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Autoreferát disertační práce

Význam biosyntetické a katabolické dráhy cholesterolu u nádorových a zánětlivých onemocnění

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Disertační práce bude nejméně pět pracovních dnů před konáním obhajoby zveřejněna k nahlížení veřejnosti v tištěné podobě na Oddělení pro vědeckou činnost a zahraniční styky Děkanátu 1. lékařské fakulty.

ABSTRAKT

Předkládaná práce se zabývá významem meziproduktů biosyntetické a katabolické dráhy cholesterolu. Cílem první části práce bylo zjistit, zda statiny (inhibitory HMG-CoA reduktasy) mají protinádorový účinek a zda se účinky jednotlivých statinů liší. Druhá část práce je zaměřena především na využití 7α-hydroxycholest-4-en-3-onu (C4), nadějného markeru aktivity cholesterol 7α-monooxygenasy (CYP7A1) a malabsorpce žlučových kyselin.

Na experimentálním modelu adenokarcinomu pankreatu jsme potvrdili protinádorové účinky statinů. Ty se však mezi jednotlivými statiny v závislosti na jejich fyzikálně-chemických vlastnostech značně lišily. Naše data ukazují, že nejpravděpodobnějším (i když ne jediným) mechanismem protinádorového účinku statinů je snížení prenylace signalizačních proteinů, převážně Ras protoonkogenu.

Podařilo se nám zavést spolehlivou metodu stanovení C4 a s její pomocí prokázat, že -203A>C polymorfismus v genu kódujícím CYP7A1 může ovlivňovat její aktivitu, že za diurnální variabilitou aktivity CYP7A1 pravděpodobně stojí insulin a že insulinová rezistence u pacientů s nealkoholovou steatosou jater dochází k poškození zpětnovazebé inhibice CYP7A1, která může následně přispět k progresi onemocnění. Konečně jsme demonstrovali důležitost laboratorního stanovení malabsorpce žlučových kyselin u pacientů s Crohnovou chorobou.

ABSTRACT

This thesis focuses on the importance of intermediate products of biosynthetic and catabolic pathway of cholesterol. The aim of the first part of the thesis is mainly to investigate, whether statins (HMG-CoA reductase inhibitors) possess antitumor properties and to compare the differences in antitumor potential of individual statins.

The other part of the thesis aims at the utilization of 7α-hydroxycholest-4-en-3-one (C4), a promising marker of cholesterol 7α-monooxygenase (CYP7A1) activity and bile acid malabsorption.

We demonstrated antitumor effect of statins on an experimental model of pancreatic cancer. Individual statins, however, differed significantly in their efficacy, depending on their physico-chemical properties. Our data suggests, that the most likely (but not the only) mechanism of antitumor effect of statins is decreased prenylation of signaling proteins, especially Ras protooncogene.

We set up a reliable method for measurement of C4, which facilitated our research in CYP7A1 regulation. We demonstrated, that promoter polymorphism -203A>C might affect CYP7A1 activity, that diurnal variability of CYP7A1 activity might be triggered by insulin, and that insulin resistance in patients with non-alcoholic fatty liver disease impedes the feedback regulation of CYP7A1, which may lead to disease progression. Finally, we demonstrated the importance of laboratory investigation of bile acid malabsorption in Crohn's disease patients.

OBSAH

1. INTRODUCTION

1.1. Cholesterol biosynthetic pathway

1.1.1. Cholesterol biosynthesis

Cholesterol, a steroid molecule present in all mammalian cells is not only an important compound of biological membranes, but also serves a substrate for biosynthesis of steroid hormones, vitamin D and bile acids (BA). The cholesterol pool consists of ingested and synthesized cholesterol. Although all mammalian cells possess the enzymatic apparatus for its biosynthesis, in most tissues serum LDL (low density lipoprotein) cholesterol represses this synthetic pathway. The liver and the small intestine thus remain the main endogenous producers of cholesterol[1].

Cholesterol synthesis is initiated in cytoplasm, where 2 molecules of acetyl-CoA (coenzyme A) form 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA). In endoplasmic reticulum HMG-CoA reductase reduces HMG-CoA to form mevalonate, which gives rise to active isopren unit. Subsequent association of six isopren units leads to the formation of squalen and in several further steps to that of cholesterol.

1.1.2. Inhibition of a mevalonate pathway of cholesterol biosynthesis

The key regulatory enzyme of the pathway is HMG-CoA reductase. Its main endogenous down-regulators are cholesterol and glucagon, whereas insulin

is the activator. In 1970s, novel inhibitors of HMG-CoA reductase (statins) were isolated[2] and rapidly introduced in human medicine for treatment of hypercholesterolemia. Later on, it beacame evident, that they exert multiple effects on human organism, often independent on lowering of serum cholesterol levels[3]. One of the proposed mechanisms is depletion of intermediate products of the mevalonate pathway of cholesterol synthesis, namely farnesyl pyrophosphate (FPP) or geranygeranyl pyrophosphate (GGPP). Both FPP and GGPP are involved in post-translational modification of proteins. Farnesylation/geranylation is crucial for membrane associated proteins (such as growth hormones receptors, lamin A/B or ras and rho proteins), since the farnesyl/geranyl residue serves as a hydrophobic anchor, which detains the protein in the membrane[4].

1.1.3. Ras proteins

Ras proteins (also called $p21^{ras}$) belong to the most important transmission centers of growth and diferentiation signals. As a small regulatory GTPases interact in the active (*i.e.* GTP bound) form with intracellular efectors. Interaction (and signal transmission) is terminated by GTP hydrolysis. In cancer cells, mutations in the codon 12 of ras protein are often found. This mutation leads to a marked reduction of GTPase activity, resulting in protracted activation and thus in growth signal amplification[4]. Mutated *ras* genes belong to the most frequent oncogens in human tumors[5]. Statin-mediated ras inhibition thus seems to be the

mechanism of antiproliferative effects described both in experimental[6] and numerous clinical studies (for review see [7,8]). Currently, however, it is evident, that apart from ras protein inhibition, additional mechanisms play an important role.

1.2. Cholesterol catabolic pathway

1.2.1. Bile acid biosynthesis

Mammalian cells are not able to open the cyclopentaneperhydrophenantrene ring, the backbone of steroid molecules. The surplus of cholesterol is thus eliminated from human body via bile either intact or metabolized to BA. Those are formed exclusively in hepatocytes. The key regulatory step of BA fomation is cholesterol hydroxylation in position 7α, carried out by cholesterol 7α-monooxygenase (CYP7A1, 1.14.13.17). In subsequent reactions, 7α–hydroxycholesterol is converted into BA, conjugated with glycine or taurine and secreted into bile.

1.2.2. CYP7A1 regulation

The rate of BA synthesis is tightly regulated. The key regulatory enzyme in the pathway is CYP7A1. The main feedback inhibitors are BA, downregulating CYP7A1 activity via several mechanisms[9]. After binding to FXR (farnesoid X receptor) they activate SHP (small heterodimer partner), which inhibits *CYP7A1* transcription[10]. Pregnane X receptor (PXR) and fibroblast growth factor (FGF19) are additional known mediators of

BA-triggered feedback inhibition of CYP7A1[11,12]. The expression of human *CYP7A1*, unlike the murine homolog, is probably not regulated by serum cholesterol, due to the absence of a specific regulatory domain in the *CYP7A1* promoter[13]. Activity of CYP7A1 can be further modified by insulin (short-term activator, long-term inhibitor) or glucagon (inhibitor)[14,15].

1.2.3. Enterohepatic circulation of bile acids

Conjugated primary BA are secreted into small intestine, where contribute importantly to the absorption of ingested fat and hydrophobic substances. The vast majority of conjugated BA is efficiently reclaimed by ileal sodium/bile acid cotransporter in the distal ileum[16]. Resorbed BA are transported to the liver via portal vein, and resecreted into bile. Under normal circumstances, daily losses of BA do not exceed 500 mg. When the losses are much higher – typically after terminal ileum dysfunction due to resection, irradiation, inflammation, or other pathological conditions, bile acid malabsorption (BAM) occurs. High concentration of unresorbed BA in the colon can lead to diarrhoea[17] and formation of oxalate kidney stones[18] or pigment gallstones[19].

2. HYPOTHESES AND AIMS

2.1. Cholesterol biosynthetic pathway

Despite of intensive clinical investigation, the role of statins in the therapy of tumor diseases still remains unclear. Several large meta-analyses of epidemiologic

studies failed to prove anticancer effect of statin therapy[20-24]. However, the results of these meta-analyses should be interpreted with caution, since several important facts have been neglected. Among them, interpretation of pooled data not considering the type of tumor or statin used, involvement of studies focused primarily on cardiovascular but not anticancer outcomes, and short follow-up belong to the most serious omissions.

The aim of our study (see the article "**Differences in antitumor effects of various statins on human pancreatic cancer**" was therefore to compare anticancer effect of various statins on both in vitro and in vivo models of pancreatic cancer.

2.2. Cholesterol catabolic pathway

Complications related to BAM can be, to certain extent, eliminated or alleviated (*eg*. by administration of BA sequestrants). Determination of the BAM severity is a prerequisite for adequate therapy. Its availability is, however, limited. Current markers of BAM, such as determination of fecal output of BA[25], serum levels of 7α-hydroxycholesterol[26], lathosterol[27], breath tests with isotope-labeled cholylglycine[28] or cholyltaurine[29] or retention test with ^{75}Se homotaurocholic acid[30] are either extremely laborious, expensive, unreliable or even not approved for human use[31]. Since CYP7A1 mediated hydroxylation of cholesterol (key regulatory step of the BA synthesis) is negatively regulated by BA, determination of CYP7A1 activity might serve not only as a marker of BA synthesis but, importantly, as a marker of BAM. It has been demonstrated, serum levels of 7α-

hydroxycholest-4-en-3-one (C4) correlate with the rate of CYP7A1 activity[32-35] and severity of BAM[31,36]. Currently available methods for C4 determination are not suitable for routine use, due to problematic solid phase extraction (SPE) at 64°C[32,35] or the need of equipment unavailable in routine clinical laboratories[37].

The aim of our next study (see the article "**Improved HPLC analysis of serum 7α-hydroxycholest-4-en-3-one, a marker of bile acid malabsorption**") was therefore to set up and validate reliable method, which can be introduced to the clinical laboratories.

Patients with Crohn's disease suffer very often from BAM, however, its laboratory determination is still inadequate. The appropriate therapy is mostly offered only to patients after a resection of distal ileum, where BAM is expected. In our next study (see the article "**Bile acid malabsorption in inflammatory bowel disease: Assessment by serum markers**") we investigated, whether the BAM occurs also in surgically untreated patients. Additionally, we tested the possible role of FGF19 as a serum marker of BAM.

Despite of intensive research of the CYP7A1 regulation, we are still far from understanding this complex process. During the investigation of genetic determinants of CYP7A1 activity, two common polymorphisms (-203A>C and -469C>T) draw much attention. No agreement on their importance has been reached, due to conflicting results. Based on previous studies we hypothesized, that the CYP7A1 activity difference determined by these polymorphisms would be scanty (if any). Therefore, in our next study (see the

article **"CYP7A1 promoter polymorphism -203A>C affects bile salt synthesis rate in patients after ileal resection")** we examined a cohort of patients with BAM (in these patients, markedly upregulated CYP7A1 activity was expected).

The CYP7A1 activity shows significant diurnal variability, reaching its maximum soon after noon[38]. At this time, we would expect rather its decrease, caused by postprandial return of BA via portal vein. The aim of our next study (see the article "**Regulation of diurnal variation of cholesterol 7α-hydroxylase (CYP7A1) activity in healthy subjects**") was to monitor the diurnal variation of CYP7A1 activity in healthy volunteers and to identify possible responsible factors. To heighten the effect, the study has also been performed after enhancing (by administration of chenodeoxycholic acid) or repressing (by administration of cholestyramine) the enterohepatic circulation of BA.

In 2003 FGF19 was described as a novel regulator of BA synthesis[12]. Since then, its role in the regulation of liver lipid metabolism[38-40] and in sensitizing tissues to insulin has been demonstrated[41]. Increased lipid accumulation in hepatocytes underlies the non-alcoholic fatty liver disease (NAFLD) which can evolve into non-alcoholic steatohepatitis (NASH). It has been suggested, that the NAFLD to NASH progression could be triggered solely by lipid deposits in hepatocytes in the absence of other stimuli[42]. In our work (see the article "**The hepatic response to FGF19 is impaired in patients with non-alcoholic fatty liver disease and insulin resistance"**) we aimed at the role of FGF19 in regulation of metabolic processes in patients with NAFLD.

3. MATERIAL AND METHODS

C4 measurement
One mL of serum and 30 ng of internal standard (7β-hydroxycholest-4-en-3-one) were extracted by chloroform:methanol, purified on silica SPE precolumn and separated by high performance liquid chromatography (column: SGX C18, 4x250 mm, particles 4µm; mobile phase: acetonitrile:water (95:5,vol/vol), 1mL/min, 20°C; detection/reference wavelength: 241/360 nm).

FGF19 measurement
FGF19 was measured by a commercially available kit (FGF19 Quantikine ELISA kit, R&D Systems) according to the manufacturer instructions.

Genotyping of CYP7A1 -203 A>C
Genomic DNA was isolated from peripheral blood white cells by a standard salting-out method. The -203A>C polymorphism was genotyped by PCR- *Bsa*I restriction fragment length polymorphism. Primers were as follows: forward ATTAGCTATGCCCATCTTAAACAGG and reverse TAACTGGCCTTGAACTAAGTCCAC (5‘-3‘).

Cell cultures
Pancreatic cancer cell lines (CAPAN-2, MiaPaCa-2 and BxPc-3) were cultivated in appropriate media in a humidified atmosphere (5% CO2, 37°C). Methanol solutions of statins were added in the final concentration range of 0-40 µM.

Animal studies
Athymic nu/nu mice (strain CD-1) were subcutaneously xenotransplanted with human pancreatic adenocarcinoma cell line CAPAN-2 (10^7 cells). After 7-10 days of xenotransplantation, mice received either statins or placebo via gastric tube. The primary endpoint was survival time. Additionally, tumor sizes were measured every 3 days with a caliper.

Inflammatory bowel disease patients
According to the type of affection, patients were divided into following groups: ulcerative disease patients, Crohn's disease patients (CD) after moderate or extensive ileal resection, CD with ileal inflamation or CD with colonic involvement. Blood was drawn in the morning after overnight fast, serum aliquots were stored at -80°C until analyses.

NAFLD patients
According to the insulin resistance, patients were divided into two groups. After an oral fat challenge, serial blood samples were drawn (up to 7 hours after the challenge).

Control subjects (for analysis of CYP7A1 diurnal variation)
Healthy volunteers homozygous for -203 A>C variant were put on a standardized diet for 2 days. On day 1, one blood sample was drawn in the morning. On day 2, eleven blood samples were drawn in 90-min intervals. Two similar 2-day investigations were repeated after administration of either chenodeoxycholic acid (Chenofalk, 1-1.5 g/day) or cholestyramine (Questran, 16

g/day). The interval between investigations was at least 3 weeks.

4. RESULTS AND DISCUSSION

4.1. Cholesterol biosynthetic pathway

In the study "**Differences in antitumor effects of various statins on human pancreatic cancer**" we have demonstrated markedly different antitumor effect of individual statins. The best results in *in vitro* experiments have been achieved with cerivastatin, simvastatin and lovastatin, far exceeding wery veak effects of pravastatin and rosuvastatin.

The most potent antitumor substances in the *in vitro* experiments were fluvastatin, cerivastatin, and surprisingly rosuvastatin, which was nearly ineffective *in vitro*.

It is likely, the substantial difference in rosuvastatin efficiency can be explained by slow elinination of rosuvastatin – the half-life of rosuvastatin in human body is ten times higher when compared with other statins.

The discrepant antitumor potential of individual statins is, at least partially, caused by their different physico-chemical properties, affecting their pharmacokinetics. For example pravastatin, which showed a very weak effect, belongs to the most hydrophilic statins, due to the hydroxyl group in the position 3. Such molecules are not capable of passive transport across cell membranes and require specific

transporter to enter the cell[43]. Cancer cells lacking the transporter are not hit by pravastatin therapy.

BxPc-3 cell line (not carrying activation mutation in K-Ras protooncogene) responded to the statin therapy significantly less, than those with mutated K-Ras (CAPAN-2, MiaPaCa-2). Additionally, mevalonate, farnesyl pyrophosphate or geranylgeranyl pyrophosphate counteracts the antiproliferative effect of statins. Both observations are in accord with the hypothesis, that depletion of intermediate products of mevalonate pathway of cholesterol synthesis (with subsequent depletion of prenylated proteins – mainly K-Ras) is responsible for antiproliferative effects of statins.

4.2. Cholesterol catabolic pathway

In this part of our work we aimed primarilly at the study of cholesterol catabolic pathway: its function, regulation, interaction with other metabolic processes and at monitoring of its activity. We set up and validated a method of quantitative determination of C4 in biological fluids and tissues (see the article "**Improved HPLC analysis of serum 7α-hydroxycholest-4-en-3-one, a marker of bile acid malabsorption**"). By modification of the original SPE, we scaled up the linearity range more than 5 times, improved the accuracy and reproducibility, lowered the costs and above all simplified the extraction process. Introducing of this method to clinical laboratories should improve the diagnostics of BAM mainly in patients with chronic diarrhea and subsequently lead to more adequate therapy.

In our next study we investigated the BAM in CD patients (see the article "**Bile acid malabsorption in inflammatory bowel disease: Assessment by serum markers**"). As expected, BAM was quite common in CD patients and correlated well with the involvement of terminal ileum. Surprisingly, we observed quite high proportion of BAM in the patients without affection of distal ileum. On the other hand, approximately one third of patients after resection of distal ileum had normal C4 levels. Our results indicate that the BAM can not be reliably diagnosed based only on disease localization and should be measured in CD patients. When the laboratory test is not available, therapeutic attempt with BA sequestrant should be offered to symptomatic patients.

It is likely, that in the near future, laboratories not equipped with HPLC instrument (not capable of C4 determination) will use FGF19 as an alternative marker of BAM, as we demonstrated the close correlation between FGF19 and C4.

C4 not only serves as a marker of BAM, but primarily as a marker of CYP7A1 activity. In our next study (see the article **"*CYP7A1* promoter polymorphism -203A>C affects bile salt synthesis rate in patients after ileal resection"**) we did not observe any significant effect of *CYP7A1*-203A>C polymorphism on CYP7A1 activity under normal conditions. In patients with markedly upragulated CYP7A1 activity (with BAM), however, the effect of weaker -203A allele was evident. We suppose, that the functional reserve of CYP7A1 is high enough to override the effect of the polymorphism. In case of BAM, where the CYP7A1 activity probably reaches its maximum, the effect of

weaker promoter associated with -203A allele becomes apparent.

In the study "**Regulation of diurnal variation of cholesterol 7α-hydroxylase (CYP7A1) activity in healthy subjects**" we observed marked diurnal variability of CYP7A1 activity, with maxima soon after midday. Based on correlations of areas under the curve (AUC), we speculate, diurnal variability of CYP7A1 activity is driven by insulin. Some subject exhibited morning (fasting) maxima of CYP7A1 – the underlying mechanism is not clear.

Recent studies demonstrated the role of FGF19 in the regulation of lipid metabolism in hepatocytes[39-41]. Therefore we aimed at the role of FGF19 signalling in NAFLD patients (see the article"**The hepatic response to FGF19 is impaired in patients with non-alcoholic fatty liver disease and insulin resistance"**). Patients with NAFLD had elevated basal levels of triacylglyceroles (TAG) and BA, but not FGF19. Analysis of AUC_{TAG} revealed, that elevated TAG levels are caused by its slow hepatic uptake. Similar mechanism is likely to underlie elevated BA levels. After an oral fat challenge, serum BA and FGF19 reached its maxima after 1 and 2-3 hours, respectively. Next 2 hours later, we expected FGF19 mediated downregulation of CYP7A1. Surprisingly, this was observed only in insulin sensitive NAFLD patients, but not in insulin resistant ones. Patients with NAFLD thus have either defective or delayed response to FGF19. We can only speculate, the defect is localized at the level of FGF19 receptor (FGFR4).

5. CONCLUSIONS

Today, intermediate products of biochemical pathways in organism are not considered only as steps on the way to the final product, but rather as substances, that undertake multiple functions (sometimes essential).

In our work, we investigated the role of biosynthetic and catabolic cholesterol pathway. Most of the statins, inhibitors of mevalonate pathway of cholesterol synthesis, exhibited significant antitumor effects both in the *in vitro* and *in vivo* model of pancreatic cancer. However, we observed marked differences in antitumor effects of individual statins. This emphasizes the necessity of precise design of clinical studies and meta-analyses, that aim at assessing antitumor potential of statins.

In the catabolic pathway of cholesterol, C4 represents a reliable marker of CYP7A1 activity and the severity of BAM. Owing to our improved analytical method, C4 measurement can now be easily performed in most clinical laboratories. This method facilitated our research both in BAM and in CYP7A1 regulation. We found out, the severity of BAM can not be reliably estimated from the clinical findings and should be measured. We demonstrated, the -203A allele in the *CYP7A1* promotor is associated with lower activity of CYP7A1. Due to the substantial functional reserve, the effect of this allele manifests only in markedly upregulated CYP7A1 activity. Additionally, we demonstrated the role of insulin in CP7A1 regulation. Surprisingly, we observed for the first time the fasting maxima of CYP7A1. The

underlying mechanism is, however, still unclear.

In the last study, we observed defective FGF19 signalization in insulin resistant patients with NAFLD. We suppose, this defect can deregulate the lipid metabolism in hepatocytes and accelerate the disease progression.

6. REFERENCES

1. Mayes,P.A. Harperova biochemie . Murray,K.R., Granner,D.K., Mayes,P.A. & Rodwell,V.W. (eds.), pp. 279-2911998).
2. Endo,A., Kuroda,M. & Tsujita,Y. ML-236A, ML-236B, and ML-236C, new inhibitors of cholesterogenesis produced by Penicillium citrinium. *J Antibiot. (Tokyo)* 29, 1346-1348 (1976).
3. Bellosta,S., Ferri,N., Bernini,F., Paoletti,R. & Corsini,A. Non-lipid-related effects of statins. *Ann Med* 32, 164-176 (2000).
4. Krauss,G. Biochemistry of Signal Transduction and Regulation., pp. 324-349 (WILEY-VCH,2001).
5. Barbacid,M. ras genes. *Annu Rev Biochem* 56, 779-827 (1987).
6. Maltese,W.A., Defendini,R., Green,R.A., Sheridan,K.M. & Donley,D.K. Suppression of murine neuroblastoma growth in vivo by mevinolin, a competitive inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A reductase. *J Clin Invest* 76, 1748-1754 (1985).
7. Katz,M.S. Therapy insight: Potential of statins for cancer chemoprevention and therapy. *Nat Clin Pract. Oncol* 2, 82-89 (2005).
8. Vitek,L. & Lenicek,M. Cytoprotective and Antiproliferative effects of HMG-CoA reductase inhibitors. *Current Enzyme Inhibition* 2, 261-280 (2006).
9. Ramirez,M.I. *et al.* Cholesterol and bile acids regulate cholesterol 7 alpha-hydroxylase expression at the

transcriptional level in culture and in transgenic mice. *Mol Cell Biol* 14, 2809-2821 (1994).
10. Goodwin,B. *et al.* A Regulatory Cascade of the Nuclear Receptors FXR, SHP-1, and LRH-1 Represses Bile Acid Biosynthesis. *Molecular Cell* 6, 517-526 (2000).
11. Wang,L. *et al.* Redundant Pathways for Negative Feedback Regulation of Bile Acid Production. *Developmental Cell* 2, 721-731 (2002).
12. Holt,J.A. *et al.* Definition of a novel growth factor-dependent signal cascade for the suppression of bile acid biosynthesis. *Genes Dev.* 17, 1581-1591 (2003).
13. Agellon,L.B., Drover,V.A.B., Cheema,S.K., Gbaguidi,G.F. & Walsh,A. Dietary Cholesterol Fails to Stimulate the Human Cholesterol 7alpha -Hydroxylase Gene (CYP7A1) in Transgenic Mice. *J. Biol. Chem.* 277, 20131-20134 (2002).
14. Li,T. *et al.* Insulin regulation of cholesterol 7alpha-hydroxylase expression in human hepatocytes: roles of forkhead box O1 and sterol regulatory element-binding protein 1c. *J Biol Chem* 281, 28745-28754 (2006).
15. De Fabiani,E. *et al.* Coordinated control of cholesterol catabolism to bile acids and of gluconeogenesis via a novel mechanism of transcription regulation linked to the fasted-to-fed cycle. *J Biol Chem* 278, 39124-39132 (2003).
16. Craddock,A.L. *et al.* Expression and transport properties of the human ileal and renal sodium-dependent bile acid transporter. *Am J Physiol* 274, G157-G169 (1998).
17. Carey,M.C. & Duane,W.C. The Liver: Biology and Pathobiology. Arias,I.M. *et al.* (eds.), pp. 719-767 (Raven Press, Ltd., New York,1994).
18. Chadwick,V.S., Modha,K. & Dowling,R.H. Mechanism for hyperoxaluria in patients with ileal dysfunction. *N Engl J Med* 289, 172-176 (1973).
19. Vitek,L. & Carey,M.C. Enterohepatic cycling of bilirubin as a cause of 'black' pigment gallstones in adult life. *Eur J Clin Invest* 33, 799-810 (2003).
20. Bjerre,L.M. & LeLorier,J. Do statins cause cancer? A meta-analysis of large randomized clinical trials. *Am J Med* 110, 716-723 (2001).

21. Dale,K.M., Coleman,C.I., Henyan,N.N., Kluger,J. & White,C.M. Statins and cancer risk: a meta-analysis. *JAMA* 295, 74-80 (2006).
22. Hebert,P.R., Gaziano,J.M., Chan,K.S. & Hennekens,C.H. Cholesterol lowering with statin drugs, risk of stroke, and total mortality. An overview of randomized trials. *JAMA* 278, 313-321 (1997).
23. . MRC/BHF Heart Protection Study of cholesterol lowering with simvastatin in 20,536 high-risk individuals: a randomised placebo-controlled trial. *The Lancet* 360, 7-22 (2002).
24. Randomised trial of cholesterol lowering in 4444 patients with coronary heart disease: the Scandinavian Simvastatin Survival Study (4S). *The Lancet* 344, 1383-1389 (1994).
25. Otte,J.J. & Andersen,J.R. The clinical value of faecal bile acid determination in patients with chronic diarrhoea of unknown origin. *Scand. J Gastroenterol.* 21, 585-588 (1986).
26. Yoshida,T. *et al.* Simultaneous determination of mevalonate and 7 alpha- hydroxycholesterol in human plasma by gas chromatography-mass spectrometry as indices of cholesterol and bile acid biosynthesis. *J. Chromatogr.* 613, 185-193 (1993).
27. Farkkila,M.A., Kairemo,K.J., Taavitsainen,M.J., Strandberg,T.A. & Miettinen,T.A. Plasma lathosterol as a screening test for bile acid malabsorption due to ileal resection: correlation with 75SeHCAT test and faecal bile acid excretion. *Clin. Sci. (Lond)* 90, 315-319 (1996).
28. Roda,A. *et al.* Determination of 14CO2 in breath and 14C in stool after oral administration of cholyl-1-[14C]glycine: clinical application. *Clin. Chem.* 23, 2127-2132 (1977).
29. Fromm,H. & Hofmann,A.F. Breath test for altered bile-acid metabolism. *The Lancet* 2, 621-625 (1971).
30. Boyd,G.S., Merrick,M.V., Monks,R. & Thomas,I.L. Se-75-labeled bile acid analogs, new radiopharmaceuticals for investigating the enterohepatic circulation. *J Nucl. Med* 22, 720-725 (1981).
31. Sauter,G.H., Munzing,W., Ritter,C.V. & Paumgartner,G. Bile Acid Malabsorption as a Cause of Chronic Diarrhea

Diagnostic Value of 7+--Hydroxy-4-Cholesten-3-One in Serum. *Digestive Diseases and Sciences* 44, 14-19 (1999).
32. Axelson,M., Aly,A. & Sjovall,J. Levels of 7[alpha]-hydroxy-4-cholesten-3-one in plasma reflect rates of bile acid synthesis in man. *FEBS Letters* 239, 324-328 (1988).
33. Axelson,M., Bjorkhem,I., Reihner,E. & Einarsson,K. The plasma level of 7[alpha]-hydroxy-4-cholesten-3-one reflects the activity of hepatic cholesterol 7[alpha]-hydroxylase in man. *FEBS Letters* 284, 216-218 (1991).
34. Honda,A. *et al.* Significance of plasma 7alpha-hydroxy-4-cholesten-3-one and 27-hydroxycholesterol concentrations as markers for hepatic bile acid synthesis in cholesterol-fed rabbits. *Metabolism* 53, 42-48 (2004).
35. Galman,C., Arvidsson,I., Angelin,B. & Rudling,M. Monitoring hepatic cholesterol 7alpha-hydroxylase activity by assay of the stable bile acid intermediate 7alpha-hydroxy-4-cholesten-3-one in peripheral blood. *J Lipid Res* 44, 859-866 (2003).
36. Eusufzai,S., Axelson,M., Angelin,B. & Einarsson,K. Serum 7 alpha-hydroxy-4-cholesten-3-one concentrations in the evaluation of bile acid malabsorption in patients with diarrhoea: correlation to SeHCAT test. *Gut* 34, 698-701 (1993).
37. Honda,A. *et al.* Highly sensitive quantification of 7{alpha}-hydroxy-4-cholesten-3-one in human serum by LC-ESI-MS/MS. *J. Lipid Res.* 48, 458-464 (2007).
38. Jones,S. Mini-review: endocrine actions of fibroblast growth factor 19. *Mol Pharm.* 5, 42-48 (2008).
39. Tomlinson,E. *et al.* Transgenic mice expressing human fibroblast growth factor-19 display increased metabolic rate and decreased adiposity. *Endocrinology* 143, 1741-1747 (2002).
40. Bhatnagar,S., Damron,H.A. & Hillgartner,F.B. Fibroblast growth factor-19, a novel factor that inhibits hepatic fatty acid synthesis. *J Biol Chem* 284, 10023-10033 (2009).
41. Fu,L. *et al.* Fibroblast growth factor 19 increases metabolic rate and reverses dietary and leptin-deficient diabetes. *Endocrinology* 145, 2594-2603 (2004).

42. Gentile,C.L. & Pagliassotti,M.J. The role of fatty acids in the development and progression of nonalcoholic fatty liver disease. *J Nutr. Biochem.* 19, 567-576 (2008).
43. Bellosta,S., Paoletti,R. & Corsini,A. Safety of statins: focus on clinical pharmacokinetics and drug interactions. *Circulation* 109, III50-III57 (2004).

7. LIST OF PUBLISHED PAPERS

Related to thesis

With impact factor

1) Leníček M, Ďuricová D, Komárek V, Gabryšová B, Lukáš M, Šmerhovský Z, Vítek L. Bile Acid Malabsorption in Inflammatory Bowel Disease: Assessment by Serum Markers. Inflamm Bowel Dis. 2010 Accepted. **IF 4.643**
2) Kovář J, Leníček M, Zimolová M, Vítek L, Jirsa M, Piťha J. Regulation of diurnal variation of cholesterol 7alpha-hydroxylase (CYP7A1) activity in healthy subjects. Physiol Res, 59(2): 233-238, 2010. **IF 1.430**
3) Schreuder TC, Marsman HA, Leníček M, van Werven JR, Nederveen AJ, Jansen PL, Schaap FG. The hepatic response to FGF19 is impaired in patients with non-alcoholic fatty liver disease and insulin resistance. Am J Physiol – Gastr L, 298(3):G440-445, 2010. **IF 3.258**
4) 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, 49 (12): 2664-2667, 2008. **IF 4.409**
5) Leníček M, Juklová M, Zelenka J, Kovář J, Lukáš M, Bortlík M, Vítek L. Improved HPLC analysis of serum 7α-hydroxycholest-4-en-3-one, a marker of bile acid malabsorption. Clin Chem, 54 (6): 1087-1088, 2008. **IF 5.579**
6) Gbelcová H, Leníček M, Zelenka J, Knejzlík Z, Dvořáková G, Zadinová M, Poučková P, Kudla M, Baláž P, Ruml T, Vítek L. Differences in antitumor effects of various statins on human pancreatic cancer. Int J Cancer, 122(6): 1214-21, 2008.**IF 4.734**

Without impact factor

1) Gbelcová H, Leníček M, Zelenka J, Krechler T, Knejzlík Z, Dvořáková G, Zadinová M, Poučková P, Ruml T, Vítek L. Protinádorové účinky statinů na experimentálním modelu adenokarcinomu pankreatu. Čes a Slov Gastroent a Hepatol, 61(2): 75-81, 2007.
2) Leníček M, Lukáš M, Jirsa M, Kovář J, Donoval R, Bortlík M, Vítek L. Určení stupně malabsorpce žlučových kyselin a vliv polymorfismů v promotoru genu CYP7A1. Klin Biochem Metab, 14(35): 221-224, 2006.
3) Vítek L, Leníček M. Cytoprotective and antiproliferative effects of HMG-CoA Reductase Inhibitors. Current Enzyme Inhibition, 2(3): 261-280, 2006.
4) Leníček M, Lukáš M, Dudková L, Adamec S, Vítek L. Vztah malabsorpce žlučových kyselin a rizika cholelitiázy u pacientů s Crohnovou chorobou. Čes a Slov Gastroent a Hepatol, 60(2): 73-76, 2006.

Not related to thesis

With impact factor

1) Hradský O, Dušátková P, Leníček M, Bronský J, Ďuricová D, Nevoral J, Vítek L, Lukáš M, Cinek O. Two independent genetic factors responsible for the associations of the IBD5 locus with Crohn's disease in the Czech population. Inflamm Bowel Dis, 2010 Accepted. **IF 4.643**
2) Meggyesi N, Kiss LS, Koszarska M, Bortlik M, Ďuricová D, Lakatos L, Molnar T, Leníček M, Vítek L, Altorjay I, Papp M, Tulassay Z, Miheller P, Papp J, Tordai A, Andrikovics H, Lukáš M, Lakatos PL. NKX2-3 rs10883365 is associated with disease susceptibility to both Crohn's disease and ulcerative colitis while IRGM rs13361189 variant allele increased the risk for Crohn's disease in Eastern European patients. World J Gastroentero, 2010. Accepted. **IF 2.092**

3) Muchová L, Váňová K, Zelenka J, Leníček M, Petr T, Vejražka M, Sticová E, Vreman HJ, Wong RJ, Vítek L. Bile Acids Decrease Intracellular Bilirubin Levels in the Cholestatic Liver: Implications for Bile Acid-Mediated Oxidative Stress. J Cell Mol Med, 2010 May 28. [Epub ahead of print]. **IF 5.228**
4) Pedersen N, Ďuricová D, Leníček M, Elkjaer M, Bortlík M, Andersen PS, Vítek L, Davidsen B, Vewer V, Lukaš M, Munkholm P. Infliximab Dependency is Related to Decreased Surgical Rates in Adult Crohn´s Disease Patients. Eur J Gastroenterol Hepatol, 22(10):1196-1203, 2010. **IF 1.662**
5) Hradský O, Dušátková P, Leníček M, Bronský J, Nevoral J, Vítek L, Lukáš M, Ženíšková I, Cinek O. The CTLA4 variants may interact with the IL23R- and NOD2-conferred risk in development of Crohn's disease. BMC Med Genet, 11:91-99, 2010. **IF 2.840**
6) Vítek L, Novotná M, Leníček M, Novotný L, Eberová J, Petrášek J, Jirsa M. Serum bilirubin levels and UGT1A1 promoter variations in patients with schizophrenia. Psychiatry Research, 178: 449-450, 2010. **IF 2.373**
7) Jirásková A, Leníček M, Vítek L. Simultaneous genotyping of microsatellite variations in HMOX1 and UGT1A1 genes using multicolored capillary electrophoresis. Clin Biochem, 43(7-8): 697-699, 2010. **IF 2.019**
8) Dušátková P, Hradský O, Leníček M, Bronský J, Nevoral J, Kotalová R, Bajerová K, Vítek L, Lukáš M, Cinek O. Association of IL23R p.381Gln and ATG16L1 p.197Ala with Crohn's disease in the Czech population. J Pediatr Gastr Nutr, 49(4):405-410, 2009. **IF 2.183**
9) Ďuricová D, Pedersen N, Leníček M, Hradský O, Bronský J, Adamcová M, Elkjaer M, Andersen PS, Vítek L, Larsen K, Lukáš M, Nevoral J, Wewer V, Munkholm P. Infliximab Dependency in Children with Crohn´s Disease. Aliment Pharm Ther, 29(7):792-799, 2009. **IF 4.357**
10) Reiterová J, Obeidová H, Leníček M, Stekrová J, Merta M, Maixnerová D, Vítek L, Vicklický O, Tesař V. Influence of VEGF Polymorphism on Progression of Autosomal Dominant Polycystic Kidney Disease. Kidney Blood Press Res, 31(6):398-403, 2008. **IF 1.268**

11) Hradský O, Leníček M, Dušátková P, Bronský J, Nevoral J, Valtrová V, Kotalová R, Szitanyi P, Petro R, Starzyková V, Bortlík M, Vítek L, Lukáš M, Cinek O. Variants of *CARD15*, *TNFA* and *PTPN22* and susceptibility to Crohn's disease in the Czech population: high frequency of the *CARD15* 1007fs. Tissue Antigens, 71(6): 538-547, 2008. **IF 2.076**
12) Zelenka J, Leníček M, Muchová L, Jirsa M, Kudla M, Balaž P, Zadinová M, Ostrow JD, Wong RJ, Vítek L. Highly sensitive method for quantitative determination of bilirubin in biological fluids and tissues. J Chromatogr B, 867(1): 37-42, 2008.**IF 2.500**
13) Brůha R, Vítek L, Petrtýl J, Leníček M, Urbánek P, Zelenka J, Jáchymová M, Švestka T, Kaláb M, Douša M, Mareček Z. Effect of carvedilol on portal hypertension depends on the degree of endothelial activation and inflammatory changes. Scand J Gastroenterol, 41(12): 1454-63, 2006. **IF 1.869**

Without impact factor

1) Vítek L, Leníček M, Zelenka J, Kalousová M, Staňková B, Novotný L, Schreib P, Zima T, Žák A. Vztah mezi dietním příjmem alkoholu a některými metabolickými a kardiovaskulárními rizikovými faktory u zdravých mužů. Čas Lék Čes, 146(4): 367-373, 2007.
2) Brůha R, Petrtýl J, Vítek L, Leníček M, Urbánek P, Zelenka J, Jáchymová M, Švestka T, Kaláb M, Douša M, Mareček Z. Carvedilol v léčbě portální hypertenze-účinek závisí na výši aktivace zánětlivých parametrů a hladinách adhezních molekul. Čes a Slov Gastroent a Hepatol, 60(1): 31-35, 2006.
3) Leníček M, Muchová L, Vítek L. Nukleární faktor kappa B v léčbě zánětlivých a nádorových onemocnění. Čas Lék Čes, 143(10): 680-684, 2004.
4) Muchová L, Kráslová I, Leníček M, Vítek L. Gilbertův syndrom-mýty a realita. Čas Lék Čes, 143(6): 375-380, 2004.