transportation are regulated are largely unknown.

1.5.2 Cholesterol synthesis

Most of our cells can de novo synthesize cholesterol from the substrate acetate, through an enzymatic pathway where enzyme HMG-CoA reductase is the rate limiting enzyme (40, 41). However, the liver and the intestine account for the major part of whole body cholesterol synthesis (4). As mentioned above, the transcription of HMG-CoA reductase is strongly regulated by SREBP-2, and is up regulated during cholesterol depletion. Statins are competitive inhibitors of HMG-CoA reductase resulting in; reduced intracellular cholesterol levels; activation of SREBP-2 and its target genes; higher synthesis of the LDLR and thereby higher uptake of plasma LDL-C (33). After the 1970s and the discovery of mevastatin (42), several more statins with higher efficiency have been developed and today's most potent statins are atorva- and rosuvastatin. In humans the lipid lowering effect of statins is most efficient and treatment with statins reduces the risk for atherosclerosis and CHD (3, 43, 44). Since statin treatment leads to an activation of SREBP-2, PCSK9 mRNA is up regulated concomitantly with LDLR mRNA, which will counteract the statin-effect (45-47) (described in detail below and in Fig. 2). When statins were first developed, it was observed that they had limited lipid lowering effects in rodents, (48, 49) whereas dogs,

monkeys and humans responded well. It is possible that species dependent differences in the extent to which statin up regulate PCSK9 gene expression may partly explain this (50).

1.5.3 Cholesterol absorption

Although the body can synthesize cholesterol to meet its needs, we still acquire cholesterol from the diet. Depending on the diet, approximately 500 mg of dietary cholesterol is taken up via the intestine, although the intestine contains 1-1.5 g of cholesterol from both dietary and biliary origin (51, 52). In the presence of bile acids cholesterol together with other lipids can form micelles that are absorbed by the enterocyte. The Neimann-Pick C1 Like 1 (NPC1L1) protein, located at the brush border membrane in the enterocyte (53), mediates the absorption of cholesterol and plant sterols, whereas ATP binding cassette transporters G5 and G8 (ABCG5/G8) transport plant sterols and cholesterol back to the intestinal lumen (54). Plant sterols are efficiently transported back to the lumen whereas absorbed cholesterol is esterified by acyl-Coenzyme A: cholesterol acyltransferase-2 (ACAT2) and packed into chylomicrons (55). Inhibiting cholesterol absorption could lead to lower plasma LDL-C, and the lipid lowering drug Ezetimibe acts through inhibiting the cholesterol absorption by binding to NPC1L1 (56, 57). Increasing the amount of dietary plant sterols, where β-sitosterol, campesterol and stigmasterol are the most common ones (58), also lowers plasma LDL-C (59), presumably due to decreased cholesterol absorption (60).

1.5.4 Bile acid metabolism

In the liver, cholesterol can be converted to bile acids, a process involving a cascade of different enzymes, via two major pathways, the classical (neutral) and the alternative (acidic) (61). During normal circumstances the classical pathway accounts for approximately 90% of the bile acids formed, where the rate limiting enzyme is cholesterol 7α hydroxylase (CYP7A1). From the classical pathway mainly cholic acid (CA) but also chenodexoycholic acid (CDCA) are formed, whereas from the alternative pathways predominantly CDCA is formed. Before secretion 98% of the bile acids are conjugated. Approximately 500 mg of cholesterol is converted to bile acids each day, and bile acids are together with phospholipids and cholesterol the compounds of bile. Hepatic ABCG5/G8 are important for the transportation of cholesterol into the bile (62) and approximately 500-600 mg cholesterol is secreted with the bile each day (52). The

bile salt export pump (BSEP) transports conjugated bile acids into the bile. The bile is concentrated and stored in the gallbladder until food intake stimulates the gallbladder to contract, and the bile is then secreted into the intestine. The bile acids in the intestine work as detergents and facilitate the uptake of dietary fats.

More than 95% of bile acids are reabsorbed and return to the liver in an enterohepatic circulation, with 6-10 cycles per day. The major part is absorbed in the distal ileum by apical sodium-dependent bile acid transporter (ASBT) (63), also named IBAT. The remaining bile acids then enter the large intestine where they are deconjugated and converted into secondary bile acids, such as deoxycholic and lithocholic acids, by intestinal bacteria. These secondary bile acids can be taken up by passive diffusion or excreted with feces.

Loss of bile acids is efficiently compensated for by increased bile acid synthesis due to a tight feedback regulation (61, 64). Bile acids that have returned to the liver bind to the nuclear hormone receptor farnesoid X receptor (FXR). FXR represses CYP7A1 transcription through a process involving activation of small heterodimer (SHP) and liver receptor homologue 1 (LRH1). Bile acid synthesis is also regulated by fibroblast growth factor (FGF)19 (in mice FGF15), expressed in the enterocytes (65, 66). Intestinal FGF19 is transported via the blood to the liver where it binds to the hepatic FGF receptor resulting in suppression of CYP7A1 (67). Increased intestinal levels of bile acids lead to increased transcription and circulation of FGF19, whereas low intestinal levels of bile acids lead to decreased FGF19 levels and thereby stimulation of CYP7A1.

Bile acids sequestrans such as cholystyramine, bind intestinal bile acids, which are thereby excreted with feces leading to a compensatory up regulation of bile acid synthesis. The increased bile acid synthesis will deplete intracellular cholesterol levels and will subsequently increase LDLR transcription resulting in decreased plasma LDL-C (68, 69). Cholestyramine in the past a commonly used lipid lowering drug but side effects such as stomach pain are common and reduce compliance.

1.5.5 Lipid lowering treatments

CHD is a leading cause of death in western countries, and lowering plasma LDL-C reduces the risk for CHD (3, 43, 44). Statins are efficient lipid lowering agents but there

are still patients in need for other agents, due to poor response (70) or adverse side effects (71). Ezetmibe, cholestyramine (mentioned above), fibrates and nicotinic acid are other lipid lowering drugs on the market. Fibrates activate peroxisome proliferator-activated receptor alpha (PPARα), a key regulator of fatty acid synthesis and lipid metabolism leading to reduced TG synthesis (72), and fibrates are therefore used especially in hypertriglycerdiemia. Nicotinic acid has been used to improve the lipid profile since it increases HDL-C and decreases LDL-C, but it's mechanism is largely unknown, and the side effects many (73). Inhibiting; PCSK9 (as described below), apoB production (74), VLDL assembly by interaction with ACAT or MTP, and CETP are some other potential drug targets. Eprotirome is a new compound that acts as a liver specific thyromimetic, and recent results from a placebo-controlled patient study have revealed promising results with reduced plasma LDL-C and Lp(a) levels (75).

1.5.6 Hormonal regulation

Cholesterol metabolism is regulated by a broad set of hormones, where the pituitary gland is important for controlling the secretion of many of these. The pituitary gland consists of an anterior and a posterior part and is connected to the hypothalamus. The anterior pituitary secretes growth hormone (GH), thyroid stimulating hormone (TSH), adrenocorticotropic hormone (ACTH), gonadotropins, prolactin and melanocyte–stimulating hormones. The posterior part secretes oxytocin and antidiuretic hormone.

GH is a complex hormone that stimulates growth and has major implications in metabolic regulation. GH stimulates the liver to produce insulin growth factor (IGF) 1 that meditates some of GH's metabolic effects. The secretion pattern of GH is affected by several physiological situations such as sleep, exercise, fasting and feeding. GH exerts significant effects on cholesterol metabolism *in vivo* (76, 77), and GH deficiency leads to increased plasma cholesterol levels (78, 79) whereas treatment with GH decreases plasma LDL-C (80, 81), presumably due to increased LDL clearance (80) through an increased number of hepatic LDLRs (82). The secretion of GH is reduced with age in both humans and rats (83, 84), and treatment of rats with GH reverses the age dependent increase of plasma LDL-C (85). GH has also a gender differentiated secretion pattern (86, 87) that might contribute to gender dependent differences in cholesterol metabolism.

TSH stimulates the thyroid gland to produce thyroid hormones (TH) which regulate metabolism and increase the basic metabolic rate. It is well known that excess of TH decreases body weight, increases heart rate and decreases plasma LDL-C levels, presumably due to increased expression of the hepatic LDLRs (88, 89). TH also exerts major effects on bile acid metabolism (90). ACTH stimulates the adrenal cortex to synthesize gluco- and mineralocorticosteroids and androgenic steroids, a process that consumes cholesterol. ACTH stimulates the adrenal uptake of lipoproteins through increased LDLRs and SR-BI (91).

The gonadotropins, luteinizing hormone and follicle stimulating hormone (FSH), are important hormones in reproduction and stimulate ovaries and testis to produce estrogen and testosterone. 17β-estradiol (E_2) is the natural estrogen with the highest biological activity whereas the synthetic derivative 17α-ethinylestradiol is much more potent. It is well known that estrogen treatment has multiple effects on cholesterol metabolism in humans, with reduced levels of plasma LDL, apoB, Lp(a), and increased levels of plasma HDL-C and apoAI (92-96). Additionally, treatment with estrogen increases the number of hepatic LDLRs *in vivo* (97-99). Fertile women have lower plasma LDL-C than men of same age (100), but after menopause the levels increase (101) presumably partly due to reduced levels of endogenous estrogen. The effects of estrogen are usually mediated by estrogen receptors, and estrogen receptor expression is influenced by other hormones such as GH and TH (102, 103). Removal of the pituitary leads to reduced levels of hepatic estrogen receptors, but the levels can be restored with GH substitution (104). Estrogens also influence the GH-pathway by increasing the pituitary secretion of GH (105, 106).

Fasting and feeding alter the secretion of insulin and glucagon that control the plasma glucose level. Both insulin and glucagon influence cholesterol metabolism partly through induction of hepatic LDLRs (107, 108), and glucagon has also been shown to induce hepatic uptake of HDL-C by regulation of PDZK1, an posttranscriptional modulator of SR-BI (109). FGF19 and FGF21 are two recently identified hormones involved in the metabolism, FGF19 participates in the regulation of bile acid synthesis (as described above) and FGF21 is increased during prolonged fasting (110) and is involved in glucose metabolism, although its role is still not clear (111).

1.6 PCSK9

1.6.1 Gene variants

PCSK9 is the ninth member of the proprotein convertase family, and was originally named NARC-1 (112). In 2003, gain of function (GOF) mutations in *PCSK9* were related to FH in French families (113). Several different *PCSK9* GOF mutations have now been described (114), leading to dysfunctional LDLRs and thereby increased plasma LDL-C. The importance of PCSK9 in cholesterol metabolism was highlighted when loss of function (LOF) mutations in the gene for PCSK9 were found to cause hypocholesterolemia (115). When screening more than 12 000 Americans, *PCSK9* LOF variants Y142X or C697X had a prevalence of 2.6% among black individuals whereas the LOF variant R46L had a prevalence of 3.2% among white individuals. Subjects carrying LOF mutations have significantly lower plasma LDL-C levels (116). When the incidence of CHD was followed for 15 years, carriers of Y142X or C697X had 30% lower plasma LDL-C and 90% reduced risk of CHD, whereas R46L carriers had 15% lower plasma LDL-C and 50% reduced risk, as compared to non-carriers (116). These dramatic risk reductions are probably due to a life-long reduction of plasma LDL-C (117) and highlight the benefit of lowering plasma LDL-C levels at an early age.

A few individuals totally lacking PCSK9 have been reported, further supporting the importance of PCSK9 for plasma LDL-C levels. Two subjects, heterozygous for two compounded LOF mutations have been found, who have undetectable plasma PCSK9 levels and extremely low plasma LDL-C of 0.36 mM and 0.41 mM (118, 119). One woman homozygous for a PCSK9 LOF mutation has also been found, and was reported to be completely healthy, with a plasma LDL-C of 0.41 mM (120).

1.6.2 Function

Studies in genetically modified mice have revealed PCSK9 as a key regulator of LDLRs *in vivo*. When *pcsk9* was over-expressed in a mouse model the number of LDLRs was decreased without any reduction of the LDLR mRNA levels (121, 122), clearly indicating that PCSK9 regulates LDLR in a posttranscriptional manner. On the other hand mice lacking *pcsk9* had increased numbers of LDLRs and hypocholesterolemia (123). PCSK9 is secreted and circulates in the blood with biological activity, first demonstrated when the bloodstreams of a normal mouse and a *PCSK9* over-expressing mouse were connected, and the normal mice acquired a

reduced number of LDLRs (124). Additionally, when recombinant PCSK9 was given to mice, 90% of hepatic LDLRs were gone within 60 min. Recombinant PCSK9 was rapidly removed from the circulation mainly by hepatic LDLR-mediated uptake, resulting in a PCSK9 half-life of only 5 min (125).

PCSK9 is mainly expressed in the liver (126), but also in other tissues such as intestine, kidney, lung and spleen (112, 126). There is clear evidence that the main part of circulating PCSK9 is of hepatic origin and that circulating PCSK9 reflects its hepatic expression. Knocking out hepatocyte-specific *pcsk9* expression in mice resulted in a 95% reduction of circulating PCSK9 and strongly increased hepatic LDLRs (126). In addition, when knocking out hepatic PCSK9 by small interfering RNA (siRNA) in mice, rats and cynomolgus monkeys hepatic LDLRs were strongly increased and subsequently plasma LDL-C reduced (127). Hepatic LDLRs were also strongly increased and plasma LDL-C decreased following hepatic suppression of PCSK9 mRNA using antisense oligonuclieotides (128), clearly pointing to PCSK9 as an attractive drug target.

If PCSK9 also regulates extrahepatic LDLRs is not fully understood. Adrenal-LDLRs are not regulated by PCSK9 and PCSK9 is not expressed in the adrenals (125). Other extrahepatic LDLRs e.g. in adipose tissue, kidney and lung have however been reported to be moderately suppressed by exogenous PCSK9 administration (129).

1.6.3 Interaction with the LDLR

ProPCSK9 is a 76kDa protein and is synthesized in the ER containing a signal sequence, a pro-domain, a catalytic domain and a C-terminal domain. The pro-domain is auto-cleaved in the ER but remains covalently bound and the mature 62kDa PCSK9 is secreted together with its pro-domain (130). Mature PCSK9 can bind to the LDLR, via interaction of PCSK9's catalytic domain with the LDLR-EGF-A domain (131) and when pH is decreased, the affinity between PCSK9 and LDLR increases (132). The LDR-PCSK9 complex is internalized and directed to lysosomal degradation (133) but the exact mechanism for how PCSK9 leads the LDLR to lysosomal destruction is not clear. Clathrin-mediated endocytosis seems to be required (134, 135), whereas PCSK9's catalytic activity is not (136). Early studies suggested that PCSK9 could lead the LDLR to degradation through an intracellular route (130). However extracellular

PCSK9 is sufficient for the reduction of LDLRs (137), and seems to be the major pathway for PCSK9-mediated LDLR destruction *in vivo*.

The reasons why different *PCSK9* variants lead to hypo- or hypercholesterolemia are diverse. The *PCSK9*-D374Y GOF mutation, leading to severe hypercholesterolemia and reported plasma LDL-C levels of >10 mM (138, 139), binds to the LDLR with higher affinity than normal variants resulting in pronounced LDLR degradation (140). Since this mutated form of PCSK9 binds to the LDLR with higher affinity, it is also removed from plasma more efficiently leading to lower plasma PCSK9 levels than expected (141). However, PCSK9 LOF mutations often lead to reduced plasma PCSK9 levels due to impaired effects of PCSK9's synthesis, secretion or folding (119).

1.6.4 Regulation

PCSK9, LDLR and HMG-CoA reductase are all transcriptinally regulated by SREBP-2 (36, 37) and a positive correlation between PCSK9, SREBP-2, HMG-CoA reductase and LDLR mRNA levels has been shown in human liver (142). And as mentioned above, SREBP-2 is activated when the intracellular cholesterol content is low, leading to up regulation of PCSK9, LDLR and HMG-CoA reductase, and during excess of intracellular cholesterol, SREBP-2 target genes are down regulated (33-35).

Statins inhibit cholesterol synthesis, leading to the depletion of cholesterol in the cell; activation of SREBP-2; and up regulation of LDLR and PCSK9 gene transcription (46). During statin treatment more LDLRs are synthesized but in parallel more LDLRs are degraded due to higher levels of circulating PCSK9 (45). The up regulation of PCSK9 thereby counteracts the lipid lowering effect of statins, and mice lacking PCSK9 are therefore hypersensitive to statins (123).

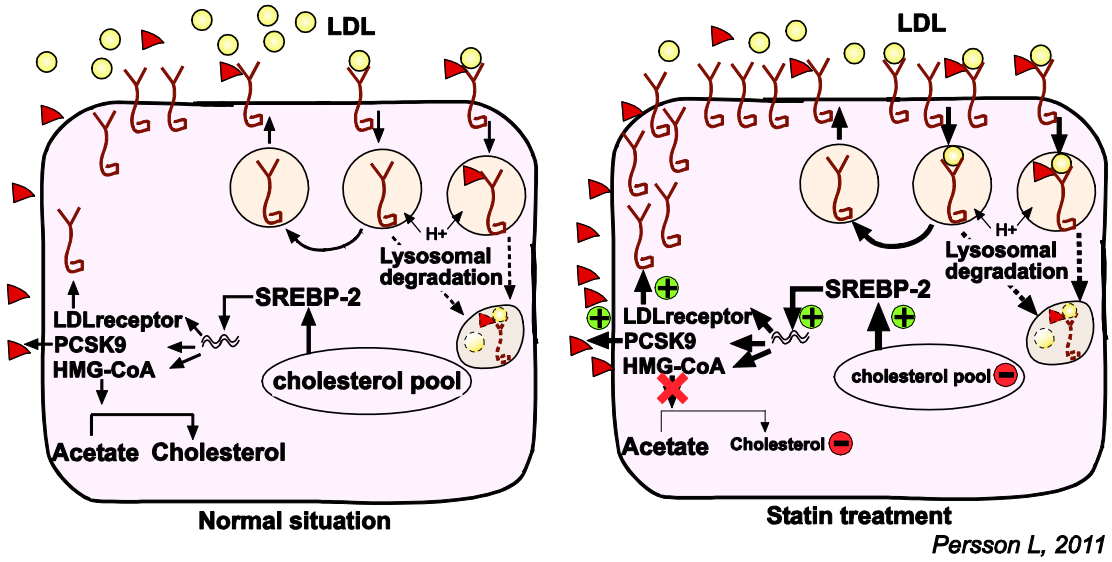

Fig. 2 Schematic picture of the statin-effects on SREBP-2, LDLR and PCSK9 in a hepatocyte

Fasting increases hepatic cholesterol (143), and fasted mice have decreased SREBP-2 levels and accordingly suppressed target genes (144) including PCSK9 (145). When PCSK9 over-expressing mice are fasted they develop hypercholesterolemia (146), indicating that these mice have low LDL catabolism due to reduced LDLR synthesis in combination with high LDLR degradation. Insulin treatment upregulates PCSK9 mRNA levels in mice (145), and declining insulin secretion may contribute to the fasting-mediated down regulation of PCSK9. Streptozotozin treated rats, a model to induce type-1 diabetes, have also decreased levels of PCSK9, presumably due to lack of insulin (147). However, healthy and diabetic patients treated with a 24h insulin infusion have unaltered serum PCSK9 levels (148).

The PCSK9 promoter contains an SRE-element (149), but whether transcription factors other than SREBP-2 regulate PCSK9 is not clear. A hepatocyte nuclear factor (HNF)-1 binding site is also present in the PCSK9 promoter (150), and *in vitro* treatment with the lipid lowering compound berberine may reduce PCSK9 through HNF-1α (150); if this binding site is of physiological relevance is unclear. *In vitro* treatment with bile acids such as CDCA down regulates PCSK9 mRNA possibly via FXR involvement (151), but CDCA treatment in humans does not affect hepatic PCSK9 mRNA levels (142). Involvement of PPARα has also been postulated though *in vitro* treatment with fibrates, which repressed PCSK9 via decreased activity in the PCSK9 promoter (152). However, fibrate treatment to humans has been shown to increase plasma PCSK9

levels (153-155), and whether the PCSK9 promoter contains any PPARα responsive elements is unknown.

Postranscriptional regulation of PCSK9 has not yet been described, although PCSK9 undergoes glycosylation, sulfation and phosphorylation modifications during its processing (130, 156, 157).

1.6.5 Relevance in humans

As mentioned, circulating PCSK9 is mainly of hepatic origin and relates to the hepatic expression of PCSK9. In humans circulating PCSK9 levels vary 100-fold between individuals, with a skewed distribution (158). Plasma PCSK9 levels correlate positively with plasma LDL-C in a large number of studies (158-163) but the association is modest. In the largest study with over 3000 subjects PCSK9 does only predict 7% of the variation in plasma LDL-C (158). The LDLR half life however is strongly related to plasma PCSK9 levels, suggesting that the circulating PCSK9 level has a strong impact on LDL-apoB catabolism (164), even though other factors such as the level of LDLR mRNA synthesis complicate the prediction of LDL-C plasma values. As described above, statins induce PCSK9 expression, and when patients are treated with statins, plasma PCSK9 levels increase dose dependently (45, 165). Combining statin with Ezetimibe further increases PCSK9 plasma levels (161, 165). Other lipid lowering drugs such as fibrates also increase plasma PCSK9 (47, 153-155).

Circulating PCSK9 levels are higher in premenopausal women compared to men (158) and plasma PCSK9 is also higher in girls than in boys (166). After menopause plasma PCSK9 increases (158, 163) which could partly contribute to the increase of plasma LDL-C in postmenopausal women. If PCSK9 is altered during ageing is not clear, plasma PCSK9 and age correlate positively but moderately in some studies (161, 163, 165) but not all (158, 160).

Regulation of the LDLR seems to be the main action of PCSK9 in cholesterol metabolism, supported by the observation that double knockout PCSK9/LDLR mice have not altered plasma lipid levels as compared to LDLR knockouts (126). It has, however, been postulated that PCSK9 is also connected to the metabolism of TGs and somehow involved in diabetes. In some studies, plasma PCSK9 levels are moderately correlated to plasma TG levels (158, 161, 163), and additionally, patients with the

PCSK9-S127P GOF mutation have an increased secretion of apoB100 (167), indicating that PCSK9 might be involved in the production or secretion of VLDL. But PCSK9 is not associated with the VLDL and LDL-apoB production (164) and does not influence the secretion rate of VLDL (122). Newly synthesized VLDL particles are efficiently taken up by hepatic LDLRs (7) and therefore it is likely that PCSK9 influences the secretion of VLDL indirectly (123), without altering VLDL production.

Plasma PCSK9 has been reported to be slightly higher in diabetic patients compared to healthy subjects (158, 162), although no such difference could be found in a recent study (168). Additionally, plasma PCSK9 correlates moderately with insulin resistance, determined as homeostasis model assessment-insulin resistance (HOMA-IR), an indicator of insulin sensitivity, and fasting glucose (158, 161, 166). Subjects with fatty liver have increased risk to develop insulin resistance and diabetes. It has been shown that plasma PCSK9 correlates positively but weakly with hepatic TG contents, measured with proton magnetic spectroscopy (158), and with gamma-glutamyltransferase (GGT) in diabetic patients (162). GGT is a marker for fatty liver as is also ALT, and plasma PCSK9 did not correlate with ALT (162). The relation between fatty liver, hepatic cholesterol content and SREBP-2 activity is not clear, and if PCSK9 is involved in the progress of diabetes or a marker for hepatic cholesterol processing is unknown.

PCSK9-LOF carriers have very low plasma LDL-C and have been reported to be healthy. When LOF carriers were studied in two large prospective studies it was concluded that these subjects do not have any increased risk of cancer or premature death (169, 170). Additional, PCSK9 knockout mice have no other phenotype than hypocholesterolemia, which indicates that PCSK9 is not critical for life, and further suggests that PCSK9 has no other function than regulating LDLR. However, it is still unclear if the PCSK9-mediated degradation of LDLRs is important during certain physiological situations or if it could be an evolutionary advantage to express PCSK9. One hypothesis is that the PCSK9-mediated degradation of LDLR via SREBP-2 is a mechanism to prevent re-uptake of newly secreted VLDL particles and thereby redirecting them to peripheral tissues (125).

1.6.6 Action on other receptors

It has been studied if PCSK9 can regulate other receptors in the LDLR family such as the VLDLR, apoER2 or LRP1. Treatment with PCSK9 *in vitro* and ex vivo reduced the levels of VLDLR and apoER2 (171), and more recent results have shown that PCSK9 knockout mice have larger adipocytes and increased levels of adipocytic VLDLRs (172). Although VLDLR contains a EGF-A domain similar to that of LDLR, binding studies have not confirmed that PCSK9 interacts with VLDLRs (131). It has also been reported that PCSK9 can bind to LRP1 with low affinity (124). Taken together, it has not been clearly shown that PCSK9 influences other members of the LDLR family.

There are some results indicating that PCSK9 might mediate a down regulation of the CD81 receptor (173), a cell surface receptor involved in the hepatitis C infection. This might suggest that PCSK9 could prevent hepatitis C infections, but this hypothesis needs further research.

1.6.7 Inhibition of PCSK9

PCSK9 has great impact on the plasma LDL-C levels and has therefore emerged as an attractive drug target. A PCSK9 inhibitor could have a pronounced cholesterol lowering effect especially in combination with statin treatment, since statins up regulate PCSK9 thus counteracting their lipid lowering effect. Statins are the most frequently used lipid lowering class of drugs globally and it is obviously of great interest to improve the efficiency of statins. Different ways to inhibit or block PCSK9 have therefore evolved, and in 2010 there were at least 30 different approved patents by ~20 companies (174). Blocking PCSK9 synthesis by siRNA (127) or antisense oligonucletides (128, 175) have proven to be efficient in rodents and non-human primates, with approximately a 50% reduction of plasma LDL-C. Other potential ways are to interfere with the binding between PCSK9 and LDLR. When mice and cynomolgus monkeys received a single injection with an interfering-antibody, plasma LDL-C was reduced by up to 80% (176). Small peptides that interact with the LDLR-EGF-A domain (177) and antibodies that prevent PCSK9 from being internalized (178) have also been developed and shown to increase LDLR expression. The development of anti-PCSK9 agents has progressed during the last few years and soon we will know how effective these compounds will be in humans.

2 AIM

The overall aim of this thesis was to increase the knowledge of PCSK9's role in cholesterol metabolism, and to investigate the regulation of PCSK9 by hormonal, dietary and diurnal influences *in vivo*.

The specific aims of the respective papers were:

I) To study the age-dependent elevation of plasma cholesterol and how hepatic PCSK9 mRNA levels are influenced by age and GH treatment in rats.

II) To study if hepatic PCSK9 is hormonally and dietary regulated in rats.

III) To gain insight into the function of PCSK9 in humans by establishing whether circulating PCSK9 is influenced by diurnal, dietary and hormonal changes.

IV) To study the role of endogenous estrogen on circulating PCSK9 and cholesterol metabolism in women.

3 MATERIAL AND METHODS

3.1 ANIMALS

Animal experiments were approved by the Karolinska Institute Institutional Animal Care and Ethic committee, Stockholm, Sweden. Rats were kept under standardized conditions with free access to water and chow and light on between 6 AM and 6 PM.

Paper I; 47 male Wistar-Hannover rats 6 and 18 months old were given Ezetimibe 3mg/kg body weight/day for 12 days, and/or bovine GH 1.5 mg/kg body weight/day for 1 week s.c.

Paper II; 62 male Sprague Dawley rats were used in four separate experiments, conducted earlier (107, 179). Rats were given 1) glucagon 400 µg x 2/day s.c. in a time course, 2) four days s.c. treatment with glucagon 400 µg x 2 and/or ethinylestradiol 5 mg/kg body weight/day, controls received vehicle, 3) four days s.c. treatment with glucagon 400 µg x 2 and/or insulin 10 IU x 2, controls received vehicle, 4) 2% cholesterol enriched diet for 8 days, controls received ordinary chow.

3.2 HUMANS

All subjects or their parents gave their informed consent to participate in the studies, which were all approved by the Ethic committees of Karolinska Institute, Stockholm, Sweden.

Paper III; 90 subjects in nine separate experiments. 1) 5 subjects followed every 90th min during 25 hrs. (180), 2) 5 subjects followed every 60th min during short-term fasting of 18 hrs. (180), 3) 10 subjects followed every 90th min. during 33 hrs. eating cholestyramine during the first 12 hrs., 4) 7 subjects before and during 48 hrs. of fasting (181), 5) 5 subjects before and during 7 days of fasting (182), 6) 17 children before and during 1-16 months of consuming a ketogenic diet (183), 7) 12 subjects followed over 8 days during 66 hrs. of fasting and 50 hrs of sleep deprivation in a crossover study, 8) 15 subjects before and after 3 weeks of increasing GH treatment, the last week 0.1 IU/kg body weight/day (80), 9) 19 subjects before and after 4 weeks of atorvastatin 80 mg/day treatment.

Paper IV; 31 female subjects scheduled for *in vitro* fertilization (IVF) -treatment, samples drawn during suppression and stimulation of endogenous estrogen production. Estrogen production was suppressed with gonadotropin releasing hormone agonist, and stimulated with a six-day long treatment of recombinant human FSH.

3.3 LIPID ASSAYS

Serum total cholesterol and TGs were determined using routine colorimetic techniques with reagents from Roche Diagnostics (Gmbh, Mannheim, Germany). Serum lipoproteins were size-fractionated by fast preformace liquid chromatography (FPLC) as previous described (184) using reagents from Roche Diagnostics (Gmbh, Mannheim, Germany). Cholesterol and TG fractions in the various lipoproteins were calculated using areas under the curves, as previously described (184). Serum ApoAI and apoB were determined using immunoturbidimetric techniques with reagents from Kamiya Biomedical Company (Seattle, WA, US).

3.4 GENE EXPRESSION

Hepatic gene expression was analyzed using Quantitative real-time PCR. Total RNA was extracted from frozen individual liver samples using Trizol reagent (Invitrogen, Carlsbad, CA). cDNA synthesis was carried out using total RNA (1 µg) using random-hexamer priming and Omniscript (QIAGEN, Valencia, CA). Quantitative real-time PCR was performed in an ABI Prism 7700 sequence detection system (ABI, Foster City, CA) using SYBR Green. Primers for each target gene are described in the papers. The comparative Ct method was used to quantify the results.

3.5 HEPATIC PROTEIN EXPRESSION

Preparation of liver membranes for the assay of LDLR by ligand blot were prepared as described previously (82), and for the assay of PCSK9 and LDLR by immunoblot as described in paper II.

3.5.1 Ligand blot to determine LDLR

In short, membrane proteins were separated on gel unreduced, blotted onto nitrocellulose filters and incubated with 125I-labeled rabbit VLDL (82), exposed on x-ray film and quantified using a Fuji Bio-imaging BAS 1800 analyzer with Multi Gauge software version 3.1 (Fuji Photo Film Co., Tokyo, Japan). The quantified bands were expressed as arbitrary units after subtraction of the background.

3.5.2 Immunoblot to determine PCSK9 and LDLR

In short, reduced membrane proteins were separated on gel, blotted onto nitrocellulose filters and incubated with the respective primary antibody. Immunoreactive proteins were visualized using secondary antibodies and the Western lightning chemiluminescence Reagent Plus (PerkinElmer Laboratories Inc., Boston, MA). Filters were exposed on x-ray film and quantified using a LAS 1000 plus imager with Multi Gauge software Science Lab 2005 version 3.1 (Fuji Photo Film Co., Tokyo, Japan). The quantified bands were expressed as arbitrary units after subtraction of the background. The antibodies used are described in paper II.

3.6 ASSAY OF 7 ALPHA-HYDROXY-4-CHOLESTEN-3-ONE (C4)

The serum concentration of C4, a plasma marker for bile acid synthesis, was assayed as described previously (185). The samples were extracted on C8 Isolute SPE columns (500 mg and 3 ml, International Sorbent Technology, Hengoed, UK) and separated by HPLC (HP 1100 series, Hewlett-Packard, Waldbronn, Germany) using deuterium labeled 7α-hydroxy-4-cholesten-3-one as internal standard and normalized for total cholesterol.

3.7 ASSAY OF CHOLESTEROL SYNTHESIS

Serum unesterified lathosterol, reflecting cholesterol synthesis was determined by isotope dilution mass spectrometry after addition of deuterium-labeled internal standard and corrected for total cholesterol, as described previously.(186)

3.8 ASSAY OF CIRCULATING PCSK9

Serum PCSK9 concentrations were in paper III measured using a PCSK9 dual–monoclonal antibody sandwich ELISA developed (159) and modified (154) by Drs. Konrad, Cao and Troutt at Eli Lilly and Company, Research Laboratories, IA, US. Serum PCSK9 concentrations were in paper IV measured using a commercially available ELISA (Cat. No. Circulex CY-8079, CycLex Co., Ltd., Japan) with quantitative sandwich enzyme immunoassay technique.

3.9 ASSAYS OF INTESTINAL CHOLESTEROL ABSORPTION

The fecal dual isotope method was used to assay intestinal cholesterol absorption in rats, as described in paper I. In short, rats received intragastric gavage containing 5 μCi

[^{14}C] and 2 μCi [5,6-^{3}H] sitostanol in corn oil, and the percentage absorbed cholesterol were calculated from the ratio of [^{14}C] / [^{3}H] in feces compared to dosing mixture.(187-189) Serum plant sterols, sitosterol and campesterol (reflecting cholesterol absorption) (188, 190) were extracted, derivatized and analyzed by gas chromatography mass spectrometry(191) using D5-campesterol/sitosterol as internal standard.

3.10 ELISA ASSAYS

Serum levels of GH, FGFs, IGF-1 were all measured using commercially available ELISA kits from R&D systems (Minneapolis, MN, US), according to manufacturer's instructions.

3.11 STATISTICS

Statistical software programs were used to calculate significant differences, see each paper for used methods.

4 RESULTS AND COMMENTS

4.1 AGE-INDUCED HYPERCHOLESTEROLEMIA IN THE RAT RELATES TO REDUCED ELIMINATION BUT NOT INCREASED INTESTINAL ABSORPTION OF CHOLESTEROL (PAPER I).

Both humans and rats present an age-dependent increase of plasma LDL-C that may contribute to the accelerating atherosclerosis that occurs during ageing. GH secretion is progressively decreased with age (83, 84) and substitution with GH completely restores plasma LDL-C to juvenile levels (85). GH exerts several important effects on cholesterol metabolism (76, 77) e.g. it decreases plasma LDL-C (80, 81) and increases hepatic LDLR protein levels (82), but if GH affects intestinal cholesterol absorption or PCSK9 is unknown.

Total cholesterol was 72% higher in old rats compared with young rats, and treatment with GH reduced LDL-C levels in old rats to levels seen in young rats. Intestinal cholesterol absorption, measured with the fecal dual isotope assay, was not changed with age and was unaffected by GH treatment. Treatment with Ezetimibe resulted in a strongly reduced cholesterol absorption in both young and old rats, as expected. Thus, the age-dependent increase of plasma LDL-C in rats does not seem to be due to increased intestinal cholesterol absorption.

The hepatic gene expression of PCSK9 or LDLR was not significantly altered between old and young rats and GH treatment increased the gene expression of PCSK9 and LDLR in both young and old rats. PCSK9 seems not to be involved in the age-induced hypercholesterolemia in rats, even though PCSK9 mRNA levels tended to be higher (18%) in old rats compared to young rats. The fact that GH increases both PCSK9 and LDLR mRNA levels could indicate that SREBP-2 is activated.

To further elucidate age-dependent metabolic changes, we assayed bile acid synthesis from C4 levels. Old rats had 50% lower bile acid synthesis as compared to young rats, but their levels were increased up to “young” levels after GH treatment. When measuring CYP7A1 mRNA levels, no significant difference between young and old rats could be detected whereas treatment with GH strongly increased the gene expression of CYP7A1, especially in young rats. Thus, an age-dependent reduction of bile acid synthesis may contribute to the age-induced hypercholesterolemia in rats, in

accordance with previous findings (85), and GH substitution can restore bile acid synthesis to "young" levels.

4.2 IMPORTANCE OF PROPROTEIN CONVERTASE SUBTILISIN/KEXIN TYPE 9 IN THE HORMONAL AND DIETARY REGULATION OF RAT LIVER LOW-DENSITY LIPOPROTEIN RECEPTORS (PAPER II)

It is well known that PCSK9 is transcriptionally regulated by SREBP-2 (36, 37), but if hormones and diets can regulate PCSK9 *in vivo* is not clear. Estrogen (107, 192, 193) treatment stimulates hepatic LDLR protein expression in rats to a degree that cannot solely be explained by the corresponding LDLR mRNA levels. Glucagon also stimulates hepatic LDLR protein expression in rats by an unclear mechanism independent of LDLR gene expression (107). Another situation with clear discrepancy between LDLR protein and mRNA levels is when rats are challenged with cholesterol. Rats receiving a cholesterol-enriched diet have increased numbers of hepatic LDLRs but decreased or unaltered LDLR mRNA levels (179, 194).

A single glucagon injection (400 μg) to rats decreased hepatic PCSK9 mRNA levels by >50%, and stimulated LDLR protein expression already after 3 hours, whereas LDLR mRNA levels were unchanged. Glucagon treatment also reduced PCSK9 proprotein levels over time, with the strongest effect after repeated injections for 98 hours. Insulin treatment (10IU twice a day for 4 days) increased PCSK9 mRNA levels, in accordance with data obtained in insulin-treated mice (145). Ethinylestradiol is known as a very powerful stimulator of hepatic LDLR protein levels, and we could show that rats treated with ethinylestradiol (5mg/kg/day) had reduced PCSK9 mRNA and proprotein levels concomitant with increased LDLR mRNA levels, resulting in 6-fold increased LDLR protein levels. Combining ethinylestradiol and glucagon resulted in a 5-fold increase in LDLR protein levels whereas LDLR mRNA levels were only slightly elevated. The strong increase of LDLR protein levels could be explained by an 80% reduction seen in PCSK9 levels. Thus, the stimulatory effect of glucagon and ethinylestradiol on hepatic LDLR protein expression can partly be explained by reduced levels of PCSK9.

Rats do not become hypercholesterolemic when fed a cholesterol-enriched diet, due to unchanged or even moderately increased hepatic LDLR protein levels (179, 194). We could show that rats fed a 2% cholesterol diet had 70% increased hepatic LDLR protein

levels, whereas LDLR mRNA levels were decreased by 30%. PCSK9 mRNA and its proprotein levels were also reduced by more than 50%, which could explain the increased number of LDLRs that presented following the cholesterol-enriched diet.

We also investigated if the hormonal and dietary effects on PCSK9 could be mediated by SREBP-2. SREBP-2 mRNA levels were reduced by 50% following both the cholesterol-enriched diet and during treatment with ethinylestradiol, indicating that hepatic intracellular cholesterol levels were increased during these two situations. Glucagon treatment only moderately reduced SREBP-2 mRNA levels, suggesting that regulation by other transcriptional factors may occur.

SREBP-2 activates its own transcription (38), but measuring nuclear levels of SREBP-2 protein may be a more accurate measurement of SREBP-2 activity. At the time of this study no antibodies against rat-PCSK9 were available and an antibody against mouse-PCSK9 was used.

4.3 CIRCULATING PROPROTEIN CONVERTASE SUBTILISIN KEXIN TYPE 9 HAS A DIURNAL RHYTHM SYNCHRONOUS WITH CHOLESTEROL SYNTHESIS AND IS REDUCED BY FASTING IN HUMANS (PAPER III).

Genetic variants of PCSK9 influence plasma LDL-C levels (113, 115, 116, 138) and the level of circulating PCSK9 relates to its hepatic expression (45, 123, 154, 158). If PCSK9 is regulated during hormonal, dietary and diurnal perturbations in humans and if such a regulation may influence plasma LDL-C levels is unknown.

We investigated whether circulating PCSK9 has a diurnal rhythm, and if this rhythm could be disturbed by a single day of cholestyramine treatment. We assayed serum PCSK9 in samples taken every 90th min during 25 hours in 5 healthy subjects. Circulating PCSK9 displayed a diurnal rhythm and varied ±15% from the mean, with a nadir between 3 and 9 PM and peaking at 4:30 AM, these variations were similar to those for serum lathosterol/c, a marker of cholesterol synthesis. Despite these variations serum cholesterol levels were stable. Depleting hepatic intracellular cholesterol levels by short-term treatment with cholestyramine abolished the diurnal rhythms of both PCSK9 and cholesterol synthesis, indicating that hepatic intracellular cholesterol levels may regulate circulating PCSK9 in humans during the day. The LDLR gene is also

regulated by hepatic intracellular cholesterol levels via SREBP-2, suggesting that a diurnal rhythm of LDLR mRNA levels may also be present. Similar diurnal variations of LDLR synthesis and degradation would stabilize LDLR numbers, resulting in unaltered serum LDL-C levels over the day, as found in our experiment.

The findings of a diurnal variation of serum PCSK9 concomitant with stable serum LDL-C levels could partly explain why circulating PCSK9 levels relate poorly to plasma LDL-C levels, as previously described (158). The 15% reduction of serum PCSK9 from the morning until 3 PM is not due to breakfast intake, since PCSK9 and cholesterol synthesis were reduced to the same extent when the subjects abstained from breakfast. The diurnal variation of serum PCSK9 stresses the importance of standardized blood samplings and the use of appropriate controls in diurnal studies.

The dynamic regulation of PCSK9 demonstrated during the diurnal phases was also prominent during fasting. Fasting for 48 hours or 7 days strongly reduced circulating PCSK9 and cholesterol synthesis. A ketogenic diet did not alter serum PCSK9 levels, suggesting that ketosis per se does no influence serum PCSK9 levels. We could also show that fasting for 18 hrs suppressed both serum PCSK9 and cholesterol synthesis by ~ 35%, and longer fasting reduced the levels even further, so that after 66 hrs of fasting serum PCSK9 and cholesterol synthesis were reduced with more than 60%. PCSK9 and lathosterol/c were strongly correlated, indicating that they may be regulated by the same mechanism, presumably hepatic intracellular cholesterol levels via SREBP-2. Reduced SREBP-2 activity would also lead to reduced synthesis of LDLRs. The combination of low LDLR synthesis and low PCSK9-mediated LDLR degradation would result in unchanged LDLR protein levels and consequently unaltered serum LDL-C, as was also found. In accordance, fasted pigs have increased hepatic cholesterol levels (143) and fasted mice have decreased activity of SREBP-2 (144), and reduced levels of PCSK9 mRNA (145).

As expected, atorvastatin treatment increased serum PCSK9 by 33%, in accordance with previous results (45, 165). GH treatment of humans has previously been shown to reduce serum LDL-C (80) and increase the number of hepatic LDLRs (82), without altering lathosterol/c (80). We could here show that serum PCSK9 levels decreased after GH-treatment. These results suggest that the GH-mediated increase of hepatic

LDLRs and decreased serum LDL-C is partly due to decreased levels of PCSK9, a regulation that may involve SREBP-2 independent pathways.

If circulating PCSK9 and lathosterol/c levels reflect the hepatic activities of PCSK9 and HMG-CoA reductase, respectively, in all situations is not fully clear and direct measurements of hepatic PCSK9, HMG-CoA reductase, SREBP-2 and intracellular cholesterol levels are highly warranted, although such measurements would require repeated liver biopsies which in our studies were impossible.

4.4 STIMULATION OF ENDOGENOUS ESTROGEN PRODUCTION IN FEMALES REDUCES CIRCULATING PCSK9 AND LDL CHOLESTEROL LEVELS (PAPER IV).

Gender differences in lipoprotein metabolism are well established; men generally have higher plasma LDL-C and lower HDL-C than females and a predominance of early atherosclerosis. During ageing, plasma LDL-C increases and postmenopausal women acquire higher plasma LDL-C then fertile women and men (101). Treatment with estrogen has multiple effects on lipoprotein metabolism, including reduced levels of LDL-C and apoB as well as increased levels of HDL-C and apoAI (92-94, 96). Reduced levels of LDL-C and apoB could be the result of an increased number of hepatic LDLRs, previously shown to occur in estrogen treated animals and humans (97-99). Little data is available on the effects of endogenous estrogen on cholesterol metabolism, and if estrogens influence PCSK9 in humans has not been studied before.

We could show that stimulation of endogenous estrogen levels resulted in distinct changes in cholesterol and lipid metabolism. As expected, total cholesterol (-13%), VLDL-C (-22%) and LDL-C (-20%) were all reduced when endogenous estrogen were high, as were apoB levels, whereas HDL-C was unchanged. Circulating PCSK9 levels were reduced by 14%, supporting the hypothesis that an increased LDLR number is partly caused by estrogen-mediated suppression of PCSK9, as previously demonstrated in rats (paper II). Since PCSK9 is co-regulated with HMG-CoA reductase (36, 37, 142) and cholesterol synthesis (paper III), we predicted that lathosterol/c may decrease when endogenous estrogens were high. However, lathosterol/c were unaltered and, if anything, increased ($p=0.09$), suggesting that (an) SREBP-2 independent mechanism(s) may be involved in the estrogen-induced reduction of PCSK9.

In contrast to HDL-C, serum apoAI levels were clearly increased when endogenous estrogens were high, presumably due to increased hepatic production of apoAI as previously reported (94-96) indicating that the latter may be an early response to estrogen stimulation, whereas increases in HDL-C may require longer exposure (92-95). Total TGs (40%), and TGs within HDL (58%) and LDL (37%) fractions were also increased. The composition changes of HDL and LDL is not likely due to CETP, since CETP activity was unaltered. These compositional changes could be due to an early decrease in the activity of hepatic lipase, a well-known consequence of estrogen treatment reported previously (94, 195, 196). Further, increased levels of TGs in VLDL were absent and may thus require longer estrogen exposure. Chronic estrogen treatment has repeatedly been shown to increase TG synthesis and apoB production (94, 96, 197), leading to higher levels of VLDL.

The stimulation of endogenous estrogen levels strongly increased the concentration of GH, in accordance with what is seen during exogenous estrogen treatment (105, 198, 199). Several effects of estrogen on cholesterol metabolism may thus actually be mediated by an increased secretion of GH. If GH regulates PCSK9 independent of SREBP-2 is unclear. We also investigated whether the acute lipid lowering effect of estrogen could be due to increased bile acid synthesis or to decreased intestinal cholesterol absorption. However, bile acid synthesis or cholesterol absorption both appeared unaltered.

In this model, endogenous estrogens were increased 40-fold after six days of FSH-stimulation. However, it could be argued that FSH treatment may also influence other hormones, including testosterone and progesterone (200, 201). Thus, some of the responses found could theoretically be mediated by other hormones. Still, this study is important since most other studies investigate the effects of exogenous estrogen (which will also interfere with other hormonal responses). In our model we investigated the effects of 40-fold elevations of endogenous estradiol levels on cholesterol metabolism. Endogenous estrogen levels vary with the menstrual cycle and during life, with high levels during pregnancy and low levels after menopause. Postmenopausal women have higher levels of LDL-C and clearly increased risk for CHD (101), whereas pregnant women have a clearly increased risk for gallstone disease. Elucidating the metabolic effects during high endogenous estrogen levels should therefore be of major importance.

5 GENERAL DISCUSSION

Studies during the last 8 years have established that PCSK9 is a key regulator of cholesterol metabolism, and it has emerged as an attractive drug target. PCSK9 initiates the degradation of LDLRs leading to reduced clearance of apoB particles. The inhibition of PCSK9 will therefore reduce plasma LDL-C. If PCSK9 is regulated by hormones, diets and during the diurnal phases has hitherto been unknown.

5.1 "PARADOXICAL" PCSK9 REGULATION IN RATS

The number of LDLRs is the net consequence of their synthesis and degradation. Though both LDLR and PCSK9 are transcriptionally regulated by SREBP-2, the regulation is complex, which is especially evident during cholesterol feeding and statin treatment of rats. When rats are fed a cholesterol-enriched diet they do not develop hypercholesterolemia, instead hepatic LDLRs are up regulated leading an improved clearance of LDL-particles (179, 194). We have shown that cholesterol-fed rats have increased LDLR numbers even though the LDLR mRNA levels are reduced, a discrepancy that can be explained by a strong down regulation of PCSK9 mRNA expression (Paper II). The transcriptional down regulation is likely exerted by SREBP-2, in accordance with what is seen in cholesterol- fed mice (149). The suppression of PCSK9 mRNA levels is, however, stronger than that of the LDLR mRNA, resulting in an induced number of hepatic LDLRs, which partly may contribute to the resistance to cholesterol when rats are exposed to a cholesterol-enriched diet. Rats are also resistant to statin therapy, and do not respond with a plasma cholesterol lowering effect (48, 49). Statins deplete the intracellular cholesterol pool, leading to an up regulation of both PCSK9 and LDLR mRNA via SREBP-2. In rats the up regulation of PCSK9 mRNA is more prominent than that of the LDLR mRNA, resulting in more LDLR degradation and a reduced expression of LDLRs (Persson L et al. unpublished). PCSK9 and LDLR are regulated by the same mechanism in humans (142), but humans are not resistant to statins or cholesterol overconsumption suggesting that the responses on PCSK9 transcription is not as sensitive as in rats. In hamsters it has been shown that rosuvastatin increases the transcription of PCSK9 via activation of both SREBP-2 and HNF1α whereas the LDLR transcription is only activated by SREBP-2, resulting in a PCSK9 predominance (50). If this HNF1α response is species specific is unknown, but statin treated human hepatoma cellines have unaltered HNF1α levels (50), however, further studies are required to elucidate the species-specific mechanisms.

5.2 DIURNAL VARIATION OF PCSK9

The transcriptional regulation of PCSK9 via SREBP-2 is also present in humans (142) and we have shown that regulation of PCSK9, presumably via SREBP-2, is present during the normal diurnal phases and during fasting-refeeding (Paper III). Circulating PCSK9 has a pronounced diurnal variation and is strongly reduced during fasting. Cholesterol synthesis is changed in parallel with serum PCSK9 in these situations, indicating that hepatic intracellular cholesterol levels and SREBP-2 activity have a diurnal variation and are reduced during fasting. Variation of SREBP-2 activity would lead to parallel changes of LDLR- and PCSK9-transcription. The variations of serum PCSK9 levels would contribute to maintain constant numbers of LDLRs and could explain why plasma LDL-C is stable during the day and during fasting. This could be one important reason for why serum PCSK9 and LDL-C correlate poorly, as previously found (158). If it is of physiologic importance to have constant plasma LDL-C during the phases of the day or beneficial in the adaptation to starvation needs further investigation.

Why the levels of PCSK9 and LDLR even out each other during the day and fasting but not during statin treatment is unclear but might illustrate the complexity of the SREBP-system (39).

5.3 HORMONAL REGULATION OF PCSK9

We have shown that PCSK9 is regulated by a broad set of hormones in vivo. Humans treated with GH have decreased serum PCSK9 levels (Paper III), which could partly explain the LDL-C -lowering effect of GH in humans (80). In contrast, GH treatment to rats increases PCSK9 mRNA levels. There are several important species differences between rodents and humans regarding the effects of GH on cholesterol metabolism. GH-treatment of rats stimulates bile acid synthesis (202), presumably leading to depletion of hepatic intracellular cholesterol content which could explain why both PCSK9 and LDLR mRNA levels were up-regulated in GH treated rats (Paper I). In contrast, GH treatment to humans does not stimulate bile acid or cholesterol -synthesis levels (80, 203), suggesting that the hepatic intracellular pool of cholesterol is unchanged, and that SREBP-2-independent mechanisms may also be involved in the regulation of PCSK9 in humans.

A progressive relative lack of GH may underlie the age-dependent serum LDL-C increase, and even though PCSK9 is probably not involved in the age-dependent plasma LDL-C increase in rats, it could still be involved in humans. It has not been fully established whether PCSK9 correlates with age in humans (158, 160, 161, 163, 165). Increased GH secretion is also part of the normal fasting response in humans, but not in rodents (204), contributing to the maintenance of a normal blood glucose level. The fasting-feeding hormones glucagon and insulin also regulate PCSK9, where glucagon decreases and insulin increases PCSK9 in rats (paper II). This is of interest since fasting leads to pronounced effects on PCSK9, and the individual role of fasting hormones such as GH and glucagon should be further explored in humans.

We have shown that endogenous estrogen reduces circulating PCSK9 in humans, in accordance with estrogen treated rats (Paper II), which will contribute to an increased clearance of apoB containing lipoproteins. An estrogen-induced reduction of PCSK9 could partly explain why postmenopausal women have higher serum levels of both PCSK9 (158) and plasma LDL-C (101). In humans, cholesterol synthesis is unchanged or, if anything, tends to increase following stimulation with estrogen (Paper IV). The fact that serum PCSK9 and cholesterol synthesis were not changed in parallel indicates that the estrogen-mediated effect on PCSK9 in humans may be independent of SREBP-2. Stimulation of endogenous estrogen resulted in a 3-fold increase of GH levels, and since GH treatment results in reduced PCSK9 levels (paper III) without altering cholesterol synthesis, it is possible that the estrogen-induced reduction of serum PCSK9 levels in humans is actually mediated by GH.

Estrogen treated rats have reduced SREBP-2 mRNA levels (paper II) and the estrogen-mediated reduction of PCSK9 in rats may be due to the previously reported increase of hepatic cholesterol levels (107). Estrogen treated rats also have a 6-10 -fold increase of hepatic LDLR numbers and an increased LDLR-mediated uptake of serum cholesterol could explain the increase of hepatic cholesterol content (107). Rats treated with estrogen respond with increased transcription of the LDLR gene, likely due to a direct estrogen-mediated increase of the LDLR promoter activity (205). If the LDLR mRNA levels are altered during endogenous estrogen stimulation in humans is unknown, but it should be noted that total serum cholesterol levels were reduced by 90% in estrogen treated rats (107), compared to a 13% reduction in our human model. This would suggest that the estrogen-induced up regulation of hepatic LDLR numbers is more

pronounced in rats compared to humans. If this is due to a dosing effect, influence of exogenous contra endogenous estrogen or species-related differences is unclear.

New results from our group have shown that circulating PCSK9 is reduced in subjects with hyperthyreosis, whereas cholesterol synthesis is unaltered (206). Thus, again showing a hormonal situation where serum PCSK9 and cholesterol synthesis are not changed in parallel, this also indicates the possibility of a SREBP-2 independent mechanism. If such a regulation is due to direct hormonal responses, or mediated by alternate regulatory pathways remains to be elucidated.

The reduction of serum PCSK9 by GH, estrogen and TH in humans may in part explain the serum LDL-C lowering effects of these hormones. The level of change of serum PCSK9 and LDL-C were, however, not significantly correlated during GH and estrogen influence. This indicates that regulation of the LDLR is complex and that several different factors might participate.

5.4 DIETARY REGULATION OF PCSK9

It is well known that different diets influence plasma cholesterol levels. As mentioned before, PCSK9 is reduced in rats following a cholesterol-enriched diet, but if PCSK9 is dietary regulated in humans is unclear. We have shown that a ketogenic (high protein and fat) diet increases plasma cholesterol levels but does not change serum PCSK9 levels (Paper III). We have also measured circulating PCSK9 in 66 patients randomized to vegan (38 patients) or non-vegan (28 patients) diet. Three and twelve months of vegan diet lead to reduced plasma LDL-C levels (207), whereas serum PCSK9 levels were unchanged (Persson L et al. unpublished). We recently studied the impact of dietary fat quality on serum PCSK9 and cholesterol metabolism. Preliminary results show that subjects supplementing their diet with unsaturated fatty acids for 10 weeks had significantly reduced serum levels of PCSK9, which could actually explain the reduced plasma cholesterol levels (Bjermo et al. manuscript). To our knowledge, this is the first example of dietary regulation of PCSK9 in humans.

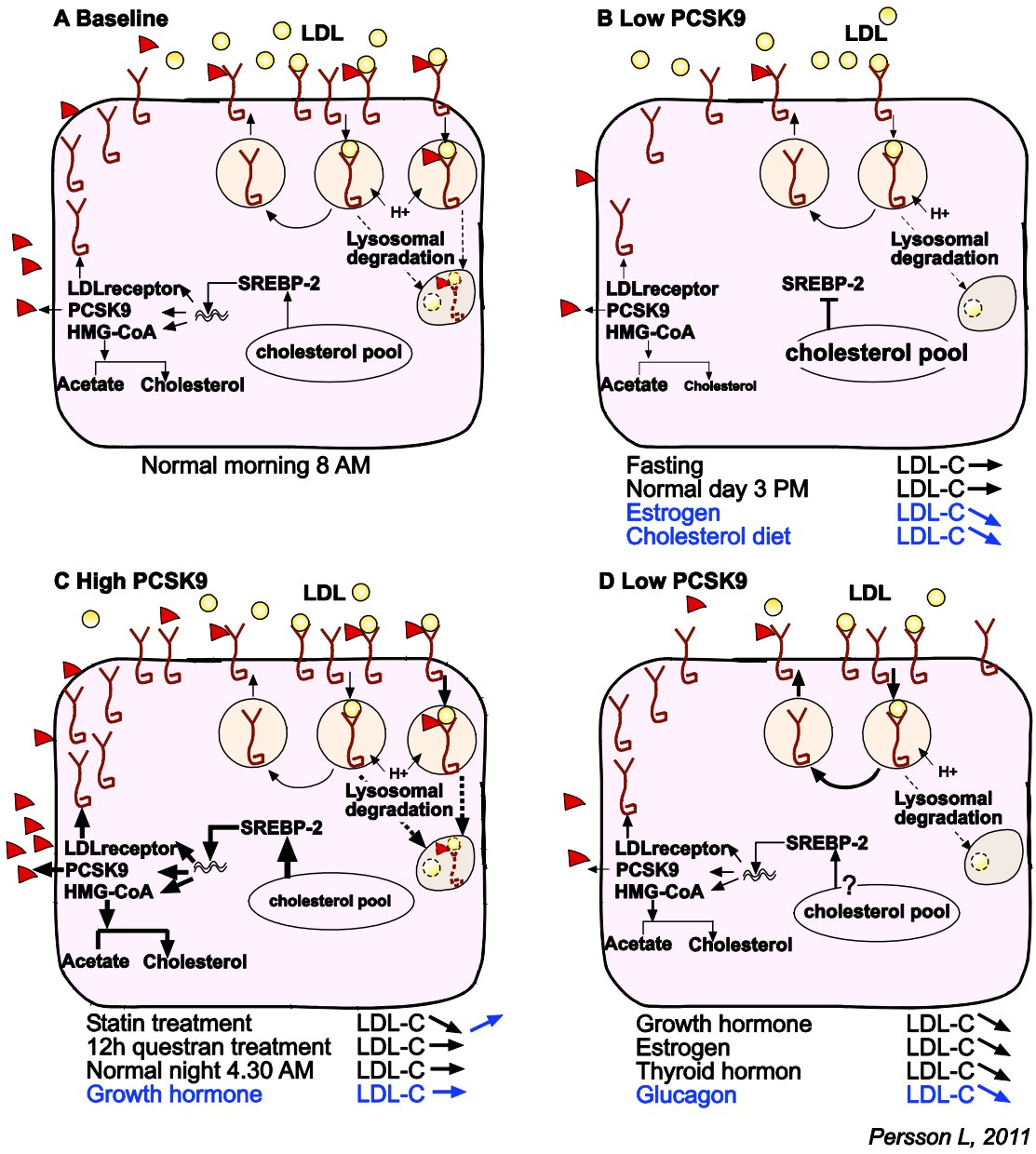

Fig. 3 Schematic picture of an hepatocyte, describing during which situations PCSK9 is altered and the resulting change of serum LDL-C. Black lines represent the human situation and blue lines the rat situation. A) Baseline, B) Low PCSK9 levels partly due to increased cholesterol pool, C) High PCSK9 levels partly due to reduced cholesterol pool, D) Low PCSK9 levels due to unknown reasons.

6 CONCLUSIONS

From our studies the following conclusions can be drawn;

I) PCSK9 is not involved in the age-dependent increase of plasma LDL-C in rats. Reduced bile acid synthesis likely contributes to the age-dependent hypercholesterolemia in rats, and treatment with GH can restore bile acid synthesis to juvenile levels. PCSK9 gene transcription is up regulated by GH, possibly via SREBP-2.

II) Hormonal and dietary regulation of hepatic LDLRs is frequently mediated by PCSK9 in rats, which explain previously reported discrepancies between LDLR mRNA and protein levels. The transcription factor SREBP-2 is partly involved in the hormonal and dietary regulation of PCSK9, although our results suggest that the glucagon-mediated suppression of PCSK9 may involve other mechanisms.

III) Circulating PCSK9 has a pronounced diurnal variation and is strongly reduced during fasting in humans. These changes are presumably related to diurnal oscillations in hepatic intracellular cholesterol levels and mediated by SREBP-2. GH treatment reduces circulating PCSK9 in humans, a regulation that may involve SREBP-2 independent mechanisms.

IV) Changes in endogenous estrogen levels exerts rapid and distinct effects on cholesterol metabolism in humans, with reduced levels of circulating PCSK9, plasma total and LDL-C, increased levels of TGs in HDL and LDL and increased apoAI levels. Some of these effects of estrogen on cholesterol metabolism could be mediated by the rapid induction of GH secretion.

We conclude that PCSK9 is regulated by hormonal, dietary and diurnal stimuli in rats and humans, and that the regulation of PCSK9 is important for the plasma LDL-C level. The hormonal regulation of PCSK9 can partly explain the cholesterol-lowering effects of GH, estrogen, glucagon and TH. Our results further suggest that such hormonal regulations may involve SREBP-2 independent mechanisms. The regulation of PCSK9 during the diurnal phases and fasting may explain why plasma LDL-C levels

are stable during these situations. We have also shown that PCSK9 can be dietary regulated, partly explaining the pronounced resistance to development of hypercholesterolemia following a cholesterol-enriched diet in the rat.

Hormonal, dietary and diurnal regulation of PCSK9 are important for the serum LDL-C levels and imply that PCSK9 is of physiological significance, which deserves attention in the use of current and novel anti-PCSK9 agents.

7 FUTURE PERSPECTIVES

PCSK9 has lately emerged as an attractive drug target and ongoing studies will soon show if it is beneficial to reduce PCSK9 in humans. From present knowledge, nothing indicates that inhibition of PCSK9 would be harmful. However, the fact that PCSK9 has a pronounced diurnal variation; is strongly reduced during fasting; and is under hormonal control shows that this system is heavily regulated during normal physiological situations in humans. PCSK9 could be important for the adaptation to starvation and for the redirection of apoB containing particles during hormonal influence, as e.g. during development and pregnancy.

To further increase our knowledge of PCSK9 it will be of great importance to elucidate the species-dependent differences, e.g. during cholesterol overload, statin- and GH-treatment, which will require an increased understanding of the SREBP-regulatory system. Mechanistic studies will be necessary to investigate the possible involvement of SREBP-2 independent mechanisms during hormonal regulation, such mechanisms may be useful in the future development of anti-PCSK9 agents.

It will be of particular interest to study the diurnal variation and effects of fasting in subjects with PCSK9 LOF mutations. If PCSK9 is important for stabilizing plasma LDL-C levels, such individuals should be expected to respond with increased plasma LDL-C levels during fasting and they may have also exhibit a diurnal variation of plasma LDL-C. The number of patients with such LOF mutations is however limited.

We will continue to study the hormonal regulation of PCSK9 in humans, e.g. by analyzing serum PCSK9 during treatment with testosterone. This will improve our understanding of the gender differences in serum PCSK9 and plasma cholesterol. We will also continue to investigate the dietary regulation of PCSK9 in humans.

8 SVENSK SAMMANFATTNING

I Sverige dör nästan 40 000 människor till följd av hjärt- och kärlsjukdomar varje år, vilket är den vanligaste dödsorsaken. Hjärt- och kärlsjukdomar orsakas av åderförkalkning som är en process av att kolesterol ansamlas och startar en inflammation i kärlväggen. Förhöjda nivåer av kolesterol i blodet ökar samt påskyndar åderförkalkningen och genom att sänka nivåerna av kolesterol i blodet minskas risken för hjärt- och kärlsjukdom. Eftersom kolesterol inte bara är skadligt utan också är en viktig byggsten i cellmembraner, hormoner och gallsyror är metabolismen av kolesterol starkt reglerad. För att kolesterol ska kunna transporteras i blodet förpackas det i lipoproteiner, där low density lipoprotein (LDL) är den vanligaste förekommande. Nivån av LDL-kolesterol bestäms till största del av hur många LDL-partiklar som tas upp av leverns LDL-receptorer, ju fler LDL-receptorer desto lägre nivåer av LDL-kolesterol i blodet.

2003 visade det sig att proteinet PCSK9 har en nyckelroll i kolesterolmetabolismen. Olika genvarianter leder till olika aktivitet av PCSK9 vilka binder till LDL-receptorn bättre eller sämre och resulterar i helt olika nivåer av LDL-kolesterol i blodet. Personer med högaktivt-PCSK9 har höga nivåer av LDL-kolesterol i blodet och en ökad risk för hjärt- kärlsjukdom. Personer med genvarianter som istället ger lågaktivt-PCSK9 har motsatt förhållande, extremt låga nivåer av LDL-kolesterol. Efter att ha följt personer med lågaktivt-PCSK9 under 15 år visade det sig att de hade 90% lägre incidens av hjärtsjukdom mot vanliga personer. Dessa personer har under hela sitt liv haft låga kolesterolnivåer och resultaten tydliggör hur viktigt det är att sänka nivåerna av LDL-kolesterol redan vid en tidig ålder.

PCSK9 bildas till största del i levern och finns cirkulerande i blodet. PCSK9 binder till LDL-receptorn och leder därmed receptorn till nedbrytning, vilket gör att färre LDL-partiklar tas upp och LDL-kolesterol i blodet ökar. Genom att hämma PCSK9 skulle nivån av LDL-kolesterol kunna sänkas, vilket gör PCSK9 mycket intressant i utvecklingen av nya kolesterolsänkande läkemedel. Statiner är dagens vanligaste läkemedel för kolesterolsänkning vilka minskar antalet dödsfall i hjärt- och kärlsjukdomar. Statiner hämmar kolesterolsyntesen och orsakar därmed brist på kolesterol i levercellen vilket resulterar i att fler LDL-receptorer bildas och därigenom

ökar upptaget av LDL-kolesterol från blodet. Brist på kolesterol i levercellen gör också att mer PCSK9 bildas och därmed att flera av de nybildade LDL-receptorerna bryts ned. PCSK9 försämrar statinernas kolesterol-sänkande effekt och att kombinera statiner med en PCSK9 hämmare skulle kunna ge starka kolesterolsänkande effekter. Människor och djur utan PCSK9 har låga nivåer av LDL-kolesterol i blodet och är helt friska. Det tyder på att PCSK9s huvudsakliga funktion består i att reglera LDL-receptorn, men om det är fysiologiskt viktigt eller en evolutionär fördel är okänt.

Målet med denna avhandling var att studera hur PCSK9 påverkas av hormoner, dieter, fasta och om PCSK9 har en dygnsrytm. Genom att studera PCSK9 får vi större förståelse för kolesterolmetabolismen. Flera läkemedel som slår ut PCSK9 utvecklas nu och vi behöver därför veta mer om detta svårförklarliga protein. Dessa studier har funnit att:

- PCSK9 regleras av en rad olika hormoner. Tillväxt hormon, östrogen och sköldkörtelhormon sänker PCSK9-nivån i blodet hos människa vilket delvis kan förklara varför dessa hormoner sänker kolesterol i plasma. Att östrogen nedreglerar PCSK9 kan vara en förklaring till varför kvinnor får högre kolesterolnivåer efter menopaus.

- Dieter påverkar PCSK9-nivåen och råttor som äter en kolesterolrik diet får minskade PCSK9-nivåer vilket delvis förklarar varför råttor inte får hyperkolesterolemi vid denna diet.

- PCSK9 har en dygnsvariation hos människa, vilket innebär att LDL-kolesterol stabiliseras under dygnet. Vid fasta sjunker PCSK9-nivån i blodet kraftigt och även i denna situation bidrar det till att LDL-kolesterol är oförändrat, om det är viktigt för förmågan att klara av svält eller för någon annan fysiologisk funktion återstår att undersöka.

Att PCSK9 har en dygnsrytm samt regleras av hormoner, dieter och fasta har stor betydelse för nivåerna av LDL-kolesterol i blodet. Det faktum att PCSK9 regleras under normala fysiologiska förhållanden antyder att PCSK9-systemet kan vara viktigt t.ex. vid anpassning till fasta och svält eller under utvecklingen. För att förstå om PCSK9 har någon avgörande fysiologisk roll krävs dock ytterligare studier.

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