Charles University
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Dynamics of changes in bile acid metabolomics in estrogen-induced cholestasis

Dynamika změn metabolomiky žlučových kyselin u cholestázy indukované estrogeny

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Souhrn

Dynamika změn metabolomiky žlučových kyselin u cholestázy indukované estrogeny

Žlučové kyseliny jsou důležitá endobiotika s množstvím exokrinních a endokrinních funkcí. V této disertační práci se věnujeme vyhodnocení třech faktorů, u kterých bylo podezření, že modifikují metabolomiku žlučových kyselin: a) vlivu metforminu u estrogeny indukované cholestázy, b) roli MRP2 (multidrug resistance-associated protein 2) proteinu v popsaném riziku častější intrahepatální cholestázy v těhotenství, c) popisu změn v důsledku excesivní kumulace železa v játrech.

Metformin byl testován s ohledem na jeho potenciální použití u žen s těhotenskou cukrovkou (GDM), u kterých je popisována vyšší incidence intrahepatální cholestázy (ICP). Jako model ICP jsme využili experimentální cholestázu navozenou u myší podáním ethinylestradiolu. Podání metforminu za této situace výrazně zvýšilo koncentrace žlučových kyselin v systémové cirkulaci, které dosáhly hodnot považovaných u těhotných žen za výrazně toxické. Tato data poukazují na zvýšenou pravděpodobnost rozvoje ICP při použití metforminu u těhotných žen.

V další studii jsme prokázali, že MRP2 transportér hraje významnou roli v biliární eliminaci žlučových kyselin a samotný genetický defekt způsobil zvyšování jejich plazmatických koncentrací u potkanů. Aplikace estrogenu MRP2 negativním potkanům vedla k mnohem výraznějšímu zvýšení plazmatických koncentrací žlučových kyselin, než jaké bylo detekováno u estrogenní cholestázy u kontrolních zvířat. Naše experimentální data tak poprvé vysvětlila mechanismy častějšího výskytu ICP u těhotných s deficitem MRP2 transportéru.

Třetím studovaným faktorem byl vliv nadbytku železa v játrech na metabolomiku žlučových kyselin. Použitým modelem byla opakovaná parenterální aplikace železa u potkanů. Následná analýza odhalila u těchto zvířat výrazně sníženou biliární sekreci žlučových kyselin v důsledku poklesu jejich syntézy redukovaným CYP7A1 enzymem a současně byla snížená jaterní exprese hlavních eliminačních transportérů, NTCP, BSEP a MRP2. Výraznější systémové kumulaci zabránilo snížení reabsorpce žlučových kyselin v ileu. Tento efekt byl pravděpodobně způsoben zvýšenou syntézou málo absorbovatelné hyodeoxycholové kyseliny změněnou bakteriální mikroflórou střeva. V této studii se tak podařilo detailně popsat změny metabolomiky žlučových kyselin vlivem kumulace železa. Tyto změny mohou přispívat k rozvíjejícímu se poškození jater, které provází ukládání železa v tomto orgánu.

Summary

Dynamics of changes in bile acid metabolomics in estrogen-induced cholestasis

Bile acids are essential endobiotics with numerous exocrine and endocrine functions. In this dissertation, we evaluate three factors that were suspected of modifying bile acid metabolomics: i) the effect of metformin in estrogen-induced cholestasis, ii) the role of MRP2 (multidrug resistance-associated protein 2) protein in the described risk of more frequent intrahepatic cholestasis in pregnancy, and iii) the excessive iron accumulation in the liver.

Metformin has been tested for its potential use in women with gestational diabetes mellitus (GDM) who have a higher incidence of intrahepatic cholestasis (ICP). As an ICP model, we used experimental cholestasis induced in mice by administration of ethinylestradiol. Administration of metformin in this situation significantly increased bile acid concentrations in the systemic circulation, which reached values considered significantly toxic in pregnant women. These data suggest that the possibility of developing ICP is accentuated when metformin is used in pregnant women.

In another study, we demonstrated that the MRP2 transporter plays a significant role in biliary bile acid elimination and that the genetic defect itself caused an increase in the plasma concentrations in rats. Estrogen administration to MRP2-negative rats resulted in a much more pronounced increase in bile acid plasma concentrations than was detected in estrogen cholestasis in control animals. Thus, for the first time, our experimental data explained the mechanisms of the more frequent occurrence of ICP in pregnant women with MRP2 transporter deficiency.

The third factor studied was the effect of excess iron in the liver on bile acid metabolomics. The model used was repeated parenteral iron administration in rats. Subsequent analysis revealed significantly reduced biliary bile acid secretion in these animals due to decreased synthesis by the reduced CYP7A1 enzyme and the hepatic expression of the crucial elimination transporters NTCP, BSEP, and MRP2. Significant systemic accumulation of bile acid was prevented by reducing reabsorption in the ileum. This effect was probably due to the increased synthesis of poorly absorbable hyodeoxycholic acid via the altered intestinal microbiome. In this study, it was possible to describe the changes in the bile acid metabolomics due to iron accumulation. These changes may contribute to the developing liver damage that accompanies iron storage in this organ.

1. Introduction

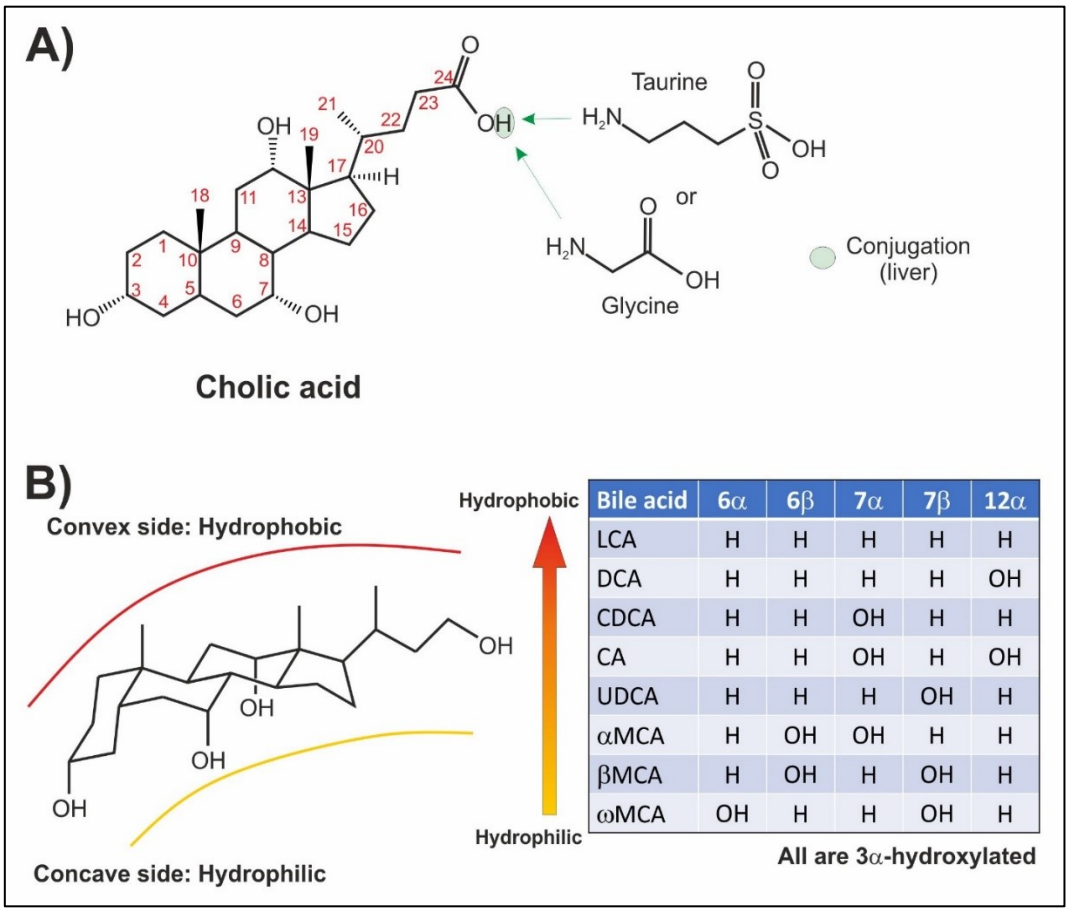
1.1. Bile acids

Bile acids play a crucial role in absorbing nutrients from the gut. They are major osmotic constituents of bile, enabling the formation of micelles and excretion of cholesterol and another lipid-soluble endo and xenobiotics from hepatocytes to the duodenum. Additionally, bile acids function also as nutrient signaling molecules that modulate lipid, and glucose homeostasis and energy balance in the organism (di Gregorio et al., 2021).These functions are enabled by the binding of bile acids to several nuclear receptors regulating intermediary metabolism such as farnesoid X receptor (FXR), vitamin D_3 receptor (VDR), constitutive androstane receptor (CAR), and pregnane X receptor (PXR), as well as the membrane G protein-coupled receptors (GPCR), Takeda G protein receptor 5 (TGR5) and sphingosine-1-phosphate receptor 2 (S1PR2). Relative to these mechanisms, bile acids, bile acid derivatives, and bile acid sequestrants proved beneficial effects in chronic liver disease, obesity, and diabetes in humans (Chiang, 2013; Choudhuri and Klaassen, 2022). On the other hand, as efficient detergents, bile acids can be highly toxic if accumulated at supraphysiological concentrations. Disorders in bile acid turnover cause cholestasis, dyslipidemia, fatty liver diseases, and cardiovascular diseases. Therefore, the content of bile acids in the organism is tightly regulated to maintain the relatively high concentrations in the biliary system and intestine and low and stable concentrations in the liver and the systemic circulation. This task is accomplished by a complex and integrated network of enzymes that synthesize bile acids from cholesterol in hepatocytes and convert them by intestinal bacteria. In addition, transporters in the liver, gallbladder and intestine ensuring that majority of bile acids recirculate between the liver and intestine via bile and portal blood. Such a complicated pathway is sensitive to numerous stimuli, but principal one is the feedback autoregulation by bile acids themselves which stimulate FXR receptor to limit synthesis and enhance biliary secretion, and subsequent fecal excretion. Taken together, it is of great clinical value to understand all aspects of bile acids homeostasis to prevent and possibly treat numerous metabolic disorders.

1.1.1. Chemistry and function of bile acids

Bile acids are saturated amphipathic molecules, meaning that they have both polar and nonpolar regions, which provide both hydrophilic and lipophilic properties. Bile acids are hydroxylated steroid carboxylic acids consisting of 24 carbons (C24) synthetized from cholesterol (C27) (Figure 1A). Several essential bile acids can be identified based on hydroxyl groups conformation. There are two primary bile acids synthetized in humans: cholic acid (CA), which is a 3α,7α,12α-trihydroxy bile acid, and chenodeoxycholic acid (CDCA), which is a 3α,7α-dihydroxy bile acid (Figure 1). CA and CDCA are then conjugated to either glycine or taurine (Figure 1), resulting in glycocholic (GCA), taurocholic (TCA) acids and glycochenodeoxycholic (GCDCA), taurochenodeoxycholic (TCDCA) acids respectively.

Figure 1: The general molecular structure of bile acids (BAs) and their amino acid conjugates. The hydroxyl groups can be in two configurations (A): either up (or out), termed beta (β; often drawn by convention as a solid line), or down, termed alpha (α; displayed as a dashed line). (B) Chair representation of the general molecular structure of bile acids. Brackets indicate the hydrophobic (convex) and hydrophilic (concave) faces. Table presents position of hydroxyl groups in the individual bile acids (di Gregorio et al., 2021).



Bile acid	6α	6β	7α	7β	12α
LCA	H	H	H	H	H
DCA	H	H	H	H	OH
CDCA	H	H	OH	H	H
CA	H	H	OH	H	OH
UDCA	H	H	H	OH	H
αMCA	H	OH	OH	H	H
βMCA	H	OH	H	OH	H
ωMCA	OH	H	H	OH	H

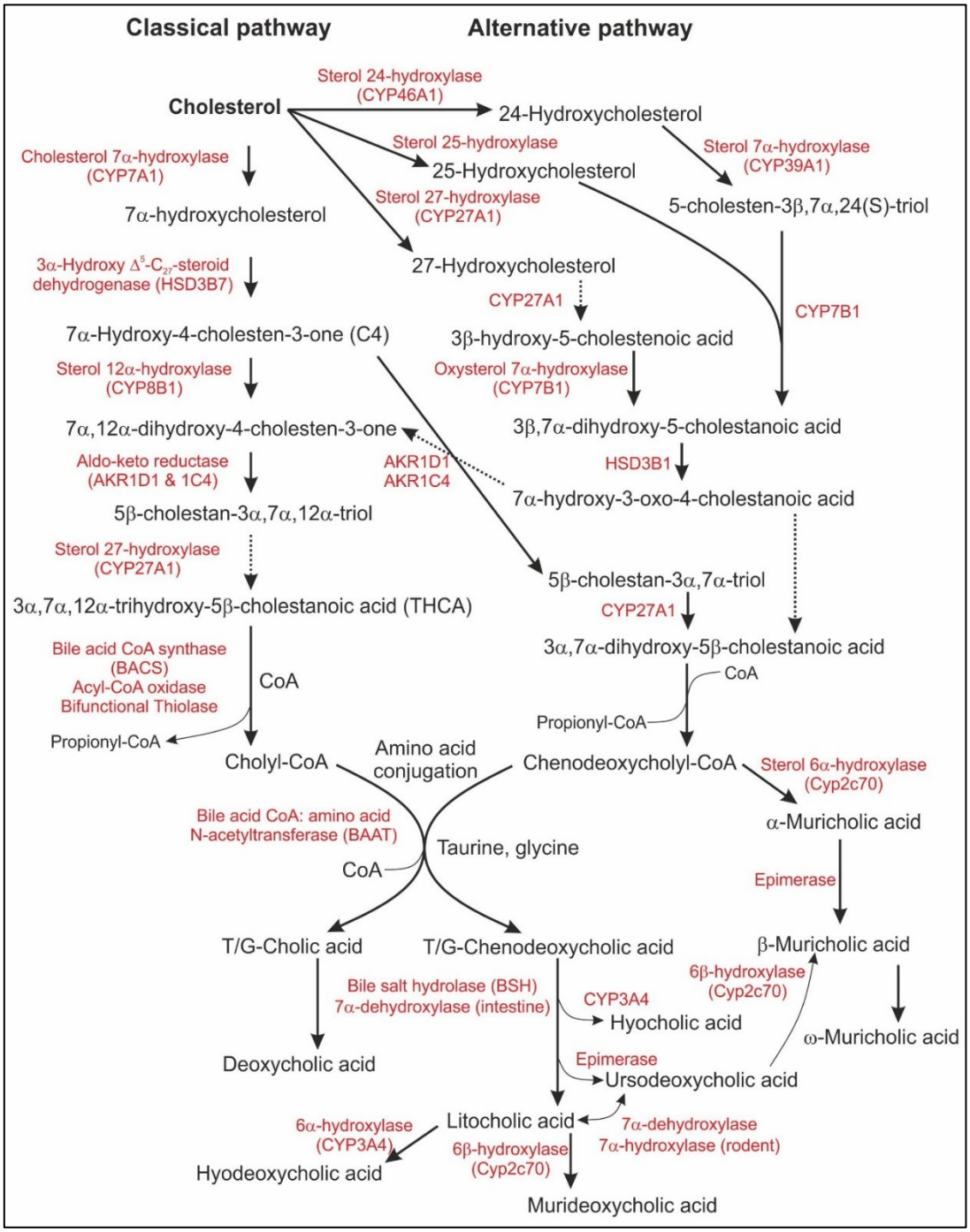
Primary bile acids produced by mice include CA, CDCA, and α-muricholic acid (α-MCA, 3α,6β,7α -trihydroxy) and β-MCA (3α,6β,7β- trihydroxy). The α-MCA and β-MCA are both synthesized from CDCA; both molecules are 6β-hydroxylated referred to as 6-OH bile acids (Figure 1). Primary bile acids are converted into secondary bile acids by the action of bacteria in the intestine. Deoxycholic acid (DCA) is formed from CA and lithocholic acid (LCA) from CDCA by 7α-dehydroxylation. Furthermore, ursodeoxycholic acid (UDCA) is formed from CDCA by epimerization of the 7α-OH of CDCA to the 7β-OH.

1.1.2. Bile acid synthesis

The liver is the only organ in the body that contains all the enzymes required for bile acids synthesis from cholesterol. The highest capacity of bile acids production is in the

perivenous (centrilobular) hepatocytes of liver lobules surrounding the central vein. The rate of bile acids synthesis is a determinant of cholesterol homeostasis since it is the main pathway for its catabolism. Multiple enzymes are involved in this complex process in various cellular compartments, such as the cytosol, endoplasmic reticulum, mitochondria, and peroxisomes (Figure 2).

Figure 2: Bile acid biosynthesis pathways. Both the classical and the alternative pathways are shown. Adapted from (Choudhuri and Klaassen, 2022).



There are two major pathways responsible for bile acids synthesis: the classic (neutral) pathway and the alternative (acidic) pathway (Figure 2). Mice and humans produce most of their bile acids through the classic pathway (Russell, 2003), which starts with the hydroxylation of steroid ring in endoplasmic reticulum, followed by dehydrogenation, reduction, isomerization and ends up by oxidation and cleavage of side chain. The alternative pathway, on the other hand, is characterized by a first hydroxylation of the cholesterol side chain, and then by a 7α-hydroxylation of the sterol nucleus (Choudhuri and Klaassen, 2022).

1.1.3. Conjugation of bile acids and conversions

A terminal step in the synthesis of bile acids involves adding an amino acid, usually glycine or taurine, via an amide linkage to the C_{24} (Figure1). In addition to decreasing toxicity, conjugation of bile acids enhances their ionization, amphipathic properties, and solubility in water. The reaction is started by Bile Acid-CoA Synthase (BACS; gene symbol *SLC27A5*) which catalyzes activation of unconjugated bile acids with coenzyme A, resulting in bile acid-coenzyme A thioesters (BACO-SCoA). After this reaction, BACO-SCoA reacts with taurine or glycine, obtaining the conjugated bile acids. This reaction is catalyzed by the cytosolic Bile Acid-CoA–amino acid N-acyltransferase (BAAT). Conjugation is a highly efficient reaction and only very low concentration of unconjugated bile acids can be detected in bile. Notably, although bile acids are primarily conjugated with taurine and glycine in all mammals, there exists remarkable species variation due to the species-specific affinities of the BAAT enzyme for taurine and glycine. These differences explain high proportion of taurine-conjugated bile acids in mice while glycine conjugates dominate in humans (He et al., 2003; Li and Dawson, 2019).

Bile acids may also be conjugated with glucuronic acid by the glucuronosyltransferases UGT 1A1, 2B4, and 2B7 at C_3, C_7 or C_{24} positions. The sterol ring of bile acids may also be conjugated with sulphate group at C_3 or C_7 position. The reaction is catalyzed by sulfotransferases SULT 2A1 and 2A8.

Intestinal bacteria in the distal part of the small intestine and especially in the large intestine play also a significant role in the metabolism of bile acids (Ridlon et al., 2006; Li and Dawson, 2019). Microbiota deconjugate bile acids by bile salt hydrolase (BSH) activity. Further metabolism by bacteria involves 7α-dehydroxylation to convert CA and CDCA to DCA and LCA, respectively. The 7α-hydroxyl group in CDCA can be isomerized to 7β-hydroxyl group producing UDCA. Consequent 7β-dehydroxylation of UDCA yields LCA (Figure 2). Humans can further metabolize LCA in enterocytes by CYP3A4, converting it into more hydrophilic (and therefore less toxic) HCA and UDCA. Rodents convert LCA back to UDCA, to hyodeoxycholic acid (6α-hydroxylation) or to murideoxycholic acid (6β-hydroxylation) (Ridlon et al., 2006). Some secondary bile acids such as DCA and to a lesser extent LCA may be reabsorbed unconjugated in the colon by passive diffusion. This pathway is minor in comparison with active reabsorption of bile acids in ileum. The majority of bile acids leaving body in stool are in unconjugated form. Similarly, only a small amount of LCA can be found in urine upon sulfur conjugation in the liver. However, during cholestasis, a higher amount of sulfated bile acids is excreted into the sinusoidal blood for renal excretion.

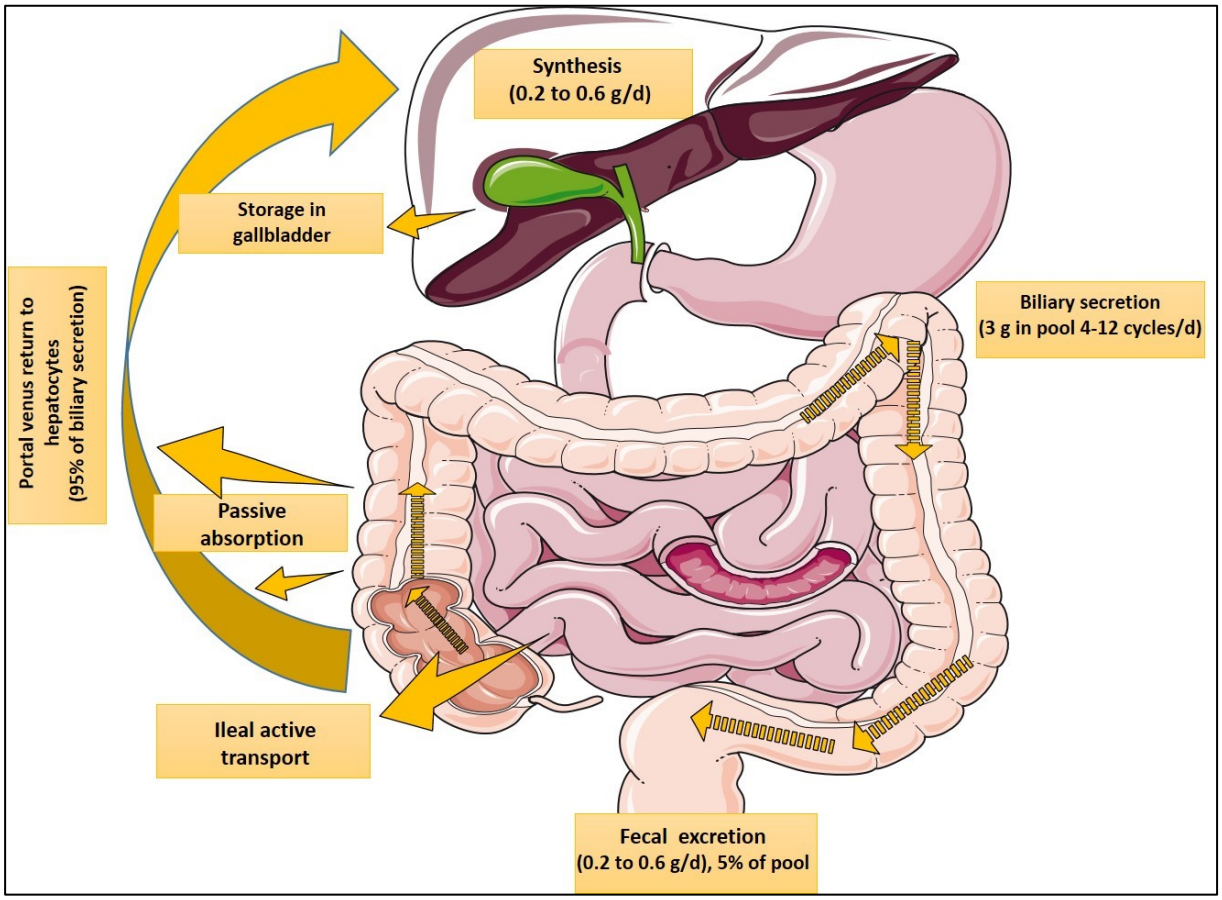
1.2. The enterohepatic circulation of bile acids

To prevent toxicity of bile acids in the systemic circulation, majority of their content in organism is restricted to intestine (~85%-90%), and gallbladder (~10%-15%). Only minor part is present in the liver (< 1 %). Massive loss of bile acids via stool elimination is prevented by their intensive reabsorption in the ileum where ~ 95% of bile acids entering the intestine via bile are reabsorbed into portal circulation. Portal blood is effectively cleared from bile acids by intensive uptake into hepatocytes, from where they are actively re-secreted into bile. Only small amount of bile acids spilled over from the liver to systemic circulation. The whole process of enterohepatic circulation of bile acids is enabled by complex network of transporting proteins at plasma membranes of hepatocytes and enterocytes (Figure 3). These events consisting of: (1) transporting bile acids from hepatocytes into bile canaliculi through bile salt export pump (BSEP) and partly also with multidrug resistance-associated protein 2 (MRP2). Bile canaliculi drain into bile ductules, then bile ducts, and eventually into the duodenum, either through a gallbladder (in mice and humans) or directly (in rats, horses, deer, whales and other animals without a gallbladder). However, even animals with gallbladders still pass a significant amount of bile directly into the duodenum without being stored there. (2) Bile acids are taken up from the distal ileum to enterocytes with sodium-dependent bile acid transporters (ASBT). (3) Ileal bile acid-binding protein (IBABP) transfers bile acids from the apical to the basolateral membrane of enterocytes. (4) The organic solute transporter α/β (OSTα/OSTβ) heterodimer exports bile acids from enterocytes into the portal blood. (5) Bile acids are taken up from sinusoidal (liver) blood into hepatocytes via sodium (Na+)-taurocholate co-transporting polypeptide (NTCP) and in minor part also with organic anion transporting polypeptides (OATP). The cycle is repeated, and it is estimated that each molecule of bile acid recycles ~4-12 times a day.

In addition to maintaining bile acid and cholesterol homeostasis, this recycling mechanism plays a critical role in maintaining the bile acid pool (Russell, 2003; Chiang, 2013). Bile acid pool size can be described as the total quantity of bile acids in the enterohepatic circulation. It is important to note that the composition of bile acids in humans and mice differs significantly. A human's highly hydrophobic bile acid pool contains 40% CA, 30% CDCA, and 20% DCA. In mice, the highly hydrophilic bile acid pool comprises about 50% CA and 50% of α- and β-MCAs. Approximately 0.5 g of bile acids are lost in the feces daily. They are replaced by de novo synthesis in the liver to maintain a constant bile acid pool. The amount of bile acid synthesized every day is about 0.5 g. Hepatic bile acid secretion into the duodenum in humans yielded a value of ~12 g/day (Lefebvre et al., 2009).

Figure 3: Enterohepatic circulation of bile acids. The human bile acid pool contains approximately 3 grams of bile acids. The gallbladder release of bile acids into the small intestine is stimulated by food consumption. The liver synthesizes approximately 0.5 g of bile acid daily to replace the fecal loss. “Figure modified with text, markings, arrows and boxes, and annotation after adaptation of stomach,

liver and intestine from Servier Medical Art by Servier, licensed under a Creative Commons Attribution 3.0 Unported License". Adapted from (Li and Chiang, 2014).



1.3. Regulation of bile acid metabolomics

The potential toxicity of bile acids requires precise control of their concentrations and total content in the organism. This is accomplished by a negative feedback mechanism which is triggered when bile acids accumulate beyond physiologic levels, typically during cholestasis. In this circumstance bile acids bind to the FXR nuclear receptor and modulate expression of target genes. Other nuclear receptors such as PXR, CAR, and VDR contribute to regulation of bile acid metabolism with less significant role than FXR, but still may be attractive therapeutic targets. Interestingly, the overall adaptive response is similar whatever the initial cause of cholestasis. In the liver, cumulating bile acids suppress the expression of their synthetic enzymes and uptake transporters, and induce expression of efflux transporters on basolateral as well as canalicular membranes. In the intestine, the expression of ASBT transporter is reduced while efflux OSTα/β is increased. However, adaptive anti cholestatic response can be modulated by numerous factors including excess of bile acids such as CDCA which repress bile acid synthesis in the liver by activation of macrophages (Kupffer cells) with release of proinflammatory cytokines (tumor necrosis factor-α, and interleukin-β1) and hepatic stellate cells with secretion of growth factors (transforming growth factor β) (Li et al., 2006; Li et al.,

2008; El Kasmi et al., 2012). These molecules activate receptors at basolateral membrane of hepatocytes and trigger protein kinase C, *c*-Jun, JNK1/2 and ERK1/2 intracellular signaling to inhibit CYP7A1 and other enzyme expression independently on FXR.

Inflammation may also trigger production of hepcidin which enhances storage of iron in the liver. We have recently demonstrated that iron depletion in rats significantly reduces plasma concentrations of bile acid due to their increased liver uptake, synthesis, and biliary secretion. We explained these changes by up-regulation of CYP7A1, CYP8B1, CYP27a1, NTCP, OATP1A1, and OATP1A4 (Prasnicka et al., 2017). Vice versa, increased plasma concentrations of bile acids have been detected during iron overload and this effect was associated with reduced CYP7A1 activity. However, other details about relationship between the iron overload and bile acid metabolomics are missing and require further clarification.

Role of precise modulation of bile acid synthesis and enterohepatic recycling is accentuated during cholestatic liver disorders that cause retention of bile acids in the liver and systemic circulation with risk of consequent toxicity. The adaptive changes in bile acids synthetizing enzymes and transporters may minimize or at least reduce liver injury. Importantly, alteration of transporters responsible for turnover of bile acids (e.g. genetic defects, hormones, drugs, or inflammation) may be even primary cause of cholestasis. Identification of mechanisms mediating and regulating bile acid metabolomics during cholestasis therefore helps to understand pathophysiology of such diseases and opens new diagnostic and therapeutic possibilities. This is important especially in pharmacologically treatable intrahepatic cholestasis, where appropriate therapy may enable prophylaxis of serious complications. Typical example of this situation is the intrahepatic cholestasis of pregnancy.

1.4. Intrahepatic cholestasis of pregnancy

The intrahepatic cholestasis of pregnancy (ICP) (Wikström Shemer et al., 2015) is the most common pregnancy-specific liver disease. It usually presents with symptoms including pruritus, abnormal liver function, and raised serum bile acid levels occurring especially in the third trimester. Besides unpleasant subjective symptoms, ICP predisposes women to cholesterol gallstones, cholecystitis, hepatitis C or even liver cirrhosis (Ropponen et al., 2006). Moreover, the cumulating bile acids threaten the fetus with higher incidence of adverse pregnancy outcomes such as iatrogenic preterm delivery, nonreassuring fetal status, meconium staining of the amnionic fluid, and stillbirth (Geenes et al., 2014). Dangerous is especially maternal plasma concentration of bile acids over 40 μM. The clinicians managing pregnancies in women with such a severe ICP may therefore prefer a strategy of labour induction prior to 37 weeks despite emerging concerns about special education needs (MacKay et al., 2010), and poorer school performance (Quigley et al., 2012) in babies born late preterm (34-36 weeks gestation). Proper therapy and prophylaxis of ICP would therefore be of highest priority. The need is further accentuated with a significant average incidence of ICP affecting ~2% of all pregnancies (0.9% in the Czech Republic) (Binder et al., 2007; Williamson and Geenes, 2014). Although the pathogenesis of ICP remains mainly unclear, there is increasing evidence suggesting that impaired transport of bile acids into bile plays the primary role (Liu et al., 2018). The expression of biliary transporters is physiologically decreasing during the pregnancy due to increased

production of cholestatic estrogens and progestins (Aleksunes et al., 2012). Consequently, the women with primary genetic defect of BSEP, MRP2 or MDR3 (Multidrug resistance protein 3; mediates biliary phospholipid secretion), are more susceptible to hormonal cholestatic insults. In agreement with this concept, predisposition factor for ICP is also genetic impairment of FXR, the principal nuclear receptor regulating the whole cascade of bile acid homeostasis. Alteration of excretory transporters leads in turn to accumulation of bile acids in the organism of mother, and consequently in the reverting of their gradient between maternal and fetal circulation.

The patterns of changes in the liver mechanisms of bile formation are currently not exactly known during ICP in humans due to obvious ethical problems (ICP is not indicated for liver biopsy), and the major source of data are relevant animal models - mainly ethinylestradiol-administered rodents. These studies demonstrated that the accumulation of bile acids under such circumstances is consistent with downregulation of hepatic bile acid basolateral (NTCP, and OATPs), and canalicular transporters (BSEP and MRP2), respectively (Geier et al., 2007). In agreement, pregnant rats have also shown reduced expression of liver NTCP, BSEP, and MRP2 transporter (Cao et al., 2001; Zhu et al., 2013; Song et al., 2014) but without additional cholestatic insult they do not develop cholestasis.

The main goal of current therapeutic strategies for ICP is the reduction of plasma bile acid concentrations in mothers through pharmacological modulation of impaired bile acid transport, and synthesis. This can be achieved by interference with bile acid regulatory signaling. Therefore, ursodeoxycholic acid (UDCA) became the first line therapy for ICP. This hydrophilic nontoxic bile acid possesses several positive mechanisms in the liver during ICP such as stimulation of bile secretion through upregulation of BSEP and MRP2 transporters at the canalicular membranes of hepatocytes, reduction of lipophilic bile acid synthesis and shift of bile acid profile in favor of less toxic, hydrophilic compounds (Tribe et al., 2010; Boyer, 2013). Upon administration to women with ICP, UDCA improves maternal itching scores and liver function tests without interfering with the fetoplacental estrogen production. UDCA is well tolerated by pregnant women and no fetal or neonatal side-effects could be detected (Joutsiniemi et al., 2014). On the other hand, it is known that UDCA has beneficial effect only in some but not all women with ICP (Williamson and Geenes, 2014). Therefore, understanding the factors which may predispose to or alleviate accumulation of bile acids during ICP is currently in the center of attention.

Previous studies have demonstrated that ICP has higher coincidence with gestational diabetes mellitus (GDM). Besides diet and insulin therapy, metformin has been approved for the treatment of GDM. This increases the chance for metformin to be administered in pregnant women with increased plasma concentrations of bile acids. Interestingly, reduction of bile acid plasma concentrations and improvement of ICP symptoms were reported in one patient after administration of metformin (Elfituri et al., 2016). The mechanism of such effect is unclear. In addition, the need to better understand the effects of metformin in ICP is accentuated by several case reports from clinical practice where metformin administration has led to cholestasis with unknown pathophysiology (Desilets et al., 2001; Nammour et al., 2003; Biyyani et al., 2009; Saadi et al., 2013). The clinical attractiveness of the topic therefore encourages further clarification of involved mechanisms.

2. Aims of the dissertation thesis

The research examined the metabolomic of bile acids during various liver pathologies impairing bile formation. The aims of the thesis included:

1- A detailed study of the potential role of metformin in regulating bile acid homeostasis and the related molecular pathways in the liver and intestine using a mouse model of the intrahepatic cholestasis of pregnancy.
2- The identification of MRP2 transporter role in the metabolomics of bile acids and the contribution of MRP2 deficiency to the pathology of estrogen-induced cholestasis.
3- The characterization of alterations in the synthesis, biliary secretion, and intestinal processing of bile acids during iron overload.

3. Results and comments

This dissertation thesis is organized as an annotated set of three research articles. The main candidate is the first author of two of these articles. All these three articles are published in international journals with impact factor. Listed below are outlines of these publications along with contributions from the candidate.

3.1. Metformin impairs bile acid homeostasis in ethinylestradiol-induced cholestasis in mice

Alaei Faradonbeh F, Sa II, Lastuvkova H, Cermanova J, Hroch M, Faistova H, Mokry J, Nova Z, Uher M, Nachtigal P, Pavek P, Micuda S. *Chem Biol Interact*. 2021; 345:109525. (IF = 5.192, Q2)

In this article, we studied modulation of bile acid metabolomics by metformin in healthy mice with intact livers as well as in mice with ethinylestradiol-induced cholestasis mitigating intrahepatic cholestasis of pregnancy (ICP). Our hypothesis was that metformin significantly affects mechanisms associated with enterohepatic recycling of bile acids and this may explain cholestasis occasionally accompanying its therapy in humans. Also, women with gestational diabetes are in greater danger of simultaneous intrahepatic cholestasis. There is therefore increased chance that metformin will be applied to women with ICP but the consequence of such situation is unknown.

For the first time, our study shows that metformin administered to mice with intact livers may accelerate enterohepatic recycling of bile acids by increasing their synthesis via induced cholesterol 7α-hydroxylase (CYP7A1) and by their increased reabsorption from the ileum via induction of the apical sodium-dependent bile acid transporter (ASBT). This knowledge may provide an explanation for the cholesterol-reducing effect of metformin observed in treated diabetic patients.

In contrast, metformin may further impair biliary secretion of bile acid in ethinylestradiol-induced cholestasis in mice via downregulation of their principal transporters in the liver: the Na^+-taurocholate cotransporting polypeptide (NTCP) and the bile salt export pump (BSEP). This effect led to a significant 3.3 times increase in the plasma concentrations of bile acids. The Simultaneous reduction in efflux MRP4 transporter at basolateral membrane of hepatocytes worsened protective capacity of these cells against cumulation of bile acids. An important implication of these findings is a warning for the careful use of metformin in individuals with ICP. Monitoring of plasma concentrations of bile acids could be recommended in this situation.

Candidate's contribution:

- Performing experiments, specifically:
  - *In vivo* part of study
  - Plasma samples preparation to biochemistry analysis

- Stool samples preparation to analysis of bile acids
- Isolation of mRNA
- cDNA synthesis for qPCR and protein isolation for WB
- Liver and ileum genes expression analysis via qPCR
- Liver and ileum protein analysis via WB

- Data analysis, interpretation of results, visualization
- Writing of the article and preparation for submission

3.2. Multidrug resistance-associated protein 2 deficiency aggravates estrogen-induced impairment of bile acid metabolomics in rats

Alaei Faradonbeh F, Lastuvkova H, Cermanova J, Hroch M, Nova Z, Uher M, Hirsova P, Pavek P, Micuda S. *Front Physiol*. 2022;13:859294. (IF = 4.134, Q1)

In this study, we focused on the role of multidrug resistance-associated protein 2 (MRP2) in the development of estrogen induced cholestasis in rats, an animal model of ICP. MRP2 as a canalicular efflux pump contributes to biliary secretion of bile acids. Previous studies in humans suggested increased incidence of ICP in women with mutation in MRP2 *(ABCC2)* gene. Therefore, we induce cholestasis by estrogen in MRP2-deficient rats to describe changes in bile acid metabolomics.

We revealed that MRP2 deficiency itself leads to elevated plasma concentrations of bile acids. This effect was caused by decreased biliary secretion of bile acids and their increased export from hepatocytes to portal blood via upregulated basolateral multidrug resistance-associated protein 3 (MRP3) and multidrug resistance-associated protein 4 (MRP4) transporters. Intestinal reabsorption of bile acids was reduced in MRP2-negative rats due to down-regulation of the apical sodium-dependent bile salt transporter (ASBT). We identified that mechanism regulating these changes in bile acid metabolomics in the liver may be activation of constitutive androstane receptor (CAR)-Nuclear factor erythroid 2-related factor 2 (NRF2) pathway by accumulating bilirubin. Modulation of this pathway opens new possibilities for future therapies.

Retention of bile acids in plasma of MRP2-deficient rats was further aggravated upon administration of ethinylestradiol. The major mechanism responsible for increased plasma bile acids concentrations in MRP2-deficient rats was their increased reverse transport from hepatocytes via induced MRP4 transporter. Interestingly, other transport mechanisms in enterohepatic recycling of bile acids were not modified by MRP2 deficiency. Instead, we detected impaired 12α-hydroxylation of bile acids due to downregulation of CYP8B1, and increased muricholic acid synthesis via up-regulated CYP2C22 enzyme. It suggests major protective mechanisms activated in this situation to limit cholestatic liver injury imposed by estrogen.

In summary, our results proved significant role of MRP2 transporter in turnover of all bile acids not only those conjugated with sulphate or glucuronic acid. Deficit of MRP2 clearly predisposes

for bile acid retention in organism. In this respect, our results present mechanisms explaining the increased incidence of ICP in MRP2-deficient women. Plasma bile acid concentration monitoring is therefore highly desirable in individuals with MRP2 deficiency because they are in the greater risk of cholestasis. Special attention should be dedicated to pregnant women with conjugated hyperbilirubinemia for early detection of ICP.

Candidate's contribution:

- Performing experiments, specifically:
  - *In vivo* part of study
  - Plasma samples preparation to biochemistry analysis
  - Stool samples preparation to analysis of bile acids
  - Isolation of mRNA
  - cDNA synthesis for qPCR and protein isolation for WB
  - Liver and ileum genes expression analysis via qPCR
  - Liver and ileum protein analysis via WB
- Data analysis, interpretation of results, visualization
- Writing of the article and preparation for submission

3.3. Iron overload reduces synthesis and elimination of bile acids in rat liver

Prasnicka A, Lastuvkova H, **Alaei Faradonbeh F**, Cermanová J, Hroch M, Mokry J, Dolezelova E, Pavek P, Zizalova K, Vitek L, Nachtigal P, Micuda S. Sci Rep. 2019 Jul 5;9(1):9780.) (IF = 4.379, Q1)

In this study we significantly extended the knowledge about possible regulatory role of iron overload on the bile acid biochemistry. Iron is essential trace element with multiple physiological functions in organism. However, iron is also highly reactive molecule contributing to generation free hydroxyl radicals which may in excess induce significant cell damage. The liver is the major storage organ for iron, and iron may be cumulated herein in excess under different pathological conditions such as genetic disorders (e.g. hereditary haemochromatosis and beta thalassaemia), or secondary to blood transfusion and haemolysis.

We utilized rat model of iron overload induced by repeated intraperitoneal administration of iron to markedly increase content of iron within the liver. This situation led to liver impairment as evaluated by plasma biochemistry or histopathological examination. Excess of liver iron reduced biliary secretion of bile acids because of downregulated NTCP, BSEP and MRP2 transporters for transcellular passage of bile acids from portal blood to bile. Simultaneous induction of MRP3 and MRP4 efflux transporters exporting bile acids from hepatocytes back to plasma, and decreased expression of CYP7A1 and CYP8B1 synthetic enzymes prevented

accumulation of bile acids in hepatocytes. Plasma concentrations of bile acids were also not modified by excess of iron due to reduced reabsorption of bile acids in the intestine. Interestingly, this change was not the consequence of altered expression of reuptake transporters, but it was related to increased synthesis of hyodeoxycholic acid which is poorly absorbed from intestine.

Collectively, our results revealed complex regulatory function of iron on the metabolomics of bile acids. Especially impaired expression of numerous transporters necessary for liver elimination of endo, and xenobiotics may contribute to iron-induced hepatotoxicity. Exact regulatory mechanisms contributing to observed changes are not known, but our data suggested that activation of iron response element and oxidative stress may be responsible. Modulation of these mechanism may offer therapeutic targets to alleviate hepatotoxicity before iron could be effectively reduced in the liver.

Candidate's contribution:

- Performing experiments, specifically:
  - *In vivo* part of study
  - Plasma samples preparation to biochemistry analysis
  - Stool samples preparation to analysis of bile acids
  - Isolation of mRNA
  - cDNA synthesis for qPCR and protein isolation for WB
  - Liver and ileum genes expression analysis via qPCR
  - Liver and ileum protein analysis via WB
- Assisted in data analysis, interpretation of results
- Assisted in writing of the article and preparation for submission

4. Discussion

4.1. Metformin and estrogen-induced cholestasis

Detailed characterization of all mechanisms associated with intrahepatic cholestasis of pregnancy may help to identify factors that contribute to or prevent cumulation of bile acids with consequent toxicity to mother and fetal organs. In our research, we, therefore, used a relevant rodent *in vivo* model of estrogen-induced intrahepatic cholestasis to study factors and interventions which are relevant for clinical practice.

Metformin is the most used antidiabetic agent in the therapy of type 2 diabetes mellitus (T2DM) which has been approved for the therapy of gestational diabetes mellitus (GDM). Its relationship with the biochemistry of bile acids is interesting because few case reports of cholestasis have been described in clinical practice. Of note, GDM can coincide with ICP, and metformin is considered an alternative therapy for GDM. Finally, a case report appeared describing the reduction of bile acid concentrations in one patient with simultaneous GDM and ICP. However, metformin worsens α-naphthyl isothiocyanate (ANIT) intrahepatic cholestasis. We, therefore, hypothesized that metformin alters bile acid synthesis and transport.

First, we tested the effect of metformin in mice with intact livers. Total plasma concentrations of bile acids were not changed by metformin in healthy animals, but the drug increased biliary secretion of bile acids due to their increased synthesis. This was consistent with increased hepatic CYP7A1 expression and increased plasma, biliary and fecal content of 12α-hydroxy bile acids representing enhanced neutral bile acid synthesis. Increased delivery of bile acids to the intestine via bile contrasted with unchanged fecal excretion of bile acids which indicates that metformin increased reabsorption of bile acids in the ileum. It is consistent with increased expression of ASBT bile acid reuptake transporter in the ileum. The practical implication of this finding might be the clarification of the cholesterol-reducing effect of metformin via activation of its conversion to bile acids, and also by facilitated excretion into bile due to increased bile acid-mediated bile flow. Intriguing question is, how metformin affects these pathways because protein expression of CYP7A1 changed without mRNA alteration suggesting a post-transcriptional mechanism.

Multiple mechanisms regulate CYP7A1 expression, but an essential one is suppressed gene expression of this enzyme by activation of FXR receptor. In one study, gene expressions of *Cyp7a1*, *Cyp8b1*, *Slc10a1* (Ntcp), and *Abcb11* (Bsep) were not changed by metformin when a suboptimal oral dose of 80 mg/kg/day was administered. In contrast, an intravenous dose of 100 mg/kg/day of metformin that highly overcomes the low bioavailability of this agent, may induce CYP7A1 (Chen et al., 2017).We, therefore, focused on the essential regulatory pathways activated by metformin to produce a therapeutic antidiabetic effect, which is NAD^+-dependent deacetylase SIRT1 and AMP-activated protein kinase (AMPK). Both these pathways are coordinately activated in fasted states to produce energy while they are reduced in the refed situation. Both molecules were significantly induced by metformin in our control, as well as cholestatic animals consistent with optimal selection of applied dose. However, the discrepancies in the modulation of bile acid metabolomics by metformin in control and

cholestatic animals suggest different roles of AMPK and SIRT1 in the intact and cholestatic liver.

Mice with *Sirt1* knockout or transgenic mice overexpressing human *SIRT1* have similar bile acid concentrations in plasma, bile acids pool size, and content in the liver, and intestine. The changes can be identified only in spectra of bile acids where livers from *Sirt1* knockout mice contain a lower amount of CA, αMCA, and HDCA, and a higher amount of βMCA than wild-type controls. The *Sirt* knockout mice have also reduced mRNA expression of FXR targets, *Cyp8b1*, and *Cyp7b1* with no other changes detected in the bile acid related enzymes or transporters in the liver and ileum. On the other hand, activation of SIRT1 by SRT1720 in heathy mice has no influence on gene expression of liver or ileum molecules responsible for the turnover of bile acids (Kulkarni et al., 2016).We detected a similar absence of changes in gene expression of these genes. Collectively, it may indicate that SIRT1 has a secondary role in the regulation of BA turnover, or rather its importance raises in a pathological situation where the basal activity of SIRT1 and especially FXR is changed.

We detected reduced SIRT1 activity in our ethinylestradiol group which may encourage the administration of SIRT1 activating agents. In support, administration of SIRT1 activator SRT1720 was protective in estrogen-induced cholestasis via enhancement of FXR pathway (Yu et al., 2016) with consequent induction of FXR, BSEP, MRP2, NTCP, and CYP7A1. SRT1720 also significantly repressed the release of proinflammatory cytokines such as IL-6, and TNF-α provoked by ethinylestradiol. A similar beneficial effect of SIRT1-FXR activation was observed by the same research group in ANIT-induced cholestasis (Yu et al., 2017), and also by Kulkarni et al (2016) in hyperchloremia induced by cholic acid diet. Herein, the content of SIRT1 was reduced in three mouse models of cholestasis – 1% cholic diet, bile duct ligation, and *Mdr2* knockout strain (the model of primary sclerosing cholangitis) and Sirt1720 repressed CYP7A1 and CYP27A1(Kulkarni et al., 2016). In contrast, SIRT1 was significantly induced in livers of cholestatic patients, bile duct-ligated mice, and *Mdr2* knockout mice in another study (Blokker et al., 2019). Mice with overexpressed SIRT1 showed exacerbated liver impairment while knockout of this receptor or its blockade showed improvement in BDL (bile duct ligation) and DDC (3,5-diethoxycarbonyl-1,4-dihydrocollidine) cholestasis (Blokker et al., 2019). The role of SIRT1 in cholestasis is therefore questionable. Our result of increased bile acids plasma concentrations in ethinylestradiol-treated mice administered with metformin which showed significant SIRT1 induction supports rather detrimental effects of SIRT1 in this type of cholestasis.

The role of AMPK in the regulation of bile acid metabolomics seems more pronounced compared with SIRT1. The crucial assumption is that AMPK activation inhibits the transcriptional activity of FXR due to decreased recruitment of FXR coactivators to promotors of its target genes (Lien et al., 2014). This was clearly seen when metformin was administered to mice with cholestasis induced by taurocholic acid where AMPK activation repressed expression of FXR target genes such as *Nrob2*, *Abcb11*, or *Abcc2*. Plasma concentrations of bile acids were consequently increased. A less obvious effect was detected in ANIT-induced cholestasis where metformin reduced only mRNA of BSEP and SHP, but the increase in plasma concentrations of bile acids was massive. Similar to SIRT1, no effect of AMPK was noticed on

bile acid homeostasis in control animals indicating that active FXR is necessary for this regulatory function. In contrast, activated AMPK may promote FXR-RXR heterodimer assembly to the BSEP promoter as suggested by chromatin immunoprecipitation methods. The AMPK may therefore increase BSEP expression, while BSEP induction is blunted in the *Ampk* knockout mice followed by the cumulation of bile acids in the liver (Chopra et al., 2011). AMPK signaling may also promote canalicular trafficking of BSEP (Homolya et al., 2014), and plasma and liver BA levels are substantially higher in AMPK-downregulated than in wild-type mice (Woods et al., 2011). Unlike all those studies, significant induction of AMPK by metformin in our study had no influence on BSEP protein localization or FXR target gene transcription in control or cholestatic animals. Variability in response of bile acid metabolomics to AMPK activation suggests that activating agents, their dosage, timing and pathological status produce dynamic changes which are not easy to clarify.

Irrespective of the regulatory mechanism, the major finding of this research was that metformin worsens estrogen-induced cholestasis by a significant reduction of biliary secretion of bile acids which was not sufficiently compensated by reduced reabsorption in the ileum. Plasma concentrations of bile acids therefore significantly increase in metformin administered mice with estrogen-induced cholestasis. This increase was proportional to the majority of bile acids and it was produced by the downregulation of NTCP, and BSEP transporters crucial for portal uptake, and biliary secretion of bile acids, respectively. We, therefore, analyzed multiple pathways regulating bile acid metabolomics, and upon exclusion of their contribution, we concluded that isolated changes at the protein levels of these transporters suggest that metformin may modulate the turnover of bile acid-related proteins. In support of this hypothesis, we performed a series of analyses and detected activation of autophagy. The effect deserves further studies.

4.2. Role of Mrp2 in the development of estrogen-induced cholestasis

Increased incidence of ICP in patients with a mutation in the MRP2 *(ABCC2)* gene was demonstrated in several clinical studies, but the mechanism was unknown because MRP2 is considered as a transporter for biliary secretion of sulfate, and glucuronic acid-conjugated bile acids, mainly TCDA and LCA, which are minor in the whole bile acid pool. Previous studies rather excluded the contribution of MRP2 to the transport of unconjugated or taurine/glycine monoconjugated bile acids (Takikawa et al., 1991; Verkade et al., 1993), but the major limitation of these studies was the impossibility to analyze individual bile acids by sophisticated mass-spectrum analytical method. Only one recent study analyzed bile acids in MRP2-deficient animals by such a method and reported increased relative peak areas suggesting an increase in plasma concentrations of bile acids. The impact of this study was limited because absolute concentrations of individual acids were not measured. Implementation of the LC-MS method validated for quantification of 26 different bile acids in our study changed the scope of detection and enabled our detailed analysis of individual bile acids in MRP2-deficient animals.

The absence of MRP2 in our control TR rats was clearly associated with reduced net biliary secretion of bile acids, TMCA, and TCDCA. This means that also dominant bile acids not conjugated with sulfate or glucuronic acid are substrates for MRP2. The second factor which may reduce the biliary secretion of bile acids in MRP2-deficient animals is increased efflux from hepatocytes to portal blood via increased MRP4 transporter, which shares substrates specificity with MRP2. Similar upregulation was previously reported also for MRP3 (Oswald et al., 2006; Gavrilova et al., 2007), another basolateral transporter with similar characteristics to MRP4. Finally, we detected repressed expression of *Slco1a4*, a nonselective uptake transporter for bile acids at the basolateral membrane of hepatocytes (Zhang et al., 2013). Retention of bile acids was further confirmed by activation of adaptive response based on repression of CYP7A1. All these changes were regulated transcriptionally, therefore we focused on the analysis of the involved transcription factors. The character of changes in MRPs suggested the involvement of the Constitutive androstane receptor (CAR) in this kind of regulation. Consequent analysis of target genes indeed confirmed that observed changes are related to significant activation of the Car-Nrf2 (Nuclear factor erythroid 2-related factor 2) pathway by cumulating bilirubin in control MRP2-deficient rats. Nrf2 plays a central role in antioxidative defense response, and activation of this protective pathway was confirmed by upregulation of its target genes mediating synthesis of glutathione and increasing liver content of glutathione. An important implication of this finding is that therapeutic activation of the Car-Nrf2 pathway, which demonstrated a protective effect in several liver pathologies may be a useful approach only in organisms with functional MRP2 transporter.

Described combination of mechanisms reducing the portal-to-bile transport led to increased plasma concentration of bile acids in MRP2 deficient animals rendering their organism more susceptible to the cumulation of bile acids during the cholestatic challenge. This was confirmed in our ethinylestradiol-administered MRP2-deficient rats which developed distinct increase in plasma concentrations of bile acids. Interestingly, biliary secretion or stool excretion of bile acids were not changed in these animals indicating that canalicular secretion of bile acids in the liver or their reabsorption in the ileum were not affected. In agreement, detailed molecular analysis revealed that three principal mechanisms may be behind such aggravation of hypercholanemia (increased plasma concentrations of bile acids), namely reduced expression of basolateral uptake *SLCO* transporters, increased MRP4-mediated efflux from hepatocytes to blood, and induced synthesis of muricholic acids due to upregulation of CYP2C22 enzyme and downregulation of CYP8B1. This was consistent with increased content of α/βMCA found in the stool and bile, which may prevent further reduction of bile acids biliary secretion by ethinylestradiol.

Several mechanisms are responsible for the induction of cholestasis by estrogens. The crucial one is activation of nuclear estrogen receptor ERα which in turn activates inflammatory response through JNK-NF-κB pathway, reduces the content of RXRα receptor crucial for dimerization and activation of FXR, CAR or PXR, and also directly suppresses adaptive anti-cholestatic response by binding and blocking promotor sequences of FXR and its downstream bile acid-related genes. This blockade can be further worsened by interaction of activated ERK1/2 and JNK1/2 with transcription factors hepatocyte nuclear factor 1 and 4α (HNF) to

block expression of CYP7A1, CYP8B1, or NTCP. In agreement with this concept, we detected repression of NTCP, bile acids synthetic enzymes, RXRα, and induction of NF-κB in estrogen-treated wild-type rats. MRP2 deficient rats responded to ethinylestradiol with a significant decrease in Nrf2-mediated protective activity and a more pronounced reduction in RXRα. MRP2 is required for the elimination of conjugated metabolites of the estrogen such as ethinylestradiol-17-α/ß-glucuronide. Therefore, more intensive effect in TR rats may be the consequence of ethinylestradiol cumulation due to impaired MRP2 activity (Zamek-Gliszczynski et al., 2011). Notably, MRP4 expression was increased in estrogen-treated TRs despite reduced activity of CAR-NRF2 pathway which plays central position in MRP4 regulation together with the peroxisome proliferator-activated receptor α (PPARα) and the aryl hydrocarbon receptor (AHR). Interestingly, the content of MRP4 is increased in the kidney of female mice (Maher et al., 2005), and it is repressed in ovariectomized mice while it can be induced by estrogen. It is therefore possible that estrogen may directly increase also Mrp4 liver expression. This effect should be further validated (Wen et al., 2015).

For the first time, we also identified activation of another compensatory mechanism in the ileum of MRP2-deficient rats, i.e. reduced intestinal reabsorption. This mechanism can be generally detected during cholestasis to alleviate the overload of bile acids in the system (Zhang et al., 2018). Indeed, we have detected significant downregulation of ASBT uptake transporter. Such a reduction is usually the consequence of increased FXR activation in the ileum, but most sensitive FXR-target genes such as *Fgf15* and *Nr0b2* we suppressed as well. The activity of FXR was therefore reduced, most probably by reduced reabsorption of FXR agonists, which was indeed detected for CDCA. Another possibility is that estrogens may directly repress both *Slc10a2* and *Fgf15* as recently reported in ovariectomized mice with estradiol replacement (Pinteur et al., 2021).

4.3. Influence of iron overload on bile acid metabolomics

It was known that the accumulation of iron in the liver, which accompanies certain genetic or metabolic disorders, impairs conversion of cholesterol into bile acids, but the mechanisms of this effect were not thoroughly studied. Thus, we analyzed bile acid metabolomics and enterohepatic recycling in rats with iron overload (IO). The excess of iron reduced bile acid synthesis with consequent reduction of their biliary secretion. The increase in plasma concentrations of bile acids was prevented by their reduced intestinal reabsorption.

The decreased synthesis of bile acids during IO was suggested previously as the consequence of decreased CYP7A1 expression. However, we identified also significantly induced 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMG-CoA reductase), the rate-limiting enzyme for the synthesis of cholesterol, the precursor for bile acids. Other studies including our described increased plasma concentrations of cholesterol when the iron is excessively stored in the liver (Brunet et al., 1999; Coppin et al., 2007). Our results suggest that both, increased cholesterol synthesis, and its reduced metabolism may be involved in the induction of hypercholesterolemia. Based on these findings we may suggest that during

disorders of lipid metabolism such as nonalcoholic steatohepatitis where iron may be increasingly stored in the liver, the rational approach to reducing cholesterol may be the administration of statins, the HMG-CoA reductase -blocking drugs. Indeed, we have previously shown that pravastatin may increase CYP7A1 expression with simultaneous blockade of cholesterol synthesis in rats (Kolouchova et al., 2011).

The reduced expression of CYP7A1 was the major cause of reduced biliary secretion of bile acids in iron overloaded rats as confirmed by simultaneous reduction of all major bile acids. The most abundant bile acid in bile was TCA and its decrease was the most prominent in iron-administered group. This was consistent with simultaneous repression of CYP8B1. Both these enzymes are regulated mainly by intestinal FXR-FGF15 and liver FXR-SHP pathways. Marked reduction of liver SHP and absence of change in intestinal FGF15 however contradicted involvement of these pathways. Instead, we detected activation of iron response proteins 1 and 2 (IRP1, IRP2). The IRPs are activated by iron depletion and by binding to promoter regions they induce expression of liver iron uptake proteins such as divalent metal transporter 1 and transferrin receptor 1 and repress expression of efflux transporter ferroportin 1. The excess of iron has the opposite effect. Interestingly, IRP2 could bind to the promoter region of CYP7A1 increasing its expression. Increased liver content of iron may therefore repress CYP7A1 by reducing IRP2.

Notably, iron overload reduced hepatocyte transporters essential for transcellular passage of bile acids from the portal to the biliary systems such as NTCP, BSEP, and MRP2. This effect may contribute to the observed reduction of biliary secretion of bile acids. The regulation of these transporters is complicated and include a transcriptional as well as significant post-transcriptional control. Our results show, that posttranscriptional mechanisms prevailed in the downregulation of these three transporters during iron overload. The AMPK and cAMP are two principal pathways enhancing turnover a membrane trafficking of major liver transporters for bile acids. Unfortunately, at the time of study, we haven't available the method for their detection. Recent studies have indeed shown that iron overload reduces AMPK activity (Tan et al., 2013; Chen et al., 2020; Kim et al., 2020). The reduced cAMP during iron overload is not so decisively described (Shaw et al., 1995). We therefore speculate that AMPK reduction in parallel to significant oxidative stress caused downregulation of NTCP, BSEP, MRP2. However, downregulation of NTCP/BSEP/MRP2 and upregulation of MRP3/MRP4 transporters increased disposition of bile acid in systemic circulation and compensated their reduced synthesis and intestinal reabsorption to maintain stable concentrations in plasma. In addition, changes in MRPs together with induction of heme oxygenase-1 explained increased concentrations of bilirubin in plasma of rats with iron overload.

Our study described, for the first time, reduced reabsorption of bile acids in ilea of iron overloaded rats. This finding was consistent with reduced biliary delivery of bile acids to the intestine and an unchanged fecal output of bile acids. The reduced reabsorption compensated for reduced biliary secretion of bile acids. Thus, plasma concentrations of bile acids remained unaffected. Analysis of intestinal transporters for bile acids failed to identify any change. We therefore anticipate, that reduced reabsorption of bile acids was the consequence of significant

intestinal (Eyssen et al., 1999) synthesis of poorly soluble HDCA, which consequently persisted in the intestinal lumen.

5. Conclusions

The first study showed that the antidiabetic drug metformin significantly affects bile acid homeostasis, manifested by increased biliary secretion in mice with healthy livers. This increase is mainly due to 12α-hydroxy bile acids, which is related to the observed inducing effect of metformin on the CYP7A1 enzyme. At the same time, metformin upregulated ASBT transporter in the ileum, with a consequent increase in bile acid reabsorption. The increased cholesterol breakdown due to increased bile acid conversion explains the decrease in cholesterol levels observed in patients treated with metformin. Administration of metformin during estrogen-induced cholestasis significantly increased the accumulation of bile acids in the plasma due to their impaired biliary secretion. The reduction in the expression of NTCP and BSEP, the major transporters for bile acid permeation from the portal circulation to bile, was responsible for this phenomenon. At the same time, protective signaling in the gut was inhibited due to reduced reabsorption of FXR receptor agonists. Therefore, the findings of our study point to the possibility of increasing plasma concentrations of bile acids when metformin is administered to pregnant women with a predisposition to cholestasis. In this case, monitoring bile acid plasma concentrations and possible treatment discontinuation would be appropriate.

The second study confirmed the essential role of the MRP2 transporter in bile acid turnover. The MRP2 transporter deficiency caused an increase in the plasma concentrations of these endobiotics due to a decrease in their biliary secretion. The hepatocyte defense response was triggered by activation of the Constitutive androstane receptor-Nuclear factor erythroid 2-related factor 2 cascade with consequently enhanced expression of the basolateral MRP3 and MRP4 transporters. MRP2 transporter deficiency worsened estrogen-induced cholestasis. In this situation, there was a sharp increase in plasma concentrations of bile acids, which was associated with a decrease in bile acid uptake into hepatocytes through repression of NTCP and *Slco1a1* transporters. At the same time, the efflux of bile acids from hepatocytes back into the bloodstream was increased. Thus, our results provided a mechanistic explanation for the predisposition to cholestasis during pregnancy in women with a mutation in the gene encoding the MRP2 protein.

The third study elucidated changes in bile acid homeostasis during the excessive hepatic iron accumulation observed during various liver pathologies. We revealed that iron overload reduced biliary bile acid secretion due to downregulation of the major transporters, NTCP, BSEP, and Mrp2. Significant oxidative stress led to the suppression of CYP7A1 and CYP8b1 with a consequent decrease in bile acid synthesis and their increased return to the blood via induced MRP3 and MRP4 transporters. Iron probably induces these changes due to the inhibition of iron-responsive elements. However, total plasma bile acid concentrations did not increase as their intestinal reabsorption was reduced. The identified complex changes in liver transport may affect the kinetics of numerous endobiotics and xenobiotics, including drugs, which may contribute to further liver damage.

6. List of references

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7. List of outputs of the candidate

7.1. Original articles related to the topic of the dissertation

- **Alaei Faradonbeh F,** Sa II, Lastuvkova H, Cermanova J, Hroch M, Faistova H, Mokry J, Nova Z, Uher M, Nachtigal P, Pavek P, Micuda S. Metformin impairs bile acid homeostasis in ethinylestradiol-induced cholestasis in mice. *Chem Biol Interact*, 2021;345:109525. (IF_{2020} = 5.194, AIS Q2).
- **Alaei Faradonbeh F,** Lastuvkova H, Cermanova J, Hroch M, Nova Z, Uher M, Hirsova P, Pavek P, Micuda S. Multidrug Resistance-Associated Protein 2 Deficiency Aggravates Estrogen-Induced Impairment of Bile Acid Metabolomics in Rats. *Front Physiol*, 2022;13:859294. (IF_{2020} = 4.566, AIS Q1).
- Prasnicka A, Lastuvkova H, **Alaei Faradonbeh F,** Cermanová J, Hroch M, Mokry J, Dolezelova E, Pavek P, Zizalova K, Vitek L, Nachtigal P, Micuda S. Iron overload reduces synthesis and elimination of bile acids in rat liver. *Sci Rep*, 2019;9(1):9780. (IF_{2019} = 4.379, AIS Q1).

7.2. Original articles unrelated to the topic of the dissertation

- Lastuvkova H, **Alaei Faradonbeh F,** Schreiberova J, Hroch M, Jaroslav Mokry J, Faistova H, Nova Z, Hyspler R, Sa II, Nachtigal P, Stefela A, Pavek P, Micuda S. Atorvastatin Modulates Bile Acid Homeostasis in Mice with Diet-Induced Nonalcoholic Steatohepatitis. *Int J Mol Sci*, 2021;22(12):6468. (IF_{2020} = 5.924, AIS Q2).

7.2. Oral/poster presentations

- **Alaei Faradonbeh F,** Hana Laštůvková, Cermanová J, Hroch M, Pávek P, Mičuda S Hana Laštůvková. Role of iron depletion and Mrp2 deficiency for development of estrogen-induced cholestasis. XXV. vědecká conference LF HK a FN HK conference 2021.
- **Alaei Faradonbeh F,** Uher M, Lastuvkova H, Schreiberova J, Hroch M, Faistova H, Mokry J, Pavek P, Nachtigal P, Micuda S. Metformin impairs bile acid homeostasis in ethinylestradiol-induced cholestasis in mice. 8th European virtual EPHAR conference 2021.
- **Alaei Faradonbeh F,** Sa I, Uher M, Lastuvkova H, Cermanova J, Hroch M, Faistova H, Mokry J, Pavek P, Nachtigal P, Micuda S. Differential effect of metformin on bile

formation in healthy and cholestatic mice. 25th Interdisciplinary Toxicology Conference (Toxcon2020).

- **Alaei Faradonbeh F,** Lastuvkova H, Cermanova J, Hroch M, Nova Z, Uher M, Hirsova P, Pavek P, Micuda S. Absence of Multidrug Resistance-Associated Protein 2 Increases the Plasma Concentrations of Bile Acids in Rats with Estrogen-Induced Cholestasis. 27th Interdisciplinary Toxicology Conference (Toxcon 2022).
- Lastuvkova H, **Alaei Faradonbeh F,** Prasnicka A, Schreiberova J, Hroch M, Jaroslav Mokry J, Micuda S. Effects of atorvastatin on bile acid homeostasis in NASH mice. 8th European virtual EPHAR conference 2021.
- Lastuvkova H, **Alaei Faradonbeh F,** Schreiberova J, Hroch M, Faistova H, Mokry J, Hyspler R, Stefela A, Pavek P, Micuda S. Atorvastatin impairs bile flow in healthy mice but not in mice with diet-induced steatohepatitis. Authors respectively. 25th Interdisciplinary Toxicology Conference (Toxcon 2020).
- Prasnicka A, Lastuvkova H, J. Cermanová, Hroch M, Mokry J, Dolezelova E, **Alaei Faradonbeh F**, Žížalová K, Vítek L, Nachtigal P, Micuda S. Modification of Bile Acid Homeostasis By Iron Overload In Rats. European Atherosclerosis Society 2018.
- Prasnicka A, Lastuvkova H, **Alaei Faradonbeh F,** Cermanová J, Hroch M, Mokry J, Dolezelova E, Pavek P, Zizalova K, Vitek L, Nachtigal P, Micuda S. Iron overload reduces synthesis and elimination of bile acids in rat liver. Pharmacology day 2017.

7.3. Grant Projects

- Principal researcher: GAUK 5562/18: Role of iron depletion and Mrp2 deficiency for development of estrogen-induced cholestasis.

7.4. Scientific membership

- Czech Society for Experimental and Clinical Pharmacology and Toxicology (ČSEKFT ČLS JEP).