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Genetic determination of cholesterolemia regulation

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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:cholesterolacyltransferasy (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*. Jejich funkce v patogenezi hypercholesterolémie však i nadále zůstávají 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 (CA) 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 (LPL)) 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.

1. Introduction

Based on the results of numerous 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 cardiovascular diseases. Therefore the adequate regulation of cholesterol blood level is essential for coronary heart disease prevention.

The liver has been considered the major site of control of cholesterol homeostasis [1]. First of all, the liver clears very low density lipoproteins (VLDL), low density lipoproteins (LDL), and chylomicron remnants from circulation, synthesises cholesterol and bile acids (BA), is involved in production of nascent high density lipoproteins (HDL), and secretes cholesterol and BA to bile. Nowadays, more attention is paid also to the role of the intestine in cholesterol metabolism. The intestine influences cholesterol homeostasis at the level of cholesterol and BA (re)absorption, fecal excretion and *de novo* synthesis [2, 3]. Moreover, trans-intestinal cholesterol excretion plays a major role in disposal of cholesterol via the feces at least in mice [4].

1.1 Regulation of cholesterolemia

Hypercholesterolemia is not a manifestation of cell cholesterol accumulation. It results from imbalance between secretion and uptake of cholesterol carrying lipoproteins (mainly LDL) [5]. The mechanisms involved in the regulation of lipid secretion from hepatocytes and those involved in the lipoprotein uptake from circulation are the major factors that determine cholesterolemia.

1.1.1 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 intracellular compartmens is precisely regulated in every cell. Several 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 has represented an intricate task because cholesterol is insoluble in water and resides exclusively in cell membranes. Both cholesterol itself (mainly FC) as well as cholesterol metabolites (mainly BA and oxysterols) are involved in maintaining cholesterol homeostasis.

When the concentration of cholesterol in plasma membrane exceeds critical level, the abundant cholesterol (called „active cholesterol“) tends 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 cholesteryl ester (CE) that can be stored in intracellular lipid droplets. Excess of FC in the cell triggers binding of 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase to insulin induced gene (Insig) which, in turn, initiates ubiquitination of this protein and its subsequent degradation. Interaction of FC with sterol regulatory element binding protein (SREBP) cleavage-active protein results in suppression of SREBP signalling pathway. This leads to the very rapid downregulation of sterol synthesis within the cell [6, 7]. In mitochondria, abundant FC can be oxidized to oxysterols that can be removed relatively easily from the cell. Oxysterols are also ligands for liver X receptor (LXR) which promote transcription of ATP-binding cassette (ABC) transporters. ABCA1 and ABCG5/G8 stimulate transfer of plasma membrane cholesterol to HDL in reverse cholesterol transport. In rodents (but not in humans) LXR also upregulates cholesterol 7α-hydroxylase (CYP7A1) gene transcription and, thus, transformation of FC to BA [8].

Oppositely, when cholesterol level in the cell decreases, CE from lipid droplets can be hydrolysed to provide FC instantly. The cholesterol metabolically active pool decrease initiates the processing of SREBPs into the active forms. These transcription factors then enhance the gene expression of many of the enzymes required for cholesterologenesis as well as for the cholesterol uptake from circulation (LDL receptor (LDLR)) [7, 9].

1.1.2 Cholesterolemia regulation

High intracellular cholesterol is detrimental to the cells, and high serum cholesterol will promote the deposition of cholesterol in the arterial wall. 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) [10, 11]. 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 the LDLR. Its deficiency leads to striking hypercholesterolemia in patients with familial hypercholesterolemia.

A number of polymorphisms in several genes involved in cholesterol metabolism that are associated with higher cholesterol level has been described. Three common alleles of *APOE* encoding apolipoprotein (apo) E (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 [12, 13]. 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. Two polymorphisms of *CYP7A1* (-203A>C, rs3808607, and -469C>T, rs3824260) that are in almost complete linkage disequilibrium have been associated with variation of plasma LDL-C [14, 15]. Furthermore, it has been repeatedly demonstrated that the -203A>C polymorphism affects responsiveness of plasma cholesterol and LDL-C to dietary fat and cholesterol [16, 17].

1.2 Animal models

In some species hypercholesterolemia can be induced very easily by dietary cholesterol (rabbit, pigeon) whereas some animals are resistant to this intervention (pig, monkey, rodents). In mice and rats, BA or drugs influencing thyroid gland must be added to cholesterol containing diet to induce hypercholesterolemia.

Furthermore, there are marked differences in lipoprotein metabolism between humans and rodents. Mice and rats do not possess cholesteryl ester transfer protein (CETP) and have up to 40-fold higher LDL clearance by the liver compared to humans [1, 18]. Hence they carry most of their plasma cholesterol in HDL particles [1, 18-21]. 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 [22] or apoE [23]. More recently “humanised” mice have become available in which human *CETP* is expressed [21].

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 new genes that are involved in cholesterolemia determination, animals with

spontaneous defects in cholesterol metabolism could be very beneficial. They 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 [24].

1.2.1 Prague hereditary hypercholesterolemic (PHHC) rat

In general, rat is not an optimal model for atherosclerosis and hypercholesterolemia research. Its plasma cholesterol concentrations are lower than 2 mmol/l and the majority of cholesterol is transported in HDL [21]. To induce hypercholesterolemia it is necessary to add BA or thyrotoxic substances to diet, because cholesterol and fat are not sufficient [25-27]. Hovewer, several groups were able to produce hypercholesterolemic rats by inbreeding animals sensitive to dietary cholesterol [28-30].

The PHHC rat was obtained through selective inbreeding of parental Wistar rats [30]. 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 cholic acid (CA) and/or thyrotoxic drugs. Besides that, cholesterol accumulates in lipoproteins other than HDL, mostly in VLDL fraction [30].

Despite considerable effort, the mechanisms responsible for hypercholesterolemia development have not been determined yet. Because no difference was found in LDL clearance from the circulation between PHHC and Wistar rats, defect in LDLR quantity and/or function of PHHC rat was excluded [207]. Additionally, the absorption of dietary cholesterol does not prominently differ in both lines [30] as well as the activity of lecithin:cholesterolacyltransferase [31], lipoprotein (LPL), and hepatic lipases [32]. It has been suggested only recently that PHHC rats, when fed cholesterol, may not be able to upregulate the transcription of *Cyp7a1* [33].

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.

2. Aims

2.1 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.

2.2 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.

3. Pathogenesis of hypercholesterolemia in the PHHC rat

3.1 Materials and methods

3.1.1 Diets

The rats were fed one of the three diets: standard laboratory diet M2 (SEMED, Prague) (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.

3.1.2 Determination of VLDL clearance *in vivo*

Firstly, VLDL were isolated by ultracentrifugation [34] from the serum of PHHC rats fed CHOL diet and from Wistar rats fed the C diet. Cholesterol/triacylglycerol (TG) ratio of these VLDL were 2.22 (PHHC) and 0.83 (Wistar). Total protein in VLDL was determined [35]. VLDL were then labeled by the iodine monochloride method [36] 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 [37]. Radioactivity was subsequently measured in supernatant and the amount of radio-labelled 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 $\alpha = 0.01$.

3.1.3 Microarray analysis

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 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).

3.2 Results

3.2.1 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*. 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 (Fig. 1). 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; $P < 0.01$).

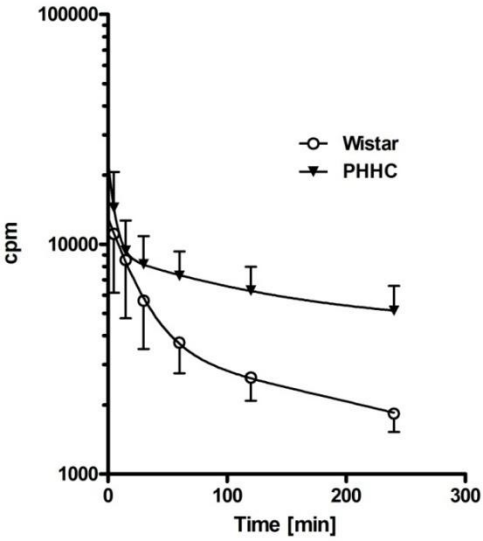

Fig. 1: Clearence of ^{125}I-VLDL from serum of PHHC and Wistar rats injected into Wistar rats.
VLDL were isolated by ultracentrifugation from sera of PHHC rats fed CHOL diet and Wistar rats fed C diet, radiolabelled [207] and injected into Wistar rats. The blood samples for determination of radioactivity were taken from tail vein.

3.2.2 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*) (Fig. 2). 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.

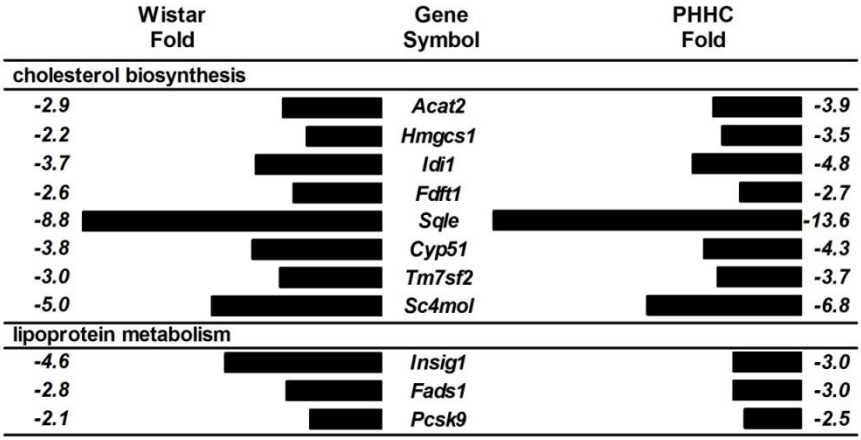

Fig. 2: Hepatic gene expression response in PHHC and Wistar rats to dietary cholesterol. The genes that were at least twofold downregulated on CHOL diet compared to both control (C, CF) diets ($P < 0.05$) are shown. Data are presented as mean of gene expression on CHOL diet compared to mean of gene expression of both control diets; expression of genes on control diets is set to 1.0.

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 (Fig. 3). The vast majority of those genes has no known connection to lipoprotein metabolism.

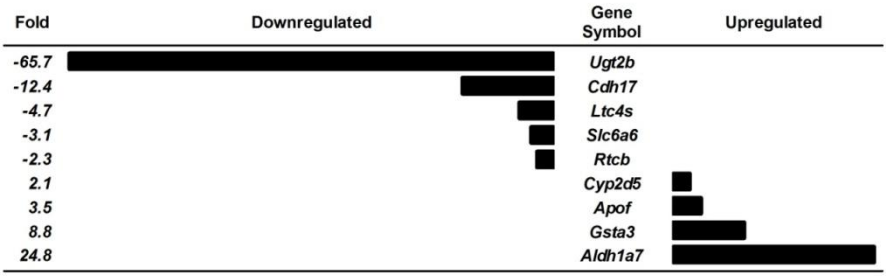

Fig. 3: Comparison of hepatic gene expression between PHHC and Wistar rats. The genes that were at least twofold down- or upregulated ($P < 0.05$) in PHHC rats compared to Wistar rats irrespective of diet are shown. Data are presented as mean of gene expression on all three diets. Gene expression of Wistar rats is set to 1.0.

The results of microarray gene expression were validated for 14 genes, 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.

3.3 Discussion

3.3.1 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 thyrotoxic drugs [25-27, 38]. While there is no difference in the lipoprotein profile between the PHHC and control Wistar rats on C diet or on CF diet, cholesterolemia rises severalfold in the PHHC rats fed CHOL diet in contrast to only a small increment in Wistar rats. In PHHC rats, cholesterol accumulates predominantly in the VLDL fraction and these VLDL carry more cholesterol than TG.

Such cholesterol-enriched VLDL originate in the liver as documented in our experiment using intravenously injected tyloxapol. 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 from Wistar rats, labelled them with ^{125}I and injected these VLDL into Wistar rats to estimate the rate of their elimination from circulation (Fig. 1). 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, do not escape from the VLDL size range, cannot be removed from circulation similarly to the remnants of normal VLDL, 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 [25-27]. A similar model of genetically determined hypercholesterolemic rat was described recently [39]. 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.

In conclusion, our data suggest that dietary cholesterol induced hypercholesterolemia in the PHHC rat is due to production of VLDL very rich in cholesterol. Such VLDL are not catabolised normally and accumulate in circulation.

3.3.2 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. 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 (Fig. 2). In both strains, most of the genes of the cholesterol biosynthetic pathway even starting from acetyl-CoA acetyltransferase 2 (*Acat2*) were downregulated. The most noticeable downregulation was observed for gene encoding squalene epoxidase (*Sqle*) in both strains, HMG-CoA reductase gene (*Hmgcr*) expression was also suppressed but it 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 [40, 41]. Similarly to *Hmgcr*, the LDL receptor gene (*Ldlr*) was downregulated only 1.5 fold, also in agreement with other findings [40]**.** We did not confirm the previous findings [33] 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, it 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 [42].

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 (Fig. 3). 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 [43]. However, mice and rats do not have CETP [44] and the role of the protein in these animals remains elusive.

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 hypercholesterolemia among patients and its genetic background is not yet fully understood. 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) 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 [45]. 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 [30, 46, 47].

To summarise, several genes are significantly up- or downregulated in PHHC rats irrespective of the diet suggestive of the fact that the response to dietary cholesterol takes place in a different genetic background.

4. The role of the -203A>C polymorphism of *CYP7A1* in cholesterolemia regulation

4.1 Materials and methods

4.1.1 Activity of promoter variants *in vitro*

4.1.1.1 Plasmid preparation

Both common variants of *CYP7A1* promoter [-469C, -203A] and [-469T, -203C] were orientedly cloned into pGL3_basic vector (Promega) in front of gene coding luciferase (*Photinus pyralis*). 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).

4.1.1.2 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). Two days after transfection, the cells were processed according manufacturer's protocol.

4.1.1.3 Statistics

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

4.1.2 Diurnal variation of CYP7A1 activity in healthy men

4.1.2.1 Subject and study design

Sixteen male volunteers were included into the study: 8 homozygotes for -203A allele of *CYP7A1* (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.

4.1.2.2 Biochemistry

Concentration of 7α-hydroxy-4-cholesten-3-one (C4) was determined by HPLC as described earlier [48] with a modification of C4 extraction procedure [49]. Cholesterol, HDL-cholesterol, TG, glucose, free fatty acids, and BA were measured using kits.

4.1.2.3 Statistics

The effect of *CYP7A1* genotype on changes of all the parameters was evaluated using ANOVA for repeated measures with one grouping factor (genotype), the data for analysis were logarithmically transformed when necessary. Corresponding post-hoc tests were carried out (GraphPad InStat). The dynamics of C4 concentration changes was then modelled using polynomial regression of 5^{th} order using JMP 10 statistical software.

4.2 Results

4.2.1 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.

Haplotypes with cytosin -203 (-203C) were expressed approximately 5fold and 3fold more in HepG2 and HuH7 cells, respectively, than haplotypes with adenosin in the same position (-203A; $P < 0.001$; Fig. 4).

The luciferase activity of antisense variants (pGL3_AC- and pGL3_CT-) was similar to the activity of elementary vector pGL3_basic.

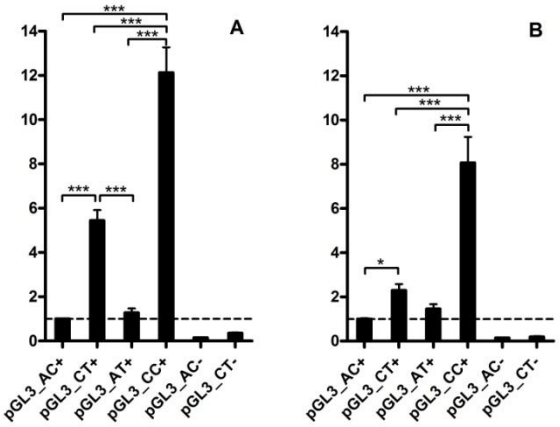

Fig. 4: Comparison of *CYP7A1* promoter activity in HepG2 (Panel A) and HuH7 (Panel B) cells.

Activity of promoter variants AC+ is set to one. Data are mean ± SEM (n = 17 – 33). * $P < 0.05$, *** $P < 0.001$ using ANOVA (the antisense variants were not analysed with post-hoc test).

4.2.2 The -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.

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 by a half after one-day treatment with CDCA ($P < 0.01$) and did not change in the control experiment in all the subjects (Tab. 1).

During the treatment with CDCA, there was no difference in AUC of C4 concentrations on the day of the study between -203A and -203C allele carriers. Importantly, no effect of the genotype on the course of C4 concentrations was detected. During the treatment with cholestyramine, area under curve (AUC) of C4 was 40 % higher in -203C than that in -203A individuals but the difference was not statistically significant. Moreover, there was also no effect of genotype on the course of C4 concentrations.

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. Cholesterol AUC after cholestyramine treatment was also decreased by 7% in comparison with control examination, although the difference was not statistically significant (Tab. 11). Neither cholestyramine nor CDCA treatment did change concentration of BA. However, the AUC of BA was increased after CDCA treatment and lowered after Q treatment in carriers of both alleles (Tab. 1).

Tab. 1: Concentration of cholesterol, 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 (AA) or -203C (CC) individuals.

			-24 h	0 h	AUC [0-15 h]
Cholesterol [mmol/l]	**AA**	ctrl	4.69 ± 1.08	4.56 ± 1.10	65.7 ± 15.6
		Q	4.84 ± 0.95	4.89 ± 1.10	67.9 ± 15.0
		CDCA	4.88 ± 0.95	4.98 ± 1.09	70.2 ± 14.6
	CC	ctrl	4.70 ± 0.65	4.71 ± 0.62	68.0 ± 9.2
		Q	4.81 ± 0.44	4.44 ± 0.42***	63.2 ± 7.0
		CDCA	4.44 ± 0.75	4.63 ± 0.61	67.7 ± 19.4
BA [µmol/l]	**AA**	ctrl	14.6 ± 7.1	12.2 ± 3.0^{a,b}	197 ± 41^{a}
		Q	12.4 ± 4.5	9.0 ± 4.7^{b}	135 ± 61^{b}
		CDCA	15.3 ± 5.2	15.8 ± 3.0^{a}	290 ± 60^{a}
	CC	ctrl	12.7 ± 4.0	10.1 ± 3.8	170 ± 51^{a,b}
		Q	10.7 ± 3.2	10.4 ± 2.2	154 ± 23^{b}
		CDCA	11.6 ± 5.1	11.8 ± 3.4	219 ± 80^{a}
C4 [µg/l]	**AA**	ctrl	16.0 ± 9.1	20.3 ± 15.9^{a}	326 ± 94^{a}
		Q	13.9 ± 8.7	70.2 ± 27.3***$^{, b}$	1317 ± 312^{b}
		CDCA	19.9 ± 13.2	9.6 ± 9.6**^{,a}	131 ± 65^{a}
	CC	ctrl	22.9 ± 26.6	24.7 ± 23.1a	270 ± 168^{a}
		Q	28.2 ± 21.1	111.3 ± 70.1***^{,b}	1835 ± 800^{b}
		CDCA	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).

Although the AUC in control experiments also did not differ, plasma C4 concentration varied during the day in the -203A allele carriers (P =

0.001) with a marked peak around 13:00. No such a distinct peak could be observed in -203C homozygotes (Fig. 5). 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: $[C4] = a_0 + a_1*(\text{time-7}) + a_2*(\text{time-14,5})^2 + a_3*(\text{time-14,5})^3 + a_4*(\text{time-14,5})^4 + a_5*(\text{time-14,5})^5$. Using the same model, no such differences between the homozygous groups could be detected after both CDCA and cholestyramine treatment.

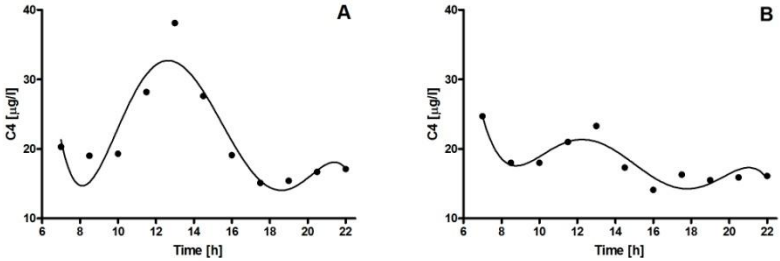

Fig. 5: Diurnal variation of C4 concentration in -203A and -203C homozygotes in control experiment. The dynamics of C4 concentration changes in -203A (Panel A) and -203C (Panel B) homozygotes.

4.3 Discussion

4.3.1 Comparison of promoter variants expression

The higher basal activity of pGL3_CT+ vectors is in perfect agreement with observations of others [50] who also found fivefold increase of transcriptional activity of -203C compared to -203A variant in HepG2 cells. Threefold higher expression 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 polymorphisms in -203 (rs3808607) and -469 (rs3824260) position in regulation of basal expression of *CYP7A1*. The substitution of C for A in -203 position resulted in five to twelvefold increase in *CYP7A1* basal expression whereas the substition of T for C led to no change or doubling expression. Therefore -203 position seems to play a critical role in modulation of basal promoter activity.

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 [51]. 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 an 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 allele.

4.3.2 The -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 (Tab. 1, Fig. 5).

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 [52]. 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 [52]. 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 bile acids and especially intestinal fibroblast growth factor-19 secretion after meal consumption are involved in dampening of CYP7A1 activity peak [53].

The diurnal variation of CYP7A1 activity and the *Cyp7a1* expression has been extensively studied in rodents. In mice, the *Cyp7a1* expression is under the control of several clock genes [54-56]. It remains to be determined whether clock genes also play a role in the regulation of bile acid 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 [52, 53] 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* [15]. 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 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.

The treatment with both cholestyramine and CDCA suppressed diurnal variation of CYP7A1 activity. Moreover, no effect of genotype on CYP7A1 activity was noted after both treatments.

Interestingly, cholestyramine treatment resulted in an immediate decrease in cholesterolemia in -203C homozygotes (Tab. 1) whereas cholesterol level 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. Nevertheless, such 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 [57].

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 the -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.

5. Conclusions

5.1 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.

5.2 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.

6. References

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7. Abbreviations

ABC	ATP-binding cassette
ACAT	acyl-CoA:cholesterol acyltransferase
ANOVA	analysis of variance
apo	apo(lipo)protein
AUC	area under curve
BA	bile acids
BMI	body mass index
C	control (diet)
C4	7α-hydroxy-4-cholesten-3-one
CA	cholic acid
CDCA	chenodeoxycholic acid
CE	cholesteryl ester
CETP	cholesteryl ester transfer protein
CF	control fat (diet)
CHOL	cholesterol (diet)
CoA	coenzyme A
ctrl	control (experiment)
CYP7A1	cholesterol 7α-hydroxylase
FC	free (unesterified) cholesterol
HDL	high density lipoprotein
HMG	3-hydroxy-3-methyl-glutaryl
HPLC	high-performance liquid chromatography
IDL	intermediate density lipoprotein
Insig	insulin induced gene
LDL	low density lipoprotein
LDL-C	cholesterol in LDL
LDLR	LDL receptor
LPL	lipoprotein lipase
LXR	liver X receptor
mRNA	messenger ribonucleic acid
MTP	microsomal TG transfer protein
PPAR	peroxisome proliferator-activated receptor
PHHC	Prague hereditary hypercholesterolemic
Q	Questran® (cholestyramine)
RNA	ribonucleic acid
qPCR	quantitative real-time polymerase chain reaction
VLDL	very low density lipoprotein
VLDL-C	cholesterol in VLDL
SREBP	sterol regulatory element binding protein
TG	triacylglycerol

8. List of publications

8.1 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

8.2 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

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