Charles University

Faculty of Medicine in Hradec Králové

DISSERTATION THESIS

Fatemeh Alaei Faradonbeh **2022**

Charles University
Faculty of Medicine in Hradec Králové

Doctoral Study Programme
Medical Pharmacology

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

Dynamika změn metabolomiky žlučových kyselin u cholestázy indukované estrogeny

Fatemeh Alaei Faradonbeh M.Sc.

Supervisor: Prof. MUDr. Stanislav Mičuda, Ph.D.

Hradec Králové, 2022

Declaration

I declare hereby that this disseertation thesis is my own original work and that I indicated by references all used information sources. I also agree with depositing my dissertation in the Medical Library of the Charles University, Faculty of Medicine in Hradec Králové and with making use of it for study and educational purposes provided that anyone who will use it for his/her publication or lectures is obliged to refer to or cite my work properly.

I give my consent to availability of my dissertation's electronic version in the information system of the Charles University.

Hradec Králové, 2022 Signature of the autho

Acknowledgments

I would like to express my gratitude to everyone who has helped me and contributed to my effort during my Ph.D. studies. I am grateful to Prof. Stanislav Mičuda, my Ph.D. advisor, for his excellent guidance during my doctoral studies. I am thankful for his professional and kind advice during my Ph.D. project and his tremendous support in my professional progress.

A special thank you goes to Hana Laštůvková and Kristýna Trubačová for their everyday support, both scientific and non-scientific. Also, I would like to thank Jolana Schreiberova and Zuzana Nová for their research collaboration and assistance. I thank Dagmar Ježková and her colleagues for taking care of my lovely animal and Miloš Hroch and Jana Lemfeldová for assisting me with the analysis.

I thank all of my colleagues at the Faculty of Medicine in Hradec Kralove's Department of Pharmacology for their friendship and scientific support, as well as for creating a nice and friendly working environment.

Last, but not least, my warm and heartfelt thanks go to my family for their tremendous support and hope they had given to me. Without that hope, this thesis would not have been possible. Thank you all for the strength you gave me. I love you all.

The work was supported by grants Progres Q40/05, SVV 260543/2020, GAUK 5562/18, GACR 19-14497S, and by the ERDF-Project PERSONMED No. CZ.02.1.01/0.0/0.0/16_048/0007441.

Table of Contents

Abbreviations

ABC	ATP-binding cassette
ABCC2	ATP-binding cassette transporter sub-family C-member 2
ABCC3	ATP-binding cassette transporter sub-family C-member 3
ABCC4	ATP-binding cassette transporter sub-family C-member 4
ABCC5	ATP-binding cassette transporter sub-family C-member 5
ABCC6	ATP-binding cassette transporter sub-family C-member 6
AHR	Aryl hydrocarbon receptor
AKR1D1	Aldo- keto reductase-family 1 member D1
AKR1C4	Aldo- keto reductase-family 1 member C4
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
ANOVA	Analysis of variance
ASBT	Ileal sodium/bile acid cotransport
ATP	Adenosine triphosphate
BA	Bile acid
BAAT	Bile acid-CoA: amino acid N-acyltransferase
BACS	Bile acid-CoA Synthase
BRIC2	Benign recurrent intrahepatic cholestasis type 2
BSEP	Bile salt export pump
CA	Cholic acid
CAR	Constitutive androstane receptor
CDCA	Chenodeoxycholic acid
CYP27a1	Sterol 27-hydroxylase
CYP3A4	Cytochrome P450 family 3, subfamily A, polypeptide 4
CYP2B1	Cytochrome P450 family 2, subfamily B, polypeptide 1
CYP2C22	Cytochrome P450, family 2, subfamily C, polypeptide 22
CYP39A1	Oxysterol 7-α-hydroxylase 2
CYP46A1	Cholesterol 24-hydroxylase
CYP7A1	Cholesterol 7α-hydroxylase
CYP7B1	Oxysterol 7α-hydroxylase

CYP8B1	Sterol 12α-hydroxylase
DCA	Deoxycholic acid
ERK	Extracellular signal-regulated kinase
Fgf15	Fibroblast growth factor 15
FGF19	Fibroblast growth factor 19
FOXA2	Forkhead box protein A2
FTF	Fetoprotein transcription factor
FXR	Farnesoid X receptor
GCA	Glycocholic acid
GDM	Gestational diabetes mellitus
GPCR	G protein-coupled receptor
HCA	Hyodeoxycholic acid
Hmg-CoA	3-hydroxy-3-methyl-glutaryl-coenzyme A
(HNF) 4α	Hepatocyte nuclear factor 4 alpha
HSD3B7	3α -hydroxy-Δ5-C27-steroid dehydrogenase
IBABP	Ileal bile acid-binding protein
IBAT	Ileal bile acid transporter
ICP	Intrahepatic cholestasis of pregnancy
IO	Iron overload
IRP	Iron response protein
JNK	c-Jun N-terminal kinases
LCA	Lithocholic acid
LC-MS	Liquid chromatography–mass spectrometry
MCA	Muricholic acid
MRP1	Multidrug resistance-associated protein 1
MRP2	Multidrug resistance-associated protein 2
MRP3	Multidrug resistance-associated protein 3
MRP4	Multidrug resistance-associated protein 4
MRP5	Multidrug resistance-associated protein 5
MRP6	Multidrug resistance-associated protein 6
NADPH	Nicotinamide adenine dinucleotide phosphate
NASH	Nonalcoholic steatohepatitis

Nrf2	Nuclear factor erythroid 2-related factor 2
NTCP	Na^+-taurocholate cotransporting polypeptide
OATP	Organic anion transporter polypeptide
OATP1A1	Organic anion transporter family member 1A1
OATP1A2	Organic anion transporter family member 1A2
OATP1A4	Organic anion transporter family member 1A4
OATP1B1	Organic anion transporter family member 1B1
OATP1B2	Organic anion transporter family member 1B2
OATP1B3	Organic anion transporter family member 1B3
OSTα	Organic solute transporter alpha
OSTβ	Organic solute transporter beta
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
PFIC2	Progressive familial intrahepatic cholestasis type 2
PPARαS	Peroxisome proliferator-activated receptor alpha
PXR	Pregnane X receptor
qRT-PCR	Quantitative reverse transcription-PCR
RXRα	Retinoid X receptor alpha
SHP	Small heterodimer partner
SIRT1	Sirtuin 1
Slco1a1	Solute carrier organic anion transporter family member 1A1
TCA	Taurocholic acid
TCDCA	Taurochenodeoxycholic acid
TDCA	Taurodeoxycholic acid
T2DM	Type 2 diabetes mellitus
TMCA	Tauro-muricholic acid
TR	Transport deficient
UDCA	Ursodeoxycholic acid
VDR	Vitamin D3 receptor

Souhrn

Žlučové kyseliny jsou důležitá endobiotika zajišťující svým emulgujícím účinkem transport lipofilních látek ve žluči a následně vstřebávání tuků ve střevě. Mimoto žlučové kyseliny vykazují řadu regulujících účinků v intermediálním metabolismu. Při jaterních onemocněních dochází často ke kumulaci žlučových kyselin, kdy po překročení fyziologických koncentrací začíná dominovat jejich toxický vliv. V játrech a ve střevě proto existuje komplexně regulovaná kaskáda enzymů a transportních proteinů, které zajišťují enterohepatální cirkulaci žlučových kyselin a změnou aktivity mohou bránit jejich excesivní kumulaci během jaterních nebo systémových onemocnění. Z toho plyne potřeba studovat faktory, které mění homeostázu žlučových kyselin, a tím přispívají k jejich toxicitě nebo jí naopak zabraňují. 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. Podstatou zjištěné kumulace žlučových kyselin bylo jejich snížené vychytávání z portální cirkulace v důsledku redukované exprese NTCP transportéru a snížení biliární sekrece redukovaným BSEP transportérem. Z těchto dat plyne doporučení, aby se při používání metforminu u těhotných žen myslelo na možnost rozvoje ICP a včas bylo zahájeno monitorování plazmatických hladin žlučových kyselin.

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. Příčinou tohoto zvýšení byla samotná absence MRP2 a současná indukce MRP3 a MRP4 efluxních transportérů na krevním pólu hepatocytů. Analýza regulačních mechanizmů označila za hlavní příčinu těchto změn aktivaci CAR-NRF2 (Constitutive androstane receptor-Nuclear factor erythroid 2-related factor 2) kaskády. Aplikace estrogenu MRP2 negativním potkanům vedla k mnohem výraznějšímu

zvýšení plazmatických koncentrací žlučových kyselin, než jaké bylo detekováno u estrogenní cholestázy u kontrolních zvířat. Příčinou tohoto zvýšení byla redukce *Slco1a1* transportéru pro vychytávání žlučových kyselin do hepatocytů a další indukce MRP4. 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

Bile acids are essential endobiotics that, due to their emulsifying effect, ensure the transport of lipophilic substances in the bile and the subsequent absorption of fats in the intestine. In addition, bile acids have numerous regulatory effects in intermediate metabolism. In liver diseases, bile acids often accumulate, and when physiological concentrations are exceeded, their toxic effects begin to dominate. Therefore, a comprehensively regulated cascade of enzymes and transport proteins in the liver and intestine ensures the enterohepatic circulation of bile acids and, by altering their activity, may prevent their excessive accumulation during liver or systemic diseases. Hence the need to study the factors that alter bile acid homeostasis and thus contribute to or prevent their toxicity. 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. The essence of the observed accumulation of bile acids was their reduced uptake from the portal circulation due to reduced expression of the NTCP transporter and reduced biliary secretion by the reduced BSEP transporter. These data suggest that the possibility of developing ICP is accentuated when metformin is used in pregnant women and that monitoring of bile acid plasma levels should be initiated in a timely manner.

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. This increase was due to the absence of MRP2 itself and concomitant induction of MRP3 and MRP4 efflux transporters on the blood pole of hepatocytes. Analysis of regulatory mechanisms identified the activation of the CAR-NRF2 (Constitutive androstane receptor-Nuclear factor erythroid 2-related factor 2) cascade as the

leading cause of these changes. 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. This increase was due to a reduction in the *Slco1a1* bile acid uptake transporter into hepatocytes and further induction of MRP4. 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 (BAs) 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 other 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 to have beneficial effects on 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 ensure 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 the 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 acid homeostasis to prevent and possibly treat numerous metabolic disorders.

1.1.1. Chemistry and function of bile acids

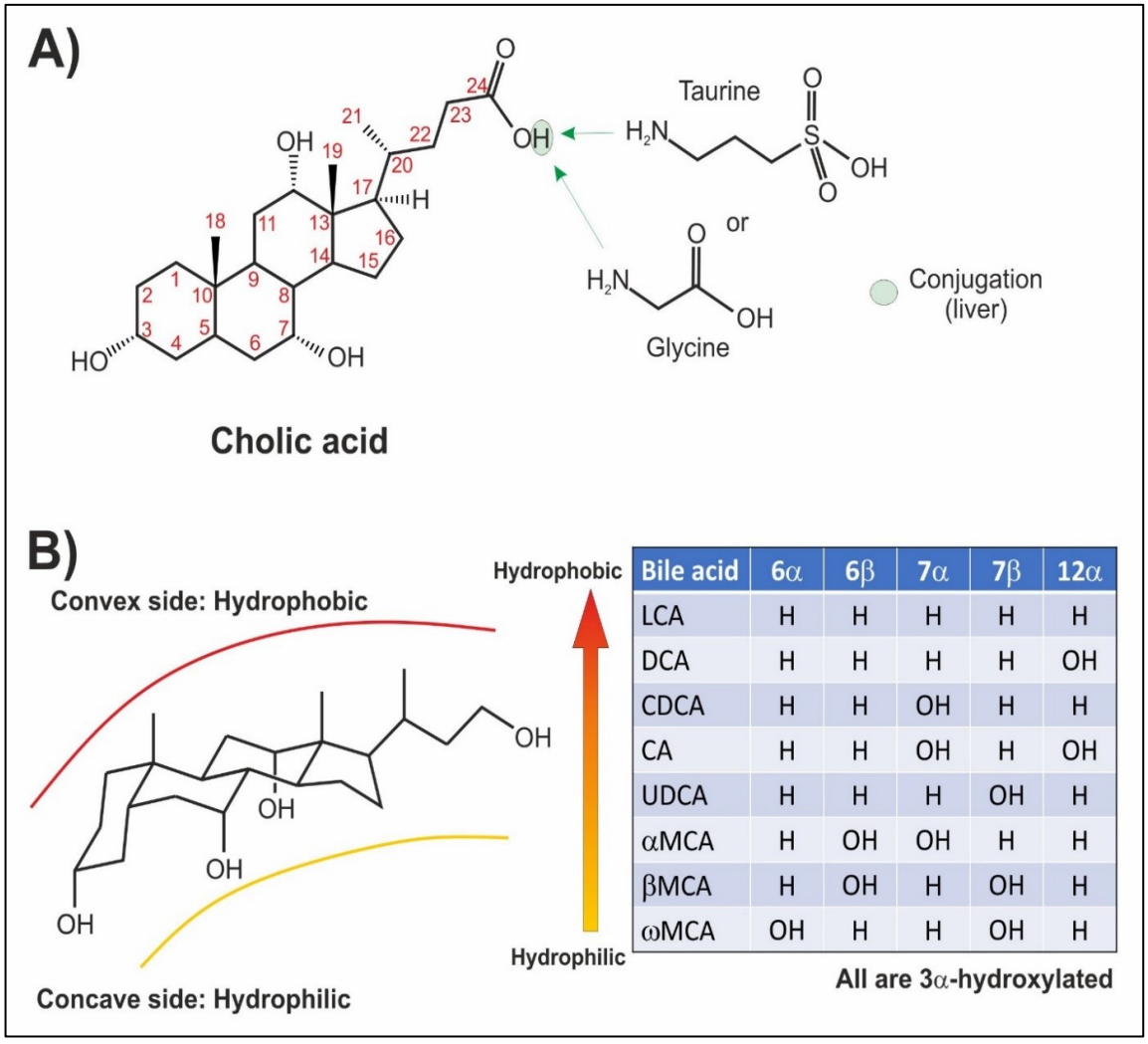
Bile acids are hydroxylated steroid carboxylic acids consisting of 24 carbons (C24) synthetized from cholesterol (C27) (Figure 1A). Four carbon rings are fused together in the steroid nucleus; three rings are 6-carbon, and one is 5-carbon with carbon C_{24} of the side chain being a part of carboxylic acid (–COOH group). All bile acids have the hydroxyl (–OH) group in the 3α-position. Further hydroxyl group may by on C_6, C_7, C_{12} either in α or β position (Choudhuri and Klaassen, 2022). Most bile acids are iso-bile acids with hydroxyl group at position 3α and hydrogen at C_5 in an β position. The 3β-OH (C_3 epimer) and 5α-H (C_5 epimer) are known as allo-bile acids. There are some species of reptile and marine organisms that produce allo-bile acids, but humans do not produce them (Shiffka et al., 2017). Bile acids have an amphipathic nature because they possess both hydrophilic (α-face, concave lower side) and hydrophobic (less hydrophilic) properties (β-face, convex upper side) (Figure 1B).

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 (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. Primary bile acids can be 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, UDCA is formed from CDCA by epimerization of the 7α-OH of CDCA to the 7β-OH. 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).

The increasing number of hydroxyl groups at C_3, C_6, C_7, and C_{12} and their localization in β position increases water solubility of bile acids. A typical example is ursodeoxycholic acid (UDCA), a 7β-OH epimer of chenodeoxycholic acid (CDCA) (Choudhuri and Klaassen, 2022). Individual bile acid therefore differs in their lipid/water solubility and can be classified according the hydrophilic-hydrophobic index reflecting

partition of each bile acid between the polar mobile phase and nonpolar column (Table 1). The value of this index increases with increasing lipophilicity of the bile acid. Conjugation of bile acids with taurine or glycine significantly increases water solubility (Figure 1A). More lipid soluble bile acids have a better potential for fat solubilization, but at the same time are more toxic.

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. Adapted from (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

All are 3α-hydroxylated

Table 1. Hydrophilic-hydrophobic index of individual bile acids

Acids	Taurine conjugate	Glycine conjugate	Unconjugated
Ursocholic	- 0.94	- 0.89	
α-Muricholic	- 0.84	- 0.79	
β-Muricholic	- 0.78	-0.73	
Ursodeoxycholic	- 0.47	- 0.43	- 0.31
Hyocholic	- 0.39	- 0.40	
Hyodeoxycholic	- 0.31	- 0.26	
Cholic	0.00	+ 0.07	+ 0.13
Chenodeoxycholic	+ 0.46	+ 0.51	+ 0.59
Deoxycholic	+ 0.59	+ 0.65	+ 0.72
Lithocholic	+ 1.00	+ 1.05	

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. There are two major pathways responsible for bile acids synthesis: the classic (neutral) pathway and the alternative (acidic) pathway. 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).

1.1.2.1. The classic bile acid synthesis pathway

This pathway starts with synthesis of 7α-hydroxycholesterol from cholesterol via the rate-limiting enzyme of whole cascade, (CYP7A1). The 7α-hydroxycholesterol is then converted to 7α-hydroxy-4-cholesten-3-one (C4) by microsomal hydroxysteroid dehydrogenase HSD3B7 (3β-hydroxy-Δ5-C27-steroid dehydrogenase). It catalyzes the epimerization of the cholesterol 3β-hydroxyl group to the 3α-hydroxyl of bile acids. The C4 is a common precursor for CA and CDCA, and C4 levels in serum are considered as a biomarker for bile acid synthesizing rates. The microsomal enzyme CYP8B1 (Sterol 12α- hydroxylase) metabolizes C4 into CA by microsomal sterol 12α-hydroxylase to form 7α,12α-dihydroxy-4-cholesten-3-one, which undergoes NADPH-dependent reductions at the C_5 and C_3 positions by aldo-keto reductase 1D1 (AKR1D1) and 1C4 (AKR1C4). AKR1C4 reduces the C_3 double bond to produce 5β-cholestan-3α,7α,12α-triol. Part of C4 bypassing CYP8B1 the C_4 is directly converted by AKR1D1, AKR1C4 into 5β-cholestan 3α,7α-diol. In the mitochondria, this is followed by oxidation of side chains (from–OH to – COOH) by CYP27A1 (Sterol 27-hydroxylase) to produce 3α,7α-dihydroxy-5β-cholestanoid acid. These intermediates are converted to bile acid-CoA thioesters by peroxisomal long-chain acyl-CoA synthase (or bile acid-CoA synthase, BACS, gene symbol *SLC27A5*), which enters peroxisomes via ABCD3 (peroxisomal transporter) for β-oxidation reaction. It cleaves a propionyl-CoA to form cholyl-CoA, and chenodeoxycholyl-CoA, respectively. The bile acid-acyl-CoAs are conjugated with amino acids taurine or glycine to form conjugated bile acids by cytosolic bile acid-CoA: amino acid N-acyltransferase (BAAT) (Figure 2). Conjugated bile acids are secreted into the bile (Choudhuri and Klaassen, 2022).

1.1.2.2. Alternative (acidic) pathway

The alternative pathway of bile acid synthesis is called "acidic" since cholesterol side chains are oxidized early in the pathway to form cholestenoic acid before the steroid nucleus is modified. The cholesterol may be initially converted by several branches of this pathway with initial hydroxylation of cholesterol at C_{27}, C_{25}, or C_{24}. These oxysterols are formed in various cells in the body, they are transported to the liver and eventually converted into C24 bile acids in hepatocytes. The presence of 27-hydroxycholesterol was also found in mice plasma (Li-Hawkins et al., 2000) and humans (Dzeletovic et al., 1995). In the liver, 27-hydroxycholesterol is metabolized to 3β-hydroxy-5-cholestenoic acid, which then

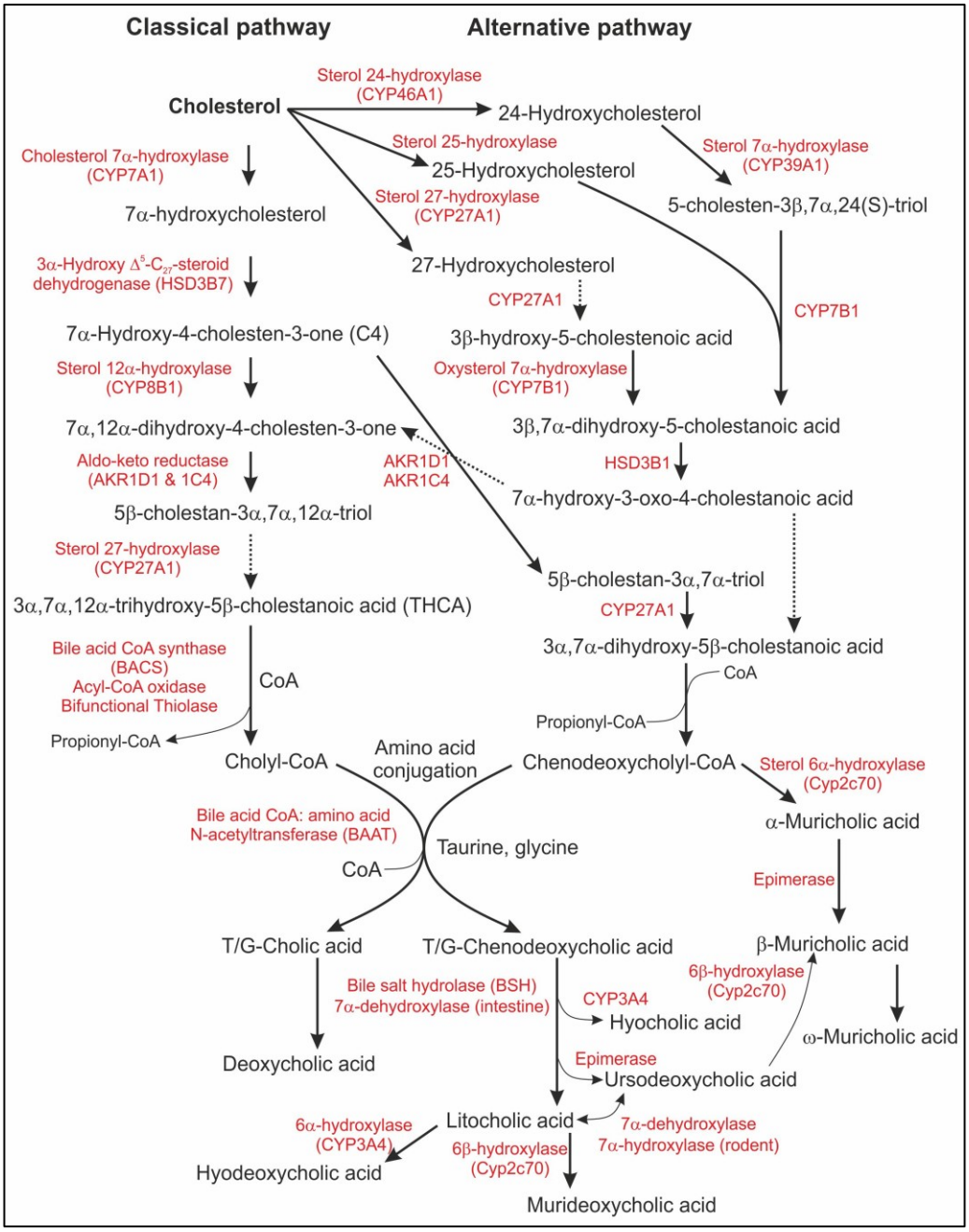
undergo 7α-hydroxylation by nonspecific microsomal oxysterol 7α-hydroxylase (CYP7B1) (Pandak and Kakiyama, 2019). A sterol 25-hydroxylase enzyme produces 25- hydroxycholesterol by hydroxylating cholesterol in endoplasmic reticulum membranes in mammals (Russell, 2003). Finally, cholesterol may be hydroxylated at C_{24} by steroid 24-hydroxylase (CYP46A1) in the brain and 24-hydroxycholesterol is then converted to 5- cholesten-3β,7α,24(S)-triol by a specific sterol-7α-hydroxylase (CYP39A1) in mouse liver. Both 25-hydroxycholesterol and 5-cholesten-3β,7α, 24 (S)-triol are then metabolized by CYP7B1 to 3β,7α-dihydroxy-5-cholestenoic acid. As reported in Schwarz et al. (Schwarz et al., 2001), mice lacking CYP46A1 or CYP27A1 exhibit no abnormalities in bile acid synthesis. However, CYP7B1 deficient mice have bile acid levels that are 50% below normal, suggesting that the alternative pathway contributes to about half of the BA pool in mice (Schwarz et al., 1998; Schwarz et al., 2001). CYP7B1 levels in female mice are low, but they do not show decreased bile acid levels (Fu et al., 2012). A Studies of bile acids in a human subject with CYP7B1 deficiency found that about 5-10% of bile acids originate from the alternative pathway (Pullinger et al., 2002). These upstream reactions in the alternative pathway feed into downstream reactions shared with the classical pathway, as seen in (Figure 2).

1.1.2.3. 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} (Figure 1). In addition to decreasing toxicity, conjugation of bile acids enhances their ionization, amphipathic properties, and solubility in water. That is mediated by BACS and BAAT enzymes (see chapter 1.1.2.1). 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; He et al., 2013; 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.

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



Intestinal bacteria in the distal part of the small intestine and especially in the large intestine also play 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

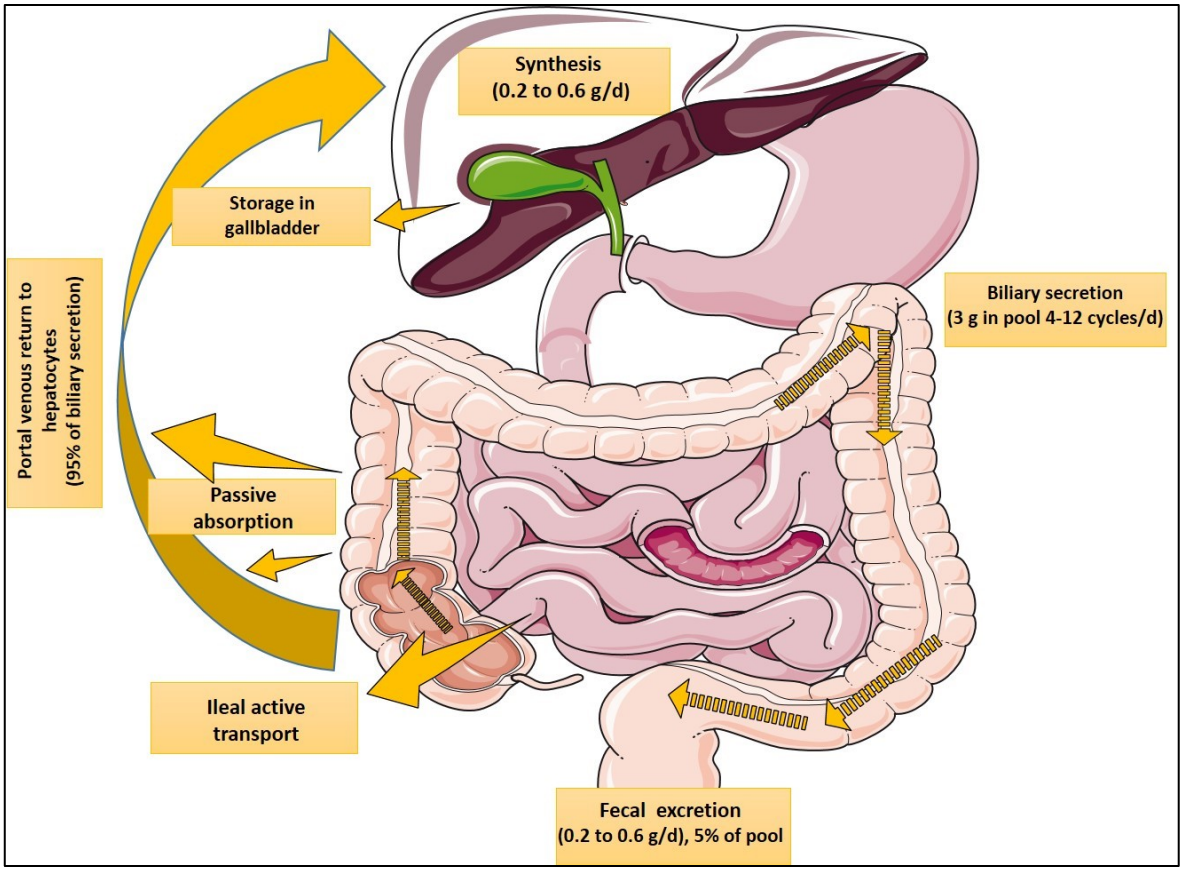
7β- hydroxyl group producing UDCA. Consequent 7β-dehydroxylation of UDCA yields LCA. Humans can further metabolize LCA in enterocytes by CYP3A4, converting it into more hydrophilic (and therefore less toxic) HCA and UDCA. Rodents convert LCA back to UDCA, to hyodeoxycholic acid (6α-hydroxylation) or 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 the active reabsorption of bile acids in the ileum. The majority of bile acids leaving the body in stool are in the unconjugated form. Similarly, only a small amount of LCA can be found in urine upon sulfur conjugation in the liver. However, a higher amount of sulfated bile acids is excreted into the sinusoidal blood for renal excretion during cholestasis.

1.2. The enterohepatic circulation of bile acids

To prevent toxicity of bile acids in systemic circulation, majority of their content in organism is restricted to the intestine (~85%-90%), and gallbladder (~10%-15%). Only a 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 is 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 a 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 a complex network of transporting proteins at the 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 species 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.

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).



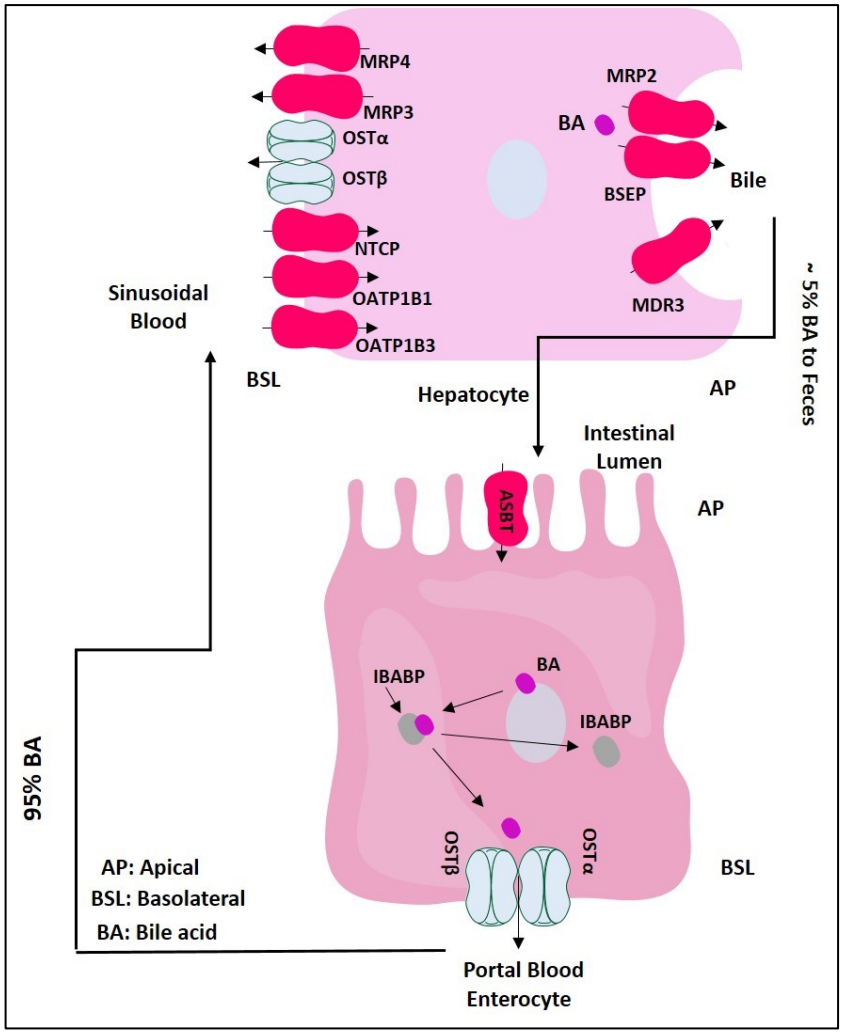
In addition to maintaining bile acid and cholesterol homeostasis, enterohepatic recycling plays a critical role in maintaining the bile acid pool (Russell, 2003; Chiang, 2013). Bile acid pool size can be described as the total quantity of bile acids in the enterohepatic circulation. It is important to 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. Hepatic bile acid secretion into the duodenum in humans yielded a value of ~12 g/day (Lefebvre et al., 2009).

1.3. Hepatic bile acid transport

About 37% of the plasma membrane of hepatocytes (sinusoidal membrane) is in direct contact with Disse's space and mediates the exchange of compounds between blood and hepatocytes. Hepatocyte bile secretory poles are surrounded by an apical (canalicular) membrane, which makes up 15% of the cell membrane. Another 50% of the cell surface is a lateral membrane composed of structures that allow hepatocytes to adhere (desmosomes and tight junctions) and communicate with each other (gap junctions) (Horak and Grabner, 1975; Marin et al., 2015). The hepatocyte sinusoidal membrane, together with the lateral membrane, is often referred to as the basolateral membrane. Circulating bile acids are efficiently taken up by the basolateral membrane. As a result, there is an 80–90% first-pass extraction rate for conjugated bile acids. Bile acids cannot cross the hepatocyte membrane by passive diffusion and require active transport (Meier and Stieger, 2002). The following paragraphs describe individual transporters necessary for enterohepatic recycling of bile acids.

Figure 4: The main bile acid transporters involved in the enterohepatic circulation. The transport of bile acids across the basolateral membrane of hepatocytes is mediated mainly by Na+-dependent taurocholate cotransporting polypeptide (NTCP) and organic anion transporting polypeptides (OATPs). Bile acid efflux across the basolateral membrane of hepatocytes may occur via the organic solute and steroid transporter (OST alpha-OST beta) and/or the multidrug resistance-associated proteins 3 and 4 (MRP3 and MRP4). The bile acid export pump (BSEP) and multidrug resistance-associated protein MRP2 are the transporters that secrete bile acids across the canalicular membrane into bile. Bile acids enter the intestinal lumen through the bile duct where they emulsify dietary lipids. Bile acids are actively reabsorbed in the distal ileum (and in cholangiocytes) via apical Na+-dependent bile acid transporter (ASBT), and are effluxed through OST alpha-OST beta. Figure modified with text, markings, arrows, and boxes, after adaptation of hepatocyte and intestine from Servier Medical Art by Servier, licensed under a Creative Commons Attribution 3.0 Unported License". Adapted from (Li and Chiang, 2014).



1.3.1. Na^+-taurocholate cotransporting polypeptide

Sodium-taurocholate transporting polypeptide (NTCP; *SLC10A1* gene) has been characterized as the major bile acid uptake transporter in the basolateral membrane of hepatocytes. In addition to conjugated bile acids, it transports unconjugated bile acids with a lower affinity (Stieger, 2011). For instance, NTCP-mediated transport accounts for over 80% of taurocholate uptake but only about 55% of cholate transport (Trauner and Boyer, 2003). Similarly, NTCP has a higher affinity for dihydroxy bile acids than trihydroxy ones (Stieger, 2011). Bile acid transport is driven by a sodium gradient produced by the ATP- dependent sodium pump, Na^+/K^+-ATPase, which is basically a cotransporter of sodium ions and bile acid molecules. The rat liver NTCP has 362 amino acids and 51 kDa, while the human liver NTCP has 349 amino acids and 50 kDa. NTCP is expressed only in mammalian hepatocytes and first appear in developing rat livers before birth. Rodent kidney,

pancreatic acinar cells, and placenta have been detected with very low levels of mRNA, but their function is unclear (Boyer, 2013). There are both endogenous compounds and xenobiotics in the spectrum of NTCP substrates, for instance, bromosulfophtalein, dehydroepiandrosterone, estrone-3-sulfate, triiodo-thyronine, thyroxine, sulindac, and rosuvastatin (Stieger 2011). Under physiological conditions, it appears that NTCP activity can be regulated by post-transcriptional mechanisms to match Na^+-dependent bile acid uptake with bile acid load. However, NTCP is primarily regulated at the level of transcription. Major task is to protect hepatocytes from toxic influence of cumulating bile acids. An adaptive response during cholestasis therefore reduces bile acid entry into the hepatocyte, essentially by suppressing *SLC10A1* gene expression regardless of the molecular mechanisms and transcription factors. There are a variety of mechanisms that can reduce NTCP transcription under pathological conditions. Essential is feedback mechanism when cumulating bile acids activate their sensor, FXR. However, factors such as inflammation, estrogen administration, pregnancy, biliary tract obstructions, and drugs or toxins may all suppress *SLC10A1* expression (Dawson et al., 2009).

1.3.2. Organic anion transporting polypeptides

Organic anion transporting polypeptides, OATPs (encoded by *SLCO* genes sub-family 21) represent a large group of transporters with an important role, especially in drug transport. According to estimates, approximately 25% of bile acids are taken up by hepatocyte basolateral sinusoidal membrane by Na^+-independent mechanisms (Trauner and Boyer, 2003). OATPs are electroneutral anion exchangers and are thought to transport bile acids and other solutes in exchange for intracellular anions such as glutathione (Li et al., 2000) and bicarbonate. OATPs have broad substrate preferences and facilitate hepatic uptake of various organic amphipathic compounds, regardless of their structures or electrical charges, typically with molecular weights greater than 300 kDa (Trauner and Boyer, 2003; Hagenbuch and Gui, 2008; Meyer zu Schwabedissen and Kim, 2009). Besides endogenous substrates such as bile salts, thyroid hormones, conjugated steroid hormones, prostaglandins, and bilirubin, the OATPs transport a wide variety of xenobiotics and environmental toxins. Human liver contains four OATPs, OATP1A2 (formerly OATPA, gene symbol *SLCO1A2*), OATP1B1 (formerly OATPC, gene symbol *SLCO1B1*), OATP1B3 (formerly OATP8, gene symbol *SLCO1B3*), and OATP2B1 (formerly OATPB, gene symbol *SLCO2B1*). It is the OATP1B1 and OATP1B3 with the highest functional importance for substrate transport into

hepatocytes. In rodents, bile acid uptake is mediated by OATP1A1, OATP1A4, and OATP1B2 (Hagenbuch and Meier, 2004; Hagenbuch and Gui, 2008; Obaidat et al., 2012).

1.3.3. Bile salt export pump

BSEP (gene symbol *ABCB11*) is a member of the *ABC* (ATP-binding cassette) family of transporters functioning as a major canalicular bile acid export pump. It enables the rate-limiting step in exporting bile acids from hepatocytes into bile canaliculi (Stieger and Geier, 2011). This step requires energy from ATP since it is performed against the significant concentration gradient. Although BSEP transports both conjugated and unconjugated bile acids, it has a greater affinity for conjugated bile acids, which markedly dominate in the liver (Mita et al., 2006). The loss of BSEP function causes the liver to accumulate bile acids, resulting in liver disorders such as PFIC2 (Progressive familial intrahepatic cholestasis type 2) and benign recurrent intrahepatic cholestasis type 2 (BRIC2). PFIC2 leads to cirrhosis and requires liver transplantation, while BRIC2 is a less severe form associated with intermittent episodes of cholestasis. The severity of the disease depends on the extent of functional impairment of BSEP. BSEP dysfunction increases the risk of intrahepatic cholestasis during pregnancy (Lam et al., 2007). In addition to bile acids, BSEP also transports drugs such as pravastatin, vinblastine, and fexofenadine (Hirano et al., 2005).

1.3.4. Multidrug resistance-associated proteins

MRPs, also known as multidrug resistance proteins, belong to the *ABCC* subfamily of transporters. Several MRPs, including MRP1 (encoded by *ABCC1* gene), MRP2 (encoded by *ABCC2* gene), MRP3 (encoded by *ABCC3* gene), MRP4 (encoded by *ABCC4* gene), MRP5 (encoded by *ABCC5* gene), and MRP6 (encoded by *ABCC6* gene), are located on the basolateral membrane of hepatocytes. Their primary function is to efflux endogenous compounds and xenobiotics from hepatocytes into the sinusoidal blood (Keppler, 2014). MRP2, is a unique member of the MRPs group since it is located on the apical membrane of hepatocytes. It was first cloned in rat hepatocytes and was known as a hepatocellular canalicular multiple organic anion transporter (cMOAT) (Büchler et al., 1996) . MRP2 is a major apical efflux pump for biliary secretion of various organic anions, including glucuronide and sulfate conjugated bile acids (Keppler, 2011). MRP2 provides a key component involved in bile acid-independent bile formation through biliary secretion of glutathione. Besides hepatocytes, MRP2 is found in large quantities at the apical plasma membranes of epithelial cells in the small intestine and renal proximal tubules (Büchler et

al., 1996). Homozygous mutations of the gene encoding MRP2 (*ABCC2*) cause Dubin-Johnson syndrome, a rare liver disorder that presents with conjugated hyperbilirubinemia (Jemnitz et al., 2010). The Eisai hyperbilirubinemic rat (EHBR) and the Groninger yellow/transporter-deficient rat (TR−) strains have been used as animal models of MRP2 deficiency, providing much information about the role of this *ABC* transporter. Recent studies indicate that bile acid plasma levels are often elevated in subjects with *ABCC2* mutations (Junge et al., 2021). It has been demonstrated that *ABCC2* variants are associated with an increased risk of intrahepatic cholestasis during pregnancy and cholestasis caused by estrogen contraceptives (Sookoian et al., 2008). The MRP3 and MRP4 are other members of *ABCC* subfamily transporting with high affinity and specificity divalent bile acids such as taurolithocholate and taurodeoxycholate through the basolateral membrane of hepatocytes. MRP3 in rodents may also transport monovalent bile acids such as taurocholate and glycocholate (Keppler, 2011). Typically, MRP3 and MRP4 are low in expression, but they rise in cholestasis or in MRP2 deficiency. Their increased expression and consequently increased extrusion of bile acids into the blood represents a compensatory mechanism to protect hepatocytes from accumulating bilirubin and bile acids (Zollner and Trauner, 2008).

1.4. Intestinal bile acid transport

A minimum amount of bile acids is lost in the feces every day, and 90-95% of them are reabsorbed in the intestine, mainly by active transport in ileum. Therefore, the amount of bile acid synthesized by the liver compensates the daily fecal loss and maintain a constant bile acid pool (Li and Chiang, 2014).

1.4.1. Apical sodium-dependent bile salt transporter

Apical sodium-dependent bile acid transporter (ASBT, gene symbol *SLC10A2*), also known as an ileal bile acid transporter (IBAT) is expressed on enterocyte apical membranes and mediates bile acid absorption from the ileum. ASBT is a member of the *SLC10* family of solute carrier proteins that requires sodium co-transport for the activity. It was first cloned from the hamster ileum (Wong et al., 1994; Wong et al., 1995). ASBT dysfunction is one of the causes of bile acid diarrhea. In animal models of cholestatic liver disease and non-alcoholic steatohepatitis (NASH), pharmacological inhibition of ASBT leads to an increased bile acid load in the colon followed by a lower bile acid pool, which improves liver histology (Al-Dury and Marschall, 2018). Human studies showed that ASBT inhibitors might be effective in patients with idiopathic chronic constipation, where they increased the number

of bowel movements. IBAT inhibitors improved itching in patients with adult and pediatric cholestatic liver diseases. Based on their mode of action, abdominal pain and diarrhea have been reported as typical adverse events of ASBT inhibitors (Al-Dury and Marschall, 2018).

1.4.2. Organic solute transporters

Organic solute transporter (OST) α/β, is the heterodimeric transporter expressed on the epithelial basolateral membranes in the intestine, kidney, liver, testes, and adrenal glands. OSTα/β allows bile acids and other steroid-like solutes to be excreted from the epithelial cells to the interstitial space, which is essential for reabsorption and homeostasis maintenance of bile acids and other hormones (Frankenberg et al., 2006; Soroka et al., 2008; Sultan et al., 2018). OSTα/β-mediated transport is driven by substrate concentration gradients along both sides of the membrane (Ballatori et al., 2005; Malinen et al., 2018). In mice, these transporters are seen in the intestine but are barely expressed in the liver, whereas human livers express OSTα/β at a higher level than in the intestine (Soroka et al., 2008). The expression of hepatic OSTα/β is significantly increased in obstructive cholestasis and primary biliary cholangitis, contributing to an adaptive response to enhance the export of cumulating bile acids from hepatocytes. It is interesting to note that OSTα/β-deficient mice are protected against cholestasis because the absence of this transporter in the ileum and renal proximal tubules increases bile acid elimination into feces and urine (Soroka et al., 2011).

1.5. 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 triggered when bile acids accumulate beyond physiologic levels, typically during cholestasis. In this circumstance, bile acids bind to the FXR nuclear receptor and modulate the expression of target genes. Other nuclear receptors such as PXR, CAR, and VDR contribute to the regulation of bile acid metabolism with a less significant role than FXR, but still may be an attractive therapeutic target. Interestingly, the overall adaptive response is similar, whatever is the initial cause of cholestasis. In the liver, cumulating bile acids suppress the expression of their synthetic enzymes and uptake transporters, and induce the expression of efflux transporters on basolateral and canalicular membranes. In the intestine, the expression of the ASBT transporter is reduced while efflux OSTα/β is increased.

However, adaptive anticholestatic response can be modulated by numerous factors given by the pathophysiology of the disease or the drug used for the therapy. The essential mechanisms are as follows.

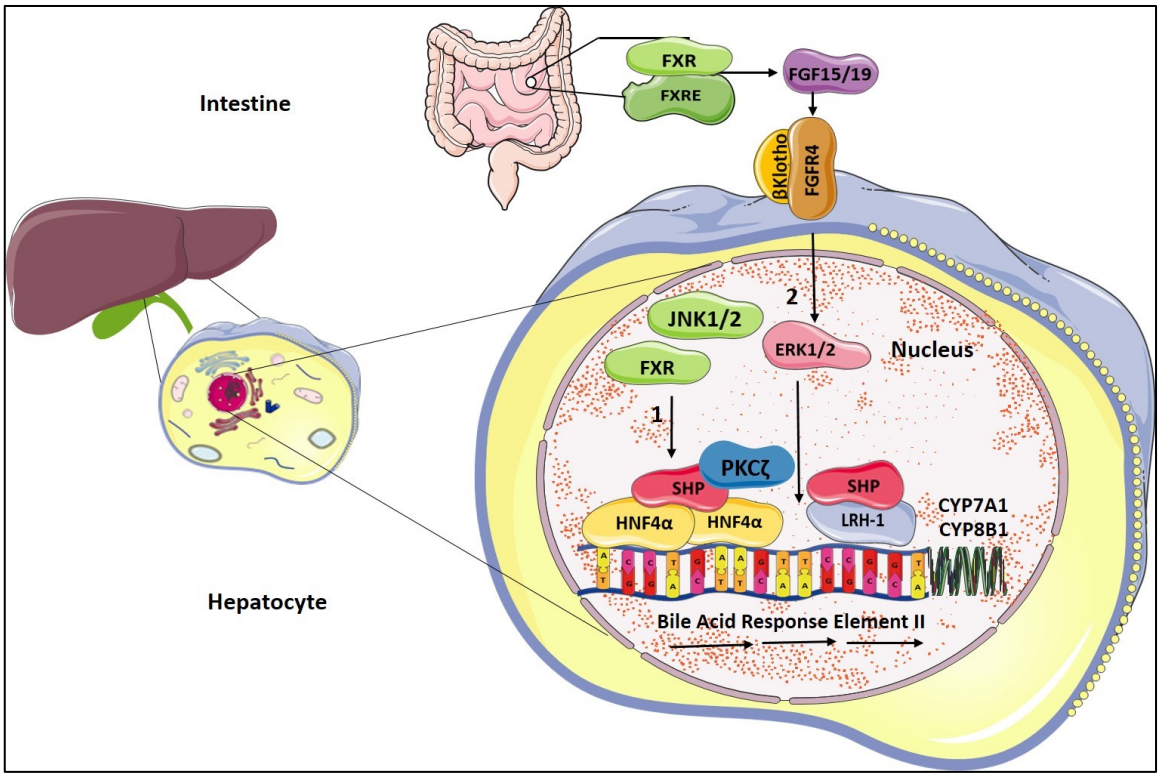
1.5.1. Farnesoid X receptor

Farnesoid X receptor (FXR; gene symbol *NR1H4*) is the nuclear transcription factor with a crucial role regulating of bile acid turnover, as revealed by numerous studies on FXR- /- mice (Petrescu and DeMorrow, 2021). FXR is the bile acid sensor with low intrinsic activity at physiological concentration of bile acids letting the synthetic pathways in the liver continuously replace bile acids lost via feces. Increasing concentrations of bile acids during liver pathologies stimulates FXR activity. The ability of individual bile acids to activate FXR differs and can be classified according to their *in vitro* binding affinity from high (left) to low (right) as follows: LCA > CDCA > Tauro CDCA > Glyco CDCA >> DCA > UDCA >> CA > Tauro CA > Glyco CA. Interestingly, the highly hydrophilic bile acids Tauro α-MCA and Tauro β-MCA act as FXR antagonists, which may render the FXR receptor inactive at physiological conditions in rodents (Sayin et al., 2013). It is, therefore, important to evaluate concentrations of individual bile acids when judging the changes in FXR target genes in different pathophysiological situations. Other natural ligands of FXR have also been identified from plant sources such as stigmasterol or guggulsterone. Both compounds are antagonists of FXR receptor.

Essentially, FXR regulates bile acid homeostasis by two principal pathways (Figure 5). **(1)** In the liver, FXR activates the expression of small heterodimer partner (SHP), the co-repressor inhibiting the transcriptional activity of a nuclear receptor liver-related homolog-1 (LRH-1) necessary for activation of hepatocyte nuclear factor 4α (HNF4α) and fetoprotein transcription factor (FTF). HNF4α and FTF activate the expression of the *Cyp8b1* gene, with FTF likely playing a more critical role. When SHP binds to FTF and/or HNF4α, an inhibitory complex is formed, acting as a transcriptional repressor of CYP8B1 (Chen et al., 2001). Interestingly, SHP seems to play a minor role in the suppression of *Cyp7a1* gene expression because bile acid-feeding still represses CYP7A1 in *Shp* null mice (Li and Chiang, 2014). It has been suggested that liver SHP regulation is sufficient in physiological situation, but the alternative pathways become important during pathological status or when the bile acid pool is depleted (Hayhurst et al., 2001; Kerr et al., 2002). **(2)** FXR is also significantly expressed in the ileum enterocytes, where its activation induces the

production of fibroblast growth factor 19 (FGF15 in mice; FGF19 in humans). The FGF19 is released into the portal blood and binds to its cognate FGF receptor 4/β-klotho (FGFR4/β-KL) complex on the hepatocyte basolateral membrane (Inagaki et al., 2005). This triggers the activation of the intracellular Extracellular Signal-Related Kinase (ERK), and Jun N- terminal Kinase (JNK) signaling pathways. The ERK and JNK both effectively reduce *CYP7A1* gene expression. A comparison of organ-specific deletions of FXR in mice confirmed that the intestinal FXR/FGF15 pathway is crucial for suppressing gene expression of both *Cyp7a1* and *Cyp8b1*, while the liver FXR/SHP pathway was most effective at suppressing *Cyp8b1* gene expression (Chiang and Ferrell, 2020). FGF19 levels in human serum peak 1.5 to 2 hours after releasing bile acids from gallbladder by cholecystokinin signaling. This reduces bile acid synthesis activated by post-prandial hyperglycemia. In agreement, cholestyramine, a bile acid sequestrant, decreases FGF19 levels by preventing the absorption of FXR agonistic bile acids to enterocytes (Lundåsen et al., 2006).

Figure 5: Bile acid feedback inhibition of bile acid synthesis. CYP7A1 and CYP8B1 are inhibited by bile acid–activated signaling, which decreases bile acid synthesis in the liver. *CYP7A1* gene promoter contains bile acid response element (BARE), a sequence with AGGTCA-like direct repeats. HNF4α and LRH1 bind to BARE and promote transcription of the *CYP7A1* gene. Bile acids activate the FXR in hepatocytes, which activates the SHP repressor. As a result of SHP interaction with HNF4α, it represses the transactivating effects thereof, a process involving chromatin remodeling enzymes and the corepressor complex (pathway 1). Enterocytes respond to bile acid- activated FXR by binding and activating FGF15 (FGF19 in humans). A number of intracellular signaling pathways are activated by FGFR4, such as ERK, protein kinase Cζ (PKCζ), and c-Jun N-terminal kinase (JNK), that are involved in repressing *CYP7A1* gene transcription (pathway 2). Figure modified with text, markings, arrows, and boxes, after adaptation of hepatocyte and intestine from Servier Medical Art by Servier, licensed under a Creative Commons Attribution 3.0 Unported License". Adapted from (Li and Chiang, 2014).



1.5.2. Pregnane X receptor

Pregnane X receptor (PXR; gene symbol *NR1I2*) is a nuclear receptor activated by xenobiotics, including drugs, steroids, and bile acids such as LCA and UDCA. The receptor is primarily expressed in the liver and intestine, and its activation increases the expression of drug-metabolizing enzymes of phase I (CYPs), phase II (conjugation enzymes), and drug efflux transporters. Activation of human PXR inhibits gene expression of *CYP7A1* by disrupting the interaction between HNF4α and PGC-1α (Peroxisome proliferator-activated receptor gamma coactivator 1-α) required for their recruitment at the *CYP7A1* promotor (Chiang, 2009; Chiang and Ferrell, 2020). This effect may not be detected in wild-type mice, where PXR activation suppresses alternative pathways of bile acid synthesis (CYP27A1 and CYP7B1) without effect on CYP7A1. Interestingly, CYP7A1 is induced by PXR agonist in CYP3A11 deficient mice. During cholestasis, PXR may reduce bile acid pool size by enhancing their degradation metabolism (induction of CYPs mediated hydroxylation, and sulfate- and glucuronide-conjugation) and biliary secretion (induction of MRP2).

1.5.3. Constitutive androstane receptor

Constitutive androstane receptor (CAR; gene symbol *NR1I3*) is a nuclear receptor sensitive to LCA and xenobiotics. LCA and another typical substrate phenobarbital activate CAR indirectly by facilitating its nuclear localization, which leads to the induction of its target genes (Dempsey et al., 2019). CAR is also predominantly expressed in the liver and intestine, and the effect is similar to PXR; it mediates the induction of drug-metabolizing enzymes and efflux transporters and represses the enzymes for bile acids synthesis (CYP8B1, and CYP7B1). Activation of CAR reduced bile acids serum concentration in healthy as well as in cholestatic mice with bile duct ligation in one study (Wagner et al., 2005). However, several other studies reported significant induction of mice CYP7A1 by CAR agonists and unchanged concentrations of bile acids in plasma (Claudel et al., 2011; Chiang, 2013; Cheng et al., 2017).

1.5.4. Vitamin D receptor

Vitamin D receptor (VDR, gene symbol *NR1I1*) is a nuclear, ligand-dependent transcription factor that is activated by 1α,25-hydroxyvitamin D3. VDR is markedly expressed in mineral-regulating organs, such as the intestine, bone, kidney, and parathyroid glands, and its primary function is the regulation of calcium and phosphate homeostasis and bone formation. The expression of VDR in the liver is low. It can be found in hepatic stellate cells and human hepatocytes while missing in mouse hepatocytes. Regarding bile acids, VDR can be activated by LCA, especially in the intestine and in the liver with following induction of drug-metabolizing P450 cytochromes such as CYP3A4, and CYP2Cs and sulfotransferases. During cholestasis, LCA levels may significantly increase in the liver, and VDR suppresses CYP7A1 expression by interaction with HNF4α. VDR also increases intestinal production of FGF15 in mice; therefore, it can reduce CYP7A1 via activation of the ERK1/2 pathway. On the other hand, VDR inhibits hepatic stellate cells activation and may reduce liver injury during cholestasis, which may thereafter increase CYP7A1 without altering serum bile acid concentration or total pool size.

1.5.5. Others

Various factors, including the excess of bile acids such as CDCA may repress bile acid synthesis in the liver by activation of macrophages (Kupffer cells) with the release of proinflammatory cytokines (tumor necrosis factor-α and interleukin-1β) and hepatic stellate

cells with secretion of growth factors (transforming growth factor β1) (Li et al., 2006; Li et al., 2008; El Kasmi et al., 2012). These molecules activate receptors at basolateral membrane of hepatocytes and triggers 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 hepcidin production, enhancing 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., 2019).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 the relationship between iron overload and bile acid metabolomics are missing and require further clarification.

The 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 the risk of consequent toxicity. The adaptive changes in bile acids synthesizing enzymes and transporters may minimize or at least reduce liver injury. Importantly, alteration of transporters responsible for the turnover of bile acids (e.g. by genetic defects, hormones, drugs, or inflammation) may be primary cause of cholestasis. Identification of mechanisms mediating and regulating bile acid metabolomics during cholestasis helps to understand the pathophysiology of such diseases and opens new diagnostic and therapeutic possibilities. This is especially important in pharmacologically treatable intrahepatic cholestasis, where appropriate therapy may enable prophylaxis of serious complications. A typical example of this situation is the intrahepatic cholestasis of pregnancy.

1.6. 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 a 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 the highest priority. The need is further accentuated by 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 (Li et al., 2016). The expression of biliary transporters is physiologically decreasing during the pregnancy due to the increased production of cholestatic estrogens and progestins (Aleksunes et al., 2012). Consequently, the women with a 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, the predisposition factor for ICP is also the 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 mother's organism, and consequently in the reverting of the bile acid 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 the 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., 2002; Zhu et al., 2013; Song et al., 2014; Song et al., 2015) 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 a 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 the accumulation of bile acids during ICP is currently at the center of attention.

Previous studies have demonstrated that ICP has a 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 metformin administration (Elfituri et al., 2016). The mechanism of such an effect is unclear. It can only be assumed that the positive effects on bile acid metabolomics originate from the ability of metformin to block the respiratory complex 1 of mitochondria in hepatocytes with a consequent increase in AMP/ATP ratio, leading to stimulation of AMP-activated protein kinase (AMPK). Concomitant accumulation of NAD^+ activates SIRT1, which deacetylates FOXA2 (Forkhead/winged-helix). FOXA2 activates the transcription of genes for the synthesis and biliary secretion of bile acids. Thus, by activation of the SIRT1-FOXA2 cascade, metformin can increase the synthesis but also the elimination of bile acids from the organism (Chen et al., 2017). Furthermore, activation of SIRT1 by SRT1720 protects against estrogen cholestasis in mice, while AMPK activation may attenuate bile acid synthesis, especially under their increased concentrations (Lien et al., 2014). Therefore, these discrepancies in the possible effects of metformin need to be studied under cholestatic conditions. 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.

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 factors. 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 the modulation of bile acid metabolomics by metformin in healthy mice with intact livers as well 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. Therefore, there is 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 explain 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. 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 the study
  - Plasma samples preparation for biochemistry analysis
  - Stool samples preparation to the 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 the biliary secretion of bile acids. Previous studies in humans suggested an increased incidence of ICP in women with a 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 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 the 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 the enterohepatic recycling of bile acids were not modified by MRP2 deficiency. Instead, we detected impaired 12α-hydroxylation of bile acids due to downregulation of CYB8B1, 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 the significant role of MRP2 transporter in the turnover of all bile acids, not only those conjugated with sulfate or glucuronic acid. A deficit

of MRP2 clearly predisposes to 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 at a greater risk of cholestasis. Particular 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 the study
  - Plasma samples preparation for biochemistry analysis
  - Stool samples preparation for the 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. Scientific Reports 2019; Sci Rep. 2019 Jul 5;9(1):9780.) (IF = 4.379, Q1)

In this study, we significantly extended the knowledge about the possible regulatory role of iron overload on the bile acid biochemistry. Iron is an essential trace element with multiple physiological functions in the organism. However, iron is also a highly reactive molecule contributing to the generation of 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 thalassemia), or secondary to blood transfusion and hemolysis.

We utilized a rat model of iron overload induced by repeated intraperitoneal administration of iron to markedly increase the content of iron within the liver. This situation led to liver impairment as evaluated by plasma biochemistry or histopathological examination. Excess 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 the excess of iron due to reduced reabsorption of bile acids in the intestine. Interestingly, this change was not the consequence of the altered expression of reuptake transporters, but it was related to the increased synthesis of hyodeoxycholic acid, which is poorly absorbed from the intestine.

Collectively, our results revealed the complex regulatory function of iron on the metabolomics of bile acids. Especially impaired expression of numerous transporters necessary for liver elimination of endo, and xenobiotic may contribute to iron-induced hepatotoxicity. Exact regulatory mechanisms contributing to observed changes are unknown, but our data suggested that the 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 the study
  - Plasma samples preparation for biochemistry analysis
  - Stool samples preparation for 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 by facilitated excretion into bile due to increased bile acid-mediated bile flow. The 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). 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. *Sirt1* 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 healthy mice does not 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 bile acids 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 SRT1720 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 the bile duct-ligated group (Blokker, Maijo 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 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 than 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 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 bile acid 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 (unpublished observation). 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 the transporter for biliary secretion of sulfate, and glucuronic acid-conjugated bile acids, mainly TDCA and LCA, which are minor in the whole bile acid pool. Previous studies instead 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 of analyzing 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 transport deficient rats was 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 activating 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 a 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 might 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 **(i)** activates

inflammatory response through JNK-NF-κB pathway, **(ii)** reduces the content of RXRα receptor crucial for dimerization and activation of FXR, CAR or PXR, and **(iii)** 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 the interaction of activated ERK1/2 and JNK1/2 with transcription factors hepatocyte nuclear factor 1 and 4α (HNF) to block the 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/β-glucuronide. Therefore, a more intensive effect in MRP2-deficient rats may be the consequence of ethinylestradiol cumulation (Zamek-Gliszczynski et al., 2011). Notably, MRP4 expression was increased in estrogen-treated MRP2-deficient rats despite reduced activity of CAR-NRF2 pathway, which plays a central position in MRP4 regulation together with the peroxisome proliferator-activated receptor a (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. Thus, 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 of bile acids. 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 the 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* were 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 the 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 also identified 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 present one, described increased plasma concentrations of cholesterol when the iron is excessively stored in the liver (Brunet et al., 1999; Coppin et al., 2007). Our present 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 lipid metabolism disorders 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 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 primary 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 the 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. However, marked reduction of liver SHP and absence of change in intestinal FGF15 contradicted the 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 includes a transcriptional as well as significant post-transcriptional control. Our results show that post-transcriptional 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 the 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). Therefore, we 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 the disposition of bile acid in systemic circulation and compensated for their reduced synthesis and intestinal reabsorption to maintain stable concentrations in plasma. In addition, changes in MRPs and induction of heme oxygenase-1 explained increased concentrations of bilirubin in plasma of rats with iron overload.

For the first time, our study described 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 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 movement 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

Al-Dury S and Marschall HU (2018) Ileal Bile Acid Transporter Inhibition for the Treatment of Chronic Constipation, Cholestatic Pruritus, and NASH. *Frontiers in pharmacology* **9**:931.

Aleksunes LM, Yeager RL, Wen X, Cui JY and Klaassen CD (2012) Repression of Hepatobiliary Transporters and Differential Regulation of Classic and Alternative Bile Acid Pathways in Mice During Pregnancy. *Toxicological Sciences* **130**:257-268.

Ballatori N, Christian WV, Lee JY, Dawson PA, Soroka CJ, Boyer JL, Madejczyk MS and Li N (2005) OSTalpha-OSTbeta: a major basolateral bile acid and steroid transporter in human intestinal, renal, and biliary epithelia. *Hepatology (Baltimore, Md)* **42**:1270-1279.

Binder T, Salaj P and Zima T (2007) Intrahepatic cholestasis in pregnancy-guidelines of treatment]. *Ceska gynekologie* **72**:94-98.

Biyyani RS, Battula S, Erhardt CA and Korkor K (2009) Metformin-induced cholangiohepatitis. *BMJ case reports* **2009**.

Blokker BA, Maijo M, Echeandia M, Galduroz M, Patterson AM, Ten A, Philo M, Schungel R, Gutierrez-de Juan V, Halilbasic E, Fuchs C, Le Gall G, Milkiewicz M, Milkiewicz P, Banales JM, Rushbrook SM, Mato JM, Trauner M, Müller M, Martínez-Chantar ML, Varela-Rey M and Beraza N (2019) Fine-Tuning of Sirtuin 1 Expression Is Essential to Protect the Liver From Cholestatic Liver Disease. *Hepatology* **69**:699-716.

Boyer JL (2013) Bile formation and secretion. *Comprehensive Physiology* **3**:1035-1078.

Brunet S, Thibault L, Delvin E, Yotov W, Bendayan M and Levy E (1999) Dietary iron overload and induced lipid peroxidation are associated with impaired plasma lipid transport and hepatic sterol metabolism in rats. *Hepatology (Baltimore, Md)* **29**:1809-1817.

Büchler M, König J, Brom M, Kartenbeck J, Spring H, Horie T and Keppler D (1996) cDNA cloning of the hepatocyte canalicular isoform of the multidrug resistance protein, cMrp, reveals a novel conjugate export pump deficient in hyperbilirubinemic mutant rats. *The Journal of biological chemistry* **271**:15091-15098.

Cao J, Stieger B, Meier PJ and Vore M (2002) Expression of rat hepatic multidrug resistance-associated proteins and organic anion transporters in pregnancy. *American journal of physiology Gastrointestinal and liver physiology* **283**:G757-766.

Claudel T, Zollner G, Wagner M and Trauner M (2011) Role of nuclear receptors for bile acid metabolism, bile secretion, cholestasis, and gallstone disease. *Biochimica et biophysica acta* **1812**:867-878.

Coppin H, Darnaud V, Kautz L, Meynard D, Aubry M, Mosser J, Martinez M and Roth MP (2007) Gene expression profiling of Hfe-/- liver and duodenum in mouse strains with differing susceptibilities to iron loading: identification of transcriptional regulatory targets of Hfe and potential hemochromatosis modifiers. *Genome Biol* **8**:R221.

Dawson PA, Lan T and Rao A (2009) Bile acid transporters. *Journal of lipid research* **50**:2340-2357.

Dempsey JL, Wang D, Siginir G, Fei Q, Raftery D, Gu H and Yue Cui J (2019) Pharmacological Activation of PXR and CAR Downregulates Distinct Bile Acid-Metabolizing Intestinal Bacteria and Alters Bile Acid Homeostasis. *Toxicological Sciences* **168**:40-60.

Desilets DJ, Shorr AF, Moran KA and Holtzmuller KC (2001) Cholestatic jaundice associated with the use of metformin. *Am J Gastroenterol* **96**:2257-2258.

di Gregorio MC, Cautela J and Galantini L (2021) Physiology and Physical Chemistry of Bile Acids. *International journal of molecular sciences* **22**.

Dzeletovic S, Babiker A, Lund E and Diczfalusy U (1995) Time course of oxysterol formation during in vitro oxidation of low density lipoprotein. *Chemistry and physics of lipids* **78**:119-128.

El Kasmi KC, Anderson AL, Devereaux MW, Fillon SA, Harris JK, Lovell MA, Finegold MJ and Sokol RJ (2012) Toll-like receptor 4-dependent Kupffer cell activation and liver injury in a novel mouse model of parenteral nutrition and intestinal injury. *Hepatology* **55**:1518-1528.

Elfituri A, Ali A and Shehata H (2016) Managing Recurring Obstetric Cholestasis With Metformin. *Obstetrics and gynecology* **128**:1320-1323.

Eyssen HJ, De Pauw G and Van Eldere J (1999) Formation of hyodeoxycholic acid from muricholic acid and hyocholic acid by an unidentified gram-positive rod termed HDCA-1 isolated from rat intestinal microflora. *Appl Environ Microbiol* **65**:3158-3163.

Frankenberg T, Rao A, Chen F, Haywood J, Shneider BL and Dawson PA (2006) Regulation of the mouse organic solute transporter alpha-beta, Ostalpha-Ostbeta, by bile acids. *American journal of physiology Gastrointestinal and liver physiology* **290**:G912-922.

Fu S, Fan J, Blanco J, Gimenez-Cassina A, Danial NN, Watkins SM and Hotamisligil GS (2012) Polysome profiling in liver identifies dynamic regulation of endoplasmic reticulum translatome by obesity and fasting. *PLoS genetics* **8**:e1002902.

Gavrilova O, Geyer J and Petzinger E (2007) In vivo relevance of Mrp2-mediated biliary excretion of the Amanita mushroom toxin demethylphalloin. *Biochimica et biophysica acta* **1768**:2070-2077.

Geenes V, Chappell LC, Seed PT, Steer PJ, Knight M and Williamson C (2014) Association of severe intrahepatic cholestasis of pregnancy with adverse pregnancy outcomes: a prospective population-based case-control study. *Hepatology* **59**:1482-1491.

Geier A, Wagner M, Dietrich CG and Trauner M (2007) Principles of hepatic organic anion transporter regulation during cholestasis, inflammation and liver regeneration. *Biochim Biophys Acta* **1773**:283-308.

Hagenbuch B and Gui C (2008) Xenobiotic transporters of the human organic anion transporting polypeptides (OATP) family. *Xenobiotica; the fate of foreign compounds in biological systems* **38**:778-801.

Hagenbuch B and Meier PJ (2004) Organic anion transporting polypeptides of the OATP/ SLC21 family: phylogenetic classification as OATP/ SLCO superfamily, new nomenclature and molecular/functional properties. *Pflugers Archiv : European journal of physiology* **447**:653-665.

Hayhurst GP, Lee YH, Lambert G, Ward JM and Gonzalez FJ (2001) Hepatocyte nuclear factor 4alpha (nuclear receptor 2A1) is essential for maintenance of hepatic gene expression and lipid homeostasis. *Molecular and cellular biology* **21**:1393-1403.

He D, Barnes S and Falany CN (2003) Rat liver bile acid CoA:amino acid N-acyltransferase: expression, characterization, and peroxisomal localization. *Journal of lipid research* **44**:2242-2249.

He XJ, Zuo HC, Chen L, Sun TS, Mao GS, Luan GM, Feng SQ, Zhong N, Ao Q, Huang Q, Liu ZH, Luan Z, Man Y, Zheng ZC, Rao YJ, Fan ZH, Xiang GH, Wang YL, Wang HL, Lu YJ, Lv XF, Geng TC, Xing HY, Li T, Huang HY, IANR IAN and Neurorestoratology CA (2013) Standard Recommendations for the Application of Chinese Clinical Cell Therapy for Neurorestoration (2012). *Cell Transplant* **22**:S5-S10.

Hirano M, Maeda K, Hayashi H, Kusuhara H and Sugiyama Y (2005) Bile salt export pump (BSEP/ABCB11) can transport a nonbile acid substrate, pravastatin. *The Journal of pharmacology and experimental therapeutics* **314**:876-882.

Homolya L, Fu D, Sengupta P, Jarnik M, Gillet JP, Vitale-Cross L, Gutkind JS, Lippincott-Schwartz J and Arias IM (2014) LKB1/AMPK and PKA control ABCB11 trafficking and polarization in hepatocytes. *PLoS One* **9**:e91921.

Horak W and Grabner G (1975) [Physiology of bile secretion. *Acta medica Austriaca* **2**:73-81.

Chen HJ, Sugiyama M, Shimokawa F, Murakami M, Hashimoto O, Matsui T and Funaba M (2020) Response to iron overload in cultured hepatocytes. *Sci Rep* **10**:21184.

Chen Q, Yang X, Zhang H, Kong X, Yao L, Cui X, Zou Y, Fang F, Yang J and Chang Y (2017) Metformin impairs systemic bile acid homeostasis through regulating SIRT1 protein levels. *Biochim Biophys Acta Mol Cell Res* **1864**:101-112.

Chen W, Owsley E, Yang Y, Stroup D and Chiang JY (2001) Nuclear receptor-mediated repression of human cholesterol 7alpha-hydroxylase gene transcription by bile acids. *Journal of lipid research* **42**:1402-1412.

Cheng S, Zou M, Liu Q, Kuang J, Shen J, Pu S, Chen L, Li H, Wu T, Li R, Li Y, Jiang W, Zhang Z and He J (2017) Activation of Constitutive Androstane Receptor Prevents Cholesterol Gallstone Formation. *The American journal of pathology* **187**:808-818.

Chiang JY (2009) Bile acids: regulation of synthesis. *Journal of lipid research* **50**:1955-1966.

Chiang JY (2013) Bile acid metabolism and signaling. *Comprehensive Physiology* **3**:1191-1212.

Chiang JYL and Ferrell JM (2020) Bile acid receptors FXR and TGR5 signaling in fatty liver diseases and therapy. *American journal of physiology Gastrointestinal and liver physiology* **318**:G554-g573.

Chopra AR, Kommagani R, Saha P, Louet JF, Salazar C, Song J, Jeong J, Finegold M, Viollet B, DeMayo F, Chan L, Moore DD and O'Malley BW (2011) Cellular energy depletion resets whole-body energy by promoting coactivator-mediated dietary fuel absorption. *Cell Metab* **13**:35-43.

Choudhuri S and Klaassen CD (2022) Molecular Regulation of Bile Acid Homeostasis. *Drug metabolism and disposition: the biological fate of chemicals* **50**:425-455.

Inagaki T, Choi M, Moschetta A, Peng L, Cummins CL, McDonald JG, Luo G, Jones SA, Goodwin B, Richardson JA, Gerard RD, Repa JJ, Mangelsdorf DJ and Kliewer SA (2005) Fibroblast growth factor 15 functions as an enterohepatic signal to regulate bile acid homeostasis. *Cell metabolism* **2**:217-225.

Jemnitz K, Heredi-Szabo K, Janossy J, Ioja E, Vereczkey L and Krajcsi P (2010) ABCC2/Abcc2: a multispecific transporter with dominant excretory functions. *Drug metabolism reviews* **42**:402-436.

Joutsiniemi T, Timonen S, Leino R, Palo P and Ekblad U (2014) Ursodeoxycholic acid in the treatment of intrahepatic cholestasis of pregnancy: a randomized controlled trial. *Archives of gynecology and obstetrics* **289**:541-547.

Junge N, Goldschmidt I, Wiegandt J, Leiskau C, Mutschler F, Laue T, Ohlendorf J, Stalke A, Hartleben B, Stindt J, Keitel V, Baumann U and Pfister ED (2021) Dubin-Johnson Syndrome as Differential Diagnosis for Neonatal Cholestasis. *Journal of pediatric gastroenterology and nutrition* **72**:e105-e111.

Keppler D (2011) Multidrug resistance proteins (MRPs, ABCCs): importance for pathophysiology and drug therapy. *Handbook of experimental pharmacology*:299-323.

Keppler D (2014) The roles of MRP2, MRP3, OATP1B1, and OATP1B3 in conjugated hyperbilirubinemia. *Drug metabolism and disposition: the biological fate of chemicals* **42**:561-565.

Kerr TA, Saeki S, Schneider M, Schaefer K, Berdy S, Redder T, Shan B, Russell DW and Schwarz M (2002) Loss of nuclear receptor SHP impairs but does not eliminate negative feedback regulation of bile acid synthesis. *Developmental cell* **2**:713-720.

Kim HY, Kwon WY, Park JB, Lee MH, Oh YJ, Suh S, Baek YH, Jeong JS and Yoo YH (2020) Hepatic STAMP2 mediates recombinant FGF21-induced improvement of hepatic iron overload in nonalcoholic fatty liver disease. *Faseb j* **34**:12354-12366.

Kolouchova G, Brcakova E, Hirsova P, Sispera L, Tomsik P, Cermanova J, Hyspler R, Slanarova M, Fuksa L, Lotkova H and Micuda S (2011) Pravastatin modulates liver bile acid and cholesterol homeostasis in rats with chronic cholestasis. *J Gastroenterol Hepatol* **26**:1544-1551.

Kulkarni SR, Soroka CJ, Hagey LR and Boyer JL (2016) Sirtuin 1 activation alleviates cholestatic liver injury in a cholic acid-fed mouse model of cholestasis. *Hepatology* **64**:2151-2164.

Lam P, Pearson CL, Soroka CJ, Xu S, Mennone A and Boyer JL (2007) Levels of plasma membrane expression in progressive and benign mutations of the bile salt export pump (Bsep/Abcb11) correlate with severity of cholestatic diseases. *American journal of physiology Cell physiology* **293**:C1709-1716.

Lefebvre P, Cariou B, Lien F, Kuipers F and Staels B (2009) Role of bile acids and bile acid receptors in metabolic regulation. *Physiological reviews* **89**:147-191.

Li-Hawkins J, Lund EG, Turley SD and Russell DW (2000) Disruption of the oxysterol 7alpha-hydroxylase gene in mice. *The Journal of biological chemistry* **275**:16536-16542.

Li J and Dawson PA (2019) Animal models to study bile acid metabolism. *Biochimica et biophysica acta Molecular basis of disease* **1865**:895-911.

Li J, Xing Y, Zhou ZX, Yao WJ, Cao RY, Li TM, Xu ML and Wu J (2016) Microbial HSP70 peptide epitope 407-426 as adjuvant in tumor-derived autophagosome vaccine therapy of mouse lung cancer. *Tumor Biol* **37**:15097-15105.

Li L, Meier PJ and Ballatori N (2000) Oatp2 mediates bidirectional organic solute transport: a role for intracellular glutathione. *Molecular pharmacology* **58**:335-340.

Li T and Chiang JY (2014) Bile acid signaling in metabolic disease and drug therapy. *Pharmacological reviews* **66**:948-983.

Li T, Jahan A and Chiang JY (2006) Bile acids and cytokines inhibit the human cholesterol 7 alpha-hydroxylase gene via the JNK/c-jun pathway in human liver cells. *Hepatology* **43**:1202-1210.

Li T, Ma H and Chiang JY (2008) TGFbeta1, TNFalpha, and insulin signaling crosstalk in regulation of the rat cholesterol 7alpha-hydroxylase gene expression. *J Lipid Res* **49**:1981-1989.

Lien F, Berthier A, Bouchaert E, Gheeraert C, Alexandre J, Porez G, Prawitt J, Dehondt H, Ploton M, Colin S, Lucas A, Patrice A, Pattou F, Diemer H, Van Dorsselaer A, Rachez C, Kamilic J, Groen AK, Staels B and Lefebvre P (2014) Metformin interferes with bile acid homeostasis through AMPK-FXR crosstalk. *J Clin Invest* **124**:1037-1051.

Lundåsen T, Gälman C, Angelin B and Rudling M (2006) Circulating intestinal fibroblast growth factor 19 has a pronounced diurnal variation and modulates hepatic bile acid synthesis in man. *Journal of internal medicine* **260**:530-536.

MacKay DF, Smith GC, Dobbie R and Pell JP (2010) Gestational age at delivery and special educational need: retrospective cohort study of 407,503 schoolchildren. *PLoS Med* **7**:e1000289.

Maher JM, Slitt AL, Cherrington NJ, Cheng X and Klaassen CD (2005) Tissue distribution and hepatic and renal ontogeny of the multidrug resistance-associated protein (Mrp) family in mice. *Drug Metab Dispos* **33**:947-955.

Malinen MM, Ali I, Bezençon J, Beaudoin JJ and Brouwer KLR (2018) Organic solute transporter OSTα/β is overexpressed in nonalcoholic steatohepatitis and modulated by drugs associated with liver injury. *American journal of physiology Gastrointestinal and liver physiology* **314**:G597-g609.

Marin JJ, Macias RI, Briz O, Banales JM and Monte MJ (2015) Bile Acids in Physiology, Pathology and Pharmacology. *Current drug metabolism* **17**:4-29.

Meier PJ and Stieger B (2002) Bile salt transporters. *Annual review of physiology* **64**:635-661.

Meyer zu Schwabedissen HE and Kim RB (2009) Hepatic OATP1B transporters and nuclear receptors PXR and CAR: interplay, regulation of drug disposition genes, and single nucleotide polymorphisms. *Mol Pharm* **6**:1644-1661.

Mita S, Suzuki H, Akita H, Hayashi H, Onuki R, Hofmann AF and Sugiyama Y (2006) Inhibition of bile acid transport across Na+/taurocholate cotransporting polypeptide (SLC10A1) and bile salt export pump (ABCB 11)-coexpressing LLC-PK1 cells by cholestasis-inducing drugs. *Drug Metab Dispos* **34**:1575-1581.

Nammour FE, Fayad NF and Peikin SR (2003) Metformin-induced cholestatic hepatitis. *Endocr Pract* **9**:307-309.

Obaidat A, Roth M and Hagenbuch B (2012) The expression and function of organic anion transporting polypeptides in normal tissues and in cancer. *Annual review of pharmacology and toxicology* **52**:135-151.

Oswald S, Westrup S, Grube M, Kroemer HK, Weitschies W and Siegmund W (2006) Disposition and sterol-lowering effect of ezetimibe in multidrug resistance-associated protein 2-deficient rats. *The Journal of pharmacology and experimental therapeutics* **318**:1293-1299.

Pandak WM and Kakiyama G (2019) The acidic pathway of bile acid synthesis: Not just an alternative pathway(☆). *Liver research* **3**:88-98.

Petrescu AD and DeMorrow S (2021) Farnesoid X Receptor as Target for Therapies to Treat Cholestasis-Induced Liver Injury. *Cells* **10**.

Pinteur C, Julien B, Véga N, Vidal H, Naville D and Le Magueresse-Battistoni B (2021) Impact of Estrogen Withdrawal and Replacement in Female Mice along the Intestinal Tract. Comparison of E2 Replacement with the Effect of a Mixture of Low Dose Pollutants. *Int J Environ Res Public Health* **18**.

Prasnicka A, Lastuvkova H, Alaei Faradonbeh F, Cermanova J, Hroch M, Mokry J, Dolezelova E, Pavek P, Zizalova K, Vitek L, Nachtigal P and Micuda S (2019) Iron

overload reduces synthesis and elimination of bile acids in rat liver. *Scientific reports* **9**:9780-9780.

Pullinger CR, Eng C, Salen G, Shefer S, Batta AK, Erickson SK, Verhagen A, Rivera CR, Mulvihill SJ, Malloy MJ and Kane JP (2002) Human cholesterol 7alpha-hydroxylase (CYP7A1) deficiency has a hypercholesterolemic phenotype. *The Journal of clinical investigation* **110**:109-117.

Quigley MA, Poulsen G, Boyle E, Wolke D, Field D, Alfirevic Z and Kurinczuk JJ (2012) Early term and late preterm birth are associated with poorer school performance at age 5 years: a cohort study. *Archives of disease in childhood Fetal and neonatal edition* **97**:F167-173.

Ridlon JM, Kang DJ and Hylemon PB (2006) Bile salt biotransformations by human intestinal bacteria. *Journal of lipid research* **47**:241-259.

Ropponen A, Sund R, Riikonen S, Ylikorkala O and Aittomaki K (2006) Intrahepatic cholestasis of pregnancy as an indicator of liver and biliary diseases: a population-based study. *Hepatology* **43**:723-728.

Russell DW (2003) The enzymes, regulation, and genetics of bile acid synthesis. *Annual review of biochemistry* **72**:137-174.

Saadi T, Waterman M, Yassin H and Baruch Y (2013) Metformin-induced mixed hepatocellular and cholestatic hepatic injury: case report and literature review. *International journal of general medicine* **6**:703-706.

Sayin SI, Wahlström A, Felin J, Jäntti S, Marschall HU, Bamberg K, Angelin B, Hyötyläinen T, Orešič M and Bäckhed F (2013) Gut microbiota regulates bile acid metabolism by reducing the levels of tauro-beta-muricholic acid, a naturally occurring FXR antagonist. *Cell metabolism* **17**:225-235.

Shaw S, Eng J and Jayatilleke E (1995) Ethanol-induced free radical injury to the hepatocyte glucagon receptor. *Alcohol* **12**:273-277.

Shiffka SJ, Kane MA and Swaan PW (2017) Planar bile acids in health and disease. *Biochimica et biophysica acta Biomembranes* **1859**:2269-2276.

Schwarz M, Russell DW, Dietschy JM and Turley SD (1998) Marked reduction in bile acid synthesis in cholesterol 7alpha-hydroxylase-deficient mice does not lead to diminished tissue cholesterol turnover or to hypercholesterolemia. *Journal of lipid research* **39**:1833-1843.

Schwarz M, Russell DW, Dietschy JM and Turley SD (2001) Alternate pathways of bile acid synthesis in the cholesterol 7alpha-hydroxylase knockout mouse are not

upregulated by either cholesterol or cholestyramine feeding. *Journal of lipid research* **42**:1594-1603.

Song P, Rockwell CE, Cui JY and Klaassen CD (2015) Individual bile acids have differential effects on bile acid signaling in mice. *Toxicology and applied pharmacology* **283**:57-64.

Song X, Vasilenko A, Chen Y, Valanejad L, Verma R, Yan B and Deng R (2014) Transcriptional dynamics of bile salt export pump during pregnancy: mechanisms and implications in intrahepatic cholestasis of pregnancy. *Hepatology (Baltimore, Md)* **60**:1993-2007.

Sookoian S, Castaño G, Burgueño A, Gianotti TF and Pirola CJ (2008) Association of the multidrug-resistance-associated protein gene (ABCC2) variants with intrahepatic cholestasis of pregnancy. *Journal of hepatology* **48**:125-132.

Soroka CJ, Velazquez H, Mennone A, Ballatori N and Boyer JL (2011) Ostα depletion protects liver from oral bile acid load. *American journal of physiology Gastrointestinal and liver physiology* **301**:G574-579.

Soroka CJ, Xu S, Mennone A, Lam P and Boyer JL (2008) N-Glycosylation of the alpha subunit does not influence trafficking or functional activity of the human organic solute transporter alpha/beta. *BMC cell biology* **9**:57.

Stieger B (2011) The role of the sodium-taurocholate cotransporting polypeptide (NTCP) and of the bile salt export pump (BSEP) in physiology and pathophysiology of bile formation. *Handbook of experimental pharmacology*:205-259.

Stieger B and Geier A (2011) Genetic variations of bile salt transporters as predisposing factors for drug-induced cholestasis, intrahepatic cholestasis of pregnancy and therapeutic response of viral hepatitis. *Expert opinion on drug metabolism & toxicology* **7**:411-425.

Sultan M, Rao A, Elpeleg O, Vaz FM, Abu-Libdeh B, Karpen SJ and Dawson PA (2018) Organic solute transporter-β (SLC51B) deficiency in two brothers with congenital diarrhea and features of cholestasis. *Hepatology (Baltimore, Md)* **68**:590-598.

Takikawa H, Sano N, Narita T, Uchida Y, Yamanaka M, Horie T, Mikami T and Tagaya O (1991) Biliary excretion of bile acid conjugates in a hyperbilirubinemic mutant Sprague-Dawley rat. *Hepatology (Baltimore, Md)* **14**:352-360.

Tan TC, Crawford DH, Jaskowski LA, Murphy TL, Santrampurwala N, Crane D, Clouston AD, Subramaniam VN, Anderson GJ and Fletcher LM (2013) A corn oil-based diet

protects against combined ethanol and iron-induced liver injury in a mouse model of hemochromatosis. *Alcohol Clin Exp Res* **37**:1619-1631.

Trauner M and Boyer JL (2003) Bile salt transporters: molecular characterization, function, and regulation. *Physiological reviews* **83**:633-671.

Tribe RM, Dann AT, Kenyon AP, Seed P, Shennan AH and Mallet A (2010) Longitudinal profiles of 15 serum bile acids in patients with intrahepatic cholestasis of pregnancy. *Am J Gastroenterol* **105**:585-595.

Verkade HJ, Wolters H, Gerding A, Havinga R, Fidler V, Vonk RJ and Kuipers F (1993) Mechanism of biliary lipid secretion in the rat: a role for bile acid-independent bile flow? *Hepatology (Baltimore, Md)* **17**:1074-1080.

Wagner M, Halilbasic E, Marschall HU, Zollner G, Fickert P, Langner C, Zatloukal K, Denk H and Trauner M (2005) CAR and PXR agonists stimulate hepatic bile acid and bilirubin detoxification and elimination pathways in mice. *Hepatology (Baltimore, Md)* **42**:420-430.

Wen J, Luo J, Huang W, Tang J, Zhou H and Zhang W (2015) The Pharmacological and Physiological Role of Multidrug-Resistant Protein 4. *J Pharmacol Exp Ther* **354**:358-375.

Wikström Shemer EA, Stephansson O, Thuresson M, Thorsell M, Ludvigsson JF and Marschall HU (2015) Intrahepatic cholestasis of pregnancy and cancer, immune-mediated and cardiovascular diseases: A population-based cohort study. *Journal of hepatology* **63**:456-461.

Williamson C and Geenes V (2014) Intrahepatic cholestasis of pregnancy. *Obstetrics and gynecology* **124**:120-133.

Wong MH, Oelkers P, Craddock AL and Dawson PA (1994) Expression cloning and characterization of the hamster ileal sodium-dependent bile acid transporter. *The Journal of biological chemistry* **269**:1340-1347.

Wong MH, Oelkers P and Dawson PA (1995) Identification of a mutation in the ileal sodium-dependent bile acid transporter gene that abolishes transport activity. *The Journal of biological chemistry* **270**:27228-27234.

Woods A, Heslegrave AJ, Muckett PJ, Levene AP, Clements M, Mobberley M, Ryder TA, Abu-Hayyeh S, Williamson C, Goldin RD, Ashworth A, Withers DJ and Carling D (2011) LKB1 is required for hepatic bile acid transport and canalicular membrane integrity in mice. *The Biochemical journal* **434**:49-60.

Yu L, Liu X, Li X, Yuan Z, Yang H, Zhang L and Jiang Z (2016) Protective effects of SRT1720 via the HNF1α/FXR signalling pathway and anti-inflammatory mechanisms in mice with estrogen-induced cholestatic liver injury. *Toxicol Lett* **264**:1-11.

Yu L, Liu X, Yuan Z, Li X, Yang H, Yuan Z, Sun L, Zhang L and Jiang Z (2017) SRT1720 Alleviates ANIT-Induced Cholestasis in a Mouse Model. *Front Pharmacol* **8**:256.

Zamek-Gliszczynski MJ, Day JS, Hillgren KM and Phillips DL (2011) Efflux transport is an important determinant of ethinylestradiol glucuronide and ethinylestradiol sulfate pharmacokinetics. *Drug Metab Dispos* **39**:1794-1800.

Zhang F, Xi L, Duan Y, Qin H, Wei M, Wu Y, Li B, Zhou Y and Wu X (2018) The ileum-liver Farnesoid X Receptor signaling axis mediates the compensatory mechanism of 17α-ethynylestradiol-induced cholestasis via increasing hepatic biosynthesis of chenodeoxycholic acids in rats. *Eur J Pharm Sci* **123**:404-415.

Zhang Y, Csanaky IL, Selwyn FP, Lehman-McKeeman LD and Klaassen CD (2013) Organic anion-transporting polypeptide 1a4 (Oatp1a4) is important for secondary bile acid metabolism. *Biochem Pharmacol* **86**:437-445.

Zhu QN, Xie HM, Zhang D, Liu J and Lu YF (2013) Hepatic bile acids and bile acid-related gene expression in pregnant and lactating rats. *PeerJ* **1**:e143.

Zollner G and Trauner M (2008) Mechanisms of cholestasis. *Clin Liver Dis* **12**:1-26, vii.

7. Attachments

7.1. Attachment 1

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. 202; 345:109525. (IF = 5.192, Q2). https://doi.org/10.1016/j.cbi.2021.109525

7.2. Attachment 2

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. (IF = 4.134, Q1). https://doi.org/10.3389/fphys.2022.859294

7.3. Attachment 3

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. (IF = 4.379, Q1). https://doi.org/10.1038/s41598-019-46150-7