Charles University in Prague

Faculty of Science

Animal Physiology

Ing. Miluše Vlachová

Genetic determination of cholesterolemia regulation

Genetická determinace regulace cholesterolémie

Ph.D. Thesis

Supervisor: **RNDr. Jan Kovář, CSc.**
Laboratory for Atherosclerosis Research
Institute for Clinical and Experimental Medicine

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Souhrn

Většina hypercholesterolémií je polygenního původu, dosud však neznáme všechny geny, které cholesterolémii určují. K objevu těchto genů by mohlo napomoci studium experimentálních modelů se spontánním defektem v metabolismu cholesterolu. Cholesterolémie u lidí může být též ovlivněna řadou genových variací a polymorfismů. Některé z nich mohou ovlivnit i odpověď cholesterolémie na příjem tuků.

Pražský hereditárně hypercholesterolemický (PHHC) potkan je jedinečný model hypercholesterolémie, která je indukovaná pouze dietním cholesterolem (bez přídavku kyseliny cholové nebo léků ovlivňujících činnost štítné žlázy). Již na standardní dietě má lehce zvýšenou cholesterolémii a na podání cholesterolové diety reaguje několikanásobným vzestupem koncentrace cholesterolu v krvi (odpovídající pacientům s hypercholesterolémií). Akumulují se u něj lipoproteiny s vysokým obsahem cholesterolu, zejména lipoproteiny o střední (IDL) a velmi nízké (VLDL) hustotě.

V pokusu s tyloxapolem (inhibitorem lipoproteinové lipasy (LPL)) jsme zjistili, že PHHC potkan na cholesterolové dietě inkorporuje do VLDL dvakrát více cholesterolu než potkan Wistar, a to při stejném obsahu cholesterolu v játrech u obou kmenů. Tyto VLDL bohaté na cholesterol jsou *in vivo* katabolizovány pomaleji a hromadí se v cirkulaci. Zvýšenou inkorporaci cholesterolu do VLDL u PHHC potkana nemůžeme vysvětlit rozdíly v aktivitě enzymů acyl-CoA:cholesterol-acyltransferasy (ACAT) a mikrosomálního proteinu přenášejícího triacylglyceroly (MTP) ani v expresi genů, které tyto enzymy kódují. Mezi potkany PHHC a Wistar nebyly také nalezeny žádné rozdíly v odpovědi jaterního transkriptomu (Affymetrix GeneChip®) na cholesterolovou dietu. Identifikovali jsme ale několik genů, jejichž exprese se mezi oběma kmeny významně lišila nezávisle na dietě. Podrobněji jsme analyzovali úlohy dvou z nich, *Apof* a *Aldh1a7*, včetně jimi kódovaných proteinů. Jejich funkce v patogenezi hypercholesterolémie zůstávají však i nadále nejasné. V rámci disertační práci jsme objasnili fyziologickou podstatu hypercholesterolémie u PHHC potkana, geny odpovědné za rozvoj hypercholesterolémie se nám však určit nepodařilo.

Cholesterol-7α-hydroxylasa (CYP7A1) klíčový řídicí enzym syntézy žlučových kyselin, hraje důležitou roli v regulaci cholesterolémie. Polymorfismus -203A>C (rs3808607) v promotoru genu pro CYP7A1 se podílí na determinaci cholesterolémie a její odpovědi na dietu. Tento polymorfismus je v pevné vazbě s dalším promotorovým polymorfismem -469C>T (rs3824260).

Pomocí duálního luciferasového stanovení jsme zjistili, že exprese alely -203C (-203C, -469T) je několikrát zvýšena v porovnání s alelou -203A (-203A, -469C). Za pozorované změny je odpovědný nukleotid v pozici -203. Obě alely se nelišily v odpovědi na stimulaci inzulínem a PPARα agonisty (WY-14643 nebo fenofibrát). Dále jsme studovali diurnální variaci aktivity CYP7A1 po podání cholestyraminu (zvýšení aktivity) a kyseliny chenodeoxycholové (CDCA, snížení aktivity) u zdravých mužů homozygotních pro variantu -203A nebo -203C. Aktivita CYP7A1 byla po podání cholestyraminu zvýšena a po podání CDCA snížena. Mezi homozygotními nositeli -203A a -203C jsme však po podání obou léků nenalezli rozdíly. Aktivita CYP7A1 vykazuje v kontrolním experimentu bez lékové intervence u nositelů alely -203A diurnální variaci, zatímco u nositelů alely -203C nikoli. Rozdíly v diurnální variaci enzymové aktivity mohou přispět k vysvělení role polymorfismu *CYP7A1* v regulaci cholesterolémie a její odpovědi na dietu.

Abstract

Most types of hypercholesterolemia are of polygenic origin. Some genes related to hypercholesterolemia are known, although all genes responsible for cholesterolemia regulation have not been characterised yet. To identify these new genes, animal models with spontaneous defects in cholesterol metabolism could be very useful. Moreover, a number of variations and polymorphisms have been found to influence blood cholesterol levels in humans. Some may also affect cholesterolemia responsiveness to dietary fat.

The Prague hereditary hypercholesterolemic (PHHC) rat is a unique model of hypercholesterolemia induced by dietary cholesterol alone (without administration of cholic acid or thyrotoxic drugs). It exhibits modestly increased cholesterolemia when fed chow and responds to a diet containing cholesterol with a several-fold increase of cholesterolemia to concentrations comparable to those observed in hypercholesterolemic patients. Hypercholesterolemia in this model is characterised by accumulation of very low density lipoproteins (VLDL) and intermediate density lipoproteins (IDL) enriched by cholesterol.

In an experiment with tyloxapol (an inhibitor of lipoprotein lipase) we found that PHHC rats on a cholesterol diet incorporated twice as much cholesterol into VLDL as Wistar rats, although liver cholesterol remained the same. When labelled with ^{125}I, these cholesterol-rich VLDL of PHHC rats were catabolised *in vivo* more slowly than ^{125}I-labelled VLDL of Wistar rats and accumulated in circulation. The increased incorporation of cholesteryl esters (CE) into VLDL in PHHC rats could not be explained by differences in acyl-CoA:cholesterolacyltransferase (ACAT) or microsomal triglyceride transfer protein (MTP) activities and gene expression. Furthermore, we found no differences between PHHC and Wistar rats in the response of the hepatic transcriptome (as determined using AffymetrixGeneChip® arrays) to dietary cholesterol. However, several genes were differently expressed between both strains, independent of diet. Of those, we studied *Apof*, *Aldh1a7* and corresponding proteins in detail. We could not ascribe any role to these genes in hypercholesterolemia pathogenesis. We were able to explain the aetiology of hypercholesterolemia in the PHHC rat, although the related genetic defects need to be clarified.

Cholesterol 7α-hydroxylase (CYP7A1), a key regulatory enzyme in bile acid biosynthesis, plays an important role in cholesterolemia regulation. The -203A>C

polymorphism (rs3808607) in the CYP7A1 gene (*CYP7A1*) promoter is involved in cholesterolemia determination and its responsiveness to diet. This polymorphism is in close linkage disequilibrium with the -469C>T polymorphism (rs3824260).

Firstly, using dual luciferase assay, we demonstrated that expression of the -203C (-203C, -469T) allele was markedly increased compared to the -203A (-203A, -469C) allele, caused by the nucleotide in position -203. The alleles neither responded to stimulation with insulin nor PPARα agonists (WY-14643 or fenofibrate). Secondly, we analysed diurnal variation of CYP7A1 after enzyme activity upregulation (cholestyramine) and suppression (chenodeoxycholic bile acid, CDCA) in healthy men homozygous for the -203A or -203C allele. As expected, CYP7A1 activity was upregulated after treatment with cholestyramine and suppressed after treatment with CDCA. There were no differences between -203A and -203C homozygous subjects in the response of enzyme activity to both drugs. Importantly, in the control experiment, CYP7A1 in -203A allele carriers displayed diurnal variation, but not in -203C carriers. The differences in diurnal variations of enzyme activity may partly explain the role of the CYP7A1 polymorphism in the regulation of cholesterolemia and its responsiveness to diet.

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1. Introduction

Cholesterol, its metabolites and immediate biosynthetic precursors play essential roles in cellular membrane physiology, dietary nutrient absorption, reproductive biology, stress responses, salt and water balance and calcium metabolism. Admittedly, this structurally fascinating lipid is utterly essential to the proper functioning of cells and organisms. However, there is little doubt that the disease process responsible for the leading cause of death in the industrialised world – atherosclerosis – is a disorder in which an excess of cholesterol is a major culprit. Has evolution created a critical, life-sustaining molecule whose oversized amount is causally related by a perverse series of events involving the lifestyle of modern humans to this human disease?

In summary, nature has produced an amphipathic planar molecule that affects (directly or through modifications) an incredible array of critical biological processes. Thus, there has been strong evolutionary pressure to ensure that individuals have an adequate supply of cholesterol to reach reproductive age. Indeed, the regulatory response of cells to sterol starvation as well as to moderate elevation of cholesterol is exquisitely designed for this purpose. What nature did not plan for, nevertheless, was how to handle levels of cholesterol that exceed these limits.

Nowadays, nobody denies that hypercholesterolemia is a causative factor of coronary heart disease. However, the clear cause-and-effect relation between hyper-cholesterolemia and atherosclerosis was generally accepted only 30 years ago [1]. The first evidence came 100 years ago when Anitschkow and Ignatowski produced experimental atherosclerosis in rabbits by feeding them cholesterol [2, 3]. Thereafter, in 1938 Carl Müller described familial hypercholesterolemia as an "inborn error of metabolism" that produces high blood cholesterol and heart attacks in young people [4]. The breakthrough came in 1984 when the Lipid Research Clinics Coronary Primary Prevention Trial showed that decreasing blood cholesterol significantly reduces coronary heart disease events [5]. At that point, for the first time, decreasing blood cholesterol levels became an official national public health goal. These days, in the era of statins, there is no longer any doubt about the value of decreasing blood cholesterol levels.

Perhaps the most discussed topic over recent decades has been the genetic basis of hypercholesterolemia, which has raised the seemingly everlasting question about

monogenic and polygenic origin. Whilst few monogenic disorders exist, the majority of hypercholesterolemic patients suffer from a disease of polygenic source. However, not all of the genes responsible for development of hypercholesterolemia have been identified yet. Apparently, studies on experimental models offer the most suitable approach.

Moreover, interindividual differences in cholesterolemia reflect both environmental variation and genetic polymorphism. Even today, a plethora of variations and polymorphisms (not only in coding sequences but also in upstream and downstream regions) have been found to influence blood cholesterol levels. Some of them also affect the responsiveness of plasma cholesterol to dietary fat.

2. State of the art

2.1 Transport of cholesterol in circulation

Cholesterol is a natural sterol that belongs to lipids. The hydrophobic backbone of the molecule contrasts with the hydrophilic hydroxyl group to give cholesterol its amphipathic character. In the body, cholesterol exists as a free (unesterified) molecule (FC) or as cholesteryl esters (CE). Cholesterol could be either taken in fat food or synthesised *de novo*. Animal fats are complex mixtures of triacylglycerols (TG) with lesser amounts of cholesterol and phospholipids (PL). As a consequence, all foods containing animal fat contain cholesterol. Virtually, every mammalian nuclear cell synthesises cholesterol, in most animals the main part is being produced in extrahepatic organs [6, 7].

Cholesterol is transported through blood in lipoproteins. The lipoprotein core contains CE and TG and is enveloped by a layer of PL, FC and proteins. The proteins (apolipoproteins) are crucial for the lipoprotein assembly, lipid transport and metabolism. The lipoproteins are classified into several groups on the basis of their density and electrophoretic mobility (Table 1).

Table 1: Classificaton and properties of plasma lipoproteins [8].

Lipopro-tein class	Density (g/ml)	Diameter (Å)	Major lipid component	Lipid (%)	Protein (%)	Apolipo-protein(s)	Electropho-retic mobility
CM	< 0,95	800 - 5000	TG	98-99	1-2	B-48, E, Cs	origin
VLDL	0,95 - 1,006	300 - 800	TG	90-93	7-10	B-100, Cs	pre-β
IDL	1,006 - 1,019	250 - 350	cholesterol, TG	89	11	B-100	broad β
LDL	1,019 - 1,063	180 - 280	cholesterol	79	21	B-100	β
HDL	1,063 - 1,210	50 - 90	cholesterol	43-67	33-57	A	α

Chylomicrons (CM) transport dietary TG from small intestine through lymph into the blood. CM interact with lipoprotein lipase (LPL) and TG undergoes hydrolysis. Chylomicron remnants (CMr) are taken up by hepatocytes. Very low density lipoproteins (VLDL) deliver endogenous lipids from the liver to peripheral tissues. In the plasma, VLDL are hydrolysed in the same manner as chylomicrons and in this way, smaller denser particles originate. These intermediate density lipoproteins (IDL) are

mostly taken up by the liver and the minority can undergo further catabolism by enzymes to become low density lipoproteins (LDL). In contrast to VLDL, LDL contain mostly CE. High density lipoproteins (HDL) transport cholesterol from peripheral tissues to the liver in a process termed reverse cholesterol transport (RCT).

2.2 Pathways of cholesterol transport

There are three main sources that supply the blood circulation with cholesterol. It can enter circulation through exogenous pathway from the intestine, through endogenous pathway from the liver and through the RCT from cellular membranes.

2.2.1 Exogenous pathway

After ingestion of a meal, dietary fats are absorbed into the cells of small intestine. Pancreatic lipase hydrolyses TG in the intestinal lumen and generates monoacylglycerols and fatty acids that are absorbed into the enterocyte where TG are resynthesised.

Intestinal cholesterol comes from two main sources, from the bile and the diet. Biliary cholesterol is unesterified, whereas dietary cholesterol is partly esterified and thus must be hydrolysed before the transport into enterocytes. Bile salt micelles facilitate the cholesterol transfer across the brush border membrane via Niemann-Pick C1-like 1 protein (a cholesterol uptake transporter). In the enterocyte cholesterol is esterified by acyl-CoA:cholesterol acyltransferase (ACAT). Once esterified, cholesterol is incorporated into the nascent CM [9]. Absorbed cholesterol in lymph on low or moderate cholesterol diet comes almost equally from bile and diet [10]. In addition, enterocytic cholesterol can be transferred to HDL [11].

Apolipoprotein B48 (apoB48) is found on CM and is necessary for assembly and secretion of CM from the intestine. In the presence of cholesterol, PL and TG, apoB48 is chaperoned and lipidated by microsomal TG transfer protein (MTP) to form prechylomicrons. Otherwise apoB48 is targeted for degradation. The prechylomicrons are transported from the endoplasmatic reticulum (ER) to the Golgi complex. Within it the mature CM are formed. The fully assembled CM exit the Golgi complex in large transport vesicles to be released from the enterocytes into lymph by exocytosis at basolateral membrane [12].

The released CM contain apoB48, apoAI and apoAIV and have to acquire apoC and apoE for catabolism from HDL. After gaining apoCII (the activator of LPL), TG

are hydrolysed by LPL at the endothelial surface. The released fatty acids are entrapped into muscle for energy production and adipocytes for storage. The core of CM is reduced and PL, FC, apoCs transferred back to HDL. CMr which are enriched in CE and apoE can interact with the liver receptors and be removed from the circulation. All CM usually disappear from the circulation within 12 – 14 h after a meal [13].

2.2.2 Endogenous pathway

In the endogenous pathway, the lipids are transported from liver to peripheral tissues in the form of VLDL, IDL or LDL.

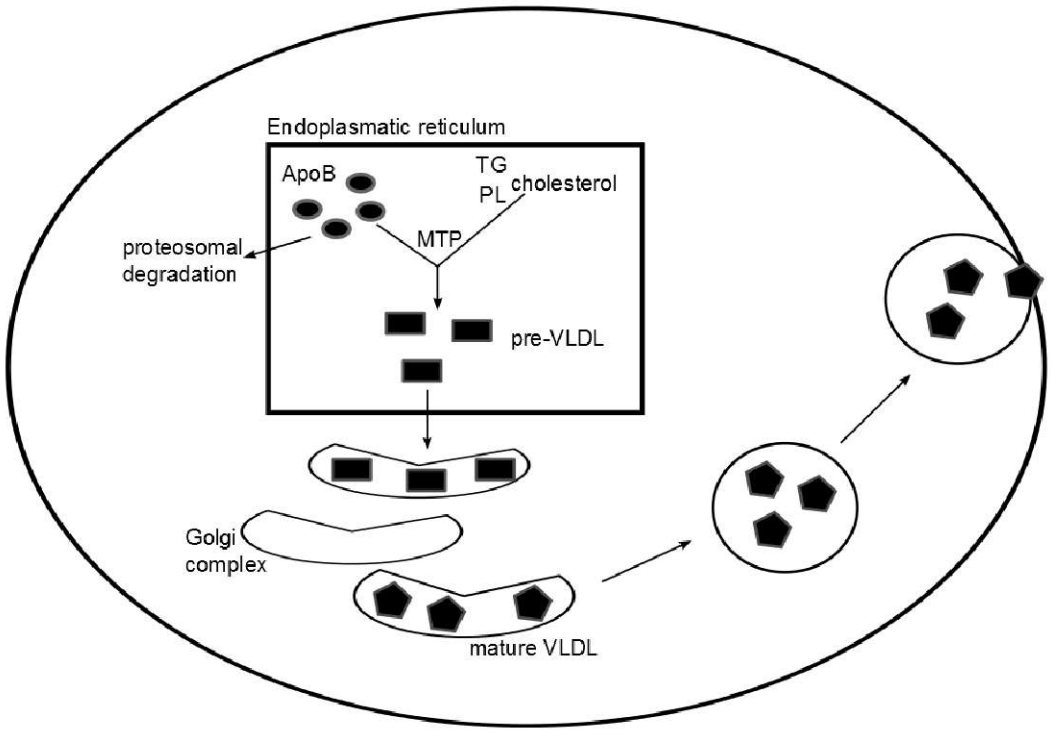

Figure 1: Biosynthesis of VLDL.

After translocation of apoB into the lumen of ER, MTP catalyses the lipidation of apoB with TG, PL and cholesterol to generate pre-VLDL. If MTP and/or lipids are missing, apoB will be targeted for proteosomal degradation. Pre-VLDL are transported to Golgi complex to form mature VLDL by fusing with lipid droplets. The mature VLDL are secreted from the cell.

Similarly to CM, the current model of VLDL assembly occurs in two steps. In the first one, the apoB100 is (post)translationally lipidated, forming pre-VLDL in the ER. The MTP catalyses this step. In the absence of lipids and MTP, apoB100 is degraded. The second step involves transfer of pre-VLDL to Golgi complex and their fusion with lipid droplets to form mature VLDL (Figure 1).

In the blood, TG of VLDL are hydrolysed by LPL to generate denser IDL. Subsequently, IDL can be taken up into the liver similarly as CMr or their TG hydrolysed by hepatic lipase to produce cholesterol rich LDL particles. The LDL are removed mainly by specific receptors from the circulation [12].

2.2.3 Reverse cholesterol transport (RCT)

HDL transport cholesterol from the peripheral tissues mostly to the liver or steroidogennic tissues. The cholesterol in the liver is excreted into the bile, whereas cholesterol supplied to adrenals, ovaries, and testes is used for the synthesis of steroid hormones. Several steps in the HDL metabolism (the RCT) are considered to have the protective function towards atherosclerosis.

The liver and the intestine synthesise and secrete apoAI as a lipid free or poorly lipidated protein into the circulation where it is further lipidated with FC and PL to form a disc-shaped nascent HDL. The lipids come either from the cells or from the lipoproteins. A plasma enzyme lecithin cholesterol acyl transferase associates with HDL and converts FC to CE which are subsequently sequestered into the particle core to form mature spherical HDL. Cholesterol is transfered to nascent and mature HDL via ATP-binding cassette (ABC) transporter A1 (ABCA1) and via ABC transporter G1 (ABCG1). Alternatively, it can be transported to HDL through scavenger receptor class B type 1 (SR-BI). In the liver, HDL cholesterol can be taken up through SR-BI which can mediate bidirectional cholesterol exchange. In humans, part of cholesterol from HDL can be transfered by cholesteryl ester transfer protein (CETP) to TG rich lipoproteins (CM, CMr, VLDL) and in this way transported also to the liver [12].

2.3 Cholesterol uptake

Receptor-mediated lipoprotein uptake is the almost exclusive way of cholesterol clearance from the blood. Plasma lipoproteins bind to specific receptors in the tissues. Besides the supply with lipids, lipoprotein receptors are transducers of extracellular signals, mediators of the endocytic uptake of steroids or scavenger receptors with a broad ligand-binding specificity [14].

The LDL receptor (LDLR) is the first discovered member of lipoprotein receptor family which also includes the VLDL receptor (VLDLR), apoE receptor 2, LDLR-related protein (LRP), as well as megalin and others (Figure 2). Whereas the LDL receptor acts in lipoprotein metabolism exclusively, the LRP and other members of this

family appear to have other distinct functions [14]. LRP and several of its ligands affect the onset of Alzheimer's disease [15]. Megalin is expressed on the apical surface of several epithelial cells (central nervous system, kidney) and plays a role in the recapture of filtered molecules at the level of the proximal tubule [16].

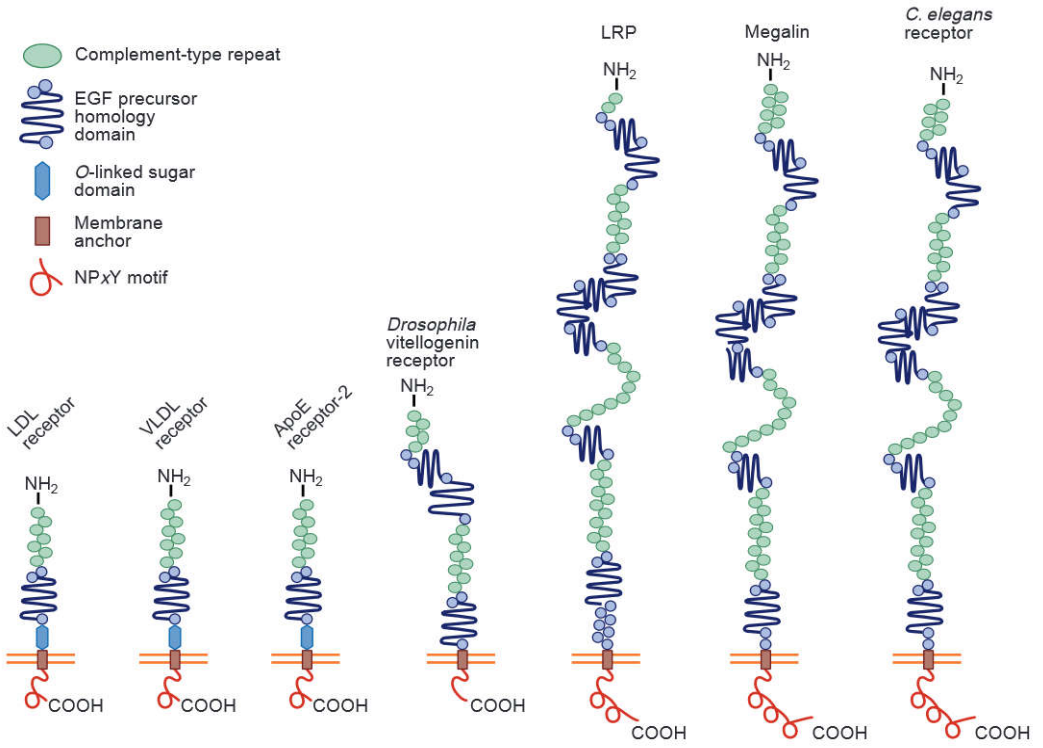

Figure 2: The LDL receptor family.
Members of the LDL receptor family share common structural motifs, including a single membrane anchor, complement-type repeats (which make up the ligand-binding domains) and epidermal growth factor (EGF) precursor homology domains (required for acid-dependent release of ligands in endosomes). NPxY designates the four-amino-acid motif — Asn-Pro-X-Tyr — that mediates clustering of the receptors into coated pits. *O*-linked sugar domains are found in some, but not all, of the receptors [14].

2.3.1 LDL receptor (LDLR)

The Nobel Prize in Physiology or Medicine 1985 was awarded jointly to Michael S. Brown and Joseph L. Goldstein "for their discoveries concerning the regulation of cholesterol metabolism". It was twelve years after they had brought the LDLR to light [17].

The LDLR is a cell surface glycoprotein with *N*- and *O*-linked oligosaccharide chains. The gene coding LDLR is split into 18 exons coding 839 amino acids (and 21 amino acids of signal peptide). The *N*-terminal domain – a crucial domain for receptor binding – is composed of seven repeats of approximately 40 amino acid and contains

a cluster of negatively charged amino acids. On the contrary, the receptor binding domains of two LDLR ligands (apoB100 and apoE) are positively charged [18-20].

About 45 minutes after synthesis, LDL receptors appear on the cell surface where they gather in coated pits. After aggregation they wait for a lipoprotein particle to arrive. Subsequently binding their ligand, endocytic vesicles are formed and internalised, followed by hydrolysis of the endocytosed lipoproteins in lysosomes and release of the lipid into the cytoplasm. The receptors are recycled back to the plasma membrane. The LDLR make one round trip into and out of the cell every 10 minutes for a total of several hundred circles in its 20 hour life-span [18].

The LDLR plays a key role in cholesterol homeostasis by mediating the cellular internalization of apoB and/or apoE containing lipoproteins, namely LDL, VLDL, and CMr [18]. The LDLR is critical for LDL removal from circulation [18, 21]. The most LDLR activity that can be identified in the live animal or human is found in the liver [6].

2.3.2 LDLR-related proteins (LRP)

LRP was discovered in 1988 as the second member of the LDL receptor family [22]. It is a multifunctional cell surface receptor that binds at least 40 different ligands. Outside of the lipoprotein uptake, its functions are also in the homeostasis of proteinases and proteinase inhibitors, cellular entry of viruses and toxins, activation of lysosomal enzymes, cellular signal transduction, and neurotransmission [23]. The absence of known functional coding mutations of the LRP gene in humans, and the lethality of the conventional knockout in mice reveal that LRP is indispensable for cellular physiology [24].

In lipid metabolism, LRP indeed works in concert with the LDLR, binds apoE-containing lipoproteins and mediates primarily the uptake of apoE-enriched CMr into hepatocytes [25].

2.3.3 VLDL receptor (VLDLR)

The VLDLR is highly expressed in the heart, muscle, adipose tissue, and brain, and is barely detectable in the liver, in which the LDLR mRNA is abundant [26]. The VLDLR is highly induced in atherosclerotic lesions [27, 28].

It was found that the VLDLR recognises apoE [26, 29]. A more detailed examination indicated that the VLDLR never binds LDL. The VLDL uptake could be

facilitated by LPL that converts particles to smaller (apoE-rich) remnants before they can become endocytosed by VLDLR [28]. Niemeier et al. showed the same mechanism for CM and their uptake by the VLDLR [30]. Nevertheless plasma lipoprotein levels strongly depends on LDLR activity in the liver, and LDLR disguised the effect of VLDLR on lipoprotein metabolism [28, 31]. Exact role of VLDLR in lipoprotein metabolism has not been clarified yet.

2.3.4 Scavenger receptor class B type 1 (SR-BI)

The SR-BI (unlike the previous receptors) is an important member of the scavenger receptor family. It is an integral glycoprotein that consists of a heavily *N*-linked glycosylated and fatty acylated protein backbone, which contains a large extracellular loop, two transmembrane domains, and short intracellular *N*- and *C*-terminal domains [32].

This receptor is highly expressed in the liver, adrenal glands, and macrophages [33]. The studies in SR-BI knockout mice showed that the molecule is responsible for the majority of the uptake of CE from HDL in hepatocytes and also contributes to the cellular internalization of native apoB-containing lipoproteins [34, 35]. Similarly, the SR-BI mediates the uptake of CE from HDLin adrenocortical cells [36].

Whereas mice predominantly transport the majority of plasma lipids in HDL, humans carry most of their cholesterol in the LDL fraction. Importantly, humans express CETP that is able to exchange CE and TG among HDL, LDL and VLDL. This is an alternative route of RCT for CE from HDL and could be the reason why the relative contribution of the SR-BI to the lipoprotein metabolism in humans is still unclear [32].

2.3.5 Non-receptor mediated uptake

Cholesterol can also move between a variety of lipid membranes via a passive, surface transfer process that does not require metabolic energy. The minor uptake processes may include fluid-phase endocytosis (without binding of lipoproteins to specific receptors) and phagocytosis (lipoproteins are probably attached to the cell surface and then engulfed by the plasma membrane). The contribution of the non-receptor mediated movements of cholesterol to the overall flux between lipoproteins and cells remains unclear.

Furthermore, cholesterol could be excreted directly from blood to feces via the intestinal mucosa in a process called trans-intestinal cholesterol excretion (TICE) [37]. Currently it is still unclear which lipoproteins are involved in this pathway and how is cholesterol targeted to the enterocyte. Using gene expression analyses, so far no membrane transporters have been identified that regulate TICE [9, 38, 39].

2.4 Cholesterol cell metabolism

In the whole animal, biosynthetic and transport mechanisms act in concert to bring about an orderly and regulated flow of cholesterol across the plasma membrane of every cell while, at the same time, preventing the abnormal accumulation of sterol within any tissue.

The cells acquire cholesterol from different sources: lipoproteins, hydrolysis of cell CE, and biosynthesis. The cholesterol elimination is more complicated. Cholesterol is virtually impossible to cleave into small molecules. Extrahepatic cells must then transfer the excess cholesterol to HDL.

2.4.1 Cholesterol from lipoproteins

Lipoproteins taken up by one of the receptors from the LDLR family are endocytosed in coated vesicles and internalised into lysosomes. The receptors recycle back to the plasma membrane to be used again. In the lysosomes, the CE are hydrolysed to FC and apolipoproteins are degraded to amino acids. The FC can be incorporated into cell membranes, reesterified to CE for storage, or leave the cell. The amount of cell cholesterol regulates the activity of 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase and the rate of LDLR synthesis [18].

The cholesterol transport via SR-BI is selective, the CE of HDL are in fact taken up without whole particle internalization. The studies in SR-BI knockout mice suggest that SR-BI is the sole molecule that is involved in the selective uptake of HDL-associated CE in the liver and adrenals [34].

2.4.2 Cholesterol biosynthesis

Cholesterol is synthesised from its precursor acetyl-CoA via a complex metabolic pathway. 18 acetyl-CoA are utilised through the action of at least 30 enzymes to synthetise one molecule of cholesterol. K. Bloch and F. Lynen were awarded the

Nobel Prize in Physiology or Medicine in 1964 for their landmark studies of the cholesterol biosynthetic pathway [40].

Cholesterol synthesis begins with condensation of three acetyl-CoA to form HMG-CoA. The next reaction, reduction of this product to mevalonate by HMG-CoA reductase, is a rate-limiting step of cholesterol synthesis [41] and the site of action for statin drugs used to treat hypercholesterolemia. Mevalonate is then converted in three reactions to isopentenyl pyrophosphate, three molecules of isopentenyl pyrophosphate condense to form farnesyl pyrophosphate. Squalene is formed from two molecules of farnesyl pyrophosphate and then cyclised to lanosterol and subsequently converted to cholesterol via many intermediates (Figure 3) [42]. The squalene oxidation was suggested to represent a possible second control point beyond HMG-CoA reductase [43].

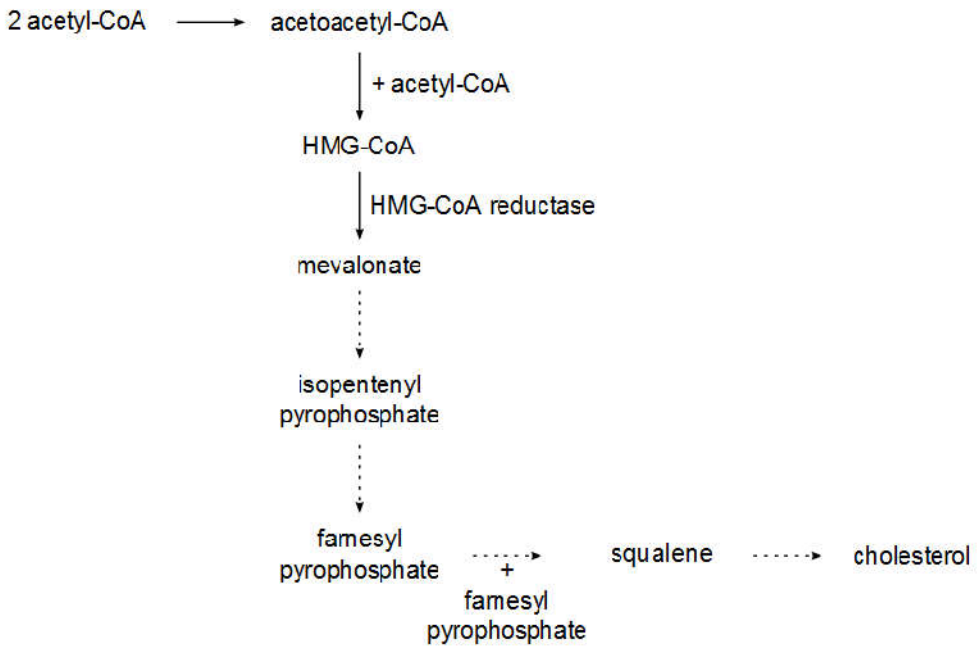

Figure 3: Cholesterol biosynthesis pathway.
Cholesterol is synthesised from its precursor unit acetyl-CoA. Two acetyl-CoA are condensed to form acetoacetyl-CoA. This product plus a third acetyl-CoA are converted to 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA). HMG-CoA is transformed to mevalonate by HMG-CoA reductase. Mevalonate is subsequently converted to isopentenyl pyrophosphate, three molecules of isopentenyl pyrophosphate condense to form farnesyl pyrophosphate. Squalene is made of two farnesyl pyrophosphate, then cyclised and eventually converted to cholesterol via many intermediates.
Solid line – direct step; dashed line – product is formed via intermediates.

In most animals the main part of cholesterol is being synthesised in extrahepatic organs [6, 7]. Hamsters, guinea pigs, rabbits, squirrel monkeys and humans have much lower synthesis rates than rats and mice [9]. The cholesterol synthesis in the liver has

been shown to vary as low as from 15 – 20 % in rabbits and guinea pigs to as high as 50 % in rats of the whole body synthesis, whilst in humans, the liver is thought to contribute only around 10 % to whole body synthesis [6].

2.4.3 Hydrolysis of cholesteryl esters (CE)

If necessary, cholesterol can be esterified and CE hydrolysed continuously. If cell needs the FC, cholesteryl ester hydrolase converts CE from lipid droplets [44].

2.4.4 Bile acid synthesis

Synthesis of BA is the predominant metabolic pathway for catabolism of cholesterol in human. Approximately 500 mg of cholesterol is converted to BA each day in the adult human liver [45, 46]. BA are increasingly being appreciated as complex metabolic integrators and signalling factors and not just as lipid solubilisers and simple regulators of BA homeostasis.

Cholesterol conversion into BA occurs via two different pathways: the classical (or neutral) and the alternative (or acidic) pathway. Cholic acid (CA) and cheno-deoxycholic acid (CDCA) represent the two main end products of these pathways. The steps leading to synthesis of primary BA include initiation (hydroxylation in position 7α), modification of the sterol ring, oxidation and shortening of the side chain, and conjugation [46, 47].

Although both pathways lead to the production of CA and CDCA, the key step, 7α-hydroxylation, is governed by two different enzymes. In the classical pathway, cholesterol is modified by cholesterol 7α-hydroxylase (CYP7A1) directly whilst in the alternative pathway cholesterol is 27-hydroxylated and after that 7α-hydroxylated. The classical pathway accounts for at least 75 % of the total BA pool in humans [46].

Three enzymes have major regulatory roles in these two pathways. CYP7A1 is the rate-limiting enzyme in the classical pathway and is partly controlled by a negative BA feedback loop whereas sterol-27 hydroxylase is the first enzyme in the alternative pathway and is not regulated by BA. Sterol 12α-hydroxylase (CYP8B1) introduces a hydroxyl group at position 12 of the steroid nucleus leading to the generation of CA. It is regulated almost CYP7A1 alike.

Majority of newly synthesised free BA (98 %) are conjugated to glycine or taurine to decrease toxicity and increase solubility for secretion into the bile. This two-step process starts with the generation of BA–CoA by BA–CoA synthase and follows

with amidation by BA-CoA:amino acid *N*-acyltransferase [46, 47]. In the intestinal lumen, gut flora deconjugates, oxidates and dehydroxylates the primary BA to generate secondary BA deoxycholic acid (DCA) and lithocholic acid (LCA). All BA can be taken back up through enterocytes into the blood and return to the liver for reuse in a process known as enterohepatic circulation. Only a small amount of BA escapes from this cycle and is further metabolised by microorganisms and excreted.

2.4.5 Synthesis of oxysterols, hormones, and others

Progestogens serve as precursors to all other human steroids. Firstly, cholesterol is converted by cholesterol side-chain cleavage enzyme to pregnenolone in the mitochondrion. This conversion is the rate-limiting step of steroid synthesis. According to the body/organ need, the production of specific steroid hormones ensues in the destined tissue (corticosteroids – adrenal cortex; estrogen and progesterone – ovary, placenta; testosterone – testes).

Cholesterol could be oxidised by a number of mitochondrial hydroxylases, (hydr)oxysterols are produced by almost all cells. In contrast to cholesterol, oxysterols can cross the blood-brain barrier. Importantly, oxysterols are regulators of cholesterol metabolism and play a significant role in the brain.

Cholesterol is also a precursor of vitamin D. Cholecalciferol is synthesised in the skin from 7-dehydrocholesterol under the action of ultraviolet B light.

2.5 Daily cholesterol intake and turnover

On a Western diet, humans synthesise an estimated 1 g of cholesterol and ingest ~400 mg a day [48]. Most nutritionists concur that excessive cholesterol intake, often construed as more than 300 mg per day, should be avoided.

The paleolithic nutritional model suggests that cholesterol consumption of preagricultural humans was probably ~480 mg/d. Nevertheless, their serum cholesterol levels were averaging ~3.2 mmol/l [49], a value subsumed within the range observed for free-living nonhuman primates (2.3 - 3.5 mmol/l) [50]. This cholesterolemia can be explain by a low total fat intake and a high ratio of polyunsaturated to saturated fat in spite of the high dietary cholesterol of gatherer-hunters [51].

Hepatic daily cholesterol turnover is 1.8 g roughly. The majority (~85 %) of cholesterol and its derivates in the bile comes originally from lipoproteins. The most cholesterol originates from CMr (0.6 g) and LDL (0.6 g). The HDL are providing 0.3 g

and 0.3 g comes from *de novo* cholesterol synthesis. Approximately 1.3 – 1.5 g of cholesterol is secreted into bile (cholesterol itself: 1.0 g and BA: 0.3 – 0.5 g), and 0.5 g of cholesterol is incorporated into VLDL [45].

2.6 Regulation of cholesterol homeostasis

Maintenance of cholesterol homeostasis requires a balance between cholesterol secretion into the blood and its uptake and copes with fluctuations in dietary cholesterol intake. The pathways involved are regulated via a complex interplay of enzymes, transport proteins, transcription factors, and non-coding RNAs.

The liver has been considered the major site of control of cholesterol homeostasis [6]. First of all, the liver clears VLDL, LDL, and CMr from circulation, synthesises cholesterol and BA, is involved in production of nascent HDL, secretes cholesterol and BA to bile. Nowadays, the importance of the intestine in cholesterol physiology has been recognised. The intestine influences cholesterol homeostasis at the level of cholesterol and BA (re)absorption, fecal excretion and *de novo* synthesis [9, 52]. At least in mice, TICE plays a major role in disposal of cholesterol via the feces [38].

A plethora of proteins and factors plays a role in the control of cholesterol level. In the next subchapters, I will characterise the major mechanisms that maintain and regulate cholesterol homeostasis. The dominant transcription factors and proteins will be shortly described. Thereafter I will summarise the regulation in the cell and blood.

2.6.1 Transcription factors

2.6.1.1 Sterol response element binding protein (SREBP)

Sterol response element binding proteins (SREBP) were identified as nuclear factors that bind the sterol response element (SRE) [53, 54]. They coordinate the synthesis of two major building blocks of membranes (fatty acids and cholesterol) through their effects on multiple genes involved in cholesterol biosynthesis and uptake via LDLR.

The SREBP family members belong to a large class of transcription factors containing a basic helix-loop-helix-leucine zipper. The SREBP family comprises three subtypes, SREBP-1a and SREBP-1c, which are generated by alternative splicing, and SREBP-2 [55, 56].

All subtypes are synthesised as inactive precursors bound to the ER. Each SREBP precursor of about 1150 amino acids is organised into three domains:

N-terminal domain with the zipper region for binding DNA, two hydrophobic transmembrane-spanning segments, and *C*-terminal domain that performs the essential regulatory function.

While the SREBP-1 proteolytic activation is largerly under the control of hormones or signal transduction systems, the SREBP-2 processing is tightly regulated by the ER cholesterol content, reflecting its deep involvement in cholesterol homeostasis [57]. The studies in the liver of rats or mice after fasting showed that the amount of the nuclear active form SREBP-1 and SREBP-2 are not the same. The amount of SREBP-1, predominantly SREBP-1c in the liver, increases enormously upon consumption of low fat/high carbohydrate compared with nonfasted rats, whereas SREBP-2 protein levels remain unaltered [58, 59].

To act as a transcription factor in cholesterol metabolism, the *N*-terminal segment of SREBP must be released from the membrane and reach the nucleus. The release is accomplished by a two-step proteolytic cascade. This process is initiated when the *C*-terminal domain of SREBP binds to SREBP cleavage-active protein (SCAP) [60, 61]. The complex formation is essential for the exit of SREBP from the ER and subsequent proteolytic activation. SCAP is both an escort for SREBP and a sensor of sterols [62]. SCAP, in turn, can bind reversibly with another ER-resident membrane protein, Insig (insulin induced gene). Addition of sterols to intact cells or to isolated membranes triggers the binding of SCAP to Insig, an event that is required for sterol-mediated inhibition of SCAP/SREBP transport [63].

When cells become depleted in cholesterol, SCAP leaves the SREBP from the ER to the Golgi complex, where two proteases reside. A membrane-bound serine protease (S1P) cleaves the SREBP molecule in the loop between their two membrane-spanning segments. Simultaneously with this change, the *N*-terminal domain of SREBP is disengaged from the membrane via a second cleavage by a zinc metalloprotease (S2P). The *N*-terminal domain translocates to the nucleus, where it activates transcription by binding to SRE in the promoter or enhancer of target genes (Figure 4) [61, 65].

When cholesterol content of the ER membrane rises, a conformational change in SCAP facilitates its binding to Insig. This interaction is required for the retention of the SCAP/SREBP complex in the ER. Altogether, SREBP are no longer processed proteolytically in the Golgi, their zipper domain cannot be released form the ER membrane, and the transcription of target genes declines.

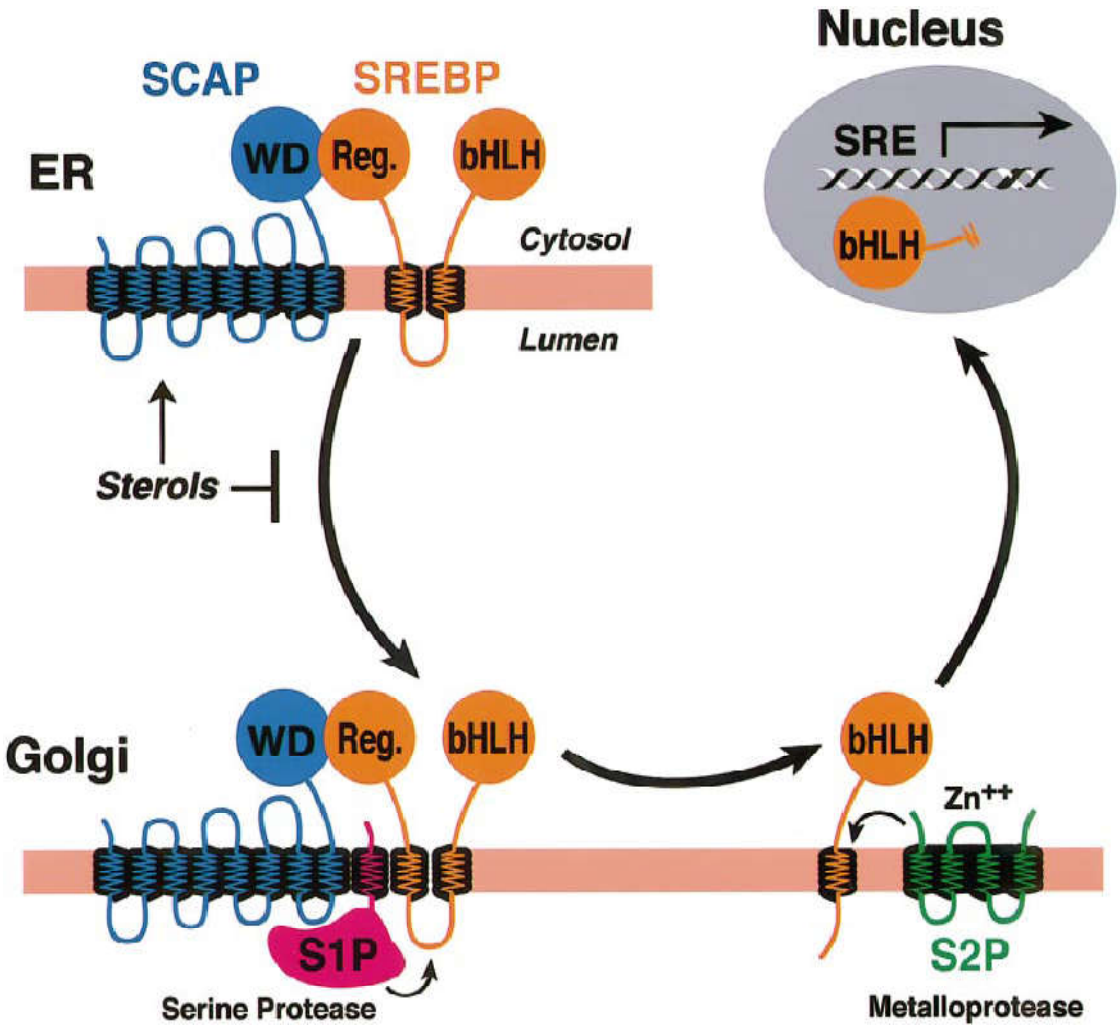

Figure 4: SREBP regulation of cholesterol metabolism.
The membrane-associated SREBP are transcriptionally inactive. In the ER, the *C*-terminal domain of the SREBP interacts with SCAP. In sterol-depleted cells, SCAP escorts the SREBP from the ER to the Golgi complex where they are processed by two membrane-associated proteases, the site 1 (S1P) and site 2 (S2P) proteases, that release the mature forms of the proteins. These transcriptionally active fragments of the SREBP are translocated to the nucleus where they bind to the promoters of SREBP target genes, including genes involved in the cholesterol synthesis and metabolism of lipids. When cholesterol builds up in the membranes of the ER, the Scap/SREBP complex is retained in the ER, the proteolytic activation of SREBPs is stopped, and the expression of SREBP target genes declines [64].

When expressed at higher than physiological levels, all three subtypes can activate genes encoding multiple enzymes in the biosynthesis of cholesterol and fatty acidas well as LDLR [66]. However, the relative activity of the three isoforms differs. SREBP-1a is a potent activator of all SREBP-responsive genes, owing to its long transactivation domain encoded by first exon. SREBP-1c differs from SREBP-1a in the alternative splicing of first exon to a shorter transactivation domain and therefore is less potent than SREBP-1a [67]. Both SREBP-1a and SREBP-1c activate genes involved in the synthesis of fatty acids. SREBP-2 encoded by a different gene, preferentially

activates cholesterologenesis and the LDLR. Genes involved in cholesterol synthesis contain SRE which are identical or relatively similar to the original SRE sequence found in the LDLR gene. Meanwhile, binding sequences of SREBP in lipogenic genes vary considerably and are tentatively designated SRE-like sequences [68].

2.6.1.2 Liver X receptor (LXR)

Liver X receptors (LXR) are nuclear receptors that regulate the metabolism of several important lipids, including cholesterol and BA. They work in complementary fashion with SREBP to maintain cholesterol homeostasis.

In 1995, the LXR were identified as members of the nuclear receptor superfamily [69] and a year after, found to be activated by oxygenated cholesterol derivatives [70]. Like other receptors in the family, LXR heterodimerise with retinoid X receptor (RXR) and bind to specific LXR response elements (LXRE) [69, 71, 72]. Two genes are known to encode LXR proteins that are activated by the same ligands. LXRα (*NR1H3*) is expressed predominantly in the liver and to a lesser extent in the kidney, small intestine, spleen, and adrenal gland [69, 71]. LXRβ (*NR1H2*) is ubiquitously expressed [69, 73, 74].

The physiological LXR ligands are oxysterols – 22(R)-hydroxycholesterol and 20(S)-hydroxycholesterol (intermediates in steroid hormone synthesis), 24(S)-hydroxy-cholesterol (the most abundant circulating oxysterol), and 24(S),25-epoxycholesterol (mainly in the liver) [76]. LXR-mediated gene regulation requires their hetero-dimerization with another nuclear receptor – RXR which is activated by 9-*cis* retinoic acid (Figure 5). The consensus LXRE is characterised by direct repeats separated by four nucleotides. In addition, LXR activity is determined by their phosphorylation, acetylation and/or SUMOylation status [75, 77-80].

The function of LXR is to maintain whole animal sterol homeostasis. To achieve this task, LXR coordinate gene expression programmes in tissue-specific fashion. LXR are activated in response to elevated cholesterol levels in cells (that results in production of oxysterols in mitochondria). By inducing the expression of genes encoding ABCA1 and ABCG1, LXR promote cholesterol efflux to apoAI and HDL [81, 82]. Further they elicit HDL metabolism via induction of genes encoding phospholipid transfer protein, LPL and CETP [83-85]. Besides promoting RCT, LXR activation may reduce cellular cholesterol uptake by enhancing the ubiquitination of the LDLR [86]. Hepatic LXR activate biosynthesis of BA, via induction of CYP7A1 in mice [76]. LXR also enhance

biliary cholesterol secretion by inducing ABCG5/ABCG8 which mediates cholesterol secretion from the liver into the bile [87, 88]. The expression of these transporters is influenced by LXR in the intestine as well to regulate cholesterol reabsorption and modulate enterohepatic recirculation of cholesterol [89]. To summarise, the LXR preserves whole cholesterol homeostasis.

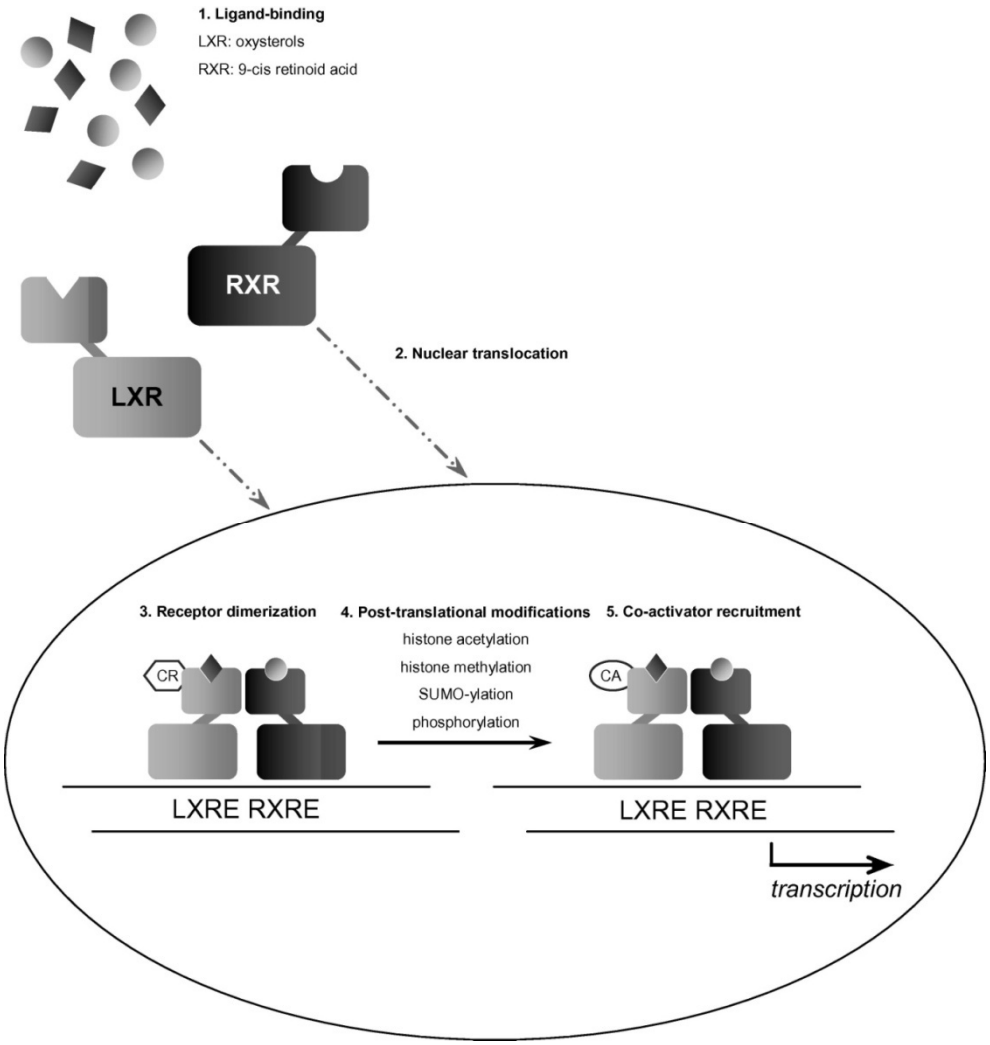

Figure 5: Regulatory steps that determine LXR transcriptional activity.
1. LXR are ligand activated by oxysterols. 2. LXR transcriptional regulation requires their translocation to the nucleus and (3.) heterodimerization with activated RXR. Both LXR and RXR bind to specific response elements in the DNA. 4. Ligand binding and posttranslational modifications alter the structural conformation of the LXR/RXR complex as (5.) a co-repressor (CR) or co-activator (CA) [75].

When phenotypes of LXRα, LXRβ andLXRαβ (double) knockout mice are compared, the expression of genes involved in hepatic cholesterol metabolism is profoundly reduced in LXRα-deficient mice. On the other hand, LXRβ knockout mice failed to show these alterations, consistent with a more prominent role of LXRα compared to LXRβ in the regulation of lipoprotein metabolism [90].

2.6.1.3 Peroxisome proliferator-activated receptor (PPAR)

Likewise the LXR, peroxisome proliferator-activated receptors (PPARs) belong to the group of ligand-activated transcription factors and form heterodimers with RXR.

The PPAR subfamily comprises of three related proteins: PPARα, PPARβ/δ, and PPARγ (known as NR1C1, NR1C2, and NR1C3, respectively). PPARα was the first member identified with PPARβ/δ and PPARγ was subsequently discovered based on sequence homology [91-93]. The PPAR response element is a direct repeat AGGTCA separated by one base pair only. The mechanism of transcription regulation by the PPAR is the same as that by the LXR.

The PPARs influence primarily fatty acid metabolism and their natural endogenous ligands are unsaturated fatty acids and eicosanoids [94, 95]. PPARα is the predominant form in the liver and plays a role in the lipoprotein clearance via the regulation of expression of genes involved in lipid metabolism. PPARβ/δ is involved in fatty acid transport and oxidation. The genes activated by PPARγ stimulate lipid uptake and adipogenesis by fat cells.

The PPARs influence cholesterolemia chiefly through the regulation of synthesis of apolipoproteins (AI, AII, AV, C), CYP7A1, LPL and LXRα. The hypolipidemic effect of fibrates is mainly a result of activation of PPARα [94, 96].

2.6.1.4 Farnesoid X receptor (FXR)

The farnesoid X receptor (FXR) is a member of nuclear receptor superfamily. Originally, FXR was described as a farnesol-activated receptor interacting with RXR, and accordingly named [97]. Later, it was identified as a receptor that is activated by BA [98-100].

The FXR is highly expressed in the liver and the intestine [97]. It can bind to and activate or repress through a large variety of response elements (FXRE) on target genes either as a monomer or as a heterodimer with RXR [97, 101, 102]. To date, more than 80 compounds have been identified as potential FXR ligands, nevertheless, BA are the most important. The potency of BA to activate FXR is CDCA > DCA > LCA > CA [100]. FXR is the chief sensor for intracellular concentration of BA, controlling their synthesis (CYP7A1, CYP8B1), conjugation, transport (membrane carriers of BA), and homeostasis.

When cellular levels of BA are already high, one of the primary functions of FXR is the suppression of *CYP7A1*. FXR does not directly bind to the *CYP7A1*

promoter but induces expression of small heterodimer partner (SHP). Positive regulation of SHP activation inhibits the activity of two other nuclear factors which then functions to inhibit transcription of the *CYP7A1* [103, 104]. Furthermore, FXR induces the expression of BA-conjugation enzymes and of the BA export transporters and simultaneously represses BA import [105].

FXR regulates gene expression of proteins involved in lipid metabolism, such as phospholipid transfer protein and apoCII (induction), and apoAI, apoCII, apoE (repression) [106-109]. In the FXR-deficient mice, serum HDL cholesterol and TG are elevated whereas SR-BI is decreased [110]. FXR also induces the expression of *SREBP-1c* in mice and *PPARα* in humans which both will modulate TG production [111].

2.6.2 Enzymes and transport proteins

2.6.2.1 HMG-CoA reductase

HMG-CoA reductase is a highly conserved, mebrane-bound enzyme. In eukaryotes, it is an ER-membrane resident consisting of two distinct domains: a hydrophobic *N*-terminal membrane anchor with sterol sensing domain (SSD) and *C*-terminal catalytic domain that extends into the cytoplasm [112]. The SSD may bind lipids and plays an important role in HMG-CoA reductase regulation [42, 112, 113].

HMG-CoA reductase catalyses the conversion of HMG-CoA to mevalonate, an important intermediate in the biosynthesis not only of cholesterol but also of essential nonsterol isoprenoids. As the primary irreversible point of the cholesterologenesis, it is advantageous to control the pathway at the beginning because a decrease in HMG-CoA activity can regulate the output of the overall pathway without accumulation of intermediates.

The complexity of the reductase regulatory system was first revealed in the late 1970s in a study with compactin (a competitive inhibitor of HMG-CoA reductase). The compactin treated cells respond to the blocking of mevalonate production by a drastic increase in reductase protein (~200fold) [114].

HMG-CoA reductase is subject of tight feedback regulation by multiple mechanisms at different levels of transcription, translation, posttranslational modification and degradation. Cholesterol has been long recognised as an important signaling molecule, however, some other intermediates and mevalonate-derived products control the reductase as well [115]. Studies with both yeast and mammalian

cells have implicated farnesyl diphosphate and farnesol as an important signaling molecule that inhibits the level of HMG-CoA reductase expression [116-118].

Sterol deficiency causes the activation of SREBPs in the liver (particularly SREBP2) that enhance the expression of genes involved in cholesterol synthesis and uptake (including HMG-CoA reductase) [66, 119]. In addition to its role in regulating HMG-CoA reductase through SREBP, Insig also regulates the reductase degradation. In sterol-replete conditions, Insig binds to the *N*-terminal region of HMG-CoA reductase and thereby recruits ubiquitin ligase and initiates ubiquitination of reductase. This ubiquitin-proteasome pathway is an obligatory process for recognition and rapid degradation of many proteins [115]. An unknown nonsterol isoprenoid controls the translational effects of HMG-CoA reductase through a poorly understood mechanism that may involve the complex 5'untranslated region (5'UTR) of the reductase mRNA [120]. A short-term regulation of HMG-CoA reductase is achieved with phosphorylation of serine in position 872 (activity decrease) by AMP-activated protein kinase and its dephosphorylation (reactivation) by protein phosphatase 2A [121, 122].

2.6.2.2 Squalene monooxygenase (SM)

Squalene monooxygenase (SM) catalyses the first oxygenation step in cholesterol biosynthesis, acting on squalene before cyclization into the basic steroid structure. The inhibition of SM activity can have possible advantage compared to inhibiton HMG-CoA reductase because it can preserve synthesis of important isoprenoids while shutting down cholesterol synthesis when the cell cholesterol levels are high [123].

Firstly, SM is controlled at the transcription level being an SREBP2 target: the SM expression is modulated by sterols, it increases under lipid-depleted conditions [124, 125]. Secondly, SM is also posttranslationally regulated by proteosomal degradation. In contrast to HMG-CoA reductase degradation, the SM degradation does not require Insig and SCAP and is mediated rather by cholesterol itself [43]. Cholesterol can bind directly to SM but the precise molecular mechanism by which cholesterol accelerates proteasomal degradation of SM still needs to be defined [126].

2.6.2.3 Cholesterol 7α-hydroxylase (CYP7A1)

CYP7A1 is a microsomal cytochrome P450 enzyme expressed only in the liver [127]. It catalyses 7α-hydroxylation of cholesterol, the first and rate-limiting step in the

classical pathway of BA synthesis. The human *CYP7A1* is localised to chromosome 8q11 where it spans about 10 kbp. The translated protein consists of 504 amino acids.

The enzyme activity is regulated principally at the transcriptional level by BA returning to the liver through enterohepatic circulation, cholesterol, steroid and/or thyroid hormones, insulin, and exhibits diurnal rhythm.

The *CYP7A1* promoter contains two highly conserved BA response elements (BARE). BARE-I binds the LXRα/RXR heterodimer and was found in rodent (rat and mouse) not in human hydroxylase promoter [128, 129]. The BARE-II contains direct repeats that bind hepatocyte nuclear factor 4 (HNF4) and liver receptor homologue 1 (LRH1). Binding of these two transcription factors is essential for basal expression of *CYP7A1* (Figure 6) [128, 130, 131].

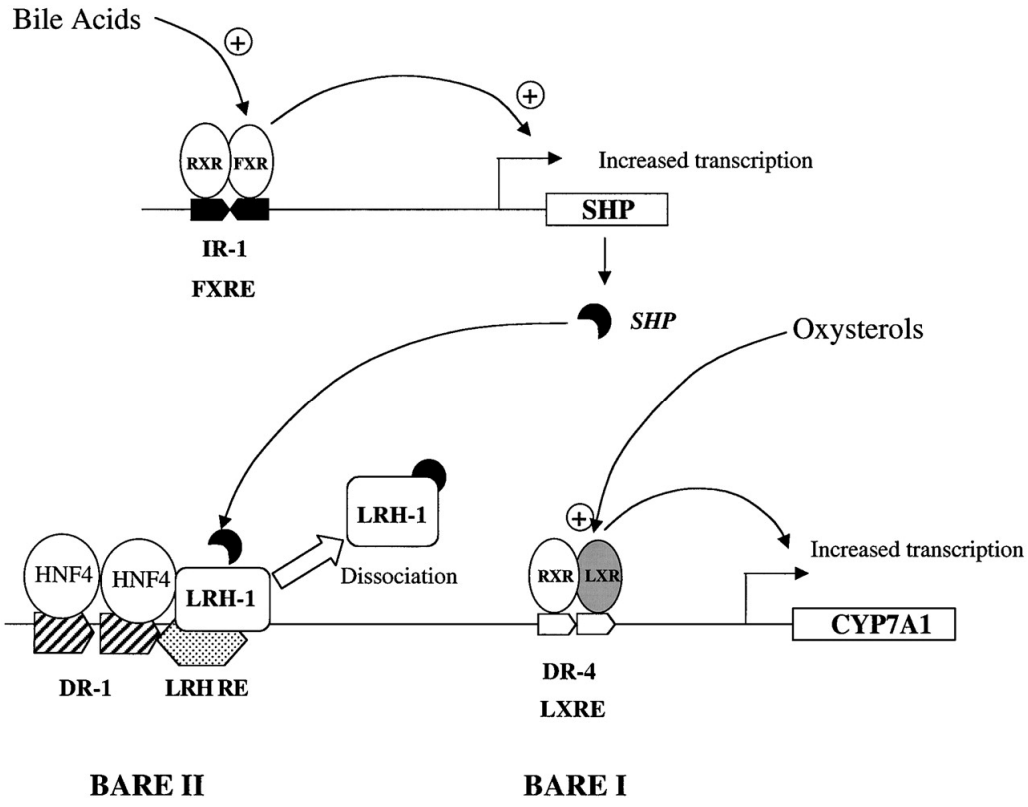

Figure 6: Transcription of *CYP7A1* via BARE.
CYP7A1 promoter contains two highly conserved BARE. The rodent (rat and mouse) *CYP7A1* contains a BARE-I, a direct repeat4 (DR4) element (not found in the human promoter), which has been shown to be the binding site for the LXRα/RXR heterodimer. Oxysterols increase *CYP7A1* transcription through the activation of LXRα. The inhibitory action of BA through FXR on *CYP7A1* transcription is mediated via BARE-II. However, *CYP7A1* promoter does not contain any FXR binding sites. BARE-II contains a conserved direct repeat 1 (DR1) element for HNF4 binding and a response element for LRH1 binding. Binding of these two factors is essential for liver-specific basal expression of *CYP7A1*. FXR binds to the inverted response 1 (IR1) element in the SHP promoter. In response to BA, SHP transcription is increased. SHP, in turns, interacts with the competence factor LRH1. This interaction represses the transcription activation by LRH1. As a result, *CYP7A1* expression is decreased [132].

When cholesterol accumulates in cell, oxysterols are generated and can drive LXRα-dependent transactivationat BARE-I and BA synthesis increases [76, 133]. Conclusive evidence has come from the characterization of LXRα knockout mice. The loss of LXRα results in the rapid accumulation of CE in the liver, because of no stimulation of the metabolic clearance of cholesterol through the synthesis of BA. As expected, a major defect in these mice is the inability to upregulate *CYP7A1* expression [133].

Conversely, excess of hydrophobic BA leads to the decline of their synthesis via FXR [104, 134] . This repression is indirect because *CYP7A1* promoter does not contain any FXR binding sites. FXR increases transcription of SHP which promotes the dissociation of coactivators linked to HNF4 and LRH1, as well as by histone deacetylation of the promoter [135]. FXR also induces fibroblast growth factor-19 (human) or -15 (mouse) in the intestine that interacts with its cognate receptor in the liver to downregulate BA production by repressing *CYP7A1* and *CYP8B1* expression through a c-Jun *N*-terminal kinase-dependent signalling cascade [136].

BA can also activate protein kinase C or induce the synthesis of inflammatory cytokines and their release from Kupffer cells. Both proteins can suppress *CYP7A1* expression [136]. Direct repeat 1 (DR1) also confers the repression of the *Cyp7a1* by fibrates mediated by PPARα. Nevertheless, PPARα does not bind to this DR1 but interferes with the binding of HNF4 [137].

The activity of CYP7A1 in rats varies diurnally, reaching the maximum during the night and falling to a minimum during the day [138, 139]. These diurnal changes seem to be regulated at the transcriptional level, and albumin site D-binding protein elements in the *Cyp7a1* promoter have been reported to be important for this regulation [140, 141]. In humans, CYP7A1 activity, as reflected by serum BA synthesis marker 7α-hydroxy-4-cholesten-3-one (C4) levels [142], shows two peaks that coincide with meal intake, reduces at night, and returns to basal levels in the morning [143].

The expression of *Cyp7a1* increases 2 – 3 times in rats administered thyroid hormone, nevertheless, the response elements have not been defined yet [144, 145]. Analyses in thyroid hormone receptor β knockout mice suggest that this receptor can be responsible for activation of CYP7A1 [146]. The administration of corticosteroids to rats leads to an increase in the CYP7A1 activity [144, 147]. In humans, *CYP7A1* promoter is strongly repressed by steroid/thyroid hormones [148, 149].

Insulin has been shown strongly inhibit the BA synthesis by downregulation of CYP7A1 and CYP8B1 [148, 149]. However, recent studies found out that short-term

treatment of physiological concentration of insulin rapidly stimulated *CYP7A1* expression in primary human hepatocytes whereas extended treatment represses human CYP7A1 [150]. In human hepatocytes, high glucose stimulated BA synthesis and induced mRNA expression of *CYP7A1*. It has been sugested that glucose increases ATP levels and in this way inhibits AMP-activated protein kinase which results in *CYP7A1* transcription stimulation [151, 152].

2.6.2.4 Sterol 12α-hydroxylase (CYP8B1)

CYP8B1 catalyses the synthesis of CA and controls the ratio of CA over CDCA in the bile that determines the hydrophobicity of the BA pool and affects the solubility of cholesterol [153]. The gene encoding CYP8B1 is unique among the cytochrome P450 genes in that it is intronless.

In BA-replete conditions, the FXR pathway is activated and *CYP8B1* transcription decreases. The mechanism is identical to that in *CYP7A1*, FXR modulates expression through both SHP and fibroblast growth factor-19 [154, 155].

Cholesterol feeding or thyroid hormone repress *Cyp8b1* expression in rats, in contrast to their stimulatory effect on *Cyp7a1*. The mechanism by which thyroid hormone represses *Cyp8b1* expression is not known [156-158].

2.6.2.5 LDL receptor (LDLR)

The LDLR take up apoB and/or apoE containing lipoproteins and is a major determinant of plasma cholesterol level. Its discovery introduced three general concepts to cell biology: receptor-mediated endocytosis, receptor recycling, and feedback regulation of receptors. (Detailed characterisation of LDLR in 2.3.1.)

In the same way as HMG-CoA reductase, the transcription of *LDLR* is under the control of SREBP2. Its transcription increases when cholesterol level in the cell is low [42]. Besides the transcription regulation through SREBP, the LDLR is also regulated by a posttranslational mechanism. Proprotein convertase subtilisin/kexin type 9 (PCSK9) binds directly to the extracellular domain of the LDLR and interferes LDLR recycling after endocytosis thereby promotion its lysosomal degradation [159]. The LXR pathway also influences the LDLR amount on the cell surface. In response to the rising cellular cholesterol concentration, LXR mediates the ubiquitination and ensuing degradation of LDLR [86].

LDL-derived cholesterol supresses cholesterol biosynthesis and uptake. Further it activates cholesterol esterification so that excess cholesterol can be stored as CE droplets in the cytoplasm.

2.6.3 Non-coding RNAs

Additionally to the classical transcriptional regulators (SREBP, LXR), a class of noncoding RNAs, termed micro RNAs (miRNA) has emerged as critical regulators of gene expression acting predominantly at the posttranscriptional level. This large family of short (22 nucleotides) RNA binds to the 3'UTR of mRNA at the seed sequence (2 – 8 nucleotides) and thereby represses gene expression. Recent studies have shown that miRNAs can also repress mRNA through binding to other regions (5'UTR, exons) [160, 161] or may even activate translation [161, 162].

The canonical pathway for the biogenesis of miRNA begins in the nucleus. After the transcription of primary long (~hundreds of nucleotides) miRNA, its hairpin structures is processed sequentially in the nucleus and cytoplasma into precursor miRNA (~70 nucleotides). In the cytoplasm, the precursor miRNA is transformed into a mature miRNA duplex by the endonuclease. One of this duplex strands associates with RNA-induced silencing complex to produce an aggregate that binds to its RNA target [163]. Experimental approaches using bioinformatics indicated that a single miRNA may simultaneously target more than 100 mRNAs [164].

Recent studies have shown key roles for miR-122 and miR-33 in regulation of lipid metabolism, and further evidence implicates miR-370 in regulation of miR-122. miR-122 is the predominant liver miRNA (70 %) [165] and plays a critical role in fat and cholesterol metabolism. Studies with antisense nucleotides in mice showed that miR-122 inhibition caused a significant decrease of expression of genes encoding enzymes involved in cholesterol biosynthesis [166, 167]. The evidence of miR-122 function using genetic deletion in mice reveals a 30 % reduction in total cholesterol (due to reduction of both LDL and HDL) and TG [168, 169].

miR-33a and miR-33b are intronic miRNAs located within SREBP gene [170, 171]. Their overexpression strongly represses *ABCA1* expression and decreases cellular cholesterol efflux to apoAI in RCT [171, 172]. Besides ABCA1, ABCG1 (in RCT), Niemann Pick C1 (transport of intracellular cholesterol), and genes involved in β-oxidation of fatty acids were described as targets of miR-33 [173, 174].

2.6.4 Regulation of cholesterolemia

Based on the results of epidemiological studies it has been established that cholesterolemia should not exceed 5.0 mmol/l. Higher level is associated with an increased risk of atherosclerosis and coronary heart disease. Therefore the adequate regulation of cholesterol blood level is essential for cardiovascular prevention.

Hypercholesterolemia is not a manifestation of cell cholesterol accumulation. It results from imbalance between secretion and uptake of cholesterol carrying lipoproteins (mainly LDL) [175]. The major factors that determine cholesterolemia are mechanisms involved in the regulation of lipid secretion from hepatocytes and those involved in the lipoprotein uptake from circulation.

2.6.4.1 Cell cholesterol homeostasis

The most significant regulatory element of cholesterol concentration in the cell is cholesterol itself. Its level in the plasma membrane and other membranes of cellular compartmens is precisely regulated in every cell. Some of regulatory mechanisms – cholesterol esterification or enzyme ubiquitination – provide fast response to cholesterol level in the cell whereas regulation through transcription factors influences cholesterol homeostasis after several hours.

The explanation of feedback regulation of cell cholesterol homeostasis represented an intricate task because cholesterol is insoluble in water and resides exclusively in cell membranes. The regulatory mechanisms involve cholesterol itself (both FC and CE) as well as cholesterol metabolites (mainly BA and oxysterols).

When the concentration of cholesterol in plasma membrane exceeds certain concentration (saturation point), the abundant cholesterol (called also „active cholesterol“) tend to escape or move to other cellular membrane compartments. It can be esterified and, in this way, the amphipathic molecule of FC is transformed into hydrophobic CE that can be stored in intracellular lipid droplets. Excess of FC in the cell triggers binding of HMG-CoA reductase to Insig which, in turn, initiates ubiquitination and subsequent degradation of this reductase. Interaction of FC with Scap in ER membranes results in suppression of SREBP signalling pathway. This leads to the very rapid downregulation of the sterol synthesis rate within the cell [115, 176]. In mitochondria, abundant FC can be oxidized to oxysterols that can be removed relatively easily from the cell. Oxysterols are also ligands for LXR which promote transcription of ABC transporters. ABCA1 and ABCG5/G8 stimulate transfer of plasma membrane

cholesterol to HDL in RCT. In rodents (but not in humans) LXR also upregulates *CYP7A1* transcription and, thus, transformation of FC to BA [177, 178].

Oppositely, when cholesterol level in the cell decreases, CE from lipid droplets can be hydrolysed to provide FC instantly. The decrease in the cholesterol metabolically active pool in ER initiates the processing of SREBPs into their active forms. These transcription factors then enhance the gene expression of many of the enzymes required for cholesterologenesis (already from acetyl-CoA C-acetyltransferase) as well as for uptake of cholesterol from circulation (LDLR) [66, 176].

2.6.4.2 Cholesterolemia regulation

High intracellular cholesterol is detrimental to the cells, and high serum cholesterol will promote the deposition of cholesterol in the arterial wall, one of the initial steps in atherogenesis. Increased cholesterol concentration in the circulation is a consequence of cell inability to remove cholesterol from the blood, or, very rarely, from increased production of cholesterol-enriched lipoproteins.

Both enviromental and genetic factors play a role in cholesterolemia regulation. Intake of saturated fat and cholesterol, physical activity are the most important enviromental factors. Data from family and twin studies indicated that genetic factors account for ~ 50 % of the interindividual variation of plasma LDL-cholesterol (LDL-C) [179, 180]. Cholesterolemia is regulated both at the level of lipoprotein cholesterol entry into circulation and at the level of cholesterol uptake by cells. Receptor-mediated lipoprotein uptake is the crucial pathway responsible for maintaning cholesterolemia. The key receptor of this process seems to be LDLR. Its deficiency leads to pronounced hypercholesterolemia in patients with familial hypercholesterolemia.

A number of small nucleotide polymorphisms (SNP) in several genes involved in cholesterol metabolism that are associated with higher cholesterol level has been described. They include genes with pivotal roles in LDL metabolism (*APOE*, *APOB*, *LDLR*, *CYP7A1*). Three common alleles of *APOE* encoding apoE (differring in amino acids in position 112 and 158) explain 5 – 10 % of variation of LDL-C. The carriers of isoform E2 have lower whereas the carriers of E4 higher cholesterol concentration compared to E3 variant carriers [181, 182]. Surprisingly, common polymorphism of *LDLR* and *APOB* contribute only little to the heritable variation in plasma LDL-C in general population. Interestingly, the allelic variations in *CYP7A1* account for 15 % of the overall variation in plasma LDL-C [183]. Two polymorphisms in the upstream

region of *CYP7A1* (-203A>C, rs3808607, and -469C>T, rs3824260) have been associated with variation of plasma LDL-C [184]. Furthermore, it was repeatedly demonstrated that -203A>C polymorphism affects responsiveness of plasma cholesterol and LDL-C to dietary fat and cholesterol [185, 186].

Since 2007, genome wide association studies have identified several chromosome loci related to atherosclerosis. Unfortunatelly, the majority of these new findings overlaps with the classical risk factors and the influence of the locus 9p21.3, the strongest genetic factor of atherosclerosis known today, is not as sufficient as has been expected [187, 188].

2.7 Animal models

The first experimental hypercholesterolemic model was a rabbit. In 1908 Ignatowski, and five years later Anitschkow showed that feeding cholesterol to rabbits resulted in hypercholesterolemia and development of atherosclerosis [2, 3]. In some species hypercholesterolemia can be induced very easily (rabbit, pigeon) whereas some animals are resistant (pig, monkey, rodents). In mice and rats, BA or drugs influencing thyroid gland must be added to cholesterol containg diet to induce hypercholesterolemia.

Furthermore, there are marked differences in lipoprotein metabolism between humans and rodents. Mice and rats do not possess CETP and have an up to 40-fold higher LDL clearance by the liver compared to humans [6, 189]. Hence they carry most of their plasma cholesterol in HDL particles [6, 189-192]. Additionally, editing of the hepatic apoB mRNA in the rodents limits apoB100 synthesis in favour of apoB48 synthesis.

For that reason many studies on cholesterol metabolism have been performed in mice with genetic deficiency of major determinants of plasma cholesterol metabolism such as the LDLR [193] or apoE [194]. More recently, “humanised” mice have become available in which human *CETP* is expressed [192].

Nevertheless, these currently used animal models can test only the function of genes already known as a candidate for a defined monogenic defect. However, most hypercholesterolemias is of polygenous origin. To discover genes that determine cholesterolemia, animals with spontaneous defects in cholesterol metabolism should aid to reveal new pathophysiological mechanisms. These experimental models can be

obtained by inbreeding of individuals with high cholesterol concentration that are more sensitive to dietary cholesterol and fat [195].

2.7.1 Watanabe-heritable hyperlipidemic (WHHL) rabbit

The WHHL rabbit has an inherited hyperlipidemic trait. It was obtained by inbreeding from a mutant discovered in 1973. It was demonstrated later that this strain represents a model of LDLR deficiency. The rabbit has LDL cholesterol abnormally increased 8 – 14fold in comparison with control rabbits [196-199]. The model was very useful for study of familial hypercholesterolemia and atherosclerosis pathogenesis [200].

2.7.2 Prague hereditary hypercholesterolemic (PHHC) rat

In general, rat is not a good model for atherosclerosis and hypercholesterolemia research. Its plasma cholesterol concentrations are commonly lower than 2 mmol/l and the majority of cholesterol is transported in HDL [192]. To induce hypercholesterolemia it is necessary to add BA or thyreotoxic substances to the diet because cholesterol and fat are not sufficient [201-203]. Hovewer, several groups were able to produce hyper-cholesterolemic rats by inbreeding animals sensitive to dietary cholesterol [204, 205].

The PHHC rat was obtained through the selective inbreeding of parental Wistar rats [206]. It has modestly increased cholesterolemia when fed chow and responds to a diet containing 2% cholesterol by a severalfold increase of cholesterolemia to concentrations comparable to those observed in hypercholesterolemic patients. Importantly, such a change in cholesterolemia is reached without the addition of CA and/or thyreotoxic drugs. Besides that, cholesterol accumulates in lipoproteins other than HDL, mostly in VLDL fraction [206].

Despite considerable effort, the mechanisms responsible for hyper-cholesterolemia development have not been determined yet. Because no difference was found in LDL clearance from the circulation between PHHC and Wistar rats (Figure 7), defect in LDLR quantity and/or function of PHHC rat was excluded [206]. Additionally, the absorption of dietary cholesterol does not prominently differ in both lines [206] as well as the activity of lecithin:cholesterol acyltransferase [207], LPL, and hepatic lipase [208]. It has been suggested only recently that PHHC rats, when fed cholesterol, may not be able to upregulate the transcription of *Cyp7a1* [209].

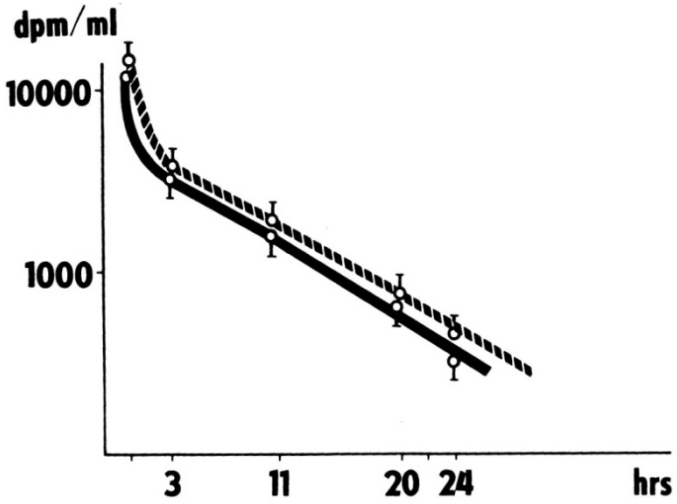

Figure 7: Clearance of ^{125}I-LDL from circulation.
LDL were isolated from Wistar rats, then radiolabelled, and intravenously injected into PHHC and Wistar rats. The radioactivity decrease of plasma ^{125}I did not differ between both lines [206].

Backcrossing of PHHC and Wistar rats revealed that hypercholesterolemia in PHHC rat is of polygenic origin. Nevertheless, genes that may be involved in the determination of hyperresponsiveness of PHHC rat to dietary cholesterol have not been idenfied up to now.

3. Aims

3.1 Pathogenesis of hypercholesterolemia in the PHHC rat (Aim I)

The PHHC rat is an established model of dietary cholesterol-induced hypercholesterolemia, although its pathogenesis is not yet fully understood. Our aims were as follows:

a) to study the production and clearance of lipoproteins in the PHHC and control Wistar rat *in vivo*,

b) to compare the response of the hepatic transcriptome to cholesterol feeding in PHHC and control Wistar rats and identify the genes involved in the determination of hyperresponsiveness of PHHC to dietary cholesterol,

c) to confirm the role of selected candidate genes in hypercholesterolemia pathogenesis.

3.2 The role of the -203A>C polymorphism of *CYP7A1* in cholesterolemia regulation (Aim II)

The -203A>C polymorphism of *CYP7A1* is involved in the determination of cholesterolemia and its responsiveness to diet, although the mechanisms behind it have not yet been explained. Our aims were as follows:

a) to study the regulation of the expression of the *CYP7A1* promoter variants *in vitro* using dual luciferase assay,

b) to examine the diurnal variation of CYP7A1 activity in homozygous carriers of the -203A and -203C allele.

4. Pathogenesis of hypercholesterolemia in the PHHC rat (Aim I)

4.1 Materials and methods

4.1.1 Rats

Male PHHC rats (bred at the Department of Experimental Medicine of the Institute for Clinical and Experimental Medicine) and control Wistar rats (AnLab, Prague, Czech Republic) weighing 260 – 460 g were used in the experiments. The studies on rats were conducted in conformity with the Institute for Laboratory Animal Research Guide for Care and Use of Laboratory Animals as incorporated in the guidelines and practices established by the Council for Animal Rights of the Institute for Clinical and Experimental Medicine.

4.1.2 Diets

The rats were fed one of the three diets: standard laboratory diet M2 (SEMED, Prague, Czech Republic) (C diet), M2 diet + 5% palm kernel oil (control fat, CF diet), and M2 diet + 5% palm kernel oil + 1% cholesterol (CHOL diet). Both PHHC and Wistar rats were kept on the above mentioned diets for three weeks.

4.1.3 Determination of VLDL production *in vivo*

Both PHHC and Wistar rats were fed C, CF or CHOL diet (n = 6 per group, six groups in total) for 3 weeks. At the end of experiment, VLDL production was measured as described previously [210] with slight modifications. The rats were anesthetised *i. p.* with Thiopental® (150 mg/kg of weight), an intravenous catheter was inserted into jugular vein and a baseline sample of blood was taken. One ml of 20% tyloxapol (Triton WR-1339, Sigma-Aldrich) was then injected through the catheter. Two hours later, the rats were sacrificed and the serum was used for TG and cholesterol determination and VLDL isolation by ultracentrifugation (3 pools per each group) [211].

4.1.4 Determination of VLDL clearance *in vivo*

Firstly, VLDL were isolated by ultracentrifugation [211] from the serum of PHHC rats fed CHOL diet and from Wistar rats fed the C diet. Cholesterol/TG ratio of these VLDL were 2.22 (PHHC) and 0.83 (Wistar). Total protein in VLDL was determined [212]. VLDL were labeled by the iodine monochloride method [213] using

^{125}I-sodium iodide (Institute of Isotopes, Budapest, Hungary). Secondly, male Wistar rats (300 – 400 g of weight) were then anesthetised i. p. with Thiopental® (150 mg/kg of weight), an intravenous catheter was inserted into jugular vein and the radiolabelled VLDL was injected. Four Wistar rats were administered autologous ^{125}I-VLDL while the other four Wistar rats were administered ^{125}I-VLDL from PHHC rats. The 0.2 ml aliquots of blood for determination of radioactivity were then taken from tail vein 5, 15, 30, 60, 120, 240 and 360 min after application. The radioactivity was measured in the serum. Human LDL (0.13 mg of lipoprotein) in saline was then added to 50 µl of each of the serum samples and the samples were precipitated with isopropanol as described previously [214]. Radioactivity was subsequently measured in supernatant and the amount of radiolabelled apoB was calculated. The half-time of apoB in VLDL was estimated from monoexponential plot of the data (least square fit) acquired from 30 to 360 min. The biexponential fit was not used because of limited amount of data. Two-sample t-test (two-tail) was used to examine whether the observed halftimes were statistically different. Normality of the data was examined via Lilliefors test (two sides) at the significance level $\alpha = 0.01$.

4.1.5 Determination of MTP and ACAT activities

The MTP activity was determined in the liver homogenate using commercial fluorimetric kit (Roar Biomedical) as recommended by the manufacturer. The ACAT activity in hepatic microsomal fraction was measured as described by others [215, 216].

4.1.6 Gene expression analysis in the liver

After the 3 week feeding period, the rats (n = 6 - 8 per group, six groups in total) were sacrificed and the samples of liver tissue were taken for RNA isolation for gene expression study. Four individual RNA samples per each group were used for microarray analysis; all samples from the study were used if the expression was validated by qPCR. The aliquots of liver and serum were used for quantification of cholesterol and TG, and lipoproteins were isolated from serum by ultracentrifugation as described [211].

4.1.7 Lipid and lipoprotein analysis

Lipoproteins were isolated by sequential ultracentrifugation as described by others [211]. Cholesterol and TG in serum and in ultracentrifugally isolated lipoproteins

were measured using enzymatic kits (Roche Diagnostics). Cholesterol and TG in the liver were determined using the same method after lipid extraction from the liver homogenate [217]. The presence of apoE in ultracentrifugally separated VLDL was confirmed as earlier described [218]. In experiment in which MTP and ACAT activities were studied, the concentration of free and total cholesterol in the liver was determined using enzymatic kit (BioVision) and CE concentration calculated as a difference.

4.1.8 RNA extraction

RNAs were produced from two liver samples of each animal. The liver samples (50 – 100 mg) were stored in RNAlater (Qiagen) immediately following dissection. Total RNA was isolated using TRIzol® (Invitrogen) according to the manufacturer's instructions. RNA concentration was determined spectrophotometrically and RNA integrity was confirmed on a 1% agarose gel electrophoresis after 2 min at 70 °C. The resulting RNA samples were stored at -80°C.

4.1.9 Microarray analysis

RNA samples passing quality control and microarray analysis of gene expression were processed by the Functional Genomics and Bioinformatics Core (Institute of Molecular Genetics, ASCR v.v.i., Prague, Czech Republic). The intactness of the 18S and 28S ribosomal subunits of RNA was analysed on Bioanalyser 2100 (Agilent Technologies). Sample quality was assessed by RNA Integrity Number (RIN score) and samples with an RIN of 7.5 or greater were passed; quantity was determined by NanoDrop ND-1000 Spectrophotometer (Nanodrop Technologies). Samples were then prepared for hybridization, hybridised, washed, strained and scanned according to the manufacturer's instructions on GeneChip®Rat Exon 1.0 ST Array (Affymetrix). Affymetrix control metrics were used to qualify the resulting data. Raw data were processed at the gene level within Affymetrix Expression Console (version 1.1) using the RMA algorithm.

4.1.10 Reverse transcription

RNA (15 μg) was treated with 2 U DNase I (Fermentas) at 37 °C for 30 min followed by the addition of 2.5mM EDTA and incubation at 65 °C for 30 min to remove trace amounts of DNA. For reverse transcription, a modified manufacturer's protocol of RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas) was used. Three

µg of purified RNA, 5 µM oligo(dT) primer and 5 µM random hexamer primer in 12 µl reaction mixture were heated to 70 °C for 5 min, then cooled on ice for 1 min and a reverse transcriptase reaction mix containing buffer (50mM Tris-HCl (pH 8.3), 50 mM KCl, 4 mM MgCl2, 10mM DTT), 20 U RNase Inhibitor and 1mM dNTP mix was added. The reaction mixture was then incubated for 5 min at 25 °C, 200 U MuLV Reverse Transcriptase was added and incubation continued at 25 °C for 10 min. The reaction mix was heated to 42 °C for 60 min, then to 70 °C for 15 min and placed on ice for 2 min. Afterwards, RNase H (2 U) was added, followed by incubation at 37 °C for 20 min and then by heating to 65 °C for 10 min. cDNA was purified via Qiaquick PCR Purification Kit (Qiagen) and dissolved in 30 µl of DEPC water. The resulting cDNA samples were quantified spectrophotometrically at 260 nm and then stored at -80 °C.

4.1.11 Quantitative real-time PCR (qPCR)

Reaction was performed for selected genes using a 7300 Real Time PCR System (Applied Biosystems) and TaqMan® Gene Expression Assays (Appendix 12.2) in combination with TaqMan® Gene Expression Master Mix, according to the manufacturer's instructions (Applied Biosystems). Twenty ng of cDNA in a 20 µl reaction volume per well were used. Reactions were performed in duplicates for each sample. All results were normalised to *Vars2l* (the lowest standard deviation of microarray analysis of gene expression among all samples) to compensate for differences in the amount of cDNA. The PCR efficiency of each gene-specific real-time PCR session was validated with a standard curve constructed from a simultaneously run serially diluted cDNA [219, 220]. The relative expression levels were quantified by using the delta-delta threshold cycle method with efficiency correction [221].

4.1.12 DNA sequencing

gDNA was extracted from peripheral leukocytes using QIAamp columns (Qiagen). The coding exons of *Apoe* and *Apof*, together with the adjacent parts of the intronic sequences, were amplified by PCR with intronic oligonucleotide primers (Appendix 12.2). The amplicons were gel-purified, extracted with QIAquick spin columns (Qiagen), and used as templates for the sequencing reaction with Big DyeTerminator Kit v3.1 (Applied Biosystems) according to the manufacturer's protocol. The products were analysed on a 3130 Genetic Analyser (Applied Biosystems). Data were evaluated using SeqScape® Software (Applied Biosystems).

4.1.13 Determination of Aldh1a7 activity

The activity of aldehyde dehydrogenase family 1, subfamily A7 (Aldh1a7) was measured in cytosol fraction of liver homogenate as described by Kathmann with slight modifications [222]. Reactions were performed at 24 °C in 60mM sodium pyrophosphate buffer (pH 8.5) with 1mM NAD^+. Enzyme activity was expressed as increase of NADH concentration in $nmol.l^{-1}min^{-1}mg^{-1}$. The initial velocities were estimated by extrapolation of differences to zero time.

4.1.14 Statistics

The data (with exception of results of VLDL clearance and the microarray experiment) were analysed by ANOVA and, if ANOVA revealed any differences, then corresponding post-hoc tests were performed (GraphPad InStat).

Differential gene expression analysis was performed in the R statistical environment [223] using the Limma package [224] which is a part of the Bioconductor project [225]. Multiple testing correction was performed using the Benjamini and Hochberg method. We considered genes to be differentially expressed if the adjusted *P* value was < 0.05.

4.2 Results

4.2.1 Lipids and lipoproteins

Feeding both Wistar and PHHC rats CHOL diet resulted in a marked accumulation of TG and cholesterol in the liver and a cholesterol increase in serum lipid and lipoproteins (Table 2, Figure 8).

While the response in the liver of both strains did not differ, the cholesterol change in serum was severalfold higher in PHHC than in Wistar rats. The increase of cholesterolemia in Wistar rats was caused by a fivefold increase of VLDL-cholesterol (VLDL-C), the cholesterolemia increase in PHHC rats was due to an approximately tenfold increase of VLDL-C and IDL-C. VLDL of PHHC rats were substantially enriched by cholesterol. Feeding both strains the CF diet had no effect (in comparison with C diet) either on concentrations of cholesterol and TG in the liver or on lipid and lipoprotein concentrations in serum.

Table 2: Lipids in the liver and serum in PHHC and Wistar rats.

	Wistar			PHHC		
	C	CF	CHOL	C	CF	CHOL
n	8	8	8	6	6	7
Liver						
cholesterol	6.1 [a]	5.8 [a]	24.3 [b]	6.0 [a]	5.6 [a]	27.6 [b]
[µmol/g]	(1.3)	(0.4)	(4.5)	(0.7)	(1.6)	(13.1)
TG	5.0 [a]	6.6 [a]	22.2 [b]	4.8 [a]	5.3 [a]	16 [b]
[µmol/g]	(0.9)	(1.6)	(3.7)	(1.1)	(1.1)	(8.8)
Serum						
cholesterol	1.68 [a]	1.98 [a,b]	2.34 [b]	2.48 [a]**	2.71 [a]**	4.24 [b]**
[mmol/l]	(0.19)	(0.26)	(0.37)	(0.21)	(0.26)	(0.24)
TG	1.22 [a]	1.24 [a,b]	1.79 [b]	0.92 *	0.95	1.42
[mmol/l]	(0.18)	(0.42)	(0.39)	(0.21)	(0.38)	(0.47)

[a,b] the same letters are assigned to the groups that do not differ within a given strain ($P < 0.05$),
*,** $P < 0.05$, $P < 0.01$ … differences between Wistar and PHHC rats on the same diet.
Data are presented as mean (SD).

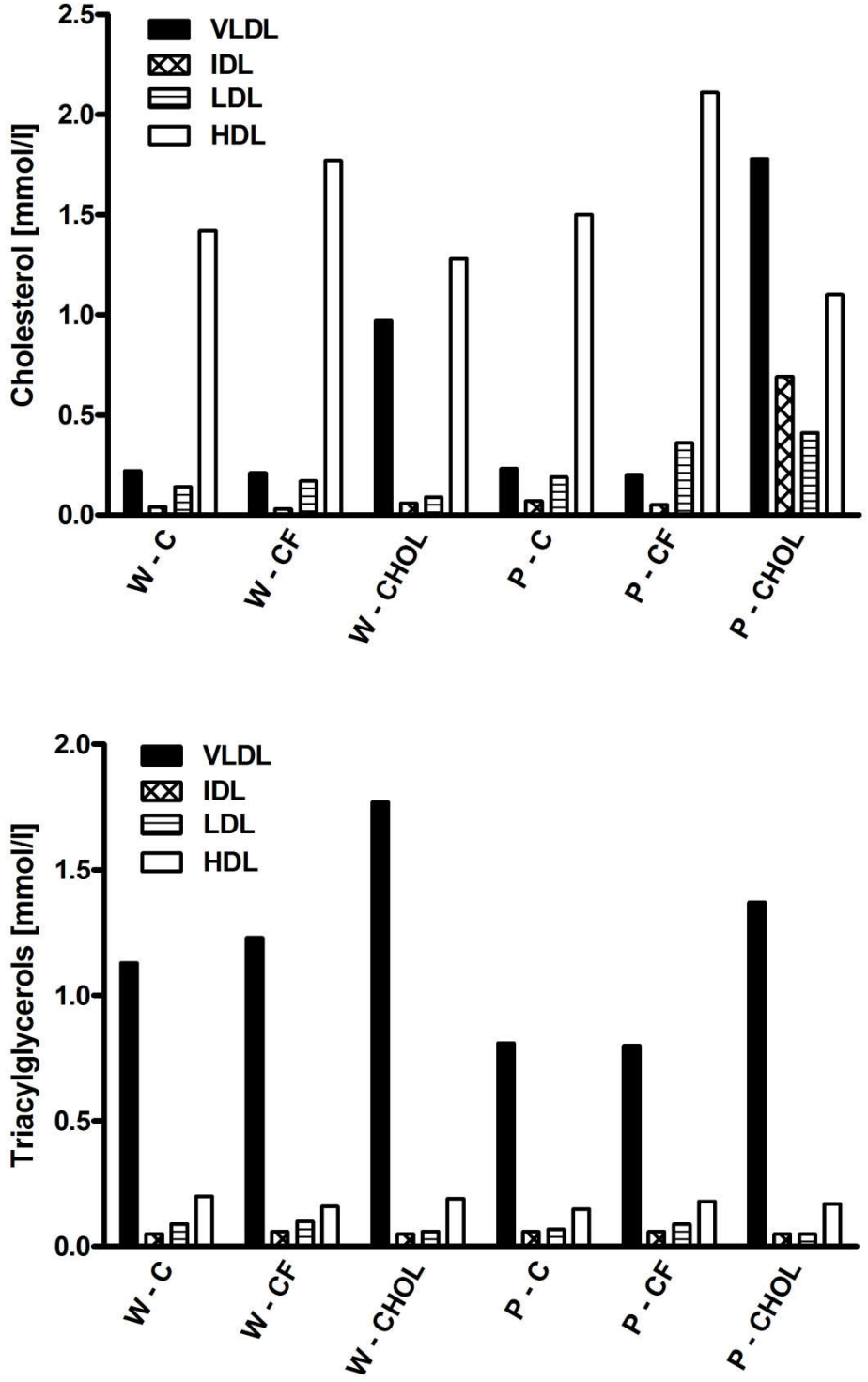

Figure 8: Cholesterol and TG in ultracentrifugally isolated lipoproteins in PHHC (P) and Wistar (W) rats.

PHHC and Wistar rats were fed C, CF or CHOL diet for three weeks. The lipoproteins were isolated from serum by ultracentrifugation (one pool per group was used) [212].

4.2.2 VLDL production *in vivo*

To estimate the rate of VLDL production *in vivo* and analyse their composition, tyloxapol (Triton WR 1339), an inhibitor of LPL, was injected to rats fed C, CF, and CHOL diet in another experiment. Two hours later, the serum TG raised several fold in all groups of animals (Figure 9). On the CHOL diet, serum cholesterol rose from 1.5 to 3.6 and from 3.2 to 7.9 mmol/l in Wistar and PHHC rats, respectively ($P < 0.001$). The increase in cholesterolemia was only modest on both control diets.

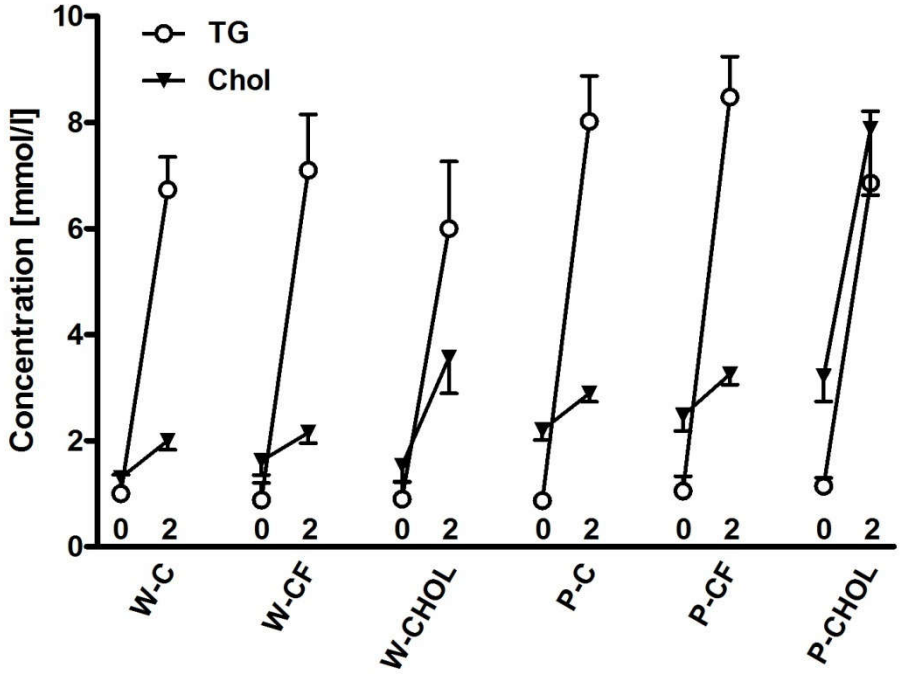

Figure 9: Cholesterol and TG in serum before (0) and 2 hours after (2) tyloxapol application. PHHC and Wistar rats were fed C, CF or CHOL diet for three weeks. At the end of the experiment, the rats were injected 1 ml of 20% tyloxapol and sacrified two hours later. Data are presented as mean ± SD. W (Wistar), P (PHHC) rat.

Importantly, the cholesterolemia increase was exclusively caused by the increment in VLDL-C. VLDL-C/VLDL-TG ratio which was ~ 0.2 in both PHHC and Wistar rats on both control diets, rose to 0.45 in Wistar rats and to 0.9 in PHHC rats on CHOL diet (Figure 10).

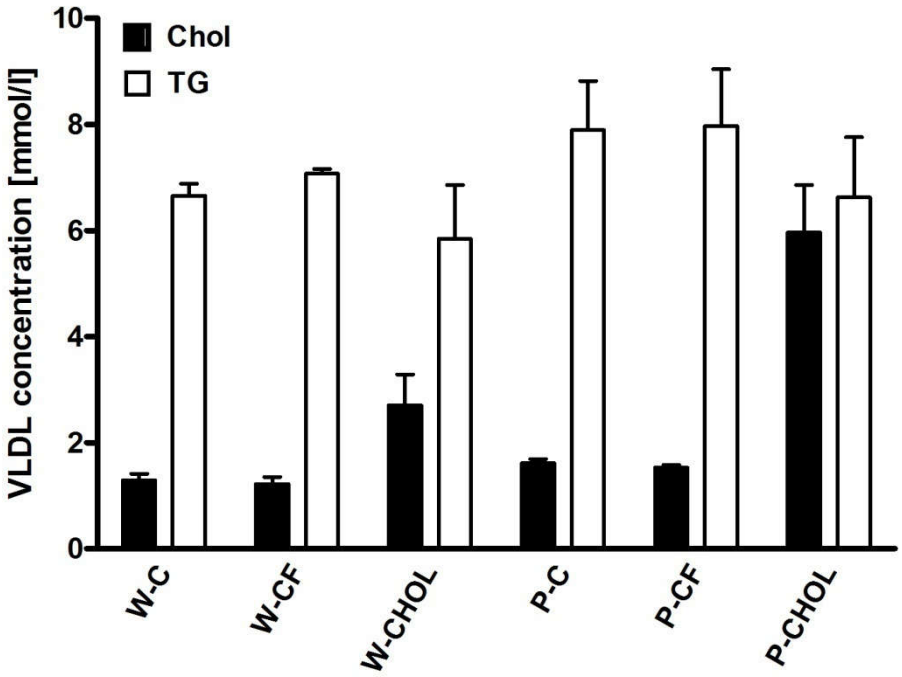

Figure 10: Concentration of cholesterol and TG in VLDL isolated from rat serum 2 hours after tyloxapol administration.
PHHC and Wistar rats were fed C, CF or CHOL diet for three weeks. At the end of the experiment, the rats were injected 1 ml of 20 % tyloxapol and sacrified two hours later. Three pools per each group of 6 rats were used for VLDL isolation. Data are presented as mean ± SD. W (Wistar), P (PHHC) rat.

4.2.3 VLDL clearance *in vivo*

To determine whether the cholesterol-rich VLDL of PHHC rats are removed from circulation at a different rate than those of Wistar rats, the rate of disappearance of radiolabelled VLDL from both Wistar and PHHC rats was compared in Wistar rats *in vivo* in another experiment. When intravenously injected into Wistar rats, the ^{125}I-VLDL from Wistar rats were cleared from serum more rapidly than the ^{125}I-VLDL from PHHC rats (Figure 11). The half time of apoB of Wistar VLDL was estimated to be more than three times shorter than that of apoB of VLDL isolated from PHHC rats (107 ± 42 min vs. 376 ± 109 min, respectively; $P < 0.01$).

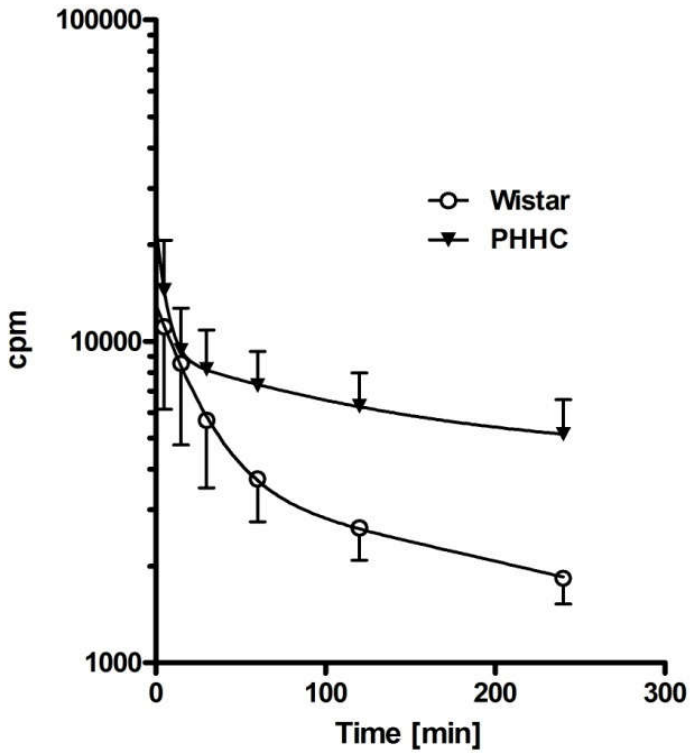

Figure 11: Clearence of ^{125}I-VLDL from serum of PHHC and Wistar rats injected into Wistar rats. VLDL were isolated by ultracentrifugation from sera of PHHC rats fed CHOL diet and Wistar rats fed C diet, radiolabelled [214] and injected into Wistar rats. The blood samples for determination of radioactivity were taken from tail vein. Data are presented as mean ± SD.

4.2.4 ACAT and MTP activities and gene expression in the liver

To determine whether the rate of VLDL assembly in the liver is affected by dietary cholesterol, the activities of two crucial proteins ACAT and MTP were measured in another experiment. The CHOL diet did not influence ACAT and MTP activities in both strains (Table 3).

Table 3: ACAT and MTP activities in the liver and FC, CE and TG concentration in the liver, plasma, and VLDL in PHHC and Wistar rats.

	Wistar		PHHC	
n	**CF 6**	**CHOL 6**	**CF 6**	**CHOL 6**
Liver				
ACAT	1.18	1.23	1.46	1.34
[nmol/mg/min]	(0.27)	(0.21)	(0.10)	(0.14)
MTP	607	604	421	554
[pmol/mg/h]	(130)	(225)	(91)	(105)
FC	7.4	7.0	6.9	6.3
[µmol/g]	(1.0)	(0.6)	(1.1)	(1.0)
CE	0.7	2.2 #	0.6	2.6 ##
[µmol/g]	(0.9)	(0.9)	(0.9)	(1.0)
Serum				
FC	0.19	0.24	0.32	0.66 ##,**
[mmol/l]	(0.09)	(0.03)	(0.05)	(0.08)
CE	1.89	2.23	2.67 *	4.28 ##,**
[mmol/l]	(0.24)	(0.36)	(0.19)	(0.73)
TG	1.96	2.59	2.32	2.30
[mmol/l]	(0.67)	(0.81)	(0.21)	(0.27)
VLDL				
FC	0.15	0.23	0.22	0.51 ##,**
[mmol/l]	(0.05)	(0.03)	(0.02)	(0.06)
CE	0.08	0.44 ##	0.13	1.53 ##,**
[mmol/l]	(0.01)	(0.08)	(0.03)	(0.17)
TG	1.55	2.08	1.75	1.90
[mmol/l]	(0.78)	(0.62)	(0.21)	(0.08)

#,## $P < 0.05$, $P < 0.01$ differences between CF and CHOL diet within the strain;
*,** $P < 0.05$, $P < 0.01$ differences between Wistar and PHHC rats on the same diet.
Data are presented as mean (SD).

CHOL diet did not influence gene expression of ACAT (*Soat1* and *Soat2*) and MTP (*Mttp*) in both strains in the same animals (Figure 12). When the expression between PHHC and Wistar rats was compared, just *Soat1* expression on CHOL diet was found to be modestly downregulated ($P < 0.05$).

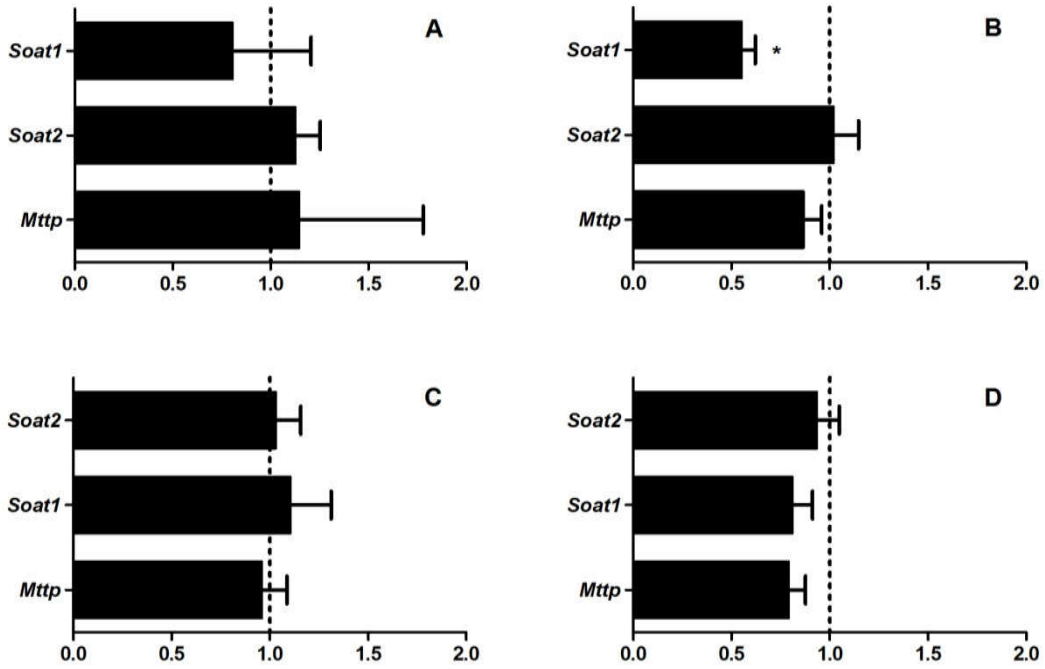

Figure 12: ACAT (*Soat1* and *Soat2*) and MTP (*Mttp*) gene expression.
Comparison of gene expression between PHHC and Wistar rats on CF (Panel A) and CHOL (Panel B) diet; influence of CHOL diet on gene expression in Wistar (Panel C) and PHHC (Panel D) rats. Gene expression of Wistar rats or control diet is set to 1.0 (dash line). * $P < 0.05$ differences between Wistar and PHHC rats on the same diet. Data are presented as mean ± SD.

4.2.5 Gene expression in the liver

Feeding both strains of the CHOL diet induced at least a twofold downregulation ($P < 0.05$) of eight genes involved in the cholesterol biosynthesis pathway and three other genes involved in the lipid metabolism (*Insig*, *Pcsk9*, and *Fads*) (Figure 13). The expression of *Ldlr*, *Hmgcr* and *Cyp7a1* was also changed but did not fulfill the strict criteria used in the study. In either strain, no gene was upregulated on the CHOL diet, and, importantly, there were no significant differences between the response of both strains to the CHOL diet. Feeding both PHHC and Wistar rats a CF diet had no effect on the expression of any of the approximately 6500 genes expressed in the liver in comparison to the C diet.

When the hepatic transcriptome of both strains was compared on the same diet using the same criteria (twofold change, $P < 0.05$), several genes were found to be expressed differently (Figure 14). Five genes were downregulated and four genes upregulated in the PHHC rats. The vast majority of those genes, except for *Apof,* has no known connection to the lipoprotein metabolism.

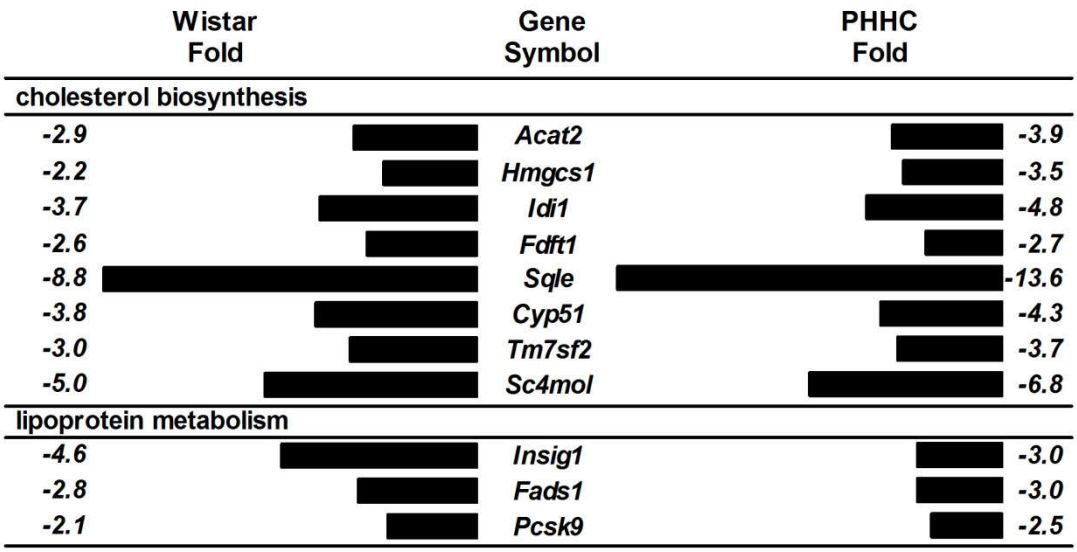

Figure 13: Hepatic gene expression response in PHHC and Wistar rats to dietary cholesterol.
The genes that were at least twofold downregulated on CHOL diet compared to both control (C, CF) diets ($P < 0.05$) are shown. Data are presented as mean of gene expression on CHOL diet compared to mean of gene expression of both control diets; expression of genes on control diets is set to 1.0.
Acat2, acetyl-CoA acetyltransferase 2, NM_001006995, *Hmgcs1*, 3-hydroxy-3-methylglutaryl-CoA synthase 1 (soluble), NM_017268, *Idi1,* Isopentenyl-diphosphate delta isomerase, NM_053539, *Fdft1*, Farnesyl diphosphate farnesyl transferase 1, NM_019238, *Sqle*, Squalene monooxygenase, NM_017136, *Cyp51*, Cytochrome P450, subfamily 51, NM_012941, *Tm7sf2*, Transmembrane 7 superfamily member 2, NM_001013071, *Sc4mol* sterol-C4-methyl oxidase-like NM_080886.1, *Insig1*, Insulin induced gene 1, NM_022392, *Fads1*, Fatty acid desaturase 1, NM_053445, *Pcsk9*, Proprotein convertase subtilisin/kexin type 9, NM_199253. All IDs are from NCBI nucleotide database.

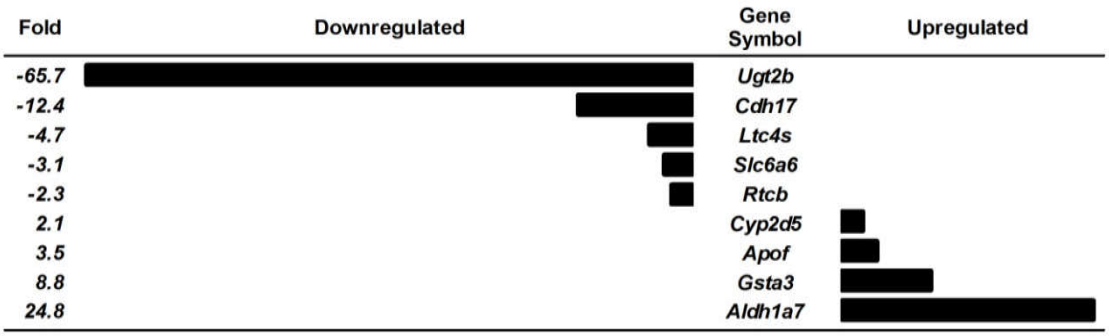

Figure 14: Comparison of hepatic gene expression between PHHC and Wistar rats.
The genes that were at least twofold down- or upregulated ($P< 0.05$) in PHHC rats compared to Wistar rats irrespective of the diet are shown. Data are presented as mean of gene expression on all three diets. Gene expression of Wistar rats is set to 1.0.
Ugt2b, UDP glycosyltransferase 2 family, polypeptide B, NM_031533, *Cdh17*, Cadherin 17, NM_053977, *Ltc4s*, Leukotriene C4 synthase, NM_053639, *Slc6a6*, Solute carrier family 6 (neurotransmitter transporter, taurine), member 6, NM_017206, *Rtcb,*RNA 2',3'-cyclic phosphate and 5'-OH ligase, NM_207614, *Cyp2d5*, Cytochrome P450, family 2, subfamily d, polypeptide 5, NM_173304, *Apof*, Apolipoprotein F, NM_001024351, *Gsta3,*Glutathione S-transferase alpha 3, NM_001009920, *Aldh1a7*, Aldehyde dehydrogenase family 1, subfamily A7, NM_017272. All IDs are from NCBI nucleotide database.

The results of microarray gene expression were validated for 14 genes (Table 4, Table 5), the validation was carried out on RNA samples from all the animals in the study. The results of qPCR did not differ from that of microarray gene expression profiling except for three of the most prominently up- or downregulated genes (*Ugt2b*, *Aldh1a7*, *Ltc4s*). The differences between both strains in the expression of these genes were found to be even more pronounced when qPCR was used for quantification.

Table 4: Validation of microarray gene expression data using qPCR.
Part I – Effect of the diet.

Gene Symbol	CHOL x CF				CHOL x C				CF x C	
	Microarray		qPCR		Microarray		qPCR		Microarray	qPCR
PHHC										
cholesterol biosynthesis										
Acat2	0.251	***	0.265	***	0.269	***	0.261	***	1.072	0.985
Hmgcr	0.482	**	0.364	***	0.517	**	0.383	***	1.073	1.054
Sqle	0.069	***	0.038	***	0.079	***	0.049	***	1.140	1.274
Ebp	0.696	**	0.530	**	0.664	***	0.517	**	0.953	0.976
lipoprotein metabolism										
Cyp7a1	3.700		3.083		4.237	*	2.901		1.145	0.941
Insig1	0.341	**	0.293	***	0.323	**	0.364	**	0.946	1.242
Ldlr	0.689	*	0.614		0.686	*	0.422	***	0.997	0.687
Wistar										
cholesterol biosynthesis										
Acat2	0.353	***	0.298	***	0.339	***	0.291	***	0.961	0.976
Hmgcr	0.654		0.409	***	0.639		0.444	***	0.978	1.086
Sqle	0.113	***	0.059	***	0.113	***	0.074	***	1.003	1.239
Ebp	0.721	**	0.632	*	0.652	***	0.618	*	1.000	0.978
lipoprotein metabolism										
Cyp7a1	2.163		2.124		2.275		2.505		1.052	1.180
Insig1	0.238	**	0.171	***	0.200	**	0.179	***	0.842	1.047
Ldlr	0.640	*	0.500	**	0.652	*	0.516	**	1.019	1.033

Data are presented as mean fold of gene expression of group of interest in comparison to control group; expression of genes on control diets is set to 1.0.
* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.
Acat2, acetyl-CoA acetyltransferase 2, NM_001006995, *Hmgcr*, 3-hydroxy-3-methylglutaryl-CoA reductase, NM_013134, *Sqle*, Squalene monooxygenase, NM_017136, *Ebp*, Emopamil binding protein (sterol isomerase), NM_057137, *Cyp7a1*, Cytochrome P450, family 7, subfamily a, polypeptide 1, NM_012942, *Insig1*, Insulin induced gene 1, NM_022392, *Ldlr*, Low density lipoprotein receptor, NM_175762. All IDs are from NCBI Nucleotide database.

Table 5: Validation of microarray gene expression data using qPCR.
Part II – Effect of the strain.

PHHC x Wistar												
Gene Symbol	**CHOL**				**CF**				**C**			
	Microarray		**qPCR**		**Microarray**		**qPCR**		**Microarray**		**qPCR**	
Ugt2b	$1.49x10^{-2}$	***	$4.79x10^{-4}$	***	$1.78x10^{-2}$	***	$9.27x10^{-4}$	***	$1.36x10^{-2}$	***	$5.19x10^{-4}$	***
Cdh17	$9.69x10^{-2}$	**	$6.48x10^{-2}$	***	$1.09x10^{-1}$	**	$8.59x10^{-2}$	***	$5.62x10^{-2}$	***	$5.81x10^{-2}$	***
Ltc4s	$1.68x10^{-1}$	***	$1.79x10^{-2}$	*	$2.53x10^{-1}$	***	$2.18x10^{-2}$	**	$2.30x10^{-1}$	***	$9.81x10^{-2}$	***
Slc6a6	$3.90x10^{-1}$	**	$1.85x10^{-1}$	*	$3.28x10^{-1}$	***	$1.67x10^{-1}$	*	$2.73x10^{-1}$	***	$3.23x10^{-1}$	*
Apof	3.40	***	5.92	*	3.20	***	5.85	**	3.81	***	7.62	*
Gsta3	11.2	***	10.2	***	6.94	***	9.80	***	8.22	***	9.76	***
Aldh1a7	19.1	***	129	**	30.6	***	208	*	24.8	***	280	***

Data are presented as mean fold of gene expression of group of interest in comparison to control group; gene expression of Wistar rats is set to 1.0.
* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.
Ugt2b, UDP glycosyltransferase 2 family, polypeptide B, NM_031533, *Cdh17*, Cadherin 17, NM_053977, *Ltc4s*, Leukotriene C4 synthase, NM_053639, *Slc6a6*, Solute carrier family 6 (neurotransmitter transporter, taurine), member 6, NM_017206, *Apof*, Apolipoprotein F, NM_001024351, , *Gsta3,*Glutathione S-transferase alpha 3, NM_001009920, *Aldh1a7*, Aldehyde dehydrogenase family 1, subfamily A7, NM_017272. All IDs are from NCBI Nucleotide database.

4.2.6 The *Apoe* and *Apof* sequencing

To exclude the possibility that hypercholesterolemia pathogenesis of PHHC rats is similar to the pathogenesis of human dysbetalipoproteinemia, the *Apoe* was sequenced in both strains and the homozygous substitution of C, instead of G, was found in position -42 (from the start of translation) in the first exon of the gene only in the PHHC rats, not in the Wistar rats (Figure 15).

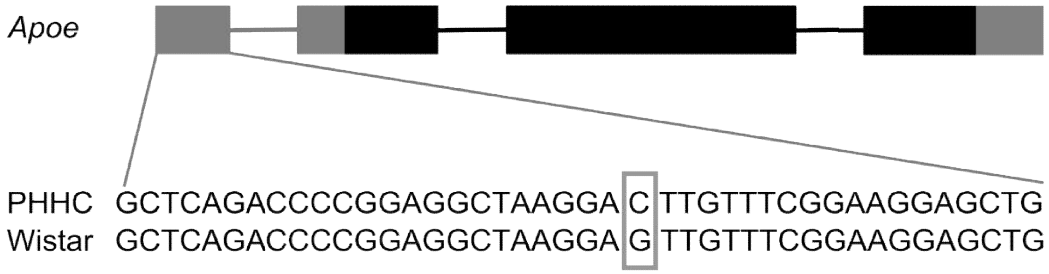

Figure 15: Sequence of first exon of *Apoe* with homozygous substitution of C instead G in PHHC rats.
Untranslated region (gray), coding region (black).

Furthermore, no substantial differences in apoE content in VLDL (evaluated as a ratio of apoE and apoB) were observed between PHHC and Wistar rats (Figure 16).

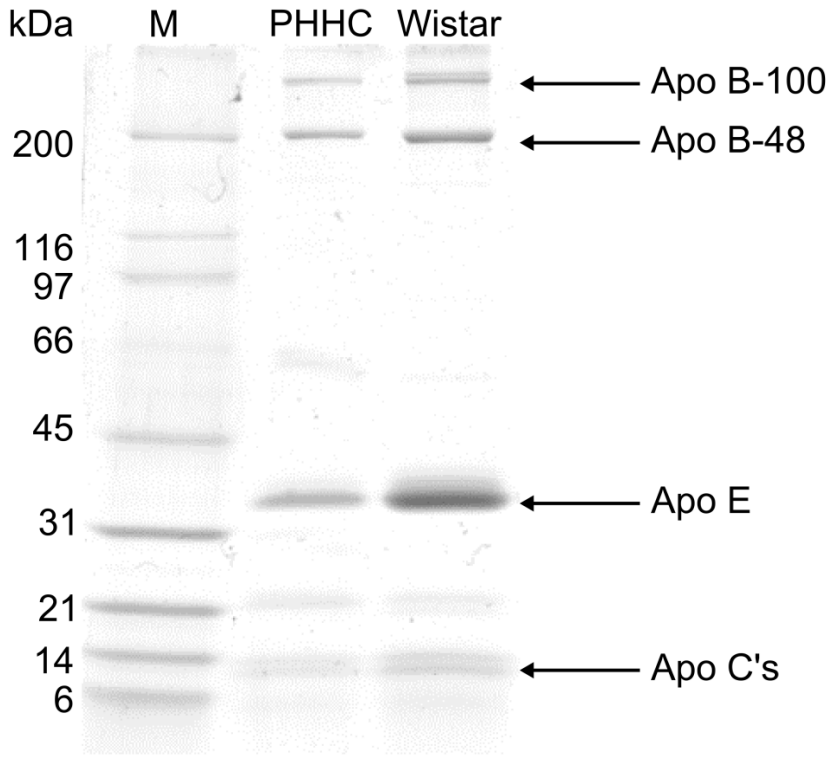

Figure 16: VLDL apolipoproteins in PHHC and Wistar rats.
VLDL were separated by ultracentrifugation [212] and apolipoproteins separated on SDS-PAGE [219].

Apof, the only gene differentially expressed in PHHC rats with a presumed role in lipoprotein metabolism, was sequenced and 17nt insertion in coding exon was found (Figure 17). The same variation of *Apof* was found in Wistar rats with 0.125 allele frequency (n = 24).

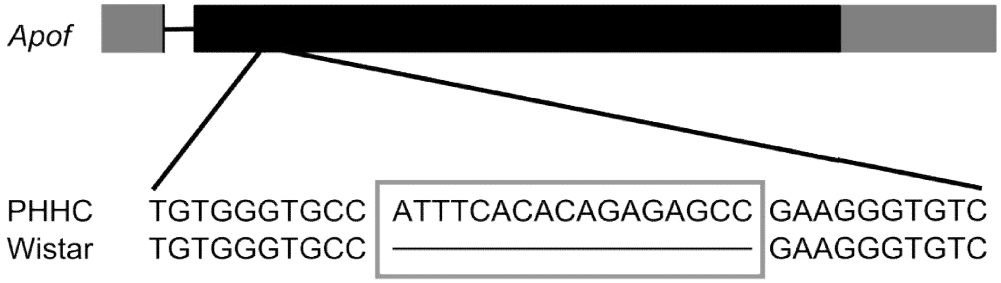

Figure 17: Sequence of a part of second exon of *Apof* with 17nt insertion in PHHC rats.
The sequence starts at nucleotide 83 (from translation start site). Untranslated region (gray), coding region (black).

4.2.7 Aldh1a7 activity and gene sequencing

Aldh1a7 specific activity was measured in both strains on C and CHOL diet to compare if the expression of *Aldh1a7* (the most upregulated gene in PHHC rats independently of the diet) correlates with its activity. Whereas the Aldh1a7 activity on the C diet did not differ between both strains, the enzyme activity of PHHC rats was increased 56 % ($P < 0.001$) then that of Wistar rats on the CHOL diet (Figure 18).

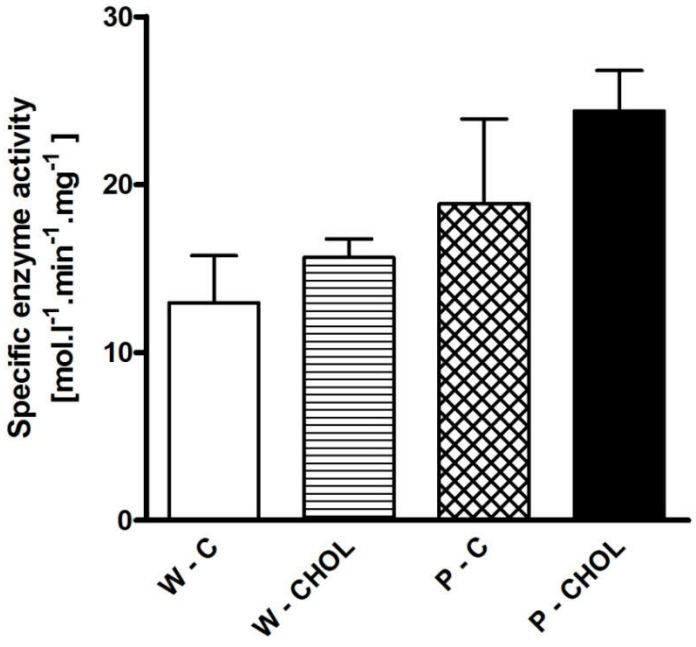

Figure 18: Specific activity of Aldh1a7 in PHHC (P) and Wistar (W) rats on C and CHOL diet.
Data are presented as mean ± SD.

To verify whether *Aldh1a7* does not contain any mutations in PHHC rat, *Aldh1a7* was also sequenced in both strains. Only already known polymorphisms were found in both strains. Because of the mRNA different splicing possibility, cDNA were also sequenced, however, the cDNA sequences of PHHC and Wistar rats did not differ.

4.3 Discussion

PHHC rat was obtained through selective inbreeding of Wistar rats hyperresponsive to cholesterol diet in the late 1970's. Even today the pathogenesis of its hypercholesterolemia has remained elusive. Our results brought the first evidence that this dietary cholesterol-induced hypercholesterolemia in PHHC rat is due to the production of VLDL abnormally enriched with cholesterol. These VLDL are apparently catabolised more slowly than normal VLDL and mount up in circulation. However, cholesterol feeding does not influence the activities of two key proteins (ACAT and MTP) which can affect the cholesterol incorporation into VLDL. Moreover, no difference between PHHC rats and control Wistar rats was observed in response of the hepatic transcriptome to dietary cholesterol. However, several genes were significantly up- or downregulated in PHHC rats irrespective of diet which is suggestive of the fact that the response to dietary cholesterol takes place in a different genetic background.

4.3.1 Response of lipid metabolism to dietary cholesterol

The PHHC rat represents a unique rat model because the hypercholesterolemia is induced only by dietary cholesterol without the need for addition of CA or thyreotoxic drugs that are usually used in the other strains to induce hypercholesterolemia [201-203, 226]. While there is no difference in the lipoprotein profile between PHHC and control Wistar rats on C diet or on CF diet, on the CHOL diet there is a much more pronounced increase in cholesterolemia in PHHC rats than in Wistar rats (Table 2). In PHHC rats, cholesterol accumulates predominantly in the VLDL fraction and these VLDL carry more cholesterol than TG. Moreover, there is also an increase in IDL-C and LDL-C whereas HDL-cholesterol concentration is not affected (Figure 8).

Such cholesterol-enriched VLDL originate in the liver as documented in our experiment using intravenously injected tyloxapol. Tyloxapol inhibits LPL which is responsible for hydrolysis of VLDL in circulation and its application results in an accumulation of VLDL in circulation. Two hours after tyloxapol application there was a comparable increase in triglyceridemia in both PHHC and Wistar rats on all the diets

(Figure 9) and it can be estimated from these data that more than 80 % of TG in VLDL are associated with particles that can be regarded as nascent VLDL. Importantly, tyloxapol injection also affects cholesterolemia. A small increment in cholesterolemia can be observed in all groups of rats fed C or CF diet and such an increment can again be ascribed to a rise in VLDL cholesterol. On a CHOL diet, cholesterolemia goes up ~2 mmol/l in Wistar rats and ~4.5 mmol/l in PHHC rats due to an increase in VLDL-C (Figure 9). Therefore, when fed cholesterol, both rat strains tend to increase the secretion of cholesterol from the liver without affecting the rate of TG secretion. Using a model of perfused liver, Fungwe et al. [227] have shown that VLDL in rats fed cholesterol are relatively enriched with cholesterol due to an increased secretion of CE. However, it is not entirely clear from our data why the cholesterol incorporation into VLDL is more than doubled in PHHC rats than in Wistar rats when fed the same amount of cholesterol and at the same cholesterol concentration in the liver.

It can be hypothesised that these cholesterol-rich VLDLproduced by PHHC rats cannot be transformed by LPL to smaller VLDL remnants as normal VLDL and, therefore, they are catabolised more slowly, accumulate in circulation and cause hypercholesterolemia. To test such a hypothesis, we isolated VLDL from PHHC rats fed cholesterol and Wistar rats, labelled them with ^{125}I and injected these VLDL into Wistar rats to estimate the rate of their elimination from circulation (Figure 11). When injected into Wistar rats, cholesterol-rich VLDL from PHHC rats were cleared more slowly than autologous VLDL. It is likely that lipoproteins originated from nascent cholesterol-rich VLDL of PHHC rats after the TG hydrolysis by LPL remain too large due to high cholesterol content and do not escape from the VLDL size range, they just become cholesterol transporting lipoproteins and cannot be removed from circulation similarly to the remnants of normal VLDL.

It can be assumed that the increased incorporation of CE into VLDL can be due to increased activity of ACAT but the enzyme activity was not affected by dietary cholesterol and also did not differ between strains (Table 3). In addition, the expression of both genes encoding ACAT (*Soat1* and *Soat2*) was also not increased after cholesterol feeding (Figure 12). These findings thus do not explain the increased incorporation of CE into VLDL in PHHC rats.

Therefore, PHHC rats respond to dietary cholesterol by the production of VLDL extremely enriched with cholesterol. Such VLDL cannot be catabolised normally and accumulate in circulation. Interestingly, the lipoprotein profile characteristic for PHHC

rats on a CHOL diet can also be observed in other rat models that use dietary CA and/or propylthiouracil to induce hypercholesterolemia [201-203]. A similar model of hypercholesterolemic rat was described recently [228]. The exact pathogenesis of hypercholesterolemia in these models have not yet been explained and it remains to be clarified whether the same mechanism as in PHHC rats – production of cholesterol-rich VLDL that cannot be normally catabolised and accumulate – is in operation in these models.

Since the lipoprotein profile of PHHC rats, especially the accumulation of cholesterol-rich VLDL and IDL, approximates that of patients with dysbetalipoproteinemia, we sequenced gene encoding apoE of PHHC rat. Nevertheless, we did not find mutation in the *Apoe* which would affect the primary structure of the protein and no substantial differences in apoE content in VLDL (evaluated as a ratio of apoE and apoB) were observed between PHHC and Wistar rats (Figure 15, Figure 16). Moreover, hepatic lipase activity, deficiency of which also results in dysbetalipoproteinemic phenotype in humans, is normal in PHHC rats [208].

4.3.2 The response of hepatic transcriptome to dietary cholesterol

To understand the genetic background of hypercholesterolemia in PHHC rats, the response of hepatic transcriptome of PHHC rats and control Wistar rats to dietary cholesterol was studied using microaarrays. We chose the liver transcriptome because the liver is the main organ regulating cholesterol concentration and also the excessive export of cholesterol in VLDL from the liver seems to play a critical role in development of hypercholesterolemia in the PHHC rat. Moreover, since cholesterol is not absorbed from the diet if not dissolved in fat during diet preparation, and because such a fat could affect gene expression, we also included a diet with fat alone (CF diet). Further, to minimise the effect of such a fat on gene expression, we used palm kernel oil which is highly saturated and does not contain cholesterol.

Feeding animals a CF diet had no effect on either the lipids in serum or the liver and, more importantly, no effect on hepatic gene expression at all. This allows us to assume that all the observed changes in hepatic transcriptome were due to the effect of dietary cholesterol because the confounding role of dietary fat was minimised.

Surprisingly, when the responses of hepatic transcriptome of PHHC and Wistar rats to dietary cholesterol were compared, no differences were found (Figure 13). In both strains, most of the genes of the cholesterol biosynthetic pathway were downregulated even starting with acetyl-CoA acetyltransferase 2 (*Acat2*). The most

noticeable downregulation was observed for gene encoding SM (*Sqle*) in both strains, whereas HMG-CoA reductase gene (*Hmgcr*) cannot be included in the list of affected genes because it did not fulfil the strict criteria we used. Such an observation is in agreement with findings that HMG-CoA reductase is regulated rather posttranscriptionally in rat [229, 230]. Similarly to *Hmgcr*, the LDLR gene (*Ldlr*) was downregulated only 1.5 fold, also in agreement with other findings [229]**.** However, the gene for LDLR chaperon, *Pcsk9,* that modulates the number of LDLR on the cell surface and is under the same transcriptional control, was found to be markedly downregulated as described in mice [231]. *Insig1*, a gene encoding an essential component of cholesterol feedback response, was also found to be downregulated in mice fed cholesterol [232, 233]. We did not confirm the previous findings [209] of increased *Cyp7a1* expression in Wistar rats in response to dietary cholesterol. However, we used only 1% cholesterol and palm kernel oil, not 2% cholesterol and lard in the diet and it cannot be excluded that the response of *Cyp7a1* expression is affected by the quantity of dietary cholesterol and the type of fat in the diet. It can be hypothesised that rat in the response to increase in cell cholesterol content firstly downregulates synthesis, and if this is insufficient, then upregulates cholesterol degradation to BA. Contradictorily BA could contribute to more effective cholesterol absorption in the intestine and their high concentrations are potentially toxic [234]. With respect to the above discussed findings, no differences in the expression of genes which could affect the incorporation of CE into VLDL during VLDL assembly (such as *Soat1*, *Soat2*, and *Mttp*) were observed.

Altogether, this data cannot explain why PHHC rats develop hypercholesterolemia whereas Wistar rats do not. Such a paradox might be explained by the fact that the same response of hepatic transcriptome to dietary cholesterol takes place against a different genetic background. Indeed, using the same criteria (2fold change, $P < 0.05$), we identified several genes that do not respond to dietary cholesterol but differ between both strains independently of the diet used. Five genes were downregulated and four upregulated in PHHC rats (Figure 14). The vast majority of those genes have no known connection with lipoprotein metabolism except *Apof*. A human ortholog of *Apof* encoded protein, apoF, is known as an inhibitor of CETP in serum [235]. However, mice and rats do not have CETP [236] and the role of the protein in these animals remains elusive. Because the upregulation may be due to the dysfunction of protein encoded by a gene, we sequenced *Apof* in both Wistar and PHHC rats and found that

PHHC rats carry a homozygous 17 nucleotide insertion in exon 2 (Figure 17). This variant also occurred in Wistar rats with allelic frequency 0.125. The role of rat apoF in lipoprotein metabolism remains to be clarified – the human ortholog inhibits the exchange of CE and TG between lipoproteins mediated by CETP [237, 238] and it may be speculated that it can play such a role even intracellularly and reduce the CE incorporation into VLDL in hepatocyte. Nevertheless, such a hypothesis remains to be tested.

To verify if the upregulation of *Aldh1a7* in PHHC correlates with a higher enzyme activity in this strain, the enzyme activity was measured. Enzyme activity increased about 50 % in PHHC rats than that in Wistar rats although the difference was statistically significant only on CHOL diet (Figure 18). These results are in contrast with the transcriptome analysis when only 25fold upregulation of *Aldh1a7* was found in PHHC rats in comparison to Wistar rats. But at the same time, when the transcriptome data were validated by qPCR, the *Aldh1a7* expression was found to be even higher (200x) in PHHC rats. Currently we do not have explanation for this contrast between activity and expression data. Gene sequencing did reveal no differences (except known polymorphisms) but the mutation may also occur in the regulation region of the gene. The physiological function of Aldh1a7 has been not known to date, and, in addition, a human ortholog does not exist. Thus it is disputable whether the clarification of role of this enzyme could explain the hypercholesterolemia pathogenesis in humans.

The other proteins encoded by some of the other affected genes may play a role in metabolic response to xenobiotics or oxidative stress (UDP glycosyltransferase 2, polypeptide B (*Ugt2b*), cytochrome P450 CYP2D5 (*Cyp2d5*), and glutathion transferase (*Gsta3*). However, their exact role in cholesterol metabolism remains enigmatic.

Importantly, these newly identified genes and their human orthologs or metabolic pathways they are involved in may become new candidate genes for human polygenic hypercholesterolemia which is the most common type of hyper-cholesterolemia among patients and its genetic background is not yet fully understood [8]. It should be stressed that models like the PHHC rat can be very useful to identify new candidate genes that could be involved in pathogenesis of hypercholesterolemia. Most animal models (knock-out or transgene animals) currently in use allow us to test only the role of genes that were already identified as the candidate genes.

Nowadays, this animal model could be useful also for study of nonalcoholic hepatic steatosis pathogenesis that is very often present in patients with insulin

resistance and type 2 diabetes [239]. This steatosis is relatively easy inducible in rats by dietary cholesterol. PHHC rat represents a perspective model for this type of studies because contrary to other rat models cholesterol can be given to rats without addition of nonphysiologic amount of BA in the diet [206, 240, 241].

In conclusion, our data suggest that dietary cholesterol induced hypercholesterolemia in the PHHC rat is due to the production of VLDL very rich in cholesterol. These VLDL are not catabolised normally and accumulate in circulation. The increased production of cholesterol-rich VLDL can be explained neither by changes in ACAT and MTP activities nor by changes in the hepatic transcriptome response to dietary cholesterol. However, several genes are significantly up- or downregulated in PHHC rats irrespective of the diet are suggestive of the fact that the response to dietary cholesterol takes place in a different genetic background.

5. The role of the -203A>C polymorphism of *CYP7A1* in cholesterolemia regulation (Aim II)

5.1 Material and methods

5.1.1 Activity of promoter variants *in vitro*

5.1.1.1 Plasmid preparation

Both common variants of *CYP7A1* promoter [-469C, -203A] and [-469T, -203C] were amplified by PCR from gDNA samples obtained in clinical part of the study. Amplified fragments with promoter sequence from -772 to +95 (from transcription start) were cloned into vector pDCV (Qiagen). Plasmids pDCV_AC and pDCV_CT served as templates for the following PCR amplification when primers with *Xho*I adapters were used for production of promoter fragments from -764 to +14. After *Xho*I cleavage the new fragments were orientedly cloned into restriction site of pGL3_basic vector (Promega) in front of gene coding luciferase (*Photinus pyralis*) and these constructs were then cloned in *Escherichia coli*. Clones with sense (pGL3_AC+ and pGL3_CT+) and antisense (pGL3_AC- and pGL3_CT-) orientation were isolated. Additionally, plasmids [-469T, -203A] and [-469C, -203C] (pGL3_AT+ and pGL3_CC+) were prepared by directed mutagenesis (Stratagene kit).

5.1.1.2 Promoter activity testing

Promoter activity was determined using luciferase reporter assay (Dual-Luciferase® Reporter Assay System, Promega). 400 µl of hepatic cell cultures (HepG2 and HuH7) grown in 12-well plate were transfected at ~70 % confluency with 500 ng of tested plasmid DNA using Tfx™-20 (ratio Tfx™-20 : DNA = 2 : 1), according to the manufacturer's instruction (Promega). Fifty ng of normalisation vector pRL-TK (Promega) was used. As a negative control served vector pGL3_basic. Two days after transfection, the cells were processed according manufacturer's protocol. Luciferase activity was assayed in Sirius Luminometer (Berthold). Transfection assays were performed in triplicate.

5.1.1.3 Stimulation

In addition to determination of basal promoter activity, its stimulation by insulin, and PPARα antagonists was studied (both Sigma-Aldrich). Tested substances were added to cultivation medium.

Insulin (1μmol/l or 10 nmol/l) was added to cells for 16 or 6 h. PPARα agonists (WY-14643 or fenofibrate) were first disolved in dimethylsulfoxide and then diluted to final concentration 100 - 300 μmol/l. They were incubated with cells for 24 h.

5.1.1.4 Statistics

Data were analysed by ANOVA and, if ANOVA revealed any differences, then corresponding post-hoc tests were performed (GraphPad InStat).

5.1.2 *In silico* analysis

Putative binding sites for transcription factors were searched using Transcription Element Search System [242]. The neighbourhood (± 20 bp) of both SNP variants (rs1023652, rs1023649, rs1125226, rs3903445, rs7833904, rs3824260, rs3808607) was analysed.

5.1.3 Diurnal variation of CYP7A1 activity in healthy subjects

5.1.3.1 Subject and study design

Sixteen male volunteers were included into the study: 8 homozygotes for -203A allele (age: 25.7 ± 3.4 years, BMI: 23.4 ± 3.5 kg/m^2) and 8 homozygotes for -203C allele (age: 25.3 ± 3.8 years, BMI: 27.2 ± 3.3 kg/m^2). Three day-long examinations were carried out in all the subjects. One of these examinations served as a control with no drug) while the other two examinations studied the effect of short-term administration of cholestyramine (Questran®, Bristol-Myers Squibb, 16 g/day) and CDCA (Chenofalk®, Dr. Falk Pharma, 1-1.5 g/day dependent on the subject weight).

One day before each of these examinations, the first blood sample was drawn at 7:00 (-24 h) and subjects received food for the whole day to standardise their intake before the study. On the day of examination, the first blood sample was drawn again at 7:00 (0 h) and the blood samples were then collected in 90-min intervals for 15 h till 22:00. Again, subjects received food for the whole day and they had to eat at exactly defined time points (breakfast 7:15, snack 9:45, lunch 12:30, snack 15:30 and dinner

17:30). The amount of food was calculated to cover their energy requirements; the diet was relatively low in fat (25 % of energy intake).

If the examination included the drug administration, the drugs were given to subjects on the day before the examination and also on the day of the examination. Questran® was given to subjects in two doses on both days: one with breakfast, the other one with dinner. Due to differences in pharmacokinetics, Chenofalk® treatment was started with dinner on the day preceeding examination, and on the day of examination, it was given to subjects in two doses at the same time as cholestyramine. The order of the examinations was randomised and they were carried out in three-week intervals at a minimum.

The study protocol was approved by the Ethical Committee of the Institute for Clinical and Experimental Medicine and all the participants gave their informed consents.

5.1.3.2 Biochemistry

Concentration of C4 was determined by HPLC as described earlier [243] with a modification of C4 extraction procedure [244]. Cholesterol, HDL-cholesterol (after precipitation of the other lipoproteins), and TG were measured using enzymatic kits (Roche Diagnostics), glucose using kits from PLIVA-Lachema Diagnostika, free fatty acids using kits from Wako Chemicals, and BA using enzymatic kits from Trinity Biotech. Insulin was determined using IRMA kits (Immunotech).

5.1.3.3 Statistics

The effect of the *CYP7A1* genotype on changes to all parameters was evaluated using ANOVA for repeated measures with one grouping factor (genotype). Data for analysis were logarithmically transformed where necessary. ANOVA for repeated measures or its non-parametric variant (the Friedman test) were then used for analysis of the pooled data from all subjects. Corresponding post-hoc tests were carried out if significant differences were detected using ANOVA or the Friedman test (GraphPad InStat). The dynamics of C4 concentration changes was then modelled using the polynomial regression of 5^{th} order using JMP 10 statistical software.

5.2 Results

5.2.1 Comparison of promoter variant expression

To determine whether two polymorphisms of *CYP7A1* that are in tight linkage disequilibrium (-203A>C and -469C>T) play a role in the regulation of *CYP7A1* transcription, fragments of these promoter variants spanning positions -772 to +95 were cloned into plasmids encoding luciferase gene and their promoter activity was then studied under different conditions.

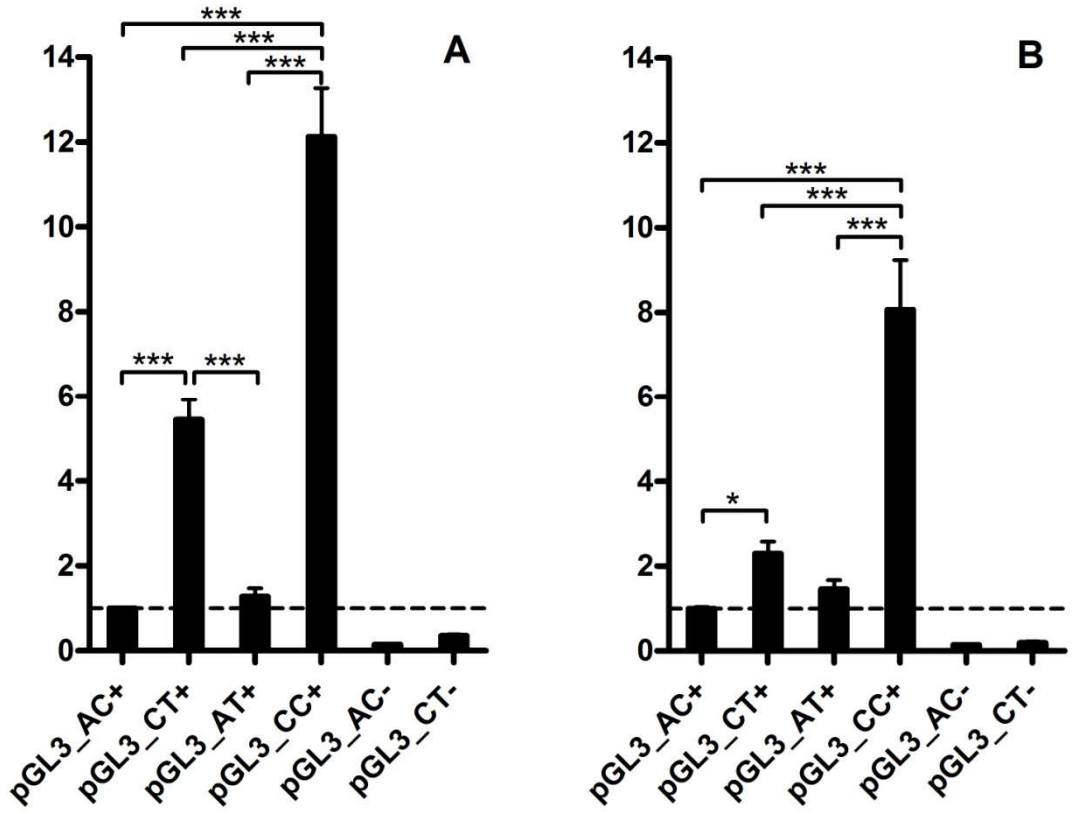

Figure 19: Comparison of *CYP7A1* promoter activity in HepG2 (Panel A) and HuH7 (Panel B) cells. Activity of promoter variants AC+ is set to one (dash line). Data are presented as mean ± SEM (n = 17 – 33). * $P < 0.05$, *** $P < 0.001$ using ANOVA with the Bonferroni post-hoc test (the antisense variants were not analysed with post-hoc test).

Haplotypes -203C were expressed approximately 5fold and 3fold more in HepG2 and HuH7 cells, respectively, than haplotypes -203A ($P < 0.001$; Figure 19). The luciferase activity of antisense variants (pGL3_AC- and pGL3_CT-) was similar to the activity of pGL3_basic vector.

Insulin stimulation was studied because insulin influences *CYP7A1* promoter activity [150] and because the different response of both common variants to insulin stimulation could explain the differences in enzyme activity during a day. Sixteen hour incubation with insulin did not affect the activity of both common variants (Figure 20). The similar results were obtained when the cells were incubated with insulin only 6 hours (data not shown).

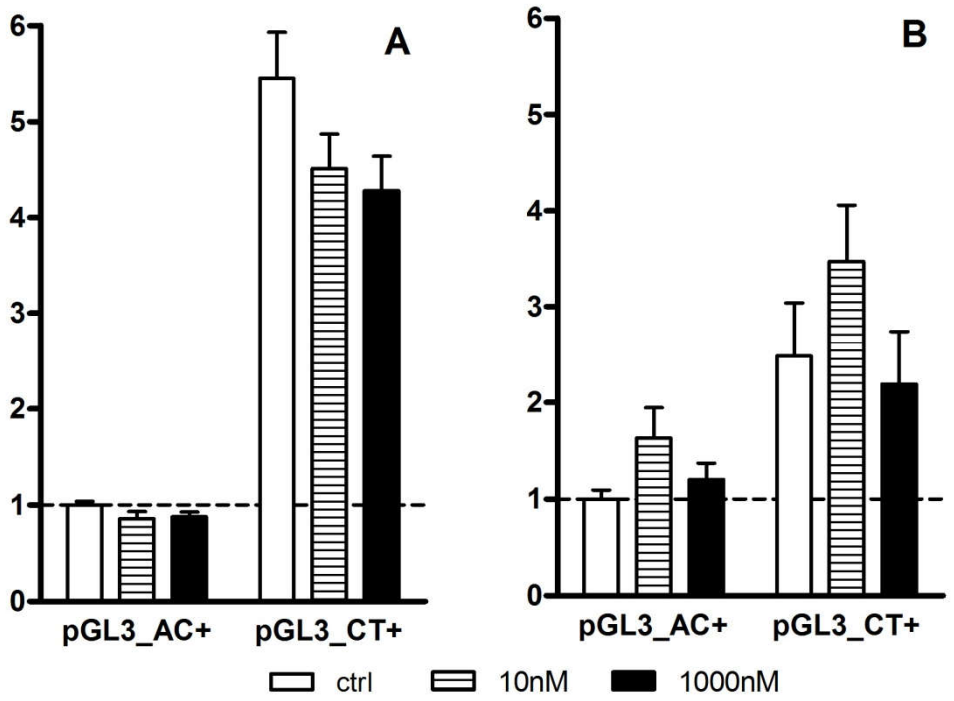

Figure 20: Effect of insulin on *CYP7A1* promoter activity.
Insulin stimulation in HepG2 (Panel A) and HuH7 (Panel B) cells after 16 hour incubation. Activity of promoter variants AC+ (without insulin stimulation) is set to one (dash line). Data are presented as mean ± SEM (n = 6 – 9). No statistically significant differences were found between control and stimulated cells.

The more pronounced decrease of cholesterolemia in C allele carriers could be due to a rise in vegetable fat intake (containing higher amount of polyunsaturated fatty acids, natural PPARα agonists) [185], therefore the PPARα agonists influence on promoter variants activity was studied. PPARα agonists affected the luciferase activity of both natural variant by the same way. Incubation with WY-14643 or fenofibrate did not differ from control (Figure 21).

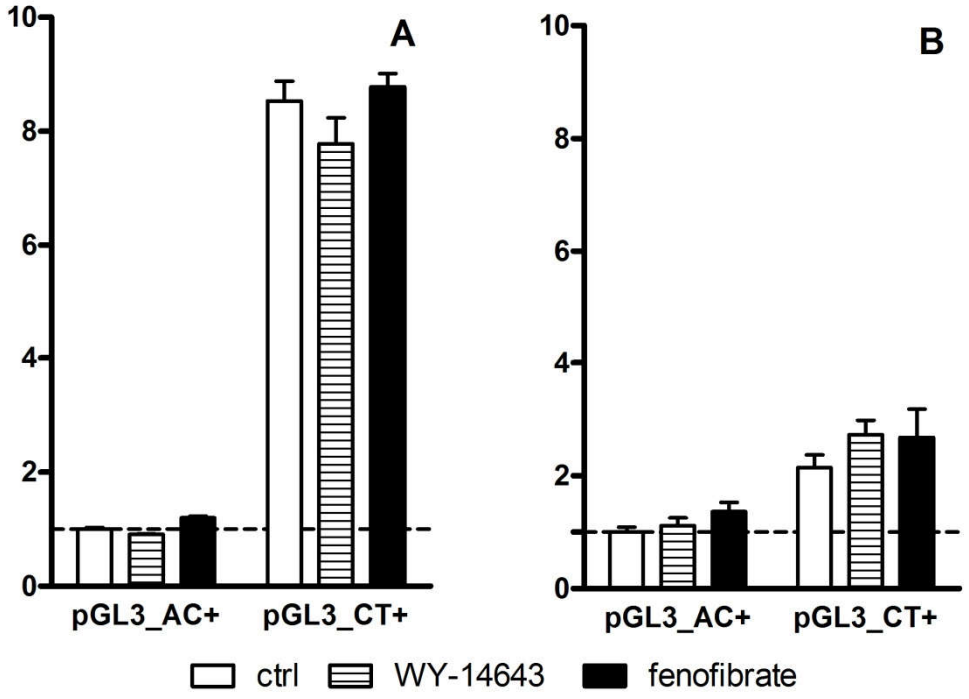

Figure 21: Effect of PPARα agonists on *CYP7A1* promoter activity.
PPARα agonist stimulation in HepG2 (panel A) and HuH7 (panel B) cells after 24 hour incubation. Activity of promoter variants AC+ (without stimulation) is set to one (dash line). Data are presented as mean ± SEM (n = 8 – 9). No statistically significant differences were found between control and stimulated experiments.

5.2.2 *In silico* analysis

To determine whether there are differences in binding of transcription factors to both variants of -203A>C polymorphism and variants of other polymorphisms that are in the tight linkage disequilibrium complex of SNP in promoter region of *CYP7A1* we carried out *in silico* analysis. The transcription binding prediction software (Transcription Element Search System) suggested a putative binding site for a glucocorticoid receptor at -203A variant (AGAA$_{-203}$CT) but not to -203C sequence. No other differences in putative transcription factor binding sites between variants of -203 and other SNP were detected.

5.2.3 -203A>C polymorphism effect on diurnal changes of serum concentrations

To address the question whether *CYP7A1* polymorphism affects diurnal changes in CYP7A1 activity, the changes in concentration of C4 – a serum marker of an enzyme activity – were measured during 15 hour period in healthy volunteers homozygous either for A or C allele. The study was carried out under standard conditions and also after stimulation or inhibition of CYP7A1 activity by cholestyramine or CDCA administration, respectively.

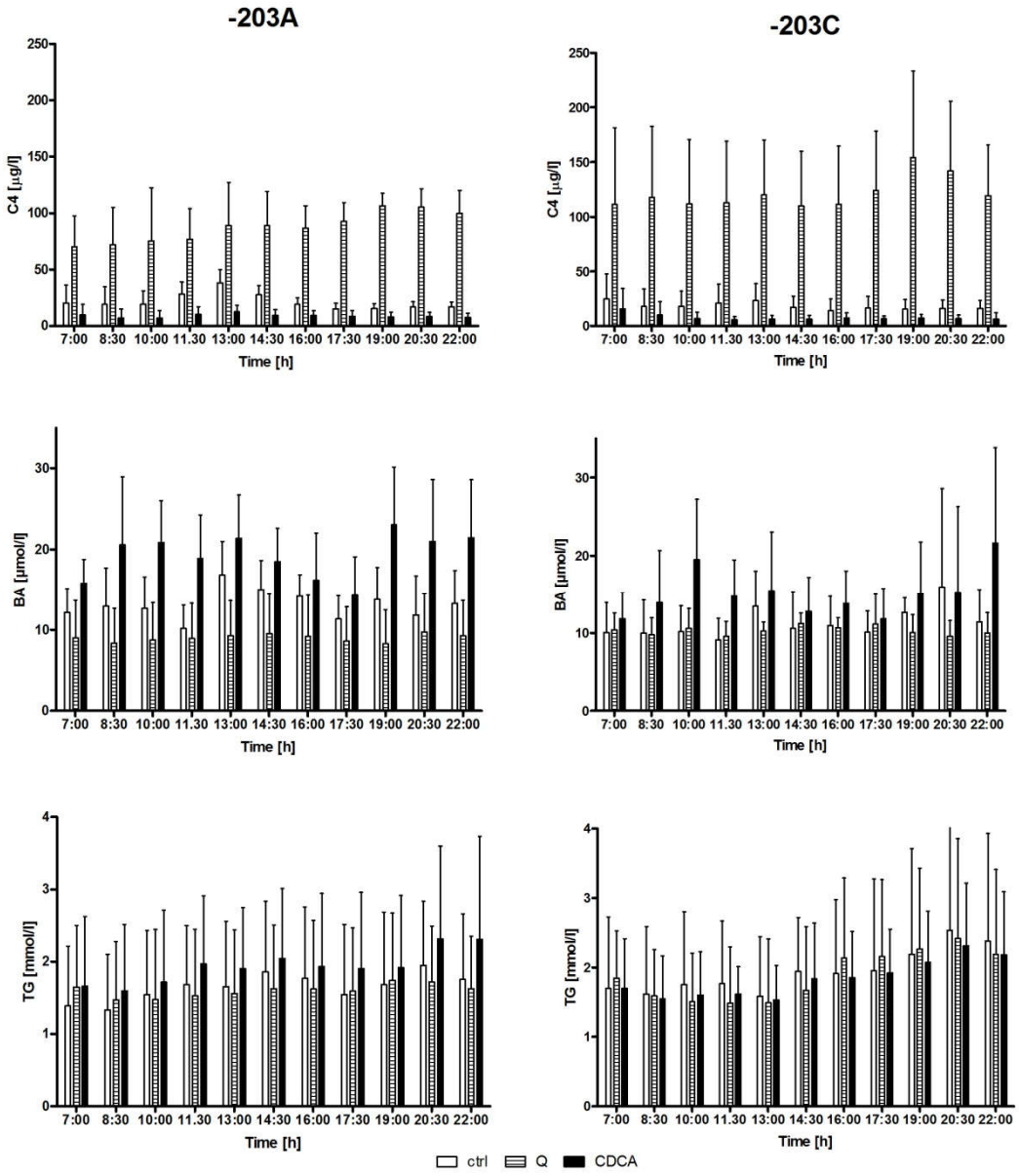

Figure 22: Diurnal variation of concentration of C4, BA, and TG in -203A and -203C homozygotes. ctrl – control experiment, Q – cholestyramine treatment, CDCA – chenodeoxycholic acid treatment. Data are presented as mean ± SD.

Table 6: Concentration of cholesterol, TG, HDL- and LDL-cholesterol, glucose, free fatty acids, insulin, BA, and C4.
Data at 7:00 on the day before the study (-24 h), at 7:00 on the day of the study (0 h) and 15-hour area under the curve of these variables (AUC [0-15 h]) in -203A or -203C.

		-203A			-203C		
		-24 h	**0 h**	**AUC [0-15 h]**	**-24 h**	**0 h**	**AUC [0-15 h]**
Cholesterol [mmol/l]	ctrl	4.69 ± 1.08	4.56 ± 1.10	65.7 ± 15.6	4.70 ± 0.65	4.71 ± 0.62	68.0 ± 9.2
	Q	4.84 ± 0.95	4.89 ± 1.10	67.9 ± 15.0	4.81 ± 0.44	4.44 ± 0.42***	63.2 ± 7.0
	CDCA	4.88 ± 0.95	4.98 ± 1.09	70.2 ± 14.6	4.44 ± 0.75	4.63 ± 0.61	67.7 ± 19.4
TG [mmol/l]	ctrl	1.48 ± 0.84	1.39 ± 0.82	24.9 ± 13.3	1.54 ± 0.65	1.70 ± 1.03	29.0 ± 16.9
	Q	1.71 ± 1.26	1.65 ± 0.85	24.0 ± 12.9	1.33 ± 0.41	1.85 ± 0.68*	28.1 ± 14.3
	CDCA	1.58 ± 0.82	1.66 ± 0.96	28.9 ± 15.0	1.40 ± 0.73	1.70 ± 0.72	27.4 ± 9.2
HDL-cholesterol [mmol/l]	ctrl	1.23 ± 0.31	1.19 ± 0.39		1.32 ± 0.29	1.32 ± 0.25	
	Q	1.28 ± 0.44	1.29 ± 0.32	---	1.42 ± 0.20	1.28 ± 0.21**	---
	CDCA	1.26 ± 0.29	1.23 ± 0.33		1.38 ± 0.24	1.32 ± 0.26	
LDL-cholesterol (equation) [mmol/l]	ctrl	2.78 ± 0.95	2.73 ± 0.96		2.68 ± 0.58	2.62 ± 0.52	
	Q	2.78 ± 0.73	2.85 ± 1.03	---	2.79 ± 0.39	2.32 ± 0.48**	---
	CDCA	2.90 ± 0.87	2.99 ± 0.92		2.43 ± 0.51	2.54 ± 0.35	
Glucose [mmol/l]	ctrl	5.06 ± 0.51	4.85 ± 0.61	76.2 ± 6.6	5.14 ± 0.52	5.08 ± 0.32	80.8 ± 8.5
	Q	5.40 ± 0.69	4.87 ± 0.47*	68.8 ± 6.0	4.94 ± 0.26	4.98 ± 0.49	73.4 ± 5.7
	CDCA	5.43 ± 0.89	4.77 ± 0.48*	72.5 ± 9.4	5.43 ± 1.11	4.81 ± 0.90	77.8 ± 6.6
Free fatty acids [mmol/l]	ctrl	0.25 ± 0.18	0.36 ± 0.20	2.63 ± 0.74	0.32 ± 0.32	0.38 ± 0.19	3.15 ± 1.91
	Q	0.30 ± 0.29	0.56 ± 0.61	3.92 ± 2.52	0.36 ± 0.21	0.35 ± 0.21	2.18 ± 0.79
	CDCA	0.24 ± 0.11	0.46 ± 0.37	2.30 ± 1.68	0.21 ± 0.13	0.37 ± 0.25*	2.51 ± 1.23
Insulin [IU/l]	ctrl	7.2 ± 6.5	4.8 ± 3.2	223 ± 127	7.8 ± 4.4	7.0 ± 3.1	296 ± 119
	Q	8.0 ± 3.8	7.5 ± 4.7	193 ± 120	6.7 ± 1.9	9.2 ± 7.2	200 ± 116
	CDCA	6.4 ± 3.1	8.8 ± 7.0	231 ± 140	11.2 ± 11.5	5.2 ± 1.8	268 ± 114
BA [µmol/l]	ctrl	14.6 ± 7.1	12.2 ± 3.0^{a,b}	197 ± 41^{a}	12.7 ± 4.0	10.1 ± 3.8	170 ± 51^{a,b}
	Q	12.4 ± 4.5	9.0 ± 4.7^{b}	135 ± 61^{b}	10.7 ± 3.2	10.4 ± 2.2	154 ± 23^{b}
	CDCA	15.3 ± 5.2	15.8 ± 3.0^{a}	290 ± 60^{a}	11.6 ± 5.1	11.8 ± 3.4	219 ± 80^{a}
C4 [µg/l]	ctrl	16.0 ± 9.1	20.3 ± 15.9^{a}	326 ± 94^{a}	22.9 ± 26.6	24.7 ± 23.1^{a}	270 ± 168^{a}
	Q	13.9 ± 8.7	70.2 ± 27.3***$^{, b}$	1317 ± 312^{b}	28.2 ± 21.1	111.3 ± 70.1***^{,b}	1835 ± 800^{b}
	CDCA	19.9 ± 13.2	9.6 ± 9.6**^{,a}	131 ± 65^{a}	36.1 ± 32.8	15.6 ± 18.8*^{,a}	112 ± 71^{a}

Data are mean ± SD. ctrl – control experiment, Q – cholestyramine treatment, CDCA – chenodeoxycholic acid treatment. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ -24 h vs. 0 h using paired t-test. ^{a,b} the same letters are assigned to the experiments that do not differ in -203A or -203C individuals (ANOVA detected differences between experiments).

As expected, CYP7A1 activity – estimated on the basis of C4 concentration measurement in the morning at 7:00 – rose several-fold after one-day treatment with cholestyramine ($P < 0.001$), dropped to a half after one-day treatment with CDCA ($P < 0.01$) and did not change in the control experiment in all the subjects (Table 6).

During the treatment with CDCA, there were no changes in plasma C4 concentrations on the day of the study in both -203A and -203C allele carriers (Figure 22). Importantly, no effect of the genotype on the course of C4 concentrations was detected. During the treatment with cholestyramine, C4 concentrations increased during the day in -203A allele carriers ($P = 0.004$). A similar but not significant trend was observed in -203C homozygotes (Figure 22). However, the genotype had no effect on the course of C4 concentrations throughout the day. AUC of C4 was 40 % higher in -203C than those in -203A individuals but the difference was not statistically significant. Similarly, AUC of C4 after Q treatment is much bigger than that on control and after CDCA treatment.

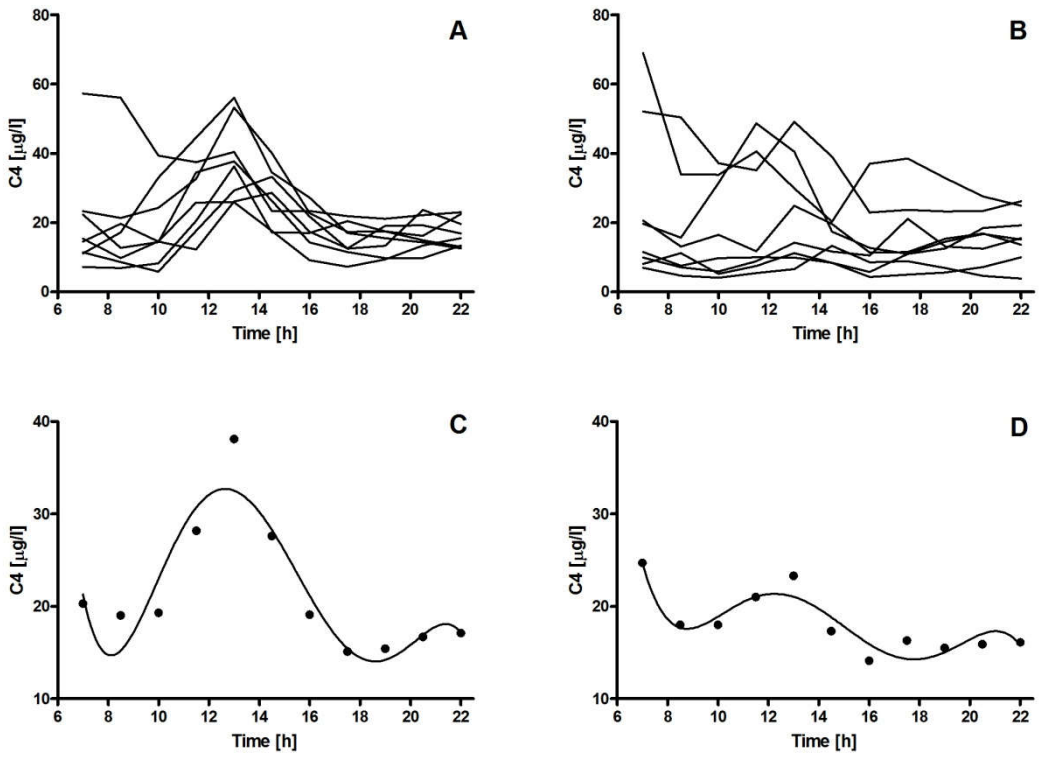

Figure 23: Diurnal variation of C4 concentration in -203A and -203C homozygotes in control experiment.
Individual changes of C4 concentration in -203A (Panel A) and -203C (Panel B) homozygotes. The dynamics of C4 concentration changes in -203A (Panel C) and -203C (Panel D) homozygotes.

In the control experiment, plasma C4 concentration varied during the day in -203A allele carriers (P = 0.001) with a marked peak around 13:00. No such a distinct peak could be observed in -203C homozygotes (Figure 23). Moreover, ANOVA for repeated measures detected a trend for the effect of the genotype on the course of C4 concentrations (P = 0.092). When the polynomial regression modelling was used to describe the course of changes in C4 concentration, statistically significant differences in two of the six compared coefficients on control diet between -203A and -203C homozygotes were found. The best fit was achieved with equation of fifth degree: $[C4] = a_0 + a_1*(time-7) + a_2*(time-14,5)^2 + a_3*(time-14,5)^3 + a_4*(time-14,5)^4 + a_5*(time-14,5)^5$. Using the same modelling, no difference between the homozygous groups was observed after both CDCA and cholestyramine treatment (Table 7).

Table 7: Parameter estimates of polynomial regression modelling.
The estimates in equation of fifth degree $[C4] = a_0 + a_1*(time-7) + a_2*(time-14,5)^2 + a_3*(time-14,5)^3 + a_4*(time-14,5)^4 + a_5*(time-14,5)^5$.

		-203A	-203C
ctrl	a_0	60.205 ± 8.963	33.191 ± 4.361
	a_1	-4.258 ± 1.158	-1.919 ± 0.563
	a_2	-0.656 ± 0.217	-0.156 ± 0.106*
	a_3	0.213 ± 0.073	0.102 ± 0.035
	a_4	0.009 ± 0.004	0.003 ± 0.001*
	a_5	-0.003 ± 0.001	-0.001 ± 0.000
Q	a_0	76.305 ± 9.652	106.056 ± 17.719
	a_1	1.395 ± 1.247	0.669 ± 2.290
	a_2	0.191 ± 0.234	1.364 ± 0.430
	a_3	0.115 ± 0.079	0.192 ± 0.145
	a_4	-0.004 ± 0.004	-0.022 ± 0.007
	a_5	-0.002 ± 0.001	-0.004 ± 0.002
CDCA	a_0	16.097 ± 2.340	4.070 ± 0.752
	a_1	-0.755 ± 0.302	0.335 ± 0.097
	a_2	-0.182 ± 0.057	-0.013 ± 0.018
	a_3	0.054 ± 0.019	-0.016 ± 0.006
	a_4	0.003 ± 0.001	0.002 ± 3.12 x10^{-4}
	a_5	-0.001 ± 2.63 x10^{-4}	-1.24 x10^{-5} ± 8.46 x10^{-5}

Data are presented as mean ± SEM.
ctrl – control experiment, Q – cholestyramine treatment, CDCA – chenodeoxycholic acid treatment.
* $P < 0.05$... -203A vs -203C using JMP statistical software

In -203C allele carriers cholestyramine treatment led to 8 % decrease of cholesterolemia as early as 24 h after first dose ($P < 0.001$). No such an effect was

observed in -203A homozygotes (Table 6). The cholesterol concentration dropped in both HDL and LDL. Cholesterol AUC after cholestyramine treatment was also decreased by 7% in comparison with control examination however the difference was not statistically significant. Cholestyramine caused statistically significant increase of TG level in -203C ($P < 0.05$), not in -203A individuals. When AUC of TG after cholestyramine treatment were compared, no differences were observed. CDCA treatment did not affect observed lipid parameters (Table 6).

Cholestyramine treatment resulted in a significant ($P < 0.05$) decrease of glucose concentration only in -203A, not in -203C individuals. Nevertheless, glucose AUC did not differ between both groups of homozygotes. CDCA treatment did not affect glycemia course. Neither cholestyramine nor CDCA treatment did change concentration of insulin and BA (Table 6). Fasting free fatty acids rose after CDCA treatment in both groups but the difference was significant in -203C individuals only. Both Q and CDCA treatment did not affect fasting BA concentrations. However the AUC of BA was increased after CDCA treatment and lowered after Q treatment in carriers of both alleles (Table 6, Figure 22).

5.3 Discussion

5.3.1 Comparison of promoter variants expression

Luciferase activity of pGL3_CT+ was approximately 5fold higher than that of pGL3_AC+ in HepG2 cells (Figure 19). The promoter activity was not influenced by insulin and PPARα agonists (Figure 20, Figure 21). No differences were found when these stimulations were compared between both *CYP7A1* promoter variants.

The higher basal activity of pGL3_CT+ vectors is in perfect agreement with observations of others [245] who also found 5fold increase of transcriptional activity of -203C compared to -203A variant in HepG2 cells. Threefold expression increase of -203C variant was also found in another cell line - HuH7. Its strenghtens the evidence that cytosin in -203 position is associated with increased basal expression.

The design of our promoter variants allowed us to distinguish the role of two SNP in -203 (rs3808607) and -469 (rs3824260) position in regulation of basal expression of *CYP7A1*. The substitution of A for C in -203 position resulted in five to twelvefold increase in *CYP7A1* basal expression whereas the substition of T for C in -469 position led to no change or doubling expression. Therefore -203 position seems to play a critical role in modulation of basal promoter activity.

It has been shown that physiological concentration of insulin stimulates *CYP7A1* expression initially and represses it afterwards [246]. On the contrary 2 or 4 hour incubation of HepG2 cells with 100nM insulin downregulated *CYP7A1* expression [247]. In our experiment we have not observed insulin effect (Figure 20). On the other hand, we measured *CYP7A1* promoter activity via dual luciferase assay not *CYP7A1* expression directly. It can be speculated that insulin influence on promoter activity manifestes much earlier. Importantly, we did not observe any differences in response of both promoter variants expression to insulin. Such an observation is in agreement with findings that three putative insulin response elements in CYP7A1 promoter did not overlap with -203 position [247].

PPARα agonists downregulated *Cyp7a1* expression in rat hepatocyte cultures [248] and longer promoter fragments inhibited stronger the reporter activities of human CYP7A1 constructs [137]. So the polyunsaturated fatty acids (that are embodied in food and are physiological activators of PPARα) might play an important role in the regulation of *CYP7A1* expression during the day. Therefore we study the effect of two PPARα agonists (fenofibrate or WY14643) on both variants via luciferase reporter assay. In our system, both fenofibrate and WY14643 did not influence *CYP7A1* promoter activity. Such findings are in contrast to those mentioned above but we used much shorter constructs (800 bp vs ~ 2 kbp) and stimulation took 24 h only (vs 42 h) [249]. It can be speculated that the similar conditions may lead to the marked changes.

The higher basal activity of -203C variant does not explain our previous observation that -203C variant is responsible for hyperresponsiveness to dietary fat and/or cholesterol [249]. Hovewer, the fact that *CYP7A1* expression can be regulated in much wider range in -203C than in -203A allele carries could be a critical precondition for more profound response of enzyme expression and activity to different stimuli and so hyperresponsiveness in individuals carrying -203C allele.

To summarise, using a dual luciferase reporter assay with promoter fragments (-716 to +14) of both *CYP7A1* variants, we found the -203C variant is expressed severalfold more than the -203A variant. However, we did not see any differences in the effects of insulin and PPARα agonists (fenofibrate and WY-14643) on the expression of both promoter variants. This may suggest that other factors may play a role in the regulation of CYP7A1 activity throughout the day.

5.3.2 *In silico* analysis

In silico analysis revealed a putative binding site for a glucocorticoid receptor in -203A allele not in -203C allele. A hypothesis that both -203 variants respond differentially to corticoid stimulation was tested using dual luciferase assay. However, no differences between -203A and -203C variants expression after incubation with cortisol or dexamethasone were observed [T. Blahová, unpublished data].

5.3.3 -203A>C polymorphism effect on diurnal changes of serum concentrations

The homozygous carriers of -203A allele display a diurnal variation of CYP7A1 activity, peaking at midday. No changes in CYP7A1 activity between 7:00 and 22:00 could be observed in homozygous carriers of -203C allele. Not surprisingly, the treatment with cholestyramine upregulates the CYP7A1 activity severalfold and, conversely, the treatment with CDCA downregulates this activity. No differences in CYP7A1 activity variation were found between -203A and -203C subjects after cholestyramine or CDCA treatment (Table 6, Figure 22, Figure 23).

Our findings that C4 concentration as a marker of CYP7A1 activity displays a peak around midday confirms the earlier findings from the study of five healthy volunteers [143]. This study has demonstrated that CYP7A1 activity has two peaks in humans: first in the early afternoon and the second before midnight. Due to our design, we can make conclusions only about the first peak. Interestingly, there is no clear mechanistic explanation for the midday peak. CYP7A1 activity increases in subjects who eat normally and also in those who are fasting before midday [143]. Such an increase is thus unlikely to be associated with food intake. Interestingly, CYP7A1 activity then falls rapidly only in subjects who eat normally and not in those who are fasting. This may suggest that increased intrahepatic flux of BA and especially intestinal fibroblast growth factor-19 secretion after meal consumption are involved in dampening of CYP7A1 activity peak [250].

The diurnal variation of CYP7A1 activity and *Cyp7a1* expression has been extensively studied in rodents. The *Cyp7a1* expression in mice falls under the control of several clock genes [251-253]. It remains to be determined whether clock genes also play a role in the regulation of BA synthesis in humans.

Given that some subjects do not display diurnal variation, it is surprising that this has gone unnoticed so far. This is likely due to the small sample size of study that

has investigated circadian variation of CYP7A1 activity [143, 250] and it might be possible that subjects homozygous for -203C allele were not included. This can even suggest that the effect of -203A allele is dominant and heterozygous subjects display diurnal variation of CYP7A1 activity.

It must be also stressed that the -203A>C polymorphism may not be the one responsible for observed differences in circadian changes in CYP7A1 activity. This polymorphism is in close linkage disequilibrium with several other polymorphisms in the *CYP7A1* [183]. In the Caucasian population (the only population in which studies of diurnal variation in CYP7A1 have been carried out), the -203A and -203C alleles are considered to be markers of haplotype blocks spanning 14 kb from the proximal promoter to the 3'-downstream region of the *CYP7A1*. Therefore, either of the polymorphisms included in these haplotype blocks may be responsible for the observed differences in diurnal variation of enzyme activity.

After cholestyramine or CDCA treatment the diurnal variation of CYP7A1 activity did not differ between -203A and -203C homozygous subjects. The effects of both treatments on CYP7A1 activity suppressed diurnal variation.

Cholestyramine treatment resulted in a immediate decrease in cholesterolemia and increase trialcylglycerolemia in -203C homozygotes (Table 6, Figure 22). Levels of cholesterol and TG in -203A carriers did not change significantly. This result indicated that -203C allele could not only increase cholesterolemia responsiveness to the diet but also to affect the responsiveness to BA sequestrants treatment. If this finding validated in clinical studies it would have a practical use and -203A>C polymorphism could be of great importance for farmogenetics. Nevertheless, these findings were not confirmed in our recent study in which no differences between -203A and -203C allele carriers in response to 4-week treatment with colesevelam (BA sequestrant) were noted [254].

At the same time, glucose concentration decreased after cholestyramine treatment only in -203A not in -203C individuals. This finding could be applied by treatment of diabetic patiens to lower cholesterolemia and glycemia with BA sequestrants [255]. Hovewer, extensive clinical studies are required for confirmation.

The major limitation of this experiment is, apart from its small size, the lack of night blood sampling. In future studies, 24-hour monitoring and inclusion of heterozygous individuals would be desirable. It should be kept in mind that C4 is only a surrogate marker of CYP7A1 activity – however, such a limitation is hard to overcome

in human studies. Additionally, -203C carriers in our cohort tended to have slightly higher BMI.

In conclusion, the -203A allele of the *CYP7A1* is associated with pronounced diurnal variation of CYP7A1 activity whereas -203C variant is not. It remains to be determined whether differences over the course of enzyme activity throughout the day between carriers of the -203A and -203C alleles can explain the dissimilar effects of these variants on cholesterolemia and its responsiveness to diet.

6. Conclusions

6.1 Pathogenesis of hypercholesterolemia in the PHHC rat (Aim I)

We demonstrated that the PHHC rat produces cholesterol-rich VLDL that cannot be catabolised normally and accumulate in circulation.

We did not find any differences in the response of the hepatic transcriptome to cholesterol feeding in PHHC and control Wistar rats. Hovewer, we detected several differently expressed genes between both strains independently of diet.

We analysed the role of some candidate genes (*Aldh1a7*, *Apof*, *Mttp*, *Soat1* and *Soat2*) in hypercholesterolemia pathogenesis.

6.2 The role of the -203A>C polymorphism of *CYP7A1* in cholesterolemia regulation (Aim II)

Using dual luciferase assay, we found that expression of the -203C allele is markedly increased in comparison with the -203A allele and that there are no differences in the response of both alleles to insulin and PPARα stimulation.

We documented that carriers of the -203A allele display diurnal variation of CYP7A1 activity whereas -203C allele carriers do not.

7. References

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8. Abbreviations

ABC	ATP-binding cassette
ACAT	acyl-CoA:cholesterol acyltransferase
Aldh1a7	aldehyde dehydrogenase family 1, subfamily A7
AMP	adenosine monophosphate
ANOVA	analysis of variance
apo	apo(lipo)protein
ATP	adenosine triphosphate
AUC	area under curve
BA	bile acids
BARE	bile acid response element
BMI	body mass index
C	control (diet)
C4	7α-hydroxy-4-cholesten-3-one
CA	cholic acid
CDCA	chenodeoxycholic acid
cDNA	deoxyribonucleic acid
CE	cholesteryl ester
CETP	cholesteryl ester transfer protein
CF	control fat (diet)
CHOL	cholesterol (diet)
CM	chylomicron
CMr	chylomicron remnant
CoA	coenzyme A
ctrl	control (experiment)
CYP7A1	cholesterol 7α-hydroxylase
CYP8B1	sterol 12α-hydroxylase
DCA	deoxycholic acid
DNA	deoxyribonucleic acid
DR1	direct repeat 1
ER	endoplasmatic reticulum
FC	free (unesterified) cholesterol
FXR	farnesoid X receptor

FXRE	FXR response element
gDNA	genomic DNA
HDL	high density lipoprotein
HMG	3-hydroxy-3-methyl-glutaryl
HNF4	hepatocyte nuclear factor 4
HPLC	high-performance liquid chromatography
IDL	intermediate density lipoprotein
Insig	insulin induced gene
LCA	lithocholic acid
LDL	low density lipoprotein
LDL-C	cholesterol in LDL
LDLR	LDL receptor
LPL	lipoprotein lipase
LRH1	liver receptor homologue 1
LRP	LDLR-related protein
LXR	liver X receptor
LXRE	LXR response element
mRNA	messenger RNA
miRNA	micro RNA
MTP	microsomal TG transfer protein
PPAR	peroxisome proliferator-activated receptor
PCSK9	proprotein convertase subtilisin/kexin type 9
PCR	polymerase chain reaction
PHHC	Prague hereditary hypercholesterolemic
PL	phospholipid
Q	Questran® (cholestyramine)
qPCR	quantitative real-time PCR
RCT	reverse cholesterol transport
RNA	ribonucleic acid
RXR	retinoid X receptor
S1P	site 1 protease
S2P	site 2 protease
SCAP	SREBP cleavage-active protein
SHP	small heterodimer partner

SM	squalene monooxygenase
SNP	single nucleotide polymorphism
SR-BI	scavenger receptor class B type 1
SRE	sterol response element
SREBP	sterol regulatory element binding protein
SSD	sterol sensing domain
SUMO	small ubiquitin-like modifier
TICE	trans-intestinal cholesterol excretion
TG	triacylglycerol
UTR	untranslated
VLDL	very low density lipoprotein
VLDL-C	cholesterol in VLDL
VLDLR	VLDL receptor
WHHL	Watanabe-heritable hyperlipidemic

9. List of tables

10. List of figures

11. List of publications and presentations

11.1 Publications related to this thesis

Kovář, J., Leníček, M., **Zimolová, M.,** Vítek, L., Jirsa, M., Piťha, J. Regulation of diurnal variation of cholesterol 7α-hydroxylase (CYP7A1) activity in healthy subjects. *Physiol Res. 2010;59(2):233-8. Epub 2009 Jun 19*. IF = 1.646

Vlachová, M., Heczková, M., Jirsa, M., Poledne, R., Kovář, J. The response of hepatic transcriptome to dietary cholesterol in Prague hereditary hypercholesterolemic (PHHC) rat. *Physiol Res. 2014;63 Suppl 3:S429-37.* IF = 1.293

Vlachová, M., Blahová, T., Lánská, V., Leníček, M., Piťha, J., Vítek, L., Kovář, J. Diurnal variation of cholesterol 7α-hydroxylase activity is determined by a common promoter polymorphism of the *CYP7A1* gene. *Croatian Med J 2016***.** *Submitted***.** IF (2014) = 1.305

11.2 Publications unrelated to this thesis

Leníček, M., Komárek, V., **Zimolová, M.**, Kovář, J., Jirsa, M., Lukáš, M., Vítek, L. CYP7A1 promoter polymorphism -203A>C affects bile salt synthesis rate in patients after ileal resection. *J Lipid Res. 2008 Dec;49(12):2664-7. doi: 10.1194/jlr.M800364-JLR200. Epub 2008 Aug 26.* IF = 4.409

Blahová, T., Peterková, L., Leníček, M., **Vlachová, M.,** Zemánková, K., Adámková, V., Vítek, L., Kovář, J. The effect of colesevelam treatment on bile acid and lipid metabolism, and glycemic control in healthy men. *Physiol Res. 2016. Accepted.* IF (2014) = 1.293

11.3 Conference presentations related to this thesis

Zimolová, M., Kovář, J., Jirsa, M.: The role of -203A/C polymorphism in the regulation of cholesterol 7alpha-hydroxylase expression. 2nd Congress of the International Society of Nutrigenetics, Geneva, Switzerland. 6. - 8. 10. 2008. *Abstract: Journal of Nutrigenetics and Nutrigenomics. 2008, 1, 289.* **Poster.**

Zimolová, M., Bohuslavová, R., Jirsa, M., Poledne, R., Kovář, J.: Regulace genové exprese v játrech u PHHC potkana. Konference Centra výzkumu chorob srdce a cév, Harrachov, 29. 9. - 1. 10. 2008. **Oral presentation.**

Zimolová, M., Bohuslavová, R., Jirsa, M., Poledne, R., Kovář J.: Genová exprese v játrech u Pražského hereditárně hypercholesterolemického potkana. XII. konference o ateroskleróze, Špindlerův Mlýn, 11. - 13. 12. 2008. *Abstract: Vnitřní lékařství. 2008, 54, 1223.* **Oral presentation**.

Zimolová, M., Kovář, J., Jirsa, M., Poledne R.: The role of -203A/C polymorphism in the regulation of cholesterol 7alpha-hydroxylase expression. XV. International Symposium on Atherosclerosis, Boston, USA, 14. - 18. 6. 2009. *Abstract: Atherosclerosis Suplements. 2009, 10, e1156.* **Poster.**

Zimolová, M., Bohuslavová, R., Iványek, R., Stránecký, V., Heczková, M., Coufalíková, M., Poledne, R., Jirsa, M., Kovář, J.: Mechanism of hypercholesterolemia in PHHC rat - transcriptome analysis. Konference Centra výzkumu chorob srdce a cév, Harrachov, 19. - 21. 10. 2009. **Oral presentation.**

Zimolová, M., Kovář, J., Bohuslavová, R., Stránecký, V., Iványek, R., Jirsa, M., Poledne, R.: Hepatic gene expression in Prague Hereditary Hypercholesterolemic (PHHC) rat. British Atherosclerosis Society Autumn Meeting, Genetics of Complex Diseases, Cambridge, Great Britain. 17. - 18. 9. 2009. *Abstract: Atherosclerosis. 2009, 207, e12.* **Poster.**

Zimolová, M., Bohuslavová, R., Stránecký, V., Iványek, R., Heczková, M., Coufalíková, M., Jirsa, M., Poledne, R., Kovář J.: Analýza jaterního transkriptomu Pražského hereditárně hypercholesterolemického (PHHC) potkana. XIII. konference o ateroskleróze, Špindlerův Mlýn, 10. - 12. 12. 2009. *Abstract: Vnitřní lékařství. 2009, 55, P16.* **Oral presentation.**

Zimolová, M., Bohuslavová, R., Stránecký, V., Iványek, R., Jirsa, M., Poledne, R., Kovář J.: Hepatic gene expression in Prague Hereditary Hypercholesterolemic (PHHC) rat - a model of polygenic hypercholesterolemia. Bridges in Life Sciences 5th Annual Scientific Meeting, RECOOP HST Consortium, Lviv, Ukraine, 9. - 11. 4. 2010. *Abstract: Biopolymers and Cell Supplemetary. 2010, 26, 69*. **Oral presentation.**

Zimolová, M., Coufalíková, M., Heczková, M., Bohuslavová, R., Stránecký, V., Iványek, R., Jirsa, M., Poledne, R., Kovář J.: Mechanism of hypercholesterolemia in Prague Hereditary Hypercholesterolemic (PHHC) rat. 78th European Atherosclerosis Society Congress, Hamburg, Germany, 20. - 23. 6. 2010. *Abstract: Atherosclerosis Supplements. 2010, 11, 127.* **Poster.**

Zimolová, M., Heczková, M., Coufalíková, M., Poledne, R., Jirsa, M., Kovář, J.: The analysis of hepatic transcriptome in Prague Hereditary Hypercholesterolemic (PHHC) rat - an experimental model of hypercholesterolemia. 33rd annual conference of European Lipoprotein Club, Tutzing, Germany. 6. - 9. 9. 2010. **Poster.**

Zimolová, M., Heczková, M., Poledne, R., Jirsa, M., Kovář, J.: Kandidátní geny pro hypercholesterolémii u PHHC potkana. Konference centra výzkumu chorob srdce a cév, Harrachov. 10. - 12. 11. 2010. **Oral presentation.**

Zimolová, M., Heczková, M., Schmiedtová, M., Poledne, R., Jirsa, M., Kovář, J.: Analýza jaterního transkriptomu Pražského hereditárně hypercholesterolemického potkana - nové kandidátní geny pro polygenní hypercholesterolémii. XIV. konference o ateroskleróze, Špindlerův Mlýn, 9. - 11. 12. 2010. **Oral presentation.**

Zimolová, M., Heczková, M., Schmiedtová, M., Poledne, R., Jirsa, M., Kovář J.: The analysis of hepatic transcriptome in Prague Hereditary Hypercholesterolemic (PHHC) rat – an experimental model of hypercholesterolemia. Bridges in Life Sciences 6th Annual Scientific Meeting, RECOOP HST Consortium, Bratislava, Slovakia, 8. - 10. 4. 2011. *Abstract: Biopolymers and Cell Supplemetary. 2011, 27, 68.* **Oral presentation.**

Zimolová, M., Poledne, R., Jirsa, M., Kovář, J.: Mechanism of hypercholesterolemia in PHHC rat. 17th Annual Scandinavian Atherosclerosis Conference, Humlebæk, Denmark. 13. - 16. 4. 2011. **Poster.**

Zimolová, M., Schmiedtová, M., Heczková, M., Poledne, R., Jirsa, M., Kovář, J.: Mechanism of hypercholesterolemia in PHHC rat. 79th European Atherosclerosis Society Congress, Göteborg, Sweden, 26. - 29. 6. 2011. *Abstract: Atherosclerosis Supplements. 2011, 12, 26.* **Poster.**

Vlachová, M., Heczková, M., Schmiedtová, M., Poledne, R., Jirsa, M., Kovář, J.: Analýza jaterního transkriptomu Pražského hereditárně hypercholesterolemického potkana - nové kandidátní geny pro polygenní hypercholesterolémii. Genetická konference GSGM 2011, Lednice, 14. - 16. 9. 2011. **Poster.**

Vlachová, M., Poledne, R., Jirsa, M., Kovář, J.: Promotorový polymorfismus a regulace aktivity cholesterol-7α-hydroxylasy (CYP7A1). XVI. konference o ateroskleróze, Špindlerův Mlýn, 6. - 8. 12. 2012. **Poster.**

Vlachová, M., Poledne, R., Jirsa, M., Kovář, J.: Mechanism of hypercholesterolemia in Prague Hereditary Hypercholesterolemic (PHHC) Rat. 13. - 16. 10. 2013, Florence, Italy. **Poster.**

Vlachová, M., Poledne, R., Jirsa, M., Kovář, J.: -203A/C polymorphism and regulation of cholesterol 7alpha-hydroxylase gene (CYP7A1) expression. 13. - 16. 10. 2013. Florence, Italy. **Poster.**

11.4 Conference presentations unrelated to this thesis

Zimolová, M., Hiebschová, L., Kovář, J.: Diagnostika deficitu glycerolkinasy. Dědičné metabolické poruchy – 26. pracovní dny, Mikulov, 11. - 13. 5. 2011. **Poster.**

12. Appendices

12.1 Publications

12.1.1 J. Kovář *et al., Physiol Res.* 59, 233 (2010)

Kovář, J., Leníček, M., Zimolová, M., Vítek, L., Jirsa, M., Piťha, J.
Regulation of diurnal variation of cholesterol 7α-hydroxylase (CYP7A1) activity in healthy subjects.
Physiol Res. 2010;59(2):233-8. Epub 2009 Jun 19.
IF = 1.646

Contribution:
I carried out the study in volunteers, performed genotype determination and carried out some biochemical analyses. I participated in manuscript preparation.

This appendix contains 6 pages in total.

12.1.2 M. Vlachová *et al.*, *Physiol Res.* 63 Suppl 3, S429 (2014)

Vlachová, M., Heczková, M., Jirsa, M., Poledne, R., Kovář, J.
The response of hepatic transcriptome to dietary cholesterol in Prague hereditary hypercholesterolemic (PHHC) rat.
Physiol Res. 2014;63 Suppl 3:S429-37.
IF = 1.293

Contribution:
In this paper, I coordinated the experiments on rat, performed RNA isolation, reverse transcription, designed and carried out quantitative real-time PCR, analysed the microarray and qPCR results, prepared figures and tables of gene expression part and together with my supervisor wrote the manuscript.

This appendix contains 9 pages in total.

12.1.3 M. Vlachová *et al., Croatian Med J* (2016)

Vlachová, M., Blahová, T., Lánská, V., Leníček, M., Piťha, J., Vítek, L., Kovář, J.
Diurnal variation of cholesterol 7α-hydroxylase activity is determined by a common promoter polymorphism of the CYP7A1 gene.
Croatian Med J 2016. Submitted.
IF (2014) = 1.305

Contribution:

I participated in study design, carried out the study in volunteers, performed genotype determination and carried out some biochemical analyses. I participated in manuscript preparation.

This appendix contains 13 pages in total

12.2 Oligonucleotides

TaqMan® Gene Expression Assays (Applied Biosystems) used for qPCR.

Accession number (NCBI database)	Gene Symbol	Assay ID
NM_001006995.1	*Acat2*	Rn01526241_g1
NM_013134.2	*Hmgcr*	Rn00565598_m1
NM_017136.2	*Sqle*	Rn00567532_m1
NM_057137.1	*Ebp*	Rn00589201_m1
NM_175762.2	*Ldlr*	Rn00598438_m1
NM_012942.1	*Cyp7a1*	Rn00564065_m1
NM_022392.1	*Insig1*	Rn00574380_m1
NM_031533.3	*Ugt2b*	Rn02349652_m1
NM_053977.2	*Cdh17*	Rn00678433_m1
NM_053639.2	*Ltc4s*	Rn01497055_g1
NM_017206.1	*Slc6a6*	Rn00567962_m1
NM_001024351.1	*Apof*	Rn01756260_g1
NM_001009920	*Gsta3*	Rn01511827_m1
NM_017272.15	*Aldh1a7*	Rn00755277_m1
NM_001107727.1	*Mttp*	Rn01522961_m1
NM_031118.1	*Soat1*	Rn00579605_m1
NM_153728.2	*Soat2*	Rn00596636_m1
NM_213563.1	*Vars2l*	Rn01531995_m1

Primers used for *Apoe* and *Apof* sequencing.

Gen	Exon	Forward primer	Reverse primer	$T^{anneling}$ [°C]	Product size [bp]
Apoe	1	gcaggggagtcctataattg	atctcctccatctgttctgacc	60	288
	2	gggggaggtaaatagacctttg	tttaccctctgagcatcaatcc	60	375
	3	tgacttccagacgcactgttg	taggtgcccagataggaggaac	60	327
	4a	cttcctcagcttctcaacttctgg	gcccaggctttgagtgac	64	699
	4b	gtatctgctgggtctgctcctc	aggcagaaacgataaactgagg	64	461
Apof	1	gtcaacattgggcacctcatc	ggatatgggaaatgcaacacag	60	299
	2a	aggggttccctgaatcttg	ccccttcctcgtcatgataata	60	866
	2b	cttcaagagctccagaaaggtg	ca gctgatttacccgaactgtg	60	945