From DEPARTMENT OF MEDICINE
Karolinska Institutet, Stockholm, Sweden

STUDIES ON CHOLESTEROL AND BILE ACID METABOLISM IN RELATION TO PLASMA LIPOPROTEINS IN HUMANS

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Stockholm 2016
Studies on cholesterol and bile acid metabolism in relation to plasma lipoproteins

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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ABSTRACT

The metabolism of cholesterol and bile acids is tightly controlled but only partially characterized. The liver is responsible for most of the clearance and catabolism of plasma cholesterol, and the hepatocyte expression of LDL receptors is central in this process. The major pathways for net excretion of cholesterol from the body are through biliary excretion as free cholesterol or after conversion to bile acids. Through activation of the nuclear receptor FXR and the G protein-coupled receptor TGR5, bile acids regulate not only their own homeostasis but also lipid, glucose and energy metabolism. The overall aim of this thesis was to further characterize the regulation of cholesterol and bile acid metabolism in relation to plasma lipoproteins.

Bile acid synthesis is regulated by negative feedback mechanisms. Bile acids mediate inhibition of the rate limiting enzyme in bile acid synthesis, CYP7A1, through activation of FXR in the liver and in the intestine via the FXR-SHP pathway and the FXR-FGF19-FGFR pathway, respectively. In paper I, we demonstrate that circulating FGF19 is markedly influenced by transintestinal flux of bile acids whereas its proposed role in the suppression of bile acid synthesis and triglyceride levels may not always apply.

PCSK9 regulates the number of LDL receptors by targeting the LDL receptors for degradation. In paper II, we show that circulating PCSK9 has a diurnal rhythm synchronous with cholesterol synthesis. We also describe how fasting strongly reduces circulating PCSK9 and reduces cholesterol synthesis, whereas plasma LDL-cholesterol is unchanged in healthy subjects. Furthermore we show that a ketogenic diet induces cholesterol synthesis and increase plasma cholesterol whereas circulating PCSK9 is unaltered.

Diet composition and eating patterns influence cholesterol metabolism. Knowledge of underlying mechanisms provides the ability to modulate regulation and homeostasis in a desired direction. In paper III, we show that a vegan diet reduces serum total cholesterol and LDL-cholesterol. In paper IV, we further explore possible mechanism(s) implicated in this dietary effect, but cannot find evidence for any involvement of PCSK9 or altered bile acid or cholesterol synthesis. In summary, the work presented in this thesis contributes to the understanding of lipid metabolism, and may in a longer term assist in the development of better treatment for lipid disorders.
LIST OF SCIENTIFIC PAPERS

I. Sjöberg BG*, Straniero S*, Angelin B, Rudling M. Effects of short and long term treatment with cholestyramine on bile acid, cholesterol and lipoprotein metabolism. Manuscript. * The two first authors contributed equally


IV. Sjöberg BG, Hafström I. Lowering of plasma cholesterol by a vegan diet is not related to changes in circulating PCSK9. Manuscript.

SCIENTIFIC PAPERS NOT INCLUDED IN THE THESIS


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<th>Full Form</th>
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<tbody>
<tr>
<td>ABCA1</td>
<td>ATP-binding cassette subfamily A member 1</td>
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<tr>
<td>ABCG5</td>
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</tr>
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<td>ABCG8</td>
<td>ATP-binding cassette subfamily G member 8</td>
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<tr>
<td>ACAT</td>
<td>acyl coenzyme A:cholesterol acyltransferase</td>
</tr>
<tr>
<td>apo</td>
<td>apolipoprotein</td>
</tr>
<tr>
<td>ASBT</td>
<td>apical sodium-dependent bile salt transporter</td>
</tr>
<tr>
<td>BA</td>
<td>bile acid</td>
</tr>
<tr>
<td>BSEP</td>
<td>bile salt export pump</td>
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<tr>
<td>C4</td>
<td>7α-hydroxy-4-cholesten-3-one</td>
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<td>chenodeoxycholic acid</td>
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<td>CETP</td>
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</tr>
<tr>
<td>CM</td>
<td>chylomicron</td>
</tr>
<tr>
<td>CVD</td>
<td>cardiovascular disease</td>
</tr>
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<td>CYP7A1</td>
<td>cholesterol 7α-hydroxylase</td>
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<td>CYP8B1</td>
<td>sterol 12α-hydroxylase</td>
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<td>extracellular signal-regulated kinase</td>
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<td>fatty acid</td>
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<tr>
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<td>FH</td>
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<td>fast performance liquid chromatography</td>
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<tr>
<td>FXR</td>
<td>farnesoid X receptor</td>
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<tr>
<td>GC-MS</td>
<td>gas chromatography-mass spectrometry</td>
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<tr>
<td>GH</td>
<td>growth hormone</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
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<td>--------------</td>
<td>-----------</td>
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<tr>
<td>HDL</td>
<td>high density lipoprotein</td>
</tr>
<tr>
<td>HMGCoA</td>
<td>3-hydroxy-3-methylglutaryl coenzyme A</td>
</tr>
<tr>
<td>HMGCoAR</td>
<td>3-hydroxy-3-methylglutaryl coenzyme A reductase</td>
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<tr>
<td>HNF-4α</td>
<td>hepatocyte nuclear factor-4α</td>
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<tr>
<td>HSPG</td>
<td>heparan sulphate proteoglycan</td>
</tr>
<tr>
<td>IBABP</td>
<td>ileal bile acid binding protein</td>
</tr>
<tr>
<td>IDL</td>
<td>intermediate density lipoprotein</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
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<tr>
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<td>LDL</td>
<td>low density lipoprotein</td>
</tr>
<tr>
<td>LDLR</td>
<td>low density lipoprotein receptor</td>
</tr>
<tr>
<td>LPL</td>
<td>lipoprotein lipase</td>
</tr>
<tr>
<td>LRH-1</td>
<td>liver receptor homolog-1</td>
</tr>
<tr>
<td>LRP</td>
<td>low density lipoprotein receptor related protein</td>
</tr>
<tr>
<td>MTP</td>
<td>microsomal transfer protein</td>
</tr>
<tr>
<td>NEFA</td>
<td>non esterified fatty acid</td>
</tr>
<tr>
<td>NPC1L1</td>
<td>Niemann-Pick C1 Like 1</td>
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<tr>
<td>NTCP</td>
<td>sodium bile acid co-transporter</td>
</tr>
<tr>
<td>OATP</td>
<td>organic anion transporting polypeptides</td>
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<tr>
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<td>organic solute transporter α</td>
</tr>
<tr>
<td>OSTβ</td>
<td>organic solute transporter β</td>
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<tr>
<td>PC</td>
<td>phosphorylcholine</td>
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<td>PCSK9</td>
<td>proprotein subtilisin kexin type 9</td>
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<td>PL</td>
<td>phospholipid</td>
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<td>PS</td>
<td>phosphatidyl serine</td>
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<tr>
<td>RA</td>
<td>rheumatoid arthritis</td>
</tr>
<tr>
<td>RCT</td>
<td>reverse cholesterol transport</td>
</tr>
<tr>
<td>SHP</td>
<td>small heterodimer partner</td>
</tr>
<tr>
<td>SRB1</td>
<td>scavenger receptor class B type 1</td>
</tr>
<tr>
<td>Acronym</td>
<td>Term</td>
</tr>
<tr>
<td>---------</td>
<td>-----------------------------------</td>
</tr>
<tr>
<td>SREBP</td>
<td>Sterol regulatory element binding protein</td>
</tr>
<tr>
<td>TG</td>
<td>triglyceride</td>
</tr>
<tr>
<td>TGR5</td>
<td>G protein-coupled bile acid receptor 1</td>
</tr>
<tr>
<td>UDCA</td>
<td>ursodeoxycholic acid</td>
</tr>
<tr>
<td>VLDL</td>
<td>very low density lipoprotein</td>
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1 INTRODUCTION

Cholesterol is an essential structural component in cell membranes where it helps to generate semipermeable barriers between cellular compartments and to regulate membrane fluidity influencing several transmembrane signaling processes. It is also a precursor of steroid hormones and bile acids. Plasma cholesterol is of major importance in the pathogenesis of atherosclerosis, and understanding of cholesterol metabolism has enabled the development of drugs and dietary treatments to reduce risk for cardiovascular events. The most important pathways for net excretion of cholesterol from the body are in the bile as free cholesterol or after conversion to bile acids. Through activation of the nuclear receptor farnesoid X receptor (FXR) and the G protein-coupled bile acid receptor 1 (TGR5) [1, 2] bile acids regulate lipid, glucose and energy metabolism. Animal studies provide valuable information; however, there are species differences. This thesis is based on human studies.

1.1 LIPID AND LIPOPROTEIN METABOLISM

In plasma, triglycerides and cholesterol are transported in lipoproteins. Cholesterol is present either as free cholesterol or combined with a long-chain fatty acid as cholesteryl esters, the storage form. Lipoprotein particles are generally spherical with a surface monolayer of phospholipids and unesterified cholesterol which surrounds a neutral lipid core consisting of triglycerides and cholesteryl esters. Lipoproteins also contain one or more apolipoproteins (apos) that provide structural stability and act as ligands for specific cell surface receptors or as activators of enzymatic reactions. Apos define the function and metabolic fate of lipoproteins. Circulating lipoproteins exchange their lipid and protein components between each other and as a result change in size, shape and density. According to their density, lipoproteins are categorized into five main groups: chylomicrons, very low density lipoproteins (VLDL), intermediate density lipoproteins (IDL), low density lipoproteins (LDL), and high density lipoproteins (HDL) (Table 1).

Lipoprotein metabolism consists of (i) the exogenous pathway, i.e. postprandial absorption of dietary lipids in the intestine and transport to peripheral tissue and the liver, (ii) the endogenous pathway, which is the transport of endogenously synthesized triglycerides and cholesterol from the liver to peripheral tissues via the VLDL-IDL-LDL pathway, and (iii) reverse cholesterol transport in which cholesterol is transported from peripheral tissues back to the liver.

1.1.1 Exogenous pathway

After a meal dietary lipids (triglycerides, phospholipids and cholesteryl esters) are emulsified with bile acids, hydrolyzed by different pancreatic lipases and inserted into mixed bile acid micelles [3-5]. Cholesterol is transported across the brush border membrane by cholesterol transporters, and free fatty acids (FFAs) are transported across the brush border membrane by passive diffusion and by fatty acid transporters. Within the enterocyte FFAs are transferred to the endoplasmatic reticulum (ER) and re-esterified into triglycerides.
The cellular mechanisms by which apoB containing lipoproteins, chylomicrons and VLDLs, are assembled are similar. However, intestinal apoB is made as a truncated form, apoB48, which is 48% of the full-length protein expressed in the liver referred to as apoB100. ApoB48 cannot bind to the LDL receptor (LDLR). The assembly of apoB containing lipoproteins is not fully defined, but three components are recognized as necessary; apoB, microsomal transfer protein (MTP), and lipids that form the neutral lipid core and membrane. The main site of regulation of assembly is believed to be degradation of the nascent apoB polypeptide [3, 6, 7]. It has been suggested that enterocytes and hepatocytes may have different methods of apoB stabilization. As the apoB protein is translated by ribosomes it crosses into the ER, and triglycerides are added co-translationally to the elongating apoB protein (ie apoB is lipidated). To further promote lipoprotein formation MTP also shuttles cholesteryl esters and phospholipids [3]. Mature chylomicrons are released into the lymph, from where they will reach the systemic blood. Each chylomicron and VLDL particle contains one single molecule of apoB. ApoB is a non-exchangeable apoprotein and remains with the lipoprotein particle until the particle is removed from the circulation through cellular uptake. The primary function of apoB containing lipoproteins is to deliver fatty acids in the form of triglycerides to muscle for ATP biogenesis and to adipose tissue for long term storage.

Table 1. Characteristics of the major lipoprotein classes.

<table>
<thead>
<tr>
<th>Lipoprotein</th>
<th>Density (g/mL)</th>
<th>Diameter (nm)</th>
<th>TG %</th>
<th>Chol %</th>
<th>PL %</th>
<th>Protein %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chylomicron</td>
<td>0.93</td>
<td>75-1200</td>
<td>80-95</td>
<td>2-5</td>
<td>3-8</td>
<td>1-2</td>
</tr>
<tr>
<td>VLDL</td>
<td>0.930-1.006</td>
<td>30-80</td>
<td>50</td>
<td>22</td>
<td>19</td>
<td>8</td>
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<tr>
<td>IDL</td>
<td>1.006-1.019</td>
<td>25-35</td>
<td>20</td>
<td>38</td>
<td>23</td>
<td>19</td>
</tr>
<tr>
<td>LDL</td>
<td>1.019-1.063</td>
<td>18-25</td>
<td>11</td>
<td>47</td>
<td>22</td>
<td>21</td>
</tr>
<tr>
<td>HDL</td>
<td>1.063-1.21</td>
<td>5-12</td>
<td>6</td>
<td>15-22</td>
<td>23-30</td>
<td>55</td>
</tr>
</tbody>
</table>

Percent composition by weight

Within the circulation, lipoprotein lipase (LPL) mediates hydrolysis of triglyceride rich lipoproteins (figure 1). LPL is primarily expressed attached to the capillary endothelium in tissues that oxidize or store fatty acids in large quantities, such as skeletal muscle, heart, and white and brown adipose tissue [8, 9]. ApoCII acts as a cofactor and apoCIII as an inhibitor of LPL. Changes in LPL expression occur through the action of hormones such as insulin, glucocorticoids and adrenaline [10]. Epidemiological studies reveal that mutations in apoCIII are linked to decreased risk for cardiovascular disease (CVD) and coronary heart disease risk in humans [11, 12].
Figure 1. Simplified schematic representation of the exogenous pathway. Absorption of dietary lipids in the intestine and transport to the peripheral tissue and the liver. TG, triglyceride; C, cholesterol; PL, phospholipids; A, apoA; B-48, apoB-48; C, apoC; E, apoE.

As LPL continuously hydrolyzes triglycerides from chylomicrons and VLDL particles the particles gradually become depleted of triglycerides, relative enriched in cholesterol, less negatively charged and small enough to enter the space of Disse. The particles lose affinity for LPL and dissociate (figure 1). The exchangeable apos CI, CII, CIII are transferred back to HDL in exchange for apoE which serves as a ligand for receptor mediated clearance by the liver (among other functions HDL particles serves as a reservoir for exchangeable apoproteins). Uptake of chylomicrons and VLDL remnants into hepatocytes are mediated by the LDL receptor (LDLR), the LDL-receptor related protein (LRP), a complex formed between LRP and heparan sulphate proteoglycan (HSPG) or HSPG alone [13]. In normal individuals, the intravascular metabolism of chylomicrons and chylomicron remnants is estimated to 10-15 and 30 minutes, respectively.

1.1.2 The endogenous pathway

To secrete triglycerides and cholesterol, hepatocytes assemble VLDL particles. In hepatocytes lipiddation of apoB100 by the action of MTP gives rise to pre VLDLs which are further processed to become triglyceride-poor VLDL2 particles. These are either transferred through the secretory pathway to be secreted from the cell, or acquire more lipids in the Golgi apparatus to become triglyceride rich VLDL1 particles [6, 14, 15] (figure 2). Similar to chylomicron formation, the assembly of VLDL is dependent on availability of lipids. Fatty acid (FA) sources for VLDL-triglyceride include (i) dietary FAs that have reached the liver.
via chylomicron uptake (ii) FAs synthesized de novo in the liver from carbohydrates, acetyl CoA and malonyl CoA [16] (iii) Non esterified FAs (NEFA) derived from adipose tissue, (iv) NEFA derived from the spillover of chylomicron triglycerides and (v) FAs stored in liver lipid droplets [15, 17].

Figure 2. Simplified schematic representation of the endogenous pathway. The endogenous pathway transport triglycerides and cholesterol from the liver to the peripheral tissues via the VLDL-IDL-LDL pathway. TG, triglyceride; C, cholesterol; VLDL, very low density lipoprotein, IDL, intermediate density lipoprotein; LDL, low density lipoprotein; LDLR, LDL receptor; A, apoA; B-100, apoB-100; C, apoC; E, apoE;

As triglycerides are removed by the action of LPL the density increases, large triglyceride rich VLDL1 particles become smaller VLDL2 and subsequently IDL (VLDL remnants). After exchange of apos with HDL, IDL can be further hydrolyzed by hepatic lipase to LDL. LDL particles are cholesteryl ester enriched lipoproteins with apoB as their only apoprotein, and are catabolized mainly by hepatic uptake through LDL receptors [15]. The LDL receptor is a cell surface receptor capable to bind and internalize lipoproteins containing apoB100 and apoE. In normal humans, the half-life of VLDL particles is 1-2 hours, and that of LDL particles 2-3 days. Accordingly, in the fasting state LDL cholesterol accounts for approximately 65-75% of total plasma cholesterol.
1.1.3 Reverse cholesterol transport and HDL metabolism

Most cells in the body are capable of synthesizing all of the cholesterol they require but not to catabolize cholesterol. The reverse cholesterol transport pathway removes cholesterol from peripheral tissue—including lipid-laden macrophages in the artery wall—to the liver for excretion into bile as unesterified cholesterol or after conversion to bile acids [18-20]. HDL has a key role in cholesterol efflux from cells, and as a transporter of cholesterol thorough plasma to the liver.

HDL formation begins when lipid-poor apoAI is secreted by the liver or intestine through interaction with the transporter ABCA1 in the hepatocyte or enterocyte. Lipid-free/lipid-poor apoAI is also continuously being generated during the remodeling of mature HDLs [18, 21]. ApoAI is the main apoprotein in HDL and participates in the formation of HDL as well as interaction with scavenger receptor B-1 (SR-B1), the principal HDL receptor. SR-B1 is an integral membrane protein primarily expressed in the liver, steroidogenic tissues, and endothelial cells. HDL heterogeneity is a consequence of constant remodeling by various factors. Two circulating proteins responsible for intravascular maturation of HDL are lecithin cholesterol acyl transferase (LCAT) and phospholipid transfer protein (PLTP). Further, hepatic lipase and endothelial lipase are important for HDL remodeling [18, 21, 22]. HDL-cholesteryl esters can be transferred by the enzyme cholesteryl ester transferase (CETP) to VLDL/IDL/LDL in exchange for triglycerides [22]. The actions of CETP result in decreased HDL-cholesterol concentrations and cholesterol enriched LDL-cholesterol. SR-B1 mediates selective removal of cholesteryl esters, TGs, phospholipids and vitamin E from the HDL core into the cell without endocytotic uptake and degradation of the whole HDL particle [23]. As mentioned above, ABCA1 mediates lipid efflux to lipid-poor apoA1. A second transporter, ABCG1, mediates further cholesterol efflux to lipidated HDL.

Epidemiological studies have shown that plasma HDL levels inversely correlate with atherosclerotic cardiovascular disease [24]. However, the failure of several clinical trials with drugs that rise HDL levels (niacin [25, 26] and CETP inhibitors [27, 28]), and studies of human genetic variants associated with HDL-cholesterol levels [29, 30] have brought the “HDL-hypothesis” [24, 31]—which posits that intervention that increase HDL-cholesterol will prevent occurrence of CHD—into question.

1.2 CHOLESTEROL METABOLISM

Cholesterol is exchanged within and between organelle membranes, and between tissues at the whole body level. Cholesterol homeostasis is under tight regulation, and cholesterol levels reflect the net effect of de novo synthesis, intestinal absorption of dietary and biliary cholesterol, circulatory clearance and excretion.

1.2.1 Regulation of synthesis

In humans, cholesterol is derived from two sources—diet and de novo synthesis. All nucleated cells can synthesize cholesterol de novo from acetyl CoA through the mevalonate
pathway, which occurs in the ER. The rate limiting step in the mevalonate pathway is the conversion of HMG-CoA to mevalonate by HMG-CoA reductase. HMG-CoA reductase is an integral membrane protein which is under regulation via a negative feedback system modulated by the SREBP pathway [32]. In mammals there are two SREBP genes, SREBP1 and SREBP2, that express three major SREBP proteins. SREBP1a and SREBP1c are produced from the same gene by use of different promoters and alternative splicing. The isoforms SREBP1a and SREBP1c are more active in transcription of genes involved in fatty acid synthesis, whereas SREBP2 is the main regulator of cholesterol metabolism (e.g. HMG-CoA reductase, LDLR and PCSK9) [33-36]. SREBP resides in the ER and is activated in sterol poor conditions. Simply, SREBP2 activation increases the transcription of gene products that function to increase cellular cholesterol levels, such as HMG-CoA reductase and the LDLR. When sterol levels are restored, cholesterol synthesis and uptake are coordinately suppressed. Cholesterol synthesis has a diurnal rhythm with a nadir during the day and peak levels during the night from midnight to 4:00, both in humans and rats [37-39]. Insulin and thyroid hormone increase HMG-CoA reductase activity whereas glucagon and glucocorticoids decrease it. HMG-CoA reductase inhibitors, statins, competitively inhibit the enzymatic activity of HMG-CoA reductase. The total cholesterol content of a human body (70 kg) is about 140 g of which slightly less than 1% (~1200 mg) turns over daily [40].

1.2.2 Cholesterol absorption

Whereas dietary triglycerides are almost completely absorbed, only 30-50% of the cholesterol present in the intestinal lumen (dietary 300-500 mg/day and biliary 600-1000 mg/day) is absorbed. Dietary and biliary cholesterol is transported across the brush border in the proximal jejunum principally via Niemann-Pick C1 Like 1 (NPC1L1) [41] and further transported to ER for esterification by the action of acyl coenzyme A:cholesterol acyltransferase 2 (ACAT2). Sitosterol and other non-cholesterol sterols are less effective substrates for ACAT2 and are preferentially secreted back into the intestinal lumen through the paired half-transporters ATP-binding cassette subfamily G member 5 and ATP-binding cassette subfamily G member 8 (ABCG5/G8). In the liver, hepatic ABCG5/G8 facilitates biliary secretion of cholesterol and plant sterols [42].

In addition to chylomicron assembly, some studies also suggest that HDL assembly and secretion by the intestine may play a role in absorption of cholesterol and phospholipids [5]. Cholesterol absorption is inhibited by drugs such as ezetimibe [43], which inhibits the activity of NPC1L1.

1.2.3 Cholesterol excretion

Most cells in the body cannot catabolize cholesterol. Small amounts of cholesterol are lost from the body through the excretion of steroid hormones in the urine, and through the sequestration of dead cells from the skin. The role of cholesterol elimination through the excretion of dead intestinal cells is less clear, as is the possible potential of active secretion of plasma lipoprotein-derived cholesterol by enterocytes, a process referred to as transintestinal
cholesterol excretion (TICE) [44, 45]. The most important pathways for net excretion from the body are through hepatic elimination, which can also be actively regulated by dietary and pharmacological treatments. This occurs either through direct secretion of free cholesterol into the bile, or after conversion of cholesterol to bile acids (figure 3 and 6).

1.2.4 The LDL receptor and PCSK9

The liver is responsible for the clearance and catabolism of plasma LDL and hepatocyte expression of LDLR is central to this process [46]. Upon LDL binding to the LDLR, the receptor-ligand complex is internalized into the hepatocyte via clathrin-coated pits. The vesicles containing the internalized LDL-LDLR complex fuse with endosomes resulting in dissociation of the LDL particles from the LDLR due to the acidic environment. Cholesterol is recovered and distributed in the cell. The LDLRs are then transported to the surface of the hepatocyte to bind and clear additional LDL [46].

PCSK9, a serine protease, enhances the post translational degradation of the LDLR. Circulating PCSK9 binds to the LDLR on the surface of the hepatocyte and the complex is internalized within the endosome. The LDL-PCSK9 complex is then routed to the lysosome for degradation, thereby preventing recycling of LDLR (figure 4). The expression of both LDLRs and PCSK9 is regulated by SREBP2 and upregulated when intracellular cholesterol levels are low. While SREBP2s increase the number of LDLRs, they also increase PCSK9 expression, resulting in an increased degradation of LDLRs and reduced clearance of LDL particles [47].

Figure 3. Simplified overview of cholesterol metabolism and enterohepatic circulation of bile acids.
Figure 4. Simplified schematic representation of the role of PCSK9 in the regulation of LDL cholesterol. Recycling of LDLRs enables efficient clearance of LDL particles. Circulating PCSK9 regulates the recycling of LDLRs by targeting the LDLR for degradation. By preventing LDLR from recycling back to the surface, PCSK9 reduces the number of LDLRs on hepatocytes, resulting in reduced LDL clearance rates and elevated levels of plasma. PCSK9 also appears to enhance degradation of the LDLR via an intracellular pathway.

PCSK9 was first connected to cholesterol metabolism when gain-of-function mutations were identified in two French families with familial hypercholesterolemia (FH) who did not have mutations in the LDLR or APOB genes [48]. Loss-of-function mutations are instead associated with low LDL-C and low lifetime risk of cardiovascular disease [49, 50]. PCSK9 is expressed predominantly in the liver, intestine, kidney and nervous system [51]. PCSK9 can promote the degradation of other cell surface receptors including structural homologs of the LDLR (VLDL receptor and apoE receptor) [52] and CD36 [53]. CD36 functions as a scavenger receptor that among other things binds oxidatively modified LDL particles, apoptotic cells and facilitates transport of long chain fatty acids into cells [54]. CD36 participates in muscle lipid utilization, gut fat absorption and triglyceride storage [53, 54].
Moreover, PCSK9 also appears to enhance the degradation of LDLRs by a yet incompletely defined intracellular pathway, which does not require PCSK9 to be secreted [55]. Levels of circulating PCSK9 are higher in women than in men, and in postmenopausal compared to premenopausal females [56, 57]. Inhibiting PCSK9 production with small interfering RNA [58] or using monoclonal antibodies [59] lowers LDL-C levels, and is currently a very active area of therapeutic development.

1.3 BILE ACID METABOLISM

1.3.1 Bile acid synthesis

The liver is crucial for metabolic homeostasis, and in the liver hepatocytes are the cells responsible for most of the synthetic and metabolic functions. Hepatocytes are polar cells with a canalicular and a sinusoidal surface [60]. Unlike capillaries elsewhere, the liver sinusoidal endothelial cells lack basal membrane and are perforated with pores (fenestrae) [61-63], that allow free egress of solutes from the sinusoidal blood to the space of Disse.

In the adult human liver approximately 500 mg cholesterol is converted into bile acids each day. Both the steroid nucleus and the side chain of cholesterol are modified during bile acid synthesis, and the result is a marked change in the physicochemical properties. The amphipathic property enables bile acids to emulsify lipids and enhance the absorption of lipids and fat soluble vitamins in the intestine. The detergent properties of bile acids are determined by the number and orientation of the hydroxyl groups and by the presence or absence of an amino acid moiety. The hydrophobicity increases as follows: ursodeoxycholic acid (UDCA), cholic acid (CA), chenodeoxycholic acid (CDCA), deoxycholic acid (DCA), and lithocholic acid (LCA) [64]. Bile acid synthesis occurs in pericentral hepatocytes and involves enzymes located in the ER, mitochondria, cytosol and peroxisomes [65, 66]. Bile acids are synthesized via two pathways, the classical or neutral, and the alternative or acidic pathway (figure 5). The classical pathway is the major pathway of synthesis and accounts for more than 90% of the total bile acid synthesis in humans. In man, two bile acids are synthesized from cholesterol in the liver: CDCA (3α, 7α-dihydroxy) and CA (3α, 7α, 12α-trihydroxy).

In the classical pathway the first and rate limiting step is the hydroxylation at the carbon C-7 mediated by cholesterol 7α-hydroxylase (CYP7A1) located in the ER. Then 3β-hydroxysteroid dehydrogenase (HSD3B7) converts 7α-hydroxycholesterol to 7α-hydroxy-4-cholesten-3-one (C4). C4 is a precursor for CA and CDCA and can be used as a serum marker for bile acid synthesis [67, 68]. C4 is converted to 7α, 12α-dihydroxy-4-cholesten-3-one by a sterol 12α-hydroxylase (CYP8B1) leading to synthesis of CA. Without 12α-hydroxylation by CYP8B1, C4 is eventually converted to CDCA. The mitochondrial sterol 27-hydroxylase (CYP27A1) catalyzes the steroid chain oxidation in both CA and CDCA synthesis. The enzyme CYP7A1 is largely determining the bile acid pool size whereas CYP8B1 is considered important for the CA/CDCA ratio in the bile acid pool [69].
In the alternative pathway cholesterol is first converted to 27-hydroxycholesterol by CYP27A1. Oxysterol 7α-hydroxylase (CYP7B1) catalyzes hydroxylation of 27-hydroxycholesterol to 3β,7α-dihydroxy-5-cholestenoic acid, which eventually is converted to CDCA. Primary bile acids synthesized from cholesterol in the liver are further metabolized into secondary and tertiary bile acids by the gut microbiota.

In the hepatocyte bile acids are conjugated to glycine or taurine preceding secretion into the bile canicula [70]. In addition to amidation, bile acids may undergo sulphation, glucuronidation, and N-acetylaminoglucosidation. Conjugation increases the amphipathicity and enhances the solubility of the molecules, which makes them impermeable to cell membranes. Several bile acid transporter proteins ensure proper excretion and uptake. Bile acid transporters have different transport affinities for various bile acids species, but also for other endogenous and exogenous compounds such as toxins and drugs [71]. Efficient hepatic excretion, intestinal reabsorption, and hepatic uptake of bile acids restrict bile acids to hepatobiliary and intestinal compartments. Bile acid transporters and bile acid synthesis is under strict and coordinated regulation via nuclear receptors.

![Diagram of Bile Acid Synthesis]

**Figure 5. Bile acid synthesis**
1.3.2 The enterohepatic circulation of bile acids

In the enterohepatic circulation, bile acids circulate between the liver and the intestine. A bile acid pool of about 2-3g circulate between 4-8 times per day dependent on dietary regimen in humans. Bile acids lost in feces (0.5-0.8g/day) are replaced by de novo synthesis from cholesterol in the liver to maintain a constant bile acid pool size.

Bile acids are actively transported into bile canaliculi by the bile salt export pump (BSEP; encoded by the ABCB11 gene). In the alkaline bile (pH 7.6-8.4) conjugated bile acids are present as ionic salts, and therefore often referred to as bile salts. Bile formation is an osmotic process and the most important solutes driving bile formation are bile salts. To protect the canicular membrane from bile acid mediated solubilization, phospholipids are excreted into bile to form mixed micelles with bile acids [72]. Cholesterol excretion into bile is mediated by the ABCGS/ABCG8 heterodimer. Bile flows in bile canaliculi which drain into bile ducts. The ducts join to form larger and larger channels, the so called biliary tree. A fraction of the bile acids are absorbed by cholangiocytes (the epithelial cells lining the biliary tract) and recycled back to hepatocytes, the cholangiohepatic shunt [73, 74]. The physiological relevance of this mechanism is unclear.

Upon delivery of chyme from the stomach into the duodenum cholecystokinin (CCK) will be released from the intestinal mucosa into plasma. CCK stimulates relaxation of the sphincter of Oddi, contraction of the gallbladder and the common bile duct to release bile acids into the intestine. In the ileum bile acids are efficiently absorbed (>95%), mainly by active transport. conjugated bile acids are transported across the apical brush border membrane by the apical sodium-dependent bile acid transporter (ASBT, SLC10A2). The bile acids are translocated to the basolateral membrane of the enterocyte by the cytosolic intestinal bile acid binding protein (IBABP) and subsequently transported across the basolateral membrane into portal blood by the heteromeric organic solute transporter (OST) OSTα-OSTβ [75, 76]. The fraction of bile acids not reabsorbed by active transport nor passive diffusion continues through the lumen and becomes subjected to bacterial modification. The major bile salt modifications by gut microbiota in the human large intestine include deconjugation, oxidation of hydroxy groups, and 7α/β-dehydroxylation [77]. In humans, when CDCA undergoes 7-dehydroxylation, LCA (3α-hydroxy) is formed. When cholic acid undergoes 7-dehydroxylation, DCA (3α, 12α-dihydroxy) is formed. Deconjugation and 7α/β-dehydroxylation of bile salts increases their hydrophobicity, thereby permitting passive absorption. Reclaimed secondary bile acids join the primary bile acids in portal blood.

The sodium-taurocholate cotransporting polypeptide (NTCP, encoded by SLC10A1) is the primary mechanism for hepatic uptake of bile acids from portal blood, which complete the enterohepatic cycle of bile acids. Preceding transport across the canicular membrane by BSEP, both recovered and de novo synthesized bile acids are conjugated. NTCP’s major physiological substrates include all the major glycine and taurine conjugated bile acids. Unconjugated bile acid are moderate or weak substrates [78]. Organic anion transporting polypeptides (OATPs) show substrate specificity for unconjugated bile acids [79]. The uptake
of bile acids from portal blood is efficient with a first pass clearance of ~80% [80] and occurs in direct proportion to concentration. The major bile acid transporters that control enterohepatic circulation are BSEP, ASBT, OSTα/OSTβ, NTCP and OATP [81] (figure 6).

1.3.3 Regulation of bile acid synthesis

Bile acid synthesis is regulated by negative feedback mechanisms. Bile acids mediate inhibition of the rate limiting enzyme in bile acid synthesis, CYP7A1, through activation of the nuclear receptor FXR in the liver and in the intestine. In the liver FXR induces a regulatory cascade involving SHP by which the expression of CYP7A1 is inhibited (figure 6). Thus, upon activation FXR transcriptionally induces small heterodimer partner (SHP, NR0B2), which acts as a corepressor to inhibit the transcriptional activity of liver related homolog-1 (LRH-1) and hepatocyte nuclear factor 4α (HNF4α) that positively regulate the expression of CYP7A1 and CYP8B1 [82, 83]. In the intestine; bile acid activated intestinal FXR induces FGF15 in mice, which is released into portal blood. In the liver, FGF15 binds to the FGFR4/β-klotho complex on the basolateral membrane of the hepatocyte to initiate intracellular signaling pathways, such as extracellular signal-regulated kinase (ERK), protein kinase C, and c-Jun N-terminal kinase (JNK) that represses Cyp7A1 mRNA [84, 85]. The human orthologue of FGF15 is FGF19.

The postulated role of FGF15/19 as an intestinal factor in the regulation of bile acid synthesis is based on several observations. Studies in rats showed that intraduodenal but not intravenous infusion of tauro CA repressed CYP7A1 mRNA expression. In mice suppression of Cyp7a1 by bile acids and an FXR agonist was still observed in shp-/- mice, indicating that the FXR/SHP/LRH1 cascade was not the only pathway mediating bile acid feedback inhibition [86, 87]. Treatment of primary human hepatocytes with FGF19 resulted in repression of CYP7A1 [88, 89]. Later it was demonstrated that FGF15 likely was this putative intestinal factor [90]. Expressed and induced by FXR in the intestine, but not in liver, FGF15 was shown to repress bile acid synthesis through a mechanism that involves FGFR4 and SHP. In humans, circulating levels of FGF19 were reduced following treatment with the bile acid sequestrant cholestyramine, and increased upon feeding CDCA [91]. Circulating levels of FGF19 exhibit peaks 90-120 min after the postprandial rise in serum bile acids, preceding the decline of bile acid synthesis. The meal-elicited FGF19 increase was abolished upon prolonged fasting [91]. In addition to repress bile acid synthesis [90], FGF15/19 is believed to regulate postprandial responses such as to repress gluconeogenesis [92, 93], and to stimulate glycogen and protein synthesis [94]. Moreover, FGF15/19 is believed to stimulate gallbladder filling [95], an effect in part mediated by relaxation of the gallbladder smooth muscle [95].

FXR is highly expressed in tissues that are exposed to bile acids, including the liver and intestine [96, 97]. FXR can be activated by both free an conjugated bile acids; the hydrophobic bile acid CDCA is the most potent ligand of FXR, followed by LCA, DCA and CA. Bile acids are also ligands for TGR5. Both FXR and TGR5 regulate various elements of glucose, lipid and energy metabolism. Both conjugated and free bile acids are known to
stimulate TGR5. Among all bile acids TLCA is the most potent TGR5 agonist, followed by TDCA, TCDA and TCA. TGR5 is highly expressed along the intestinal tract, with the highest expression found in the ileum and colon [2]. Despite that liver being a major bile acid target organ, TGR5 expression in the liver is low [98, 99]. Bile acid composition in mice and humans are very different [100]. In man the hydrophobic bile acid pool consist of 40% each of CA and CDCA, and 20% DCA. In mice the bile acid pool consists of about 50% CA and 50% MCA [69] [101].

Figure 6. Simplified overview of the regulation of bile acid synthesis, and membrane transporters in the enterohepatic circulation.
2 AIMS

The specific aims of the present thesis were:

I. To assess the importance of circulating FGF19 as a regulator of bile acid synthesis in humans and as a possible regulator of serum triglyceride levels

II. To gain insight into the function of PCSK9 in humans by establishing whether circulating levels are influenced by diurnal, dietary, and hormonal changes

III. To study the effect of a vegan diet on serum levels of oxidized LDL and atheroprotective antibodies in patients with rheumatoid arthritis

IV. To assess if changes in circulating PCSK9 or bile acid synthesis may contribute to the effects of a vegan diet
3 MATERIAL AND METHODS

3.1 SUBJECTS AND STUDY DESIGN

The studies were approved by the regional ethics committee at the Karolinska institutet, Stockholm, Sweden. Informed consent was obtained from all participants.

3.1.1 Paper I

Ten healthy volunteers (age 30±3, BMI 23±3.3) participated in the study. There were no clinical or laboratory signs of endocrine or gastrointestinal disorder, liver disease or diabetes. Nine subjects (4 men/5 women) participated in each experiment. Eight subjects participated in both.

Two experiments with the bile acid binding sequestrant cholestyramine were performed, a three week and a single day treatment, respectively. In the long term treatment, a daily dose of 4g was administrated together with breakfast the first week (1x4g). The second and third week, cholestyramine was taken together with breakfast and dinner (2x4g and 2x8g, respectively). The last dose was administrated together with breakfast day 21. Blood was collected after overnight fast day 0, 7, 14, 21-24 and 27. Day 0 and days 21-24, samples were collected also at 13:00 and 17:30. The one day drug treatment was studied in a 3-day experiment. Cholestyramine was taken together with each meal day 0 (4x4g). Blood was collected every 90th minutes for 33 hours, and in the morning after overnight fast day 2 and 3. The subjects were confined to bed between 22:30-7:00 (day0).

3.1.2 Paper II

Samples from nine different studies were analyzed. The participants were patients or healthy volunteers.

1) Five healthy subjects sampled every 90 minutes from 9:00 to 10:30 the next day [102].
2) Five healthy subjects sampled every 60 minutes from 9:00 to 16:00 during a prolonged overnight fast [102].
3) Five patients with clinically active rheumatoid arthritis (RA) sampled before and during 7 days of fasting [103].
4) Seven healthy subjects who participated in a study were blood was drawn before and after 48 hours of fasting [104].
5) Seventeen children with pharmacologically refractory epilepsy consuming a ketogenic diet, samples were collected before initiation and during 1-16 month follow-up [105].
6) Cholestyramine was administered to 10 healthy volunteers together with standardized meals during one day. Samples was drawn every 90th minute during 33 hours and after overnight fast day 2 and 3.
7) Twelve healthy volunteers followed over 8 days during 66 hours of fasting and 50 hours of sleep deprivation in a crossover study [106].

8) Fifteen healthy men received increasing doses of GH up to 0.1 IU/kg body weight/day for three weeks, and samples collected the last week were analyzed [107].

9) Nineteen subjects before and after 4 weeks of atorvastatin 80 mg/day treatment.

3.1.3 Papers III and IV

The patients and study design have been described in detail in [108]. Sixty-six patients with RA according to American college of Rheumatology (ACR) [109] criteria were enrolled in the study. The patients were eligible for inclusion if they were between 20 and 69 years of age, had disease duration between 2 and 10 years, had not tried dietary manipulations before, did not have a history of food allergy and had active disease.

The patients were on stable doses of non-steroidal anti-inflammatory drugs, oral glucocorticosteroids and disease-modifying anti rheumatic drugs (DMARDs).

None of the patients used statins or biologic medications before or during the study.

The patients were randomized to a vegan diet free of gluten (n=38) or a non-vegan diet (n=28) for 1 year. The vegan diet contained vegetables, root vegetables, nuts, fruits, buckwheat, millet, corn, rice and sunflower seeds.

3.2 SERUM PCSK9 AND FGF19 ASSAY

Serum PCSK9 levels (papers II and IV) were determined using a PCSK9 dual monoclonal antibody sandwich ELISA developed [110] and modified [111] at Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, IN, USA.

Serum FGF19 was analyzed using a commercially available ELISA (FGF19 Quantikine, R&D Systems, Minneapolis, MN, USA).

3.3 SERUM MARKERS OF CHOLESTEROL AND BILE ACID SYNTHESIS

Unesterified lathosterol, a marker of total body cholesterol synthesis, was determined by gas chromatography–mass spectrometry (GC–MS) [112], and corrected to serum total cholesterol [113] (Lathosterol/c) because lathosterol is transported by cholesterol rich lipoproteins (Papers I, II and IV).

The marker for bile acid synthesis, 7α-hydroxy-4-cholesten-3-one (C4), was in paper I determined by high performance liquid chromatography (HPLC) as described [68], and corrected to total cholesterol [102] (C4c). Serum levels of C4 was in paper IV measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS) [114], and corrected to total cholesterol [102].

Serum bile acids were determined using chemical hydrolysis with ether extraction and GC-MS (Paper I).
3.4 SERUM LIPID AND LIPOPROTEIN ANALYSES

Total cholesterol and TG were determined using colorimetric reagents (Roche/Hitachi, Mannheim, Germany).

Papers I and IV; Concentrations of lipoprotein cholesterol and TGs in serum were measured by fast protein lipid chromatography (FPLC). Lipoproteins were separated by size in a column. Reagents (cholesterol and TG) were continuously added to the eluate online followed by measurements of absorbance. The respective concentration was calculated by the area under the curve. The automated system for size-exclusion chromatography (SEC) of lipoproteins is described in detail in [115].

Paper III; Concentrations of LDL cholesterol were calculated by Friedewald’s formula based on P-cholesterol, P-triglycerides and P-HDL cholesterol.

3.5 GLUCOSE AND INSULIN ASSAYS

Serum glucose was determined using an enzymatic colorimetric assay with reagents from Roche/Hitachi, Mannheim, Germany. Insulin was determined by a commercially available ELISA (Mercodia Insulin ELISA, Mercodia, Uppsala, Sweden).

3.6 STATISTICS

Statistical analyses were performed as described in respective papers.
4 RESULTS

4.1 PAPER I

Effects of short and long term treatment with cholestyramine on bile acid, cholesterol and lipoprotein metabolism.

Aim; To assess the importance of circulating FGF19 as a regulator of bile acid (BA) synthesis and serum triglycerides (TGs) in humans.

Background: A non-tumorigenic FGF19 variant given in supraphysiological concentrations suppresses BA synthesis in humans. It is unclear to what extent physiological changes in circulating FGF19 contribute to the regulation of BA synthesis in man, relative to BA mediated effects via hepatic FXR. BA binding sequestrants, such as cholestyramine, reduce circulating levels of FGF19. Two experiments with cholestyramine were performed, a three-week treatment and a single-day treatment.

Results: To generate a stepwise reduction of circulating FGF19, weekly increasing doses of cholestyramine were administered during three weeks to nine healthy volunteers. Samples were collected before, during and after cessation of treatment. During the 3 week cholestyramine treatment, a dose dependent reduction in circulating FGF19, increased BA synthesis (17-fold) and cholesterol synthesis (4.4-fold) was observed. Total cholesterol, particularly LDL cholesterol, was reduced. Levels of serum BAs were not altered.

After cessation of drug treatment, circulating FGF19 gradually increased from suppressed to increased compare to basal value. The strongly induced BA synthesis was gradually reduced and returned to normal 6 days post cessation. Cholesterol synthesis was still increased 6 days after drug cessation, whereas serum cholesterol was reduced. Serum TG and glucose remained unchanged throughout the experiment. Total serum bile acids were not changed at 3 days after drug cessation compared to baseline, but were elevated 6 days after drug cessation.

The experimental set-up with one day’s drug treatment and sampling every 90th min for 33hrs and additional samples drawn after overnight fast day 2 and 3, revealed rapid dynamic changes: During the night after a single day of treatment, TG strongly increased, peaked and declined to be doubled before breakfast. Further, glucose and insulin transiently increased during the night. In the morning after a single day of treatment FGF19 levels was reduced by 90% compared to baseline, while BA synthesis (reflected by C4c levels) and cholesterol synthesis (reflected by lathosterol/c levels) were increased by 4 and 1.6-fold, respectively. Levels of serum BAs were unchanged, however, elevated day 2 (after overnight fast). The diurnal rhythms of BA and cholesterol synthesis were altered during the treatment and the alterations persisted throughout the following day. C4c and lathosterol levels were still elevated on days 2 and 3 compared to basal values.
Conclusion; Serum TG is not influenced by the degree of increase in BA synthesis. It is unlikely that circulating FGF19 levels mediate the BA-induced changes in serum TG. Circulating levels of FGF19 may not be the primary mediator of suppression of BA synthesis in humans.

4.2 PAPER II

Circulating PCSK9 has a diurnal rhythm synchronous with cholesterol synthesis and is reduced by fasting in humans.

The aim of the present study was to gain insight into the function of PCSK9 in humans by establishing whether circulating levels are influenced by diurnal and dietary changes.

Results: To answer these questions nine different studies, including 90 subjects, were analyzed. We first found that the levels of circulating PCSK9 showed a diurnal rhythm with a nadir between 3 pm and 9 pm and with a peak early morning. These distinct diurnal changes were similar to those observed for lathosterol, a marker for cholesterol synthesis. Despite these pronounced changes in circulating PCSK9 and cholesterol synthesis, serum cholesterol levels remained stable. To evaluate if food intake was related to the reduction of PCSK9 seen between breakfast and 4pm, samples from a study with prolonged overnight fast were analyzed. Circulating PCSK9 was reduced to similar extent between 9 am and 4 pm as when food was ingested.

Next we evaluated how a rapid and pronounced hepatic demand for cholesterol would influence circulating PCSK9. In the experiment analyzed, ten healthy subjects received cholestyramine together with standardized meals for one day. The treatment practically abolished the diurnal rhythm of PCSK9 and lathosterol the following day.

To further investigate parallel temporal changes of circulating PCSK9 and cholesterol synthesis, samples from a study designed to evaluate survival strategy at authentic outdoor conditions were analyzed. Up to 66 hours of fasting was compared with up to 50 hours of sleep deprivation in a crossover design. Lathosterol levels closely followed those of PCSK9 in both groups.

When morning samples from two experiments in which subjects had fasted for 2 or 7 days were evaluated we found that circulating PCSK9 levels were reduced by 70-80%. In parallel, serum lathosterol levels were reduced by 50-60 % . Serum cholesterol levels were unchanged, whereas serum ketone bodies increased. To exclude that ketosis per se may reduce PCSK9 levels we studied the effect of a ketogenic diet. Samples drawn in the morning after overnight fast before commencing the treatment, and at follow up, 1-16 months later, were analyzed. Although this fat and protein rich diet reduces glucose in a similar way as fasting, it did not reduce circulating PCSK9; instead lathosterol and total cholesterol levels increased by 24% and 37%, respectively.
LDLR numbers increase to similar extents during treatment with GH as with atorvastatin. However, when circulating PCSK9 was determined in samples from healthy subjects treated with increasing doses of GH and in samples from patients treated with atorvastatin, we found that GH treatment reduced circulating PCSK9 by 16% and atorvastatin treatment increased PCSK9 by 33%.

Conclusion; Throughout the day, and in response to fasting and cholesterol depletion, circulating PCSK9 displays marked variation, presumably related to oscillations in hepatic cholesterol that modify its activity in parallel with cholesterol synthesis. In addition to sterol mediated regulation, additional effects on LDL receptors may be mediated by hormones directly influencing PCSK9.

4.3 PAPER III
A vegan diet reduces serum LDL and oxidized LDL levels and raised atheroprotective natural antibodies against phosphorylcholine in patients with rheumatoid arthritis: a randomized study

Aim: To characterize the effects of a vegan diet on blood lipids, oxidized LDL and on natural atheroprotective antibodies against phosphorylcholine (anti PCs) in patients with rheumatoid arthritis.

Results: In a 12 month, randomized study, patients with rheumatoid arthritis (n=66), aged 51±10, BMI 24±4kg/m2, were assigned to a vegan diet or a well-balanced non-vegan diet. Blood was collected at baseline, after 3 and 12 months. Thirty patients in the vegan group and 28 in the non-vegan group completed at least 3 months of the diet regimen and were included in our analyses. Eight patients in the vegan group dropped out before completing 12 months of study.

In the vegan diet group, total cholesterol and LDL-cholesterol were reduced after 3 and 12 months compared to baseline, whereas TG and HDL-cholesterol did not change. OxLDL levels decreased after 3 months. When patients in the vegan diet group were separated in responders and non-responders at 12 months, the reduction in oxLDL levels was seen only in responders and was significant both at 3 and 12 months. In the non-vegan group, cholesterol and triglycerides were unaltered. In the vegan diet group IgA anti-PC levels was increased after 3 month compare to baseline. Levels of IgM anti-PC raised but did not reach statistical significance (p 0.057) following 12 month. In the non-vegan diet group, levels of IgM anti-PC were reduced after 3 and 12 months regimen.

Conclusion; a vegan diet given to patients with RA induces changes that are potentially atheroprotective and anti-inflammatory, including decreased LDL and oxLDL levels and increased anti-PC IgM and IgA levels
4.4 PAPER IV

Lowering of plasma cholesterol by a vegan diet is not related to changes in circulating PCSK9

Aim: To test the hypothesis that reduced circulating PCSK9 levels explain the reduction of LDL cholesterol following a 12 month vegan diet intervention demonstrated in paper III.

Of 66 patients with rheumatoid arthritis enrolled, 38 were randomized to a vegan diet and 28 to a non–vegan diet for a period of one year. Twenty-two patients in the vegan diet group and 25 in the non-vegan group completed the study, representing a dropout rate of 42% and 11 %, respectively, and were included in our analyses. All patients were sampled at baseline, and after 3 and 12 months on their respective diet.

In the vegan diet group, total cholesterol was reduced after both 3 and 12 months by 13% and 12%, respectively, as compared to baseline. VLDL cholesterol was reduced after 3 and 12 months by 28% and 31%, respectively. LDL cholesterol was reduced after 3 months by 7%, dropping from 2.0 (±0.81) to 1.9 (±0.70) mmol/L. HDL cholesterol increased after 12 month by 11%. No alterations in bile acid synthesis, cholesterol synthesis, or circulating PCSK9 levels were observed during the regimen. In the non-vegan group there were no significant changes in the cholesterol variables during the study.

Neither circulating PCSK9 levels nor altered cholesterol or bile acid synthesis explain the beneficial effects on serum lipid profiles observed after a vegan diet for one year in patients with rheumatoid arthritis.
5 GENERAL DISCUSSION

Cholesterol and bile acid homeostasis is under strict regulation from multiple mechanisms. The bile acid activated nuclear receptor FXR is central to maintain bile acid homeostasis via regulation of transporters in their enterohepatic circulation and of key enzymes in bile acid synthesis. In mice, numerous proteins have been proposed to be of regulatory importance following genetic gain- and loss-of-function experiments, such as FXR [85], SHP [86, 87], bile acid transporters including BSEP [116, 117] and ASBT, factors influencing conjugation [118, 119], klotho and FGF15. Additional proteins discussed as of possible relevance to the regulation of bile acid synthesis are hepatic SHP2 [120] and intestinal Diet1 [121]. The physiological importance of these pathways in humans remains to be determined.

In paper I we studied the importance of circulating FGF19 as a regulator of bile acid synthesis during different cholestyramine treatment regimens. All observations in paper I are not fully compatible with the thinking that FGF19 is the major suppressor of bile acid synthesis in humans. During the normalization period, both after a single day of cholestyramine treatment and after long-time treatment, there were notable discrepancies between serum levels of FGF19 and the level of bile acid synthesis. Although the results from studying the onset of treatment would be well compatible with the concept that lowering of FGF19 results in a stimulation of bile acid synthesis, these observations indicate that an increased synthesis may remain also when FGF19 levels are normalized. This suggests that cholestyramine treatment may induce bile acid synthesis also via more direct hepatic pathways, independent of the circulating levels of FGF19. An alternative explanation for these discrepancies between the level of bile acid synthesis and FGF19 serum levels could be that the interaction between FGF19 and its receptor FGFR4 is modulated by cofactors which could modify the effect of circulating FGF19.

In response to nutritional and hormonal signals, the liver regulates lipid and carbohydrate metabolic pathways to maintain homeostasis. In paper I, we further studied if changes in the presumed metabolic regulator FGF19 could explain the change in TG levels during cholestyramine treatment. We found that the elevated levels of TGs observed in healthy individuals developed unrelated to circulating levels of FGF19 or to the level of bile acid synthesis per se. During the night after a single day of treatment, TG strongly increased, peaked and declined to be doubled before breakfast. Further, glucose and insulin transiently increased during the night. In the morning after a single day of treatment FGF19 levels was strongly reduced compared to baseline, while BA synthesis (reflected by C4c levels) and cholesterol synthesis (reflected by lathosterol/c levels) were increased. In the experiment where weekly step wise increases of cholestyramine doses were used, circulating FGF19 was accordingly reduced while the levels of TGs, glucose or insulin were unchanged. In the one day treatment experiment, total serum bile acids were strongly reduced concomitantly with elevated levels of TGs. In addition, bile acid composition was altered with an enlarged percent of DCA, displaying a pattern similar to one described among patients with type 2 diabetes [122, 123]. Moreover, altered diurnal rhythms – non steady-state situations – may
directly or indirectly influence levels of triglycerides and glucose. Furthermore, mechanisms regulating apoB assembly and secretion are not fully defined and PCSK9 may have a regulatory role on triglyceride rich lipoprotein production both in intestine [124] and liver [125].

By analyzing samples from studies of different experimental situations we have demonstrated that fasting strongly reduces circulating PCSK9 in healthy humans. This occurs concomitantly with a suppressed cholesterol synthesis, as monitored by lathosterol concentrations. Despite these pronounced dynamic changes, LDL cholesterol levels were not reduced. SREBP-2 activates genes required to generate PCSK9, HMG-CoA and LDLR. The strong correlation between PCSK9 and lathosterol observed in our studies makes it reasonable to believe that hepatic PCSK9 and HMG-CoA reductase are regulated by a common mechanism in these situations. In contrast, during long time cholestyramine treatment and in response to a ketogenic diet, PCSK9 and HMG-CoA may not correlate. To separate transcription of genes regulated in a coordinated manner may sometimes be advantageous. The efficiency of statins would be improved if regulation of PCSK9 and LDLR were separated [126]. During statin treatment a reduction in cholesterol synthesis (enzyme activity) together with an increase in the number of LDLRs and levels of circulating PCSK9 is demonstrated. What appears as a discrepancy between expression of PCSK9, HMG-CoA reductase and LDLR may not be a discrepancy at mRNA level, as statins inhibit the enzyme thereby stimulating its gene expression due to reduced hepatic cholesterol. The relationship between circulating PCSK9 and LDLR may be more complex due to the fact that 20-40 % of plasma PCSK9 may be bound to apoB in LDL [127-129]. Thus, a reduction of hepatic LDLRs would result in a reduced clearance of PCSK9, leading to its accumulation in plasma. Accordingly, subjects with FH – particularly homozygotes - show significantly higher levels of circulating PCSK9 compared to healthy subjects [130]. Moreover, we could demonstrate that humans treated with GH had reduced serum PCSK9 levels (paper II), which could contribute to the LDL-lowering effect of GH in humans [107]. Circulating PCSK9 is also reduced by thyroid hormone, which may likely contribute to the lower plasma LDLC levels in hyperthyroidism [131]. Also increased levels of estrogen reduce both PCSK9 and LDL cholesterol in women [112], and variations in endogenous estrogen during the menstrual cycle may contribute to the intraindividual variation in PCSK9 and LDLC in normal women [57].

Diet composition and eating patterns influence cholesterol metabolism. Knowledge of underlying mechanisms provides the ability to modulate regulation and homeostasis in desired direction. A ketogenic diet (paper II) increased cholesterol synthesis by 24% and total plasma cholesterol by 37%, but did not influence levels of circulating PCSK9. A vegan diet (paper III) reduced total-C after 3 and 12 months. We further investigated the possible mechanism(s) implicated in this dietary effect, but could not find evidence for any involvement of PCSK9 or altered bile acid or cholesterol synthesis (paper IV). However, a randomized isocaloric trial for 10 weeks (HEPFAT) showed that PUFA, in contrast to SFA, decreased circulating PCSK9 in parallel with LDL cholesterol [132]. A Mediterranean diet
for 5 weeks in men with metabolic syndrome reduced LDL cholesterol and circulating PCSK9 [133]. Further studies on how diet may influence LDL metabolism through effects on PCSK9 in humans will be of great interest. Moreover, the gut microbiome responds to an altered diet [134]. The microbiome plays a part in controlling the composition of the acid pool, and hence also modulates bile acid signaling [135-137].
6 CONCLUSIONS

From the studies following conclusions can be drawn

- Circulating FGF19 is markedly influenced by the transintestinal flux of bile acids, whereas its proposed role in the suppression of BA synthesis and TG levels may not always apply.
- Circulating PCSK9 has a diurnal variation and is strongly reduced during fasting in humans. These changes may relate to diurnal oscillations in hepatic intracellular cholesterol levels.
- A vegan diet reduced levels of oxLDL and LDL-cholesterol, and raised anti-PC IgA and IgM levels in patients with RA.
- Changes in circulating PCSK9 or alterations in the synthesis of cholesterol or bile acids do not contribute to the improved lipid profile observed during a vegan diet.
7 ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to everyone who in various ways has contributed to this work. For discussions, encouragement, criticism, technical support, practical help, it was all essential. In particular, I would like to thank the following people:

First of all, my supervisor Mats Rudling for introducing me to this intriguing and interesting area of research. You have taught me a lot during these past years about how to conduct and present research – thank you!

I would like to thank my co-supervisor Bo Angelin, for all support and for generously sharing your extensive knowledge and for valuable discussions.

All co-authors: Ingiäld Hafström, Johan Frostegård, Guoqing Cao, Maria Dahlin, Ann-Charlotte Elkan, Mats Eriksson, Cecilia Gälman, Björn Kolsrud, Robert J. Konrad, Suzanne Lind, Lena Beckman (Persson), Bo Ringertz, Sara Straniero, Lars Stähle, Jason S. Troutt, Håkan Wallén and Per Åmark, for contributions to the papers in this thesis.

Lena Emtestam for all your administrative help and for always having the right answer.

Ingela Arvidsson and Lisbet Benthin at Metabollab for inestimable technical support. Britt-Marie Leijonhufvud, Katarina Hertel, Yvonne Widlund, Ewa Steninger and Sabine Sülow-Barin at Patient research center for their assistance with collection of blood samples and for taking excellent care of the participants in the studies.

A special thanks to Camilla Pramfalk and Ylva Bonde!

Friends and colleagues: Amani Al-Khaifi, Amit Laskar, Daniela Strodthoff, Johanna Apro, Moumita Ghosh, Sara Straniero, and former members of the group Lena Beckman, Thomas Lundåsen, Cecilia Gälman and Manuela Matasconi, it has been fun working with you, discussing science and many other aspects of life.

Special thanks also to Anna Ehrlund, Lisa-Mari Mörk, Lise-Lotte Vedin (I will very soon buy a new harness and a pair of climbing shoes), Patricia Humire, Per Antonsson and Eckardt Treuter for introducing me to Tomas.

All former and present colleagues and friends at Novum (none mentioned, none forgotten).

To the Ekström and Palosaari families, Birgitta and Arne thank you for a warm welcome.

Loving and supporting family; my parents Catharina and Tom, my brothers Daniel and Edvin. Thanks for your never ending love, support and energy.

Anne, Rebecka, Nick, Cornelia, Wilhelm and Neo.

Thea och Ellen, underbara älskade barn, tack för att ni finns!

Tomas, my life-companion, for endless love and support.
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