Understanding the molecular mechanisms of bile acid receptor activation for the treatment of human liver disease

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Cover illustration: Ibn Sina in medicine - by Ali J Al-Sammarraie

Ibn Sina (Avicenna) was an Arabian polymath, who is regarded as one of the most significant physicians, astronomers, thinkers and writers of the Islamic Golden Age. He is also called "the most influential philosopher of the pre-modern era". Of the 450 works he is believed to have written, around 240 have survived, including 150 on philosophy and 40 on medicine. His most famous works are *The Book of Healing*, a philosophical and scientific encyclopedia, and *The Canon of Medicine*, a medical encyclopedia, which became a standard medical textbook at many medieval universities and remained in use as late as 1650.

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ABSTRACT

Farnesoid X receptor (FXR) is a nuclear transcription factor that is activated by bile acids and regulates bile acid homeostasis, glucose and lipid metabolism. FXR activation by a ligand has been identified as a therapeutic modality for a range of liver and metabolic diseases. To date, FXR activation studies to decipher the underlying molecular mechanisms of its action have almost exclusively been conducted in mouse models, which are of limited human relevance due to species differences between mice and humans in bile acid composition, metabolism and FXR activation patterns.

The apical sodium-dependent bile acid transporter (ASBT; also known as ileal bile acid transporter (IBAT)) is pivotal for the reabsorption of conjugated bile acids from the ileum back to the liver and an important FXR target gene. IBAT inhibition results in the interruption of the enterohepatic circulation of bile acids. To date, IBAT inhibitors have been used in animal models for the treatment of non-alcoholic steatohepatitis (NASH), and in humans for the treatment of chronic constipation and severe itch associated with cholestatic liver diseases, such as primary biliary cholangitis (PBC) and pediatric liver disease.

Paper I presents an open-label pilot study with the IBAT inhibitor A4250 aiming to assess its safety and also efficacy in alleviating itch in patients with PBC. In this study, 10 patients with PBC were intended to be treated with A4250 for four weeks. Despite some subjective improvements in pruritus severity, the study was stopped prematurely because of drop-outs caused by abdominal side effects.

For **papers II** and **III** we performed a randomized, double-blind, placebo-controlled pharmacodynamic trial with the FXR agonist obeticholic acid (OCA, 25 mg/day) that was administered to patients with symptomatic gallstone or morbid obesity for 3 weeks prior to laparoscopic cholecystectomy or Roux-en-Y gastric bypass.

Paper II shows that OCA treatment increased cholesterol saturation and bile acid hydrophobicity indices as well as FGF19 in bile, all together increasing the risk of cholelithiasis. Gene analysis suggested a biliary origin of FGF19.

Paper III shows by performing ChIP-Seq that the expression of FXR-DNA binding sites was not related to OCA-treatment; rather, it seems to be predetermined by the phenotype (obese vs non-obese). In contrast, RNA-Seq indicated induction of FXR target genes by OCA.

In conclusion, our first experiment explored the interruption of the enterohepatic circulation of bile acids as a modality for pruritus management in patients with cholestatic liver disease. Given the side effects, this concept may be questionable, at least in the adult population. Our second and third studies employed pharmacologic FXR activation and provided a unique insight into gallbladder pathophysiology and mechanisms of gallstone formation and the puzzling finding that FXR-DNA binding is altered in the obese phenotype, which may underlie aberrant metabolism and liver function in obesity.

Keywords: bile acids, farnesoid X receptor, FGF19, IBAT, pruritus, obesity, gallstones, ChIP-Seq, cistromics, transcriptomics.

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SAMMANFATTNING PÅ SVENSKA

Gallsyror behövs för upptag av näring och fettlösliga vitaminer från tarmen. Intensiv forskning de senaste åren har visat att gallsyror även fungerar som endokrina, hormonliknande molekyler som kan aktivera nukleära receptorer, framförallt farnesoid X receptor (FXR). FXR är den primära gallsyresensorn och kontrollerar *de novo* gallsyresyntes genom en feedbackmekanism i levern via small heterodimer partner (*SHP)* och i tarmen genom fibroblast growth factor15 (hos mus) /19 (hos människa) (FGF15/19) som respons på gallsyrorna. Gallsyrorna reglerar på detta sätt inte bara sin egen syntes och utsöndring, utan också omsättningen av lipider och glukos, vilket har stor betydelse i patofysiologin av det metabola syndromet. Aktivering av FXR med en syntetisk ligand är en lovande ny behandling av leversjukdomar med dåligt gallflöde (kolestas) och har även visat lovande resultat vid behandling av komplikationer till det metabola syndromet såsom fettleversjukdom och typ 2 diabetes.
I våra studier har vi studerat de molekylära mekanismerna bakom FXR aktivering hos människa. Vi har använt en potent FXR agonist med namnet obetichlsyra (OCA) och genomfört två studier hos patienter med fetma och patienter med påvisad gallstenssjukdom. Patienter i båda grupperna genomgick antingen gastric bypass-kirurgi eller kolecystektomi. I våra studier har vi kunnat identifiera att FXR bindningsställen i människans DNA inte beror på huruvida receptorn aktiveras av en ligand, utan de är beroende av den metabola fenotypen (fetma vs ej fetma). Genom att undersöka lipidinnehåll och FGF19 koncentration i gallan från kolecystektomi-patienter har vi kunnat visa att behandlingen med OCA ökar risken för gallstens-bildning hos behandlade patienter. Vi har också identifierat att FGF19 utsöndras från gallblåsansepitel, ett fynd av klinisk betydelse.
Vi har också undersökt huruvida farmakologisk blockering av den fysiologiska enterohepatiska cirkulationen kan lindra besvärlig klåda hos patienter med kolestatisk leversjukdom. För att undersöka detta genomförde vi en pilotstudie med en inhibitor av gallsyreabsorption i tunntarmen genom att blockera transportproteinet som är ansvarigt för återupptag av gallsyrorna till levern – Ileal Bile Acid Transporter (IBAT). Vi använde en IBAT inhibitor med namnet A4250 hos patienter med primär biliär kolangit och svår klåda. Denna behandling ledde till en relativ minskning i klådans intensitet, men studien fick avbrytas på grund av abdominala biverkningar.

Slutligen kan vi påstå att våra studier har lett till en bättre förståelse av samspelet mellan gallsyror, FXR, IBAT och ämnesomsättningen i olika sammanhang. Dessa fynd kommer att vara av värde för framtida forskning och utveckling av målspecifika molekyler för behandling av metabola och leversjukdomar.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their roman numerals.

I. **Al-Dury S**, Wahlström A, Wahlin S, Langedijk J, Elferink R O, Ståhlman M, & Marschall HU (2018). **Pilot study with IBAT inhibitor A4250 for the treatment of cholestatic pruritus in primary biliary cholangitis.** *Scientific Reports* 2018; *8*(1):6658. doi: 10.1038/s41598-018-25214-0.

II. **Al-Dury S**, Wahlström A, Panzitt K, Thorell A, Ståhlman M, Trauner M, Fickert P, Bäckhed F, Fändriks L, Wagner M & Marschall HU. **Obeticholic acid may increase the risk of gallstone formation in susceptible patients.** *Journal of Hepatology* 2019; 71(5): 986–991. doi: 10.1016/j.jhep.2019.06.011

III. Jungwirth E, Panzitt K, **Al-Dury S**, Wahlström A, Thorell A, Ståhlman M, Fickert P, Fändriks L, Wagner M & Marschall HU. **Human FXR-DNA binding is associated to the obese phenotype.** *Manuscript.*

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ABBREVIATIONS

ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
ALP	Alkaline phosphatase
ApoC2/3	Apolipoprotein C2/C3
ApoE	Apolipoprotein E
IBAT	Ileal bile acid transporter
ATX	Autotaxin
BA	Bile acid
BAR	Bile acid receptor
BS	Bariatric surgery
BSEP	Bile salt export pump
βKL	Beta KLOTHO
C4	7 alpha-hydroxy-4-cholestene-3-one
CA	Cholic acid
CDCA	Chenodeoxycholic acid
CSI	Cholesterol saturation index
CYP7A1	Cholesterol 7 alpha hydroxylase
CYP8B1	12 alpha hydroxylase
CYP27A1	Sterol 27 hydroxylase

DCA	Deoxycholic acid
EHC	Enterohepatic circulation
FABP6	Fatty acid-binding protein subclass 6
FFA	Free fatty acids
FGF19	Fibroblast growth factor 19
FGFR	Fibroblast growth factor receptor
FXR	Farnesoid X receptor
GGT	Gamma-glutamyl transferase
GS	Gallstone surgery
HCC	Hepatocellular carcinoma
HDL	High density cholesterol
HI	Hydrophobicity index
IBAT	Ileal bile acid transporter
IBS	Irritable bowel syndrom
LDL	Low density cholesterol
LCA	Lithocholic acid
LDLR	Low density lipoprotein receptor
LP	Lipoprotein lipase
LPA	Lysophosphatidic acid
LRH-1	Liver receptor homolog 1
MRP2	Multidrug resistance associated protein 2

NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NTCP	Sodium bile acid co-transporting peptide
OATP	Organic anion transporting polypeptide
OCA	Obeticholic acid
OSTα/β	Organic solute transporter A/B
PFIC	Progressive familial intrahepatic cholestasis
PPARα	Peroxisome proliferator-activated receptor alpha
RXR	Retinoid X receptor
SHP	Small heterodrimer partner
SR-BI	Scavenger receptor class B type I
SREBP1/2	Sterol regulatory element binding protein 1/2
TG	Triglycerides
T/G BA	Taurine/glycine conjugated bile acid
TGR5	Takeda G-protein coupled receptor
UDCA	Ursodeoxycholic acid
VLDL	Very low density lipoprotein

1 INTRODUCTION

Bile acids (BAs) are unique amphipathic molecules with multiple functions (1). They are synthesized from cholesterol in the liver and are the major lipid component of bile. One of their major functions is the regulation of cholesterol metabolism. After a meal ingestion, BAs are pumped by the gallbladder and this way enter the gastrointestinal tract. BAs function as key regulators of fat emulsification and solubilization, the two rate limiting steps in the process of fat digestion and absorption of cholesterol, triglycerides (TG) and the associated fat-soluble vitamins A, D, E & K (1). BAs also act as signaling molecules by activating the two main BA sensors in the body: the nuclear receptor Farnesoid X receptor (FXR), and the cell surface receptor Takeda G-protein receptor 5 (TGR5), and different BAs act as agonists and antagonists of those receptors in a varying degree (2, 3). In recent years, BAs have been identified as key regulators of complex pathways at a systemic level ranging from their own homeostasis to cholesterol, TG, glucose and energy metabolism. Additional regulations include cell proliferation, inflammation, and tumor onset and progression (1). The heterogenicity of BA functions is therefore key to their involvement in an array of metabolic and liver diseases, such as obesity, type 2 diabetes mellitus, chronic liver and biliary diseases (4). The studies presented in this thesis aim to shed a light on the molecular mechanisms involved in BA regulation, closely looking at BA – FXR interactions and their downstream effect on various disease conditions, as well as answer some of the questions regarding the precise impact of BAs on lipid metabolism. Finally, we studied how pharmacological interruption of the enterohepatic circulation (EHC) may reduce the circulating BA pool in cholestatic patients and thus alleviate pruritus that is commonly associated with cholestatic conditions.

1.1 BILIARY TREE ANATOMY

In anatomy, the biliary tree refers collectively to the liver, gallbladder and bile ducts, and their contribution to make, store and secrete bile. It describes the path through which bile is produced, then secreted by the liver and stored in the gallbladder, then to be transported to the duodenum upon ingestion of a meal. This path is fairly common for most mammals with a gallbladder. The biliary tract starts as small biliary canaliculi running alongside the functional unit of the liver – the hepatocytes. Those canaliculi assemble into the intrahepatic bile ductule, joining a structure called portal triad, which consists of an arteriole and a venule, in addition to the bile ductule. The next structure in this order are the interlobular bile ducts, left and right hepatic ducts. Once merged, they form the common hepatic duct. This is where they exit the liver joining with the cystic duct from the gallbladder. Together they form the common bile duct, which joins the pancreatic duct, finally emptying into the duodenum through the ampulla of Vater (5) (Figure 1).

A significant amount of the produced bile is not secreted into the duodenum instantly, but rather stored in the gallbladder, which is a hollow piriform organ that lies on the cystic plate under the liver segment IVb and V. It is about 7 – 10 cm long and 2,5 cm wide at its widest point. The total stored bile volume in the gallbladder is 30 – 35 ml under normal conditions, but it can hold up to 350 ml if the cystic duct is obstructed. The gallbladder is supplied by the cystic artery, a branch of the right hepatic artery and is innervated sympathetically via the celiac plexus and parasympathetically via hepatic branch of vagus nerve. It also receives sensory fibers from the phrenic nerve (6).

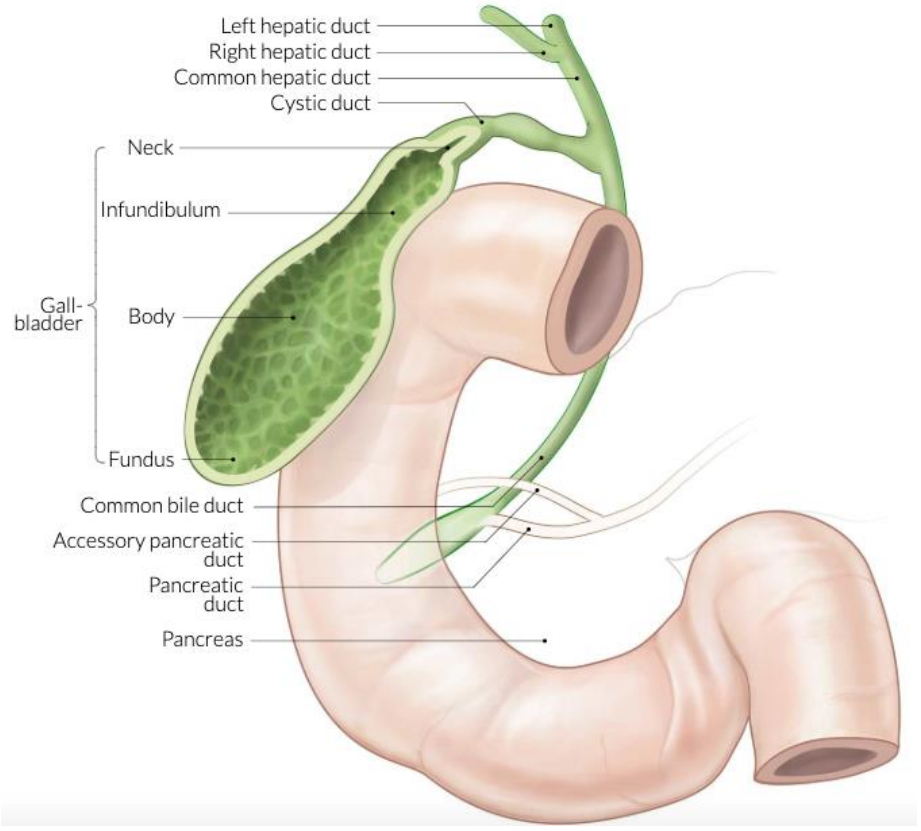

Figure 1. Anatomy of the biliary tree. The right and left hepatic ducts join to form the common hepatic duct. The cystic duct joins the common hepatic duct to form the common bile duct. The main pancreatic duct drains into the common bile duct. The common bile duct opens into the descending part of the duodenum. Illustration by Ali J Al-Sammarraie.

1.2 BILIARY TREE PHYSIOLOGY

One of the many functions of the liver is to secrete bile, normally between 600 and 1000 ml / day (7) . Prior to recognizing BAs hormonal actions, bile was thought to fulfill only two important functions. First, it plays an important role in fat digestion and absorption through emulsification of large fat particles and the absorption of the digested fat end products through the intestinal mucosal membrane. Second, it serves as a mean for excretion of several important waste products from the blood, especially bilirubin, xenobiotics, and it is virtually the only way for the disposal of excessive cholesterol (8).

1.2.1 BILE PRODUCTION, SECRETION & STORAGE

Bile is secreted in two stages by the liver from biliary canaliculi until it reaches its final destination. In the first stage, large amounts of BAs, cholesterol, phospholipids and other organic constituents are secreted. During the flow of this thick bile through the bile ducts, another wave of secretion is added to the initial bile. This additional secretion is a watery solution of sodium and bicarbonate ions and it increases the total quantity of bile by as much as an additional 100% (9).
There is a continuous secretion of bile by hepatocytes, with varying diurnal rhythms (10), but most of it is normally stored in the gallbladder until needed in the duodenum upon stimulation by a fatty meal. Hepatic bile is composed of 97% water, 0,7% BAs, 0,2% bilirubin, 0,5% fats including cholesterol, free fatty acids (FFA) and lecithin, and a small concentration of inorganic salts (8, 11) (Table 1).

	Liver Bile	Gallbladder Bile
Water	97.5 g/dl	92 g/dl
Bile salts	1.1 g/dl	6 g/dl
Bilirubin	0.04 g/dl	0.3 g/dl
Cholesterol	0.1 g/dl	0.3 to 0.9 g/dl
Fatty acids	0.12 g/dl	0.3 to 1.2 g/dl
Lecithin	0.04 g/dl	0.3 g/dl
Na^+	145 mEq/L	130 mEq/L
K^+	5 mEq/L	12 mEq/L
Ca^{++}	5 mEq/L	23 mEq/L
Cl^-	100 mEq/L	25 mEq/L
HCO_3^-	28 mEq/L	10 mEq/L

Table 1. Composition of bile in the liver and gallbladder. Copied with permission from Guyton and Hall, Textbook of medical physiology (12th edition), 2019. Philadelphia, PA: Elsevier.

When a fatty meal is ingested, the gallbladder contracts and releases its content into the duodenum within 30-40 minutes after meal ingestion (8). The mechanism of gallbladder emptying is rhythmical contractions of the wall of the gallbladder. The most potent stimulus for causing the gallbladder contractions is the hormone cholecystokinin (CCK). It is released upon the presence of fats, peptides and aromatic amino acids in the proximal duodenum (Figure 2) (12, 13). Under fasting conditions, there is a constant low circulating concentration of CCK. It increases within 20 min of meal stimulation, and then declines gradually only to reach a second peak after 1.5–2 hours (14). Of note, BAs serve as the most important luminal regulator of CCK release in humans, thus controlling its response to dietary stimulants (15).

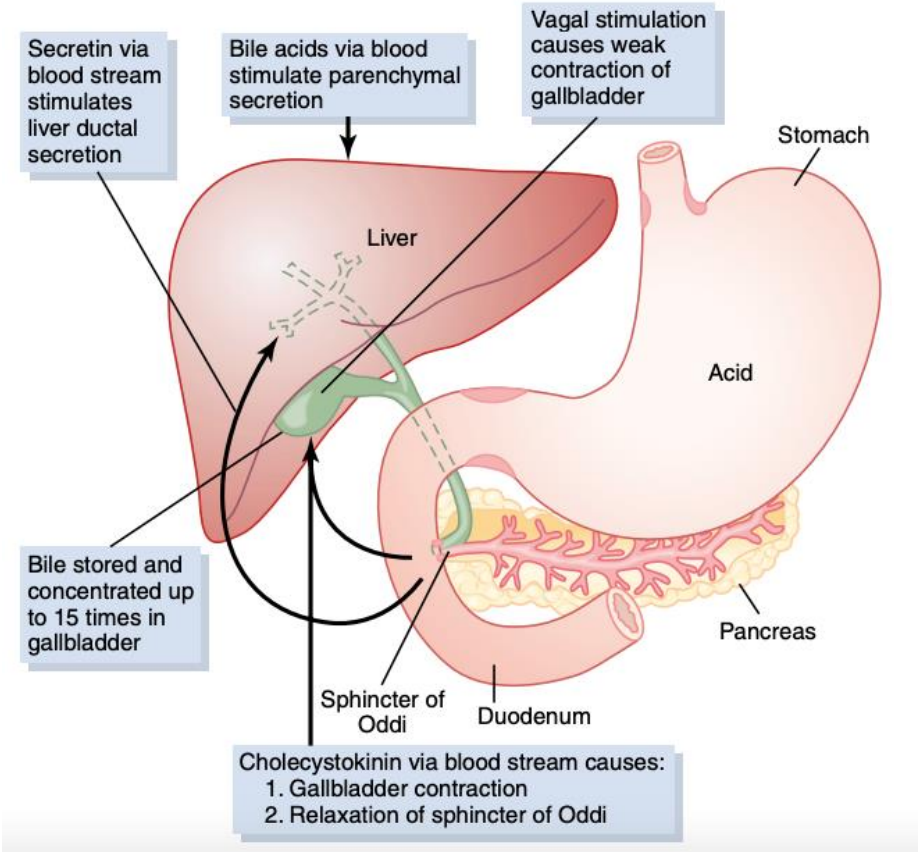

Figure 2. Liver secretion and gallbladder emptying during meal ingestion. Copied with permission from Guyton and Hall, Textbook of medical physiology (12th edition), 2019. Philadelphia, PA: Elsevier.

1.3 BILE ACIDS OVERVIEW

BAs are amphipathic pleiotropic molecules synthesized in the liver from cholesterol (1). They activate three nuclear receptors; FXR, Pregnane X receptor (PXR) and Vitamin D Receptor (VDR) and one G-protein coupled receptor (TGR5) (16). BAs have different affinities to their receptors, with a varying degree of agonistic and antagonistic activity (16). In addition, BAs control gut bacteria overgrowth and protect the intestinal epithelial barrier (17). BAs therefore regulate not only on their own synthesis, but also play a role in metabolic homeostasis, glucose and lipid metabolism, inflammation and even liver regeneration and carcinogenesis (18).

Common BAs (C-24 BAs) are composed of 24 carbon atoms and consist of four steroid rings (ABCD) with a hydrophobic hydrocarbon side, and a hydrophilic face containing various numbers of hydroxyl groups (Figure 3). At the free edge of D, there is a five-carbon acidic side chain that is subsequently amidated with glycine or taurine in an approximate 3:1 ratio in humans. This chemical structure gives BAs their unique amphipathic structure, enabling the formation of micelles, as well as digestion and absorption of dietary lipids and fat-soluble vitamins (18). Naturally, surrounding pH, presence, orientation and position of hydroxyl groups on the steroid ring are decisive factors determining BAs solubility in the following order from hydrophobic to hydrophilic: LCA > DCA > CDCA > CA > UDCA > MCA (19). Thanks to those small structural differences, different BAs have a different affinity to their receptors, from strong agonists to strong antagonists (20, 21).

1.3.1 BILE ACID SYNTHESIS

BAs are synthesized in the liver in a lengthy process consisting of at least 17 enzymatic reactions in two main pathways, the classic, or neutral pathway (up to 75% of all BAs) and the alternative, acidic pathway (responsible for the remaining 10 - 25% in humans and mice, respectively) (9, 22, 23). The rate-limiting enzyme for the formation of BAs in the classic pathway is a cytochrome P450 enzyme called 7α-hydroxylase (CYP7A1). This enzyme catalyzes the hydroxylation of the cholesterol ring at the C7 position and determines the amount of BAs to be produced. Of note, disruption in CYP7A1 activity in mice leads to

abnormal lipid secretion, skin pathologies and behavioral irregularities (24). 7α-Hydroxycholesterol is then converted to the primary BA chenodeoxycholic acid (CDCA). CDCA is then converted to another primary BA: Cholic acid (CA) through the action of 12α-hydroxylase (CYP8B1), and the ratio between those two BAs is one of the determinants of BA pool hydrophobicity (25).
The alternative pathway is initiated by sterol-27-hydroxylase (CYP27A1). The 27-hydroxycholesterol formed is further hydroxylated by oxysterol 7α-hydroxylase (CYP7B1), generating CDCA as the sole primary BA (17, 23, 24). In addition to CDCA and CA, mice also produce alpha and beta muricholic acids (α/βMCA) from CDCA and ursodeoxycholic acid (UDCA) respectively, by adding a hydroxyl group at the C-6 position. For a long time, the enzymes catalyzing the conversion of CDCA to MCA were unknown. It was commonly assumed that the enzyme CYP3A11 catalysed 6-hydroxylation of BAs, but in our previous study with Cyp3a11$^{-/-}$ mice we discovered that CYP3A11 was not essential for the formation of murine BAs (26). Recently, the enzyme CYP2C70 has been identified as the enzyme responsible for the 6-hydroxylation of CDCA to αMCA and UDCA to βMCA (27). By inhibiting this enzyme, one may come closer to producing the long sought human-like BA pool in mice. Unfortunately, livers of those humanized mice had a higher rate of baseline inflammation and injury, making this animal model relatively unsuitable for the study of liver disease associated with BA dysregulation (28). Therefore, those significant BA differences between humans and mice represent a major hurdle when attempting to interpret new findings.

Once synthesized, BAs are conjugated with either glycine or taurine (so-called bile salts) rendering them more hydrophilic, thereby facilitating micelle formation in the acidic environment of the duodenum (18, 29). Conjugated BAs can no longer diffuse freely through the membrane, but require an active transporter to move them across membranes. Bile salt export pump (BSEP, ABCB11) is the key hepatic transporter for BAs, while other transporters are responsible for the movement of phospholipids (ABCB4, known as MDR3 and MDR2 in humans and mice, respectively) and cholesterol (ABCG5/ABCG8) (30). Loss of these transporters can lead to significant morbidities. An example of this is the loss of BSEP in progressive familial intrahepatic cholestasis type 2 (PFIC-2), where bile salts in the liver accumulate to toxic levels (31).

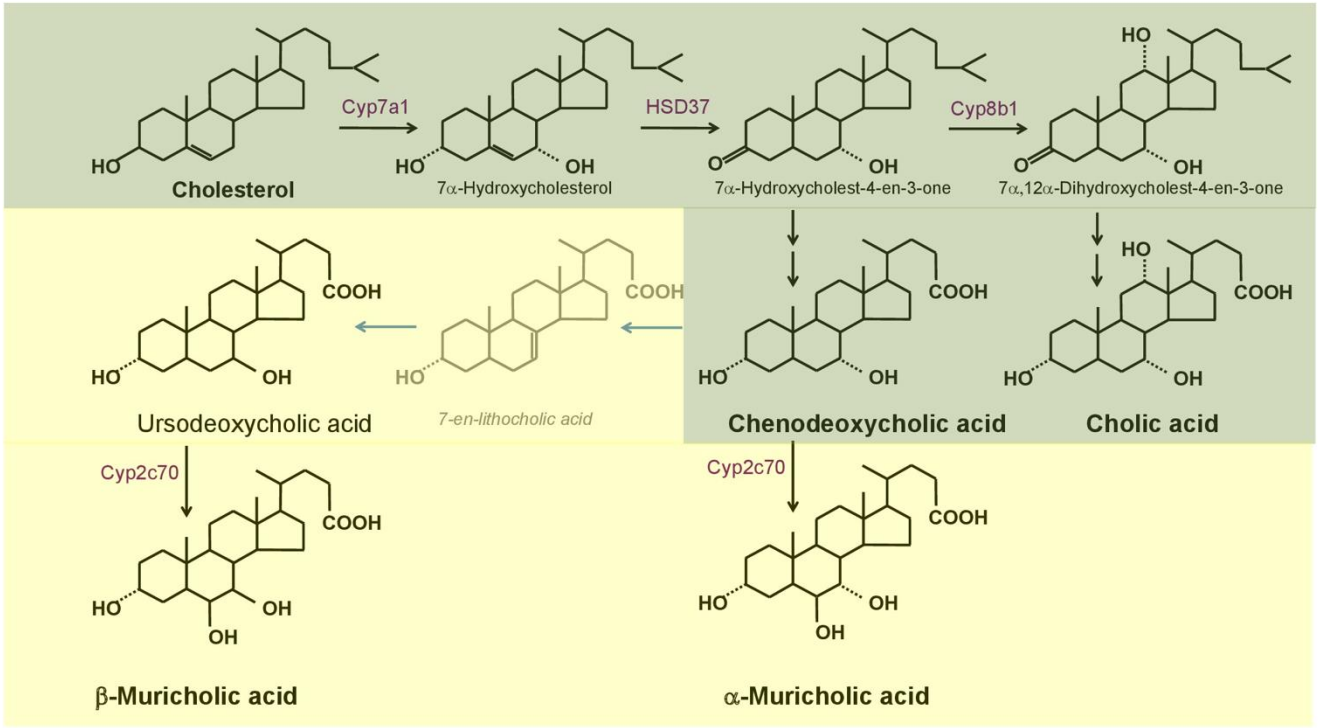

Figure 3. Bile acid synthesis in the liver. BA synthesis from cholesterol requires many enzymatic reactions in different subcellular compartments of the hepatocyte. The rate limiting enzyme is cholesterol 7α-hydroxylase (CYP7A1). Its activity is reflected by serum 7 α-hydroxy-4-cholesten-3-one (C4) which is formed by the action of 3β-hydroxy-Δ^5-C27-steroiddehydrogenase/isomerase (HSD3B7). In humans (shown in green), the primary BAs are chenodeoxycholic acid (CDCA) and cholic acid (CA); the ratio between them is determined by 12α-hydroxylase (CYP8B1), which is required for the formation of CA. In rodents (shown in yellow), there are additional primary BAs; ursodeoxycholic acid (UDCA) and α/β-muricholic acids (α/βMCA). α/βMCAs are generated by 7β-hydroxylation of CDCA and UDCA by cytochrome CYP2C70 while the mechanism of epimerization of CDCA toward UDCA still is unknown. BAs are conjugated with glycine- or taurine (in humans) and with taurine (in rodents) before excretion into bile. Murine BA profiles are much more hydrophilic than human BA profiles and have substantially different activation proprieties of the nuclear BA receptor farnesoid X receptor (FXR). While CDCA is the strongest natural activator of FXR, taurine-conjugated α/βMCAs are natural antagonists of FXR, which one needs to keep in mind when translating BA-related metabolic data from rodents to humans.

1.3.2 ENTEROHEPATIC CIRCULATION

The human liver synthesizes and secretes on average 2 – 4 g of BAs per day (8), of which 95% are reabsorbed by an active transport process through the intestinal mucosa in the distal ileum via the ileal bile acid transporter (IBAT) present on the enterocyte brush border (32) (Figure 4). Some unconjugated BAs also pass the intestinal mucosa by diffusion. Once in the enterocyte, they are carried from apical to the basolateral membrane where they are effluxed into the portal blood by the heterodimeric transporter called organic solute transporters A/B (OSTα/OSTβ) (33). Upon reaching the liver, they are absorbed almost entirely back into the hepatocytes via the sodium taurocholate co-transporting polypeptide (NTCP/SLC10A1, for conjugated BAs) and organic anion transporters (OATPs, for unconjugated BAs) and then re-secreted into the bile via BSEP (22, 32). On average, BAs make the entire circuit some 17 times before being carried out in the feces (32). BAs that escape reabsorption reach the colon and are deconjugated by gut bacteria and further converted into secondary BAs. The small quantities of BAs lost into the feces are replenished by new amounts formed continually by the hepatocytes. Microbial actions on primary BAs includes mainly their 7-dehydroxylation, thus producing secondary BAs; lithocholic acid (LCA) from CDCA and deoxycholic acid (DCA) from CA. The same reaction on the primary murine αMCA and βMCA results in the formation of murideoxycholic acid (MDCA). In contrast to mice, UDCA in humans is a secondary BA representing about 5% of total BAs and is formed by 7α/β-isomerization of CDCA, which can be performed by *Clostridium absonum* (34, 35). Microbial biotransformation of BAs leads to a more hydrophobic BA pool, which facilitates their elimination in the feces (17). The biotransformation products in the colon are largely excreted in feces, but some are passively absorbed, returned to the liver, conjugated and secreted in bile (36, 37). The only exception is LCA, which is a very hydrophobic, cytotoxic BA. In rodents LCA is detoxified by hydroxylation, while in humans it undergoes sulfation in the enterocyte prior to being effluxed back into the intestinal lumen and thus restricting its intestinal absorption and enhancing its fecal elimination (38, 39).

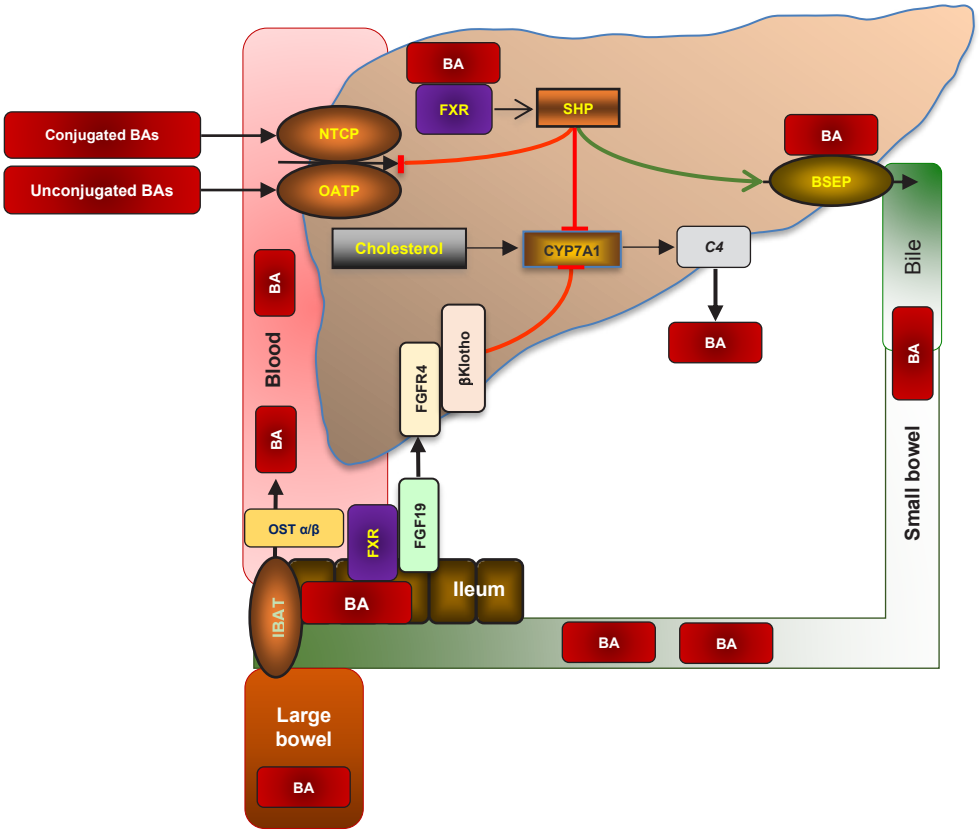

Figure 4. Enterohepatic circulation of BAs. Glycine- or taurine- conjugated BAs are excreted via the bile salt export pump (BSEP) into bile in which they reach the duodenum. About 95% of conjugated BAs are reabsorbed at the apical border of the enterocyte in the terminal ileum via the apical sodium-dependent BA transporter (ASBT, SLC10A2; also known as IBAT, ileal BA transporter). Inside the enterocyte, BAs are reversibly bound to fatty acid-binding protein subclass 6 (FABP6) and shuttled from the apical to the basolateral membrane. BAs are then pumped out of the enterocyte through OSTα/β receptors and recirculated to the liver via the portal vein from which conjugated BAs are reabsorbed via the sodium-dependent taurocholate co-transporting peptide (NTCP, SLC10A1), whereas unconjugated BAs are reabsorbed via organic anion-transporting peptides (OATPs). This recycling process is called enterohepatic circulation. The rate-limiting enzyme of BA synthesis from cholesterol, CYP7A1, is controlled by two different negative feed-back pathways that are both regulated by binding of BAs to its nuclear receptor, FXR: (1) within the liver, upon activation by its ligand, FXR induces transcription of SHP, which in turn directly interacts with liver-related homolog-1 (LRH-1), a competent transcription factor for Cyp7a1, and inhibits the transcriptional activity of LRH-1, repressing Cyp7a1; and (2) from the ileum via increased formation of fibroblast growth factor 19 (FGF19, FGF15 in rodents), which circulates to the liver in portal blood, binds to the heterodimer FGFR4-receptor/β-klotho, and triggers a signalling cascade to inhibit CYP7A1.

1.3.3 REGULATION OF BILE ACID SYNTHESIS

BAs regulate their own synthesis through their action as endogenous ligands on the nuclear receptor FXR (40-42). Different BAs activate FXR in the following order of their agonist capacity CDCA > DCA = LCA > CA (40, 41). Murine primary BAs TαMCA and TβMCA serve as naturally occurring FXR antagonists, as well as UDCA in humans, in pharmacological doses (21, 43). FXR is expressed in several tissues, most abundantly in the liver and ileum, which are the major sites that regulate BA homeostasis (44, 45). FXR can also be found in the kidneys, ovary, thymus, heart, eyes, spleen and testes, but its function in those tissues is not fully understood (46, 47).

The impact of FXR activation in the liver and intestine on *de novo* BA synthesis targeting CYP7A1 is illustrated in Figure 4. (48-50).

In addition to its role in BA synthesis, FXR also regulates the intracellular hepatic concentration of BAs by acting on their transporters. FXR controls the amounts of BAs reentering the liver through the portal vein from intestines by inhibiting NTCP and OATPs. (51). FXR also induces BA transporters OSTα/β at the hepatocyte's basolateral membrane thus helping BAs efflux into the circulation (52). FXR also enhances BAs and phospholipids efflux from the hepatocytes into the gallbladder by an upregulation of BSEP and MRP2 (53-55). Collectively, FXR activation protects hepatocytes from toxic levels of BAs. In addition to this, FXR activation protects the enterocytes from toxic levels of BAs by downregulating IBAT on the apical membrane of enterocytes, reducing the absorption of BAs into enterocytes (56).

1.3.4 FXR AND LIPID METABOLISM

The generation of $FXR^{-/-}$ mice shed light on an important role of this receptor in the regulation of lipid metabolism (57). $FXR^{-/-}$ mice have increased BA concentrations and a distinct proatherogenic lipid profile characterized by increased hepatic and serum TG and cholesterol levels. The increase in total serum cholesterol in $FXR^{-/-}$ mice was reflected in increased levels of VLDL, LDL (57), and reduced clearance rate of HDL cholesteryl ester (58). The association between BA and lipid metabolism sparked an interest in the therapeutic application of BAs already in 1980s (59). The mechanism of action was and still is largely unknown,

and is a question that we addressed in our studies in this thesis. For instance, when CDCA was used in the past to treat cholesterol gallstones, a concomitant decrease in TG levels was observed. CDCA consequently was suggested as a potential drug for the treatment of hypertriglyceridemia (59-61). In contrast, administering bile-sequestering resins to patients with dyslipidemia led to an undesirable increase in plasma TG and VLDL production levels (62, 63). Given the proposed dyslipidemic phenotype of the $FXR^{-/-}$ mouse, FXR is currently thought to mediate much of the BA effects of lipid metabolism. This hypothesis is supported by reports identifying an array of lipid-modulating proteins as direct or indirect downstream targets of FXR. For instance, FXR activation induces the expression of apolipoprotein C2 (ApoC2), which is an obligate cofactor for lipoprotein lipase (LP), thus activating it to release free fatty acids from TG (64). On the other hand, FXR suppresses the expression of apolipoprotein C3 (ApoC3), which normally functions as an inhibitor LP activity (65). As a result, FXR modulation of these apolipoproteins may reduce plasma TG levels by promoting LP-mediated TG hydrolysis in VLDL and chylomicrons. In addition, FXR represses the expression of stearoyl regulatory binding protein 1c (SREBP-1c), stearoyl-CoA desaturase-1 (SCD-1) and acyl-CoA synthetase short chain family member 2 (ACSS2), which are essential regulators of fatty acid and TG synthesis (66). FXR also enhances TG clearance by increasing fatty acid oxidation through an upregulation of pyruvate dehydrogenase kinase (PDK4), which promotes utilization of fat rather than glucose as an energy source (67). FXR also upregulates peroxisome proliferator–activated receptor α (PPARα); a nuclear receptor that plays an essential role in lipid and glucose metabolism; thus promoting hepatic fatty acid oxidation, at least in humans (68, 69). From a clinical perspective, treatment with the FXR agonist OCA resulted in increased TG clearance, but also an undesirable increase in LDL and a concomitant decrease in HDL (70). Further analysis of lipoprotein sub-particles recently showed that LDL increase is due to an increase in less atherogenic small VLDL and large-buoyant LDL particles (71).

1.3.5 FXR AND GLUCOSE METABOLISM

BA mediated activation of FXR impacts glucose metabolism by repressing hepatic gluconeogenesis and improving intracellular insulin

sensitivity. It has previously been shown that treatment of *db/db* diabetic mice with CDCA or other specific FXR agonists leads to a downregulation of some of the genes involved in gluconeogenesis, such as glucose-6-phosphatase (*G6Pase*) and phosphoenolpyruvate carboxykinase (*Pepck*), followed by an improvement in insulin sensitivity and decrease in glycemia (72-74). This has been confirmed by the finding that overexpression in FXR improves insulin sensitivity in diabetic mice, while its deficiency leads to glucose intolerance (74). It is speculated that the site of FXR activation plays a role in glucose homeostasis. This is highlighted by the interesting finding that specific hepatic FXR deletion in obese mice failed to improve glycemia and insulin sensitivity, while a double FXR/SHP deletion resulted in an improved glycemic control, lipid profile, and reduced adiposity (75, 76). These results suggest that this effect can be mediated via SHP, or driven by mechanisms that involve the inhibition of intestinal FXR signaling, as shown upon treatment with the intestine-specific FXR inhibitor glycine-β-muricholic acid (Gly-MCA) (73, 77). In contrast, it has been shown that intestinal-specific activation of FXR with fexaramine has beneficial metabolic effects. However, the effects of fexaramine appear to be driven in part by the activation of TGR5, whereas Gly-MCA works exclusively through the inhibition of intestinal FXR, with no effects on TGR5 signalling. Fexaramine induces changes in BA profiles, with a dramatic increase in LCA, a strong endogenous agonist for TGR5. TGR5-induced glucagon-like peptide-1 (GLP-1) led to an increase in energy expenditure, thermogenesis and an overall improvement in insulin sensitivity (20, 78). Thus, BA modulation of glucose metabolism is complex and multifaceted, depending not only on their receptors, but also the degree and site of their action.

1.3.6 FXR ACTIVATION IN THERAPY

Given all the beneficial effects of FXR activations that have been mentioned before, treatment with FXR ligands has emerged as an attractive therapeutic option for various hepatobiliary and metabolic diseases. The biggest steps in this area have been made in cholestatic liver diseases, such as PBC, which is associated with bile duct damage due to pathological accumulation of toxic BAs, leading to cholestasis, chronic inflammation, fibrosis and cirrhosis (79). Non-alcoholic fatty liver disease (NAFLD), non-alcoholic steatohepatitis (NASH), type 2

diabetes and obesity have also been evaluated as areas where FXR activation might have a therapeutic potential (80). The best studied drug so far is the semi-synthetic agonist Obeticholic Acid (OCA, 6α-ethyl-chenodeoxycholic acid, INT-747, now commercially known as Ocaliva). OCA is derived from CDCA and it possesses a 100-fold higher affinity to FXR than CDCA (81). OCA, due to its anticholestatic and anti-inflammatory properties, has been provisionally approved as a second line treatment in patients with PBC that are considered UDCA-non responders (82, 83). Six-weeks treatment with OCA in patients with type 2 diabetes and NAFLD has also shown to improve insulin sensitivity by almost 25%, and to reduce liver enzymes and body weight (84). OCA is currently being evaluated as an effective modality for the treatment of NASH with liver fibrosis. The results so far have been promising, with a histological decrease in steatosis, hepatocellular ballooning and lobular inflammation, as well a decrease in serum liver enzymes (70). The interim analysis from the largest trial with OCA in NASH has shown an improvement in fibrosis, but NASH resolution has not been achieved yet (85). In addition to OCA, several non-steroidal FXR ligands, as well as FGF19 analogues are being evaluated as potential therapeutic options for hepatobiliary and metabolic diseases. Those compounds are discussed in further detail in the discussion section.

1.4 IBAT IN BILE ACID METABOLISM

IBAT is responsible for the reabsorption of 95% of BAs in the terminal ileum at the enterocyte's apical membrane (86, 87). It is the ileal form of the ASBT/*SLC10* sodium/bile salt cotransporters family, which is not only expressed in ileal enterocytes, but is also found in proximal renal tubule cells, large cholangiocytes and gallbladder epithelial cells (88). In this thesis, we focus only on the ileal ASBT, referred to as IBAT. Mutations in the *IBAT* gene have been described in primary BA malabsorption, an intestinal disorder that presents with congenital bile salt diarrhea (89, 90). More commonly, BA malabsorption is seen in patients with Crohn's disease engaging the terminal ileum and/or colon, as well as postoperative malabsorption after intestinal resection (89, 91). IBAT has thus emerged as a pharmaceutical target for the treatment of lipid disorders but also for drug targeting strategies based on the coupling of drugs to BAs in order to enhance intestinal absorption and hepatic uptake (92, 93). When ileal reuptake of BAs is functionally impaired or pharmacologically blocked with an IBAT inhibitor, circulating BAs decrease, and fecal BA excretion increases. Decreased BA activation of ileal FXR results in reduced formation of FGF19 and, in turn, increased CYP7A1 expression and *de novo* BA synthesis (94). Enhanced consumption of cholesterol due to its conversion to BAs is compensated both by increased *de novo* biosynthesis, increased hepatic expression of low-density lipoprotein receptor (LDLr) and apolipoprotein b (ApoB) clearance, resulting in lowered circulating LDL cholesterol (95).

1.4.1 CHOLESTATIC PRURITUS

Pruritus is the unpleasant sensation that provokes the desire to scratch. It may be seen with PBC, primary sclerosing cholangitis (PSC), intrahepatic cholestasis of pregnancy, biliary obstruction, chronic viral hepatitis, cirrhosis, prolonged drug-induced cholestasis, and inherited cholestasis syndromes (eg, progressive familial intrahepatic cholestasis and benign recurrent intrahepatic cholestasis) (96). It has been reported that 25-70% of patients with PBC experience a varying degree of itch, and sometimes can be so severe that it affects daily activities (97).
The pathogenesis of pruritus in cholestasis is unknown, but several hypotheses have been proposed (98, 99). The earliest described theory

is BA accumulation, where elevated levels of BAs in the skin act as pruritogens. Observations in favor of this theory include the recovery of BAs from the skin surface of affected patients, although the reliability of the methods used to detect the BAs is uncertain (100). Also, there is an apparent lack of correlation between the presence or severity of pruritus and concentrations of BAs in skin of patients with chronic cholestasis in the most carefully done study that investigated this question (101). In some cases, it has been found that administering BAs can induce pruritus (70, 102, 103). There are speculations that pruritus is not caused by BAs, per se, but their elevated levels are toxic to hepatocyte membranes, causing a leakage of some pruritogenic content into the bloodstream, rather than direct BA effect on nerve endings (104).

Another theory is the endogenous opioids theory. There is evidence that increased opioid tone leads to pruritus. Administration of opioid drugs in healthy individuals induces pruritus, probably via a central action (105). Furthermore, endogenous opioid levels are usually elevated in patients with chronic liver disease (106). This assumption is the rationale behind using opioid antagonists (such as Naloxone) as second- or third-line treatment for intractable cholestatic itch (107, 108).

The latest theory discusses elevations in lysophosphatidic acid levels (LPA) (109, 110). LPA is a phospholipid that is formed by the action of a molecule called autotaxin (ATX), which functions as an enzyme that cleaves a choline group from lysophosphatidylcholine. It has been shown that patients with cholestatic pruritus had significantly increased serum concentrations of LPA and ATX activity compared to controls (111). In addition, injection of LPA induced a scratch response in mice (112). Of note, the antibiotic Rifampicin is recommended second-line treatment of severe cholestatic itch (see below), and it has been shown that its mode of action is partially mediated by an inhibition of ATX activity, and also by hydroxylating BAs to stimulate their renal excretion, collectively resulting in relief from itch (113, 114).

1.4.2 CURRENT TREATMENT OF PRURITUS

Itch has historically been a difficult symptom to treat pharmacologically. Antihistamines have been used for a long time to treat cholestatic itch with a varying degree of success in up to 60% of patients (115). On the other hand, limiting the amount of circulating BAs by the administration of BA binding resins (BA sequestrants) is an established treatment modality for cholestatic pruritus. They lower BA levels by inhibiting their absorption in the intestine. Despite the sparse evidence of their efficacy and poor tolerability profile (bloating, constipation), unpalatable taste and the need to use in larger amounts (up to 4 g four times daily), Cholestyramine and Colestipol remain to be the only licensed agents for the treatment of PBC-related pruritus (116). Previously mentioned Rifampicin is used as second-line therapy for cholestatic pruritus has a success rate of about 50% in clinical practice but is hampered by hepatotoxic side effects (117). Other drug therapies including opiate antagonists (as third-line therapy), selective serotonin uptake inhibitors and gabapentin are less well-documented (118). Nasobiliary drainage effectively relieves from cholestatic pruritus but is invasive and uncomfortable (119). This lack of effective treatments led to the investigation of IBAT inhibitors as a new therapeutic approach in patients with intractable itch.

1.4.3 IBAT INHIBITORS IN PRURITUS

Given the proposed causality between high BA load and cholestatic pruritus, it has been suggested that blocking EHC via a pharmacological inhibition of IBAT, thus decreasing BA load might play a role in itch alleviation in susceptible patients. This is discussed in details in the appendix.

2 AIMS

The main goal of this thesis is to understand the molecular mechanism regulating the BA receptor FXR and how activation and/or deactivation of FXR can have a downstream effect on BA synthesis, gallstone development and lipid metabolism as well as the impact of the interruption of EHC on pruritus in liver disease. We therefore aimed to achieve the following endpoints:

1. To assess the efficacy and safety of the pharmacological interruption of the EHC with an IBAT inhibitor A4250, and how this might improve symptomatic pruritus in patients with PBC.
   Paper I

2. To determine how FXR activation by its agonist OCA impacts serum and biliary lipids and FGF19, and whether this might lead to an increased risk of gallstone formation in susceptible patients.
   Paper II

3. To study how BAs and FXR interactions modulate BA turnover in two different populations, explore how FXR activation affects lipid metabolism, and finally identify global binding sites of FXR in human DNA.
   Paper III

3 PATIENTS AND METHODS

3.1 STUDY INTRODUCTION AND PARTICIPANT ELIGIBILITY

The investigations presented in this study are based on data collected from groups of patients with PBC (**Paper I**), ultrasound-verified gallstone disease (**Paper II**) and gallstone disease and morbid obesity (**Paper III**). Patients' age and BMI varied between groups (discussed later in this chapter). Patients were recruited at two centers – Sahlgrenska University Hospital, Gothenburg, Sweden (**Paper I, II, III**), Karolinska University Hospital, Stockholm, Sweden (**Paper I**) and Ersta Hospital, Stockholm, Sweden (**Paper II, III**).

Studies started January 2015 (**Paper I**) and September 2013 (**Paper II & III**) and were concluded by October 2016 (**paper I**) and April 2016 (**Paper II & III**). All participants gave oral and written informed consents prior to the start of the studies.

Inclusion criteria for **Paper I** were: diagnosis of PBC or PBC-autoimmune hepatitis overlap as established according to AASLD/EASL definitions (116). Patients had to be classified as UDCA non-responders defined as > 6 months of therapy with UDCA and with serum ALP >1.67 ULN at the time of enrolment and having been on treatment with BA sequestrant cholestyramine at a dose > 4 g twice daily or colestipol ≥ 5 g twice daily for at least 3 months, with a VAS-itch of at least 30/100 mm during the day before baseline (Visit 2). Women of childbearing capacity were allowed to participate provided a negative serum pregnancy test upon inclusion in the trial and the use of highly effective birth control or abstinence during the study. Exclusion criteria were other liver diseases than PBC and other reasons for itching such as atopic dermatitis or other primary skin diseases, jaundice of extrahepatic origin, cancer, chronic kidney disease, and chronic severe infection.

As for **Paper II, III**; inclusion criteria in the obesity group were: BMI ≥35 kg/m^2. In the gallstone group: symptomatic, ultrasound verified gallstone disease. Exclusion criteria were chronic liver disease other

than NAFLD, previous gastric or small bowel surgery, inflammatory bowel disease, uncontrolled diabetes mellitus, thyroid or endocrine abnormality, pregnancy, liver enzyme elevations or alkaline phosphatase / bilirubin above 2 x ULN (upper limit of normal) the day before start of medication, psychiatric disease and / or unwillingness to comply with the study protocol.

3.2 ETHICAL APPROVAL AND FUNDING

The study in **paper I** was approved by the Regional Ethical Review Board in Gothenburg (Dnr 795-14) and the study took place under the supervision of the Swedish Medical Products Agency (EudraCT 2014-004070-42).
The studies in **papers II & III** were approved by the Regional Ethical Committee in Gothenburg (Dnr 199/11; 2011-06-08) and the Swedish Medical Products Agency (EudraCT 2011-0008-13-37; 2012-05-07).
All studies were performed in accordance with the protocol, good clinical practice, and all relevant guidelines and regulations in accordance with the declaration of Helsinki.
All studies in this thesis were exploratory, investigator – initiated studies. In **paper I**, the study medication A4250 (0.75 mg and 1.5 mg capsules) was provided by Albireo AB, Gothenburg, Sweden.
In **paper II & III**, the study medication (OCA 25 mg and a matching placebo) was provided by Intercept Pharmaceuticals, Inc. San Diego, CA, USA.

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3.3 SUBJECTS AND STUDY DESIGN

Paper I: *A4250PBCPruritus* is an open-label, exploratory, phase IIa study to demonstrate the safety and efficacy of IBAT inhibitor A4250 in patients with primary biliary cholangitis (PBC) and cholestatic pruritus. Ten patients with PBC, nine females, one male, 54.9 ± 14.3 years of age were included, eight of them treated with cholestyramine 4–8 g/day and two of them with colestipol 5–10 g/day. All those patients were on continuous UDCA treatment and were classified as non-responders with ALP >1.67 ULN for more than one year. Participants were after a two-week wash out of the BA sequestrant treated with either 0.75 mg (n = 4) or 1.5 mg (n = 5) of A4250 for four weeks. UDCA was continued during the study period. The primary objective of this study is to assess the safety and tolerability of A4250, as determined by the occurrence of treatment-emergent serious adverse events. Other objectives were to demonstrate the efficacy of A4250 on pruritus variables and changes in quality of life questionnaires (QoL) and lysophosphatidic acid formation, as well as evaluation of changes in pharmacodynamic parameters of BA metabolism, such as serum and fecal BAs, C4 and FGF19 assessments and assessment of surrogate markers of cholestatic liver disease such as alkaline phosphatase, transaminases and bilirubin (Figure 5).

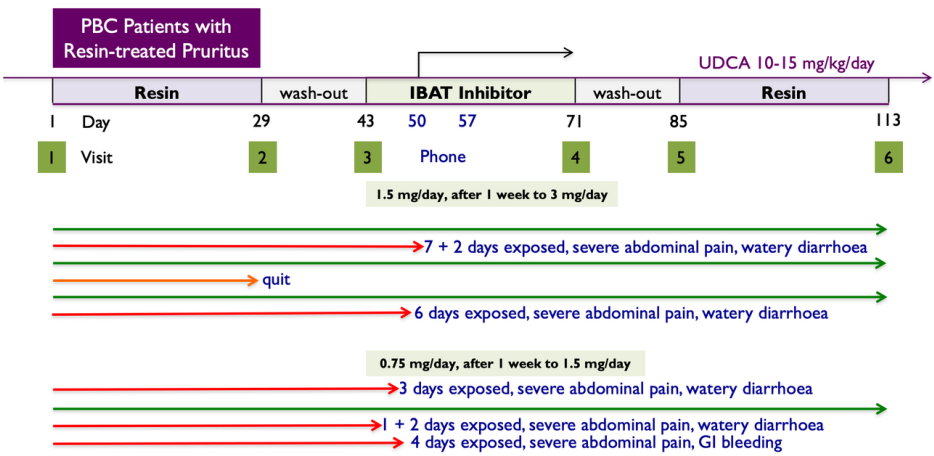

Figure 5. A4250PBCpruritus study flow scheme and individual outcome of the ten participating patients. Patients were divided into a group that received a higher dose of A4250 (1.5 mg to 3 mg per day) and a second group after amending the protocol receiving a lower dose of A4250 (0.75 mg to 1.5 mg per day).

Paper II: In this study, twenty otherwise healthy patients of the gallstone arm (OCAGS) of the Obeticholic acid in bariatric surgery and gallstone surgery (OCABSGS) study described below, were administered 25 mg/day OCA or a matching placebo for three weeks until the day before surgery. This group consisted of 80% female, 20% male, aged 50 ± 13 and 48 ± 8 years in the placebo and OCA group, respectively, BMI < 30 kg/m^2. Serum samples from days 1 and 21 were analysed for routine liver tests and biochemistry. BAs, their synthetic marker C4, FGF19 and a complete lipid profile were also assessed. During surgery, one-centimetre long harmonic-knife biopsies from the liver and the whole bile-filled gallbladder were collected for gene expression analysis and transcriptomics. Gallbladder bile was aspirated with a syringe and was sampled for the measurements of biliary lipids (cholesterol, phospholipids, BAs) and the calculation of the cholesterol saturation index and hydrophobicity index (Figure 6).

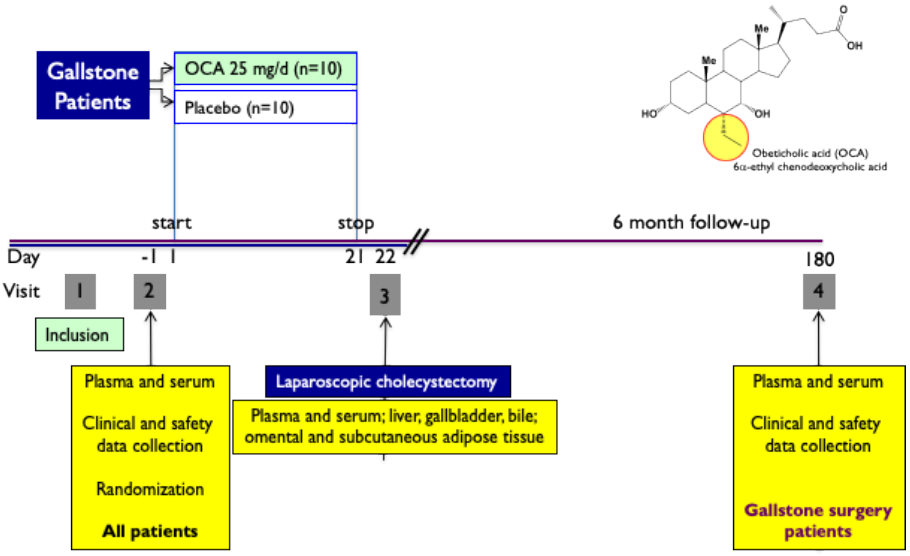

Figure 6. Flow scheme of the OCAGS arm of OCABSGS (Figure 7) showing the different timepoints of data collection for both OCA and placebo patients (n = 10 / group). In OCAGS study the focus is on Day 1 and 21. Data from Day 180 is discussed further in paper III.

Paper III: Obeticholic acid in bariatric surgery and gallstone surgery (OCABSGS) is a placebo-controlled double-blind randomized trial. It comprises in addition to the patients of the OCAGS study a bariatric surgery group of patients. Overall, forty patients were recruited in this

study divided into two arms in the following fashion: 20 otherwise healthy morbidly obese patients (BMI > 40 kg/m^2) scheduled for bariatric surgery, and 20 otherwise healthy patients with gallstones were administered 25 mg/day OCA or a matching placebo for three weeks until the day before surgery. In the bariatric surgery group that did not receive the usual preoperative low-caloric powder diet, serum from days 1 and 21 were analysed for routine liver tests and biochemistry, BAs, a complete lipid profile, serum BAs, C4 and FGF-19. For the evaluation of insulin resistance and possible pre-diabetes, plasma was taken for the estimation of HOMA – IR index and oral glucose tolerance tests (OGTT) were performed at days 1 and 21. At surgery, a liver biopsy (0.5-1 g) and a white adipose tissue (WAT) specimen (1 cm^2) were taken and immediately frozen in liquid nitrogen for mRNA and protein preparation for quantitative RT-PCR and Western blot analysis, respectively. In the gallstone surgery group, samples were collected as mentioned in paper II. At day 180, blood tests, BAs, C4 and FGF19 were collected again to assess the impact of respective surgeries on BA metabolism in each group (Figure 7).

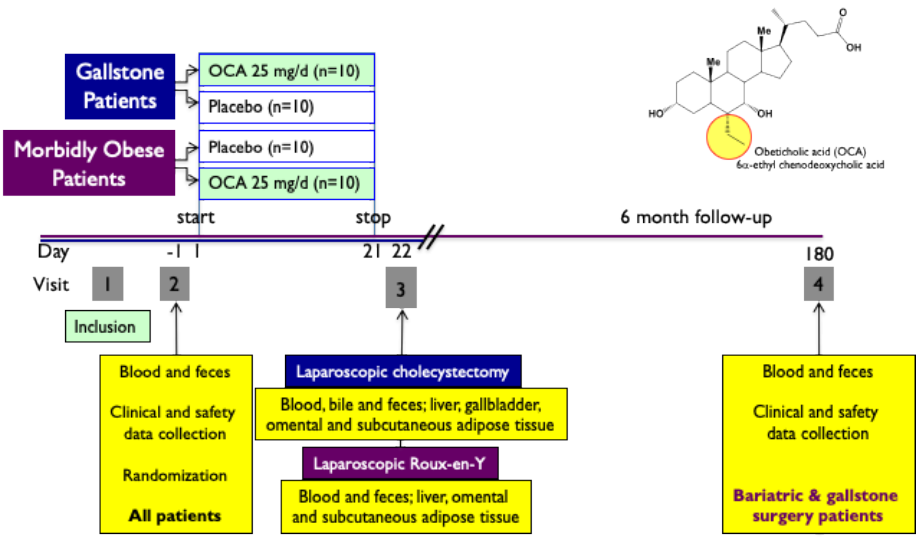

Figure 7. Flow scheme OCABSGS showing the different timepoints of data collection for both OCA and placebo patients (n = 20 / group) at Day 1, 21 and 180.

3.4 PERFORMED ANALYSES

Paper I:

Biochemistry: Blood was drawn from patients for biochemical analysis at all visits and serum liver tests (AST, ALT, ALP, Bilirubin) and lipids (serum total cholesterol, HDL-cholesterol, LDL-cholesterol, triglycerides) were analysed by routine clinical chemistry. Serum autotaxin activity was measured by a previously published homemade assay (109).

QoL: Patients were instructed to use a paper diary daily to indicate medications, number of bowel movements. They filled out the following questionnaires:

- Assessing stool consistency according to the Bristol Stool Chart (120).
- Assessing abdominal pain (0 = none, 1 = moderate, 2 = severe).
- Assessing pruritus severity according to the visual analogue score (VAS, 0–100 mm scale) (121)
- Pruritus severity according to the PBC-40 itch domain (122).
- Pruritus severity according to the 5-D itch scale (123).

Serum BA and C4 analysis: the extraction and analysis of BAs were based on a previously published method for the targeted determination of BAs by UPLC-MS (124). BAs were extracted from 50 µl of plasma using protein precipitation with 10 volumes of internal standard-containing methanol. After vortexing and centrifugation, the supernatant was evaporated and reconstituted in 200 µl of methanol/water (1:1). BAs (5µl injected) were separated using water with 7.5 mM ammonium acetate and 0.019% formic acid at a pH 4.5 as mobile phase A, and acetonitrile with 0.1% formic acid as phase B. The separation was made using gradient elution on a Kinetex C18 column (2.1x100 mm with 1.7 µm particles) (Phenomenex, Torrance, CA, USA) kept at 60 ºC. The gradient started with 1 minute of isocratic elution with 25% B, which was increased to 35% over the next 4 minutes. During the next 9.5 minutes B increased 5 from 35% to 95%. After one minute of isocratic elution the gradient was quickly returned to 25% B and the column was

equilibrated for 2.5 min to give a total runtime of 18 minutes per sample. The flow rate was 400 µl/min. Detection was made on a QTRAP 5500 instrument (Sciex, Toronto, Canada) with MRM in negative mode. BA standards were obtained from Sigma-Aldrich (Sweden, TCA, TUDCA, TCDCA, TDCA, TLCA, GCA, GCDCA, GDCA, GLCA, CA, UDCA, CDCA, DCA, LCA) and Astra Zeneca (Sweden, OCA, TOCA, GOCA). BA and C4 data are given in nanomolar concentrations.

FGF19: concentrations were measured using 100 µL of plasma and subsequent quantitative enzyme-linked immunosorbent assay with the human FGF19 Quantikine ELISA kit (R&D Systems, MN, USA) according to the manufacturer's instructions. All samples were assayed in duplicate and intra- and inter-assay coefficients of variation were <10%.

Paper II:

Biochemistry: Blood biochemistry including a complete blood count, liver enzymes (AST, ALT, ALP, GGT, Bilirubin), blood lipids (total cholesterol, HDL, LDL, triglycerides), BMI, fasting glucose and HOMA-IR in a fasted state at Day 1 and 21.

Serum BAs, C4 and FGF 19: same method as in paper I.

Biliary BAs: bile was thawed on ice, 5 µL of bile was used for the measurement of all bile components (BAs, phospholipids and cholesterol) in a similar manner to serum BAs. Concentrations measured were given in micromolar; 1000x higher than in serum.

Biliary cholesterol and phospholipids: for the quantification of cholesterol and phospholipids in bile. 5 µL of bile was thawed and pipetted into separate tubes. The desired components were then rapidly separated and quantitated using high-performance liquid chromatography (HPLC), followed by evaporative light-scattering detection (ELD) as described by Homan et al. (125).

Biliary FGF19: bile was diluted 1000x with water and FGF19 measurement using ELISA was performed in a similar manner to serum FGF19 in paper I.

pH measurement in bile samples: the pH of human bile samples was measured with a micro-combination pH-electrode (model PHR-146, Lazar Research Laboratories, Inc., Los Angeles, CA) coupled with Thermo Russell pH meter (model RL 150). The electrode was calibrated by putting the pH meter in millivolt mode and by dipping the pH electrode into pH7 buffer HI7007 (Hanna Instruments, RI, USA). After 5 minutes the pH 7 was adjusted to 0mV via potentiometer in the bias cylinder (as shown in figure). Later, pH electrode was calibrated either at pH 4 or pH 10 using standard buffers HI7004 and HI7010 respectively (Hanna instruments RI, USA). 50µl of human bile samples was aliquoted in custom made pH chamber comprised of 500µl screw cap polypropylene tubes (Cat # 72.730.006, Sarstedt, Germany) punched with 3mm diameter hole into the cap for insertion of pH probe as shown in figure. All pH measurements were performed in triplicate (126).

Microscopic analysis of bile: bile was thawed on ice. 100µL of bile from each sample in the OCA and placebo group was pipetted over to new tubes and was centrifuged at 12000 rpm. A drop of the bile sample and the pellet were then examined for the presence and morphology of crystals using white light microscopy.

Cholesterol saturation index (CSI) of gallbladder bile was calculated for each individual patient according to the critical tables by Carey for the calculation of saturation indices in native bile (127). Using those tables, it was possible to calculate the lithogenic index representing per cents of cholesterol saturation in human bile. In this method, two key physiochemical variables were important to determine the equilibrium in cholesterol solubility of bile: bile salt to lecithin ratio and total lipid concentration (bile salt + lecithin + cholesterol). Those were identified to be in the range of 0.085 – 0.45 for molar bile salt – lecithin ratio and 0.3 – 30 g/dL for total lipid concentration at 37 ^{o}C. We then employed the BA values measured by UPLC/MS-MS to determine individual CSI and calculated the mean values for OCA and placebo, respectively.

Hydrophobicity indices (HI) of gallbladder BAs were defined for each individual sample using the quantitative analysis of the composite hydrophilic-hydrophobic balance of a mixture of BAs by Heuman (19). This index is based on the logarithms of bile salt capacity factors determined using reversed phase high performance liquid chromatography (HPLC). It was standardized arbitrarily to set indices at values between 0 to 1, 0 being most hydrophilic for TCA and with increasing hydrophobicity towards 1 for TLCA. We used those indices in a standard curve that in addition to the compounds tested by Heuman also included OCA and its conjugates. Thus, were able to define their HI and finally to calculate the mean HIs for OCA and placebo groups, respectively.

RNA isolation and qPCR from human liver and gallbladder tissue were performed to determine FGF19 expression levels. RNA was isolated following the RNease protocol (Qiagen, RNeasy Mini kit) including DNAse I digest on column. RNA concentrations were measured by Nanodrop and 1 μg of RNA was subjected to cDNA synthesis (Superscript III, Invitrogen). qPCR of FGF19 gene was performed on a Light Cycler (Roche) with Luna Universal qPCR Master Mix (NEB). OSTα was used as control gene.

Paper III:

Biochemistry, BAs, C4, FGF19 data as for papers I & II.

Multi-omics analysis: Gene expression levels in the liver and adipose tissue were measured using transcriptomics (RNA-seq), as described in further detail in Paper III. Volcano plots were used to measure fold changes in mRNA expression and thus assess FXR binding strength in the respective groups (128).

ChIP-Seq: Chromatin immunoprecipitation coupled with high throughput DNA-sequencing (ChIP-Seq) was used to identify FXR binding sites in the genome of liver and gallbladder tissue biopsies taken from subjects in the study. In this process genomic DNA is crosslinked with formaldehyde to stabilize DNA and protein interactions, in our case FXR binding to its binding sites in the whole genome. Chromatin is then

sheared in small parts (~300-500 bp) by sonication. Fragments with FXR are selectively enriched and purified by immunoprecipitation with an FXR antibody. Crosslinking is then reversed, and DNA fragments are sequenced and mapped to the genome (129) (Figure 8). Once ChIP-seq was performed, we performed a principal component analysis (PCA). PCA is a technique for reducing the dimensionality of large data sets (such as ChIP-seq data sets), thus increasing interpretability but at the same time minimizing information loss. It does so by creating new uncorrelated variables that successively maximize variance (130).

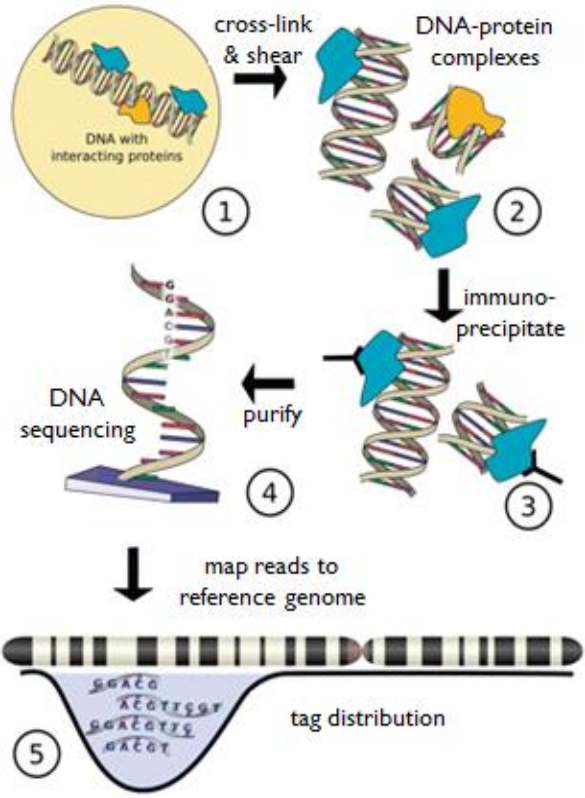

Figure 8. Main steps involved in Chromatin immunoprecipitation coupled with high throughput DNA-sequencing (Chip-seq) as described in text.

3.5 STATISTICS

All statistics are described in the corresponding papers. In paper I, no formal statistics were performed due to the too low number (n=4) of study participants finishing the study per protocol. All questionnaires were evaluated manually and fed into Microsoft Excel sheets to produce the graphs included in the publication. In papers II & III, Microsoft Excel and Graph Pad Prism software were used for statistical analyses. In general, the design of all experiments was based on examining two separate groups of patients with different treatments over a certain period of time. Thus, significances of differences between control and treated groups or within a single group were analysed using paired and unpaired t-tests, respectively, after confirming equal distribution of data points. More complex statistics was employed in paper III given the vast amount of data that was generated from ChiP-seq and RNA-seq. Here, principal component analysis (PCA) was performed on BS and GS groups for cistromics and Volcano plots were used to compare transcriptomics in OCA and placebo treated groups, respectively.

4 RESULTS

4.1 PAPER I

Pilot study with IBAT inhibitor A4250 for the treatment of cholestatic pruritus in primary biliary cholangitis

Aims

This paper aimed to assess the safety, tolerability, and potential improvements of pruritus after treatment with oral A4250 in patients with PBC and BA sequestrant pre-treated cholestatic pruritus in an open-label pilot study format.

Findings

Efficacy
One patient dropped out at the study start. The remaining nine patients exposed to A4250 (0.75 mg, $n = 4$; 1.5 mg, $n = 5$) reported a rapid (within 48 h) improvement in pruritus that in particular restored normal sleep at night and reduced daily embarrassment caused by the itch in the four patients who finished the whole 4-week treatment period with A4250. Also scratch marks on the skin diminished in the patients who had this sign of severe pruritus. Those positive changes were well documented in the PBC40 itch domain, 5-D itch scale and VAS score.

Safety
Five of the nine patients exposed to A4250 withdrew already during the first week due to diarrhea and abdominal pain. This can be partially explained by the mechanism of action of IBAT inhibitors and have been reported in other studies with IBAT inhibitors.

Metabolic investigations
A4250 did not induce any changes in liver enzymes or blood lipids. Serum BA levels did not differ between patients who finished the study per protocol and those who interrupted the treatment. There was an enrichment of UDCA in their bile due to their concomitant UDCA treatment. Similar results apply for serum FGF19 levels. C4 levels, on

the other hand increased substantially, denoting higher rate of BA synthesis. Autotaxin activity decreased in 3 out of the 4 patients that were treated with A4250 until the end of the study.

4.2 PAPER II

Obeticholic acid may increase the risk of gallstone formation in susceptible patients

Aims

This paper aimed in a double-blind placebo control trial fashion to explore how FXR activation by a steroidal semi-synthetic ligand would impact gallbladder lipid composition shifting it into a gallstone prone milieu, as well as attempt to detect FGF19 of previously unknown biliary origin.

Findings

Metabolic investigations
Treatment with OCA lead to a decrease in BA synthesis through FXR activation in the ileum and liver. This was reflected by their decreased measured concentration in serum, as well a substantial drop in their synthetic marker C4. OCA and its conjugates were discoverable in serum of patients receiving the drug. Serum FGF19 levels also increased 3 folds in the serum of patients receiving OCA due to ileal FXR activation. Another finding was a slight elevation in serum LDL cholesterol probably due to downregulation of LDL-receptors in the liver, as well as a slight elevation in serum ALP levels.

Effect of OCA on cholesterol saturation index (CSI)
Upon treatment with OCA, there was a significant drop in total lipid concentration in bile. This drop was caused primarily by a decrease in biliary BAs due to decreased synthesis, while the other main biliary lipid components (cholesterol and phospholipids) remained unchanged, as reflected by their unchanged biliary concentrations and unchanged gene expression levels of their biliary transporters. The lower amount of

gallbladder BAs in proportion to total biliary lipids lead to a substantially higher cholesterol saturation index in the bile of patients treated with OCA.

Effect of OCA on hydrophobicity index (HI)
In the OCA group, OCA and its conjugates (TOCA, GOCA) represented approximately 14 – 19% of total gallbladder BAs. UPLC characteristics of OCA and its conjugates revealed a highly hydrophobic character, eluting closely to TLCA. This then in its turn shifted the gallbladder BA composition into a more hydrophobic nature compared to placebo.

Effect of OCA on biliary FGF19
Treatment with OCA induced a robust increase of FGF19 concentrations in gallbladder bile as compared to placebo. RNA-sequencing revealed a high expression of FGF19 in the gallbladder and gene analysis using qPCR from gallbladder wall epithelium in comparison to liver indicated a biliary origin of FGF19.

4.3 PAPER III

Human FXR-DNA binding is associated to the obese phenotype

Aims

This study aims to employ a multi-omics approach to decipher FXR-activated pathways in humans. Cistromics were performed with the goal to identify genome-wide FXR binding sites in human DNA by using ChIP-seq.

Findings

Metabolic investigations
In both groups, similar biochemical changes were seen after OCA treatment at day 21 as mentioned in paper II (Table 2). Efficient FXR activation by OCA increased circulating FGF19 and supressed BA synthesis marker C4 and endogenous BA synthesis. There was a slight and transient elevation in ALP and LDL cholesterol and a decrease in

triglycerides. At the six-months control, biochemical improvements were noticed in the BS group, as expected: decreases in BMI, serum cholesterol and ALT and improvements in insulin sensitivity as shown by decreases in HOMA-IR. Further changes in BA, C4 and FGF19 levels were noticed and can be attributed to surgical manipulation of the GI tract. In the BS group, there was an increase in circulating BAs and FGF19 level after 6 months, which may reflect increased efficiency of enterohepatic circulation following intestinal rerouting after bariatric surgery (131). On the other hand, in GS patients, the absence of the gallbladder decreases the sequestrations of BA pool in the biliary tree between meals, so that hepatic bile secretion into the intestine is more continuous and total fasting BA concentration and C4 levels are therefore increased (132). There is also a prolonged exposure time of primary BAs to small intestinal bacteria, thereby enhancing the conversion of CA to DCA, which has a low affinity to FXR (133).

Multi-omics analysis

In our RNA-seq we noticed that there was a clear separation between OCA and placebo treated patients. A total of almost 400 genes were differentially regulated by OCA treatment in the obese condition, of which approx. 300 were induced. When the ChIP Seq data and the RNA Seq data were integrated we could identify approximately 100 direct FXR gene targets, which were induced selectively stronger in the obese conditions than in the non-obese condition. This demonstrates that predetermined background specific FXR binding leads to a background specific gene signature after FXR ligand activation.

FXR Chip-Seq

We observed that FXR binding is primarily not directed by ligand availability. Instead, occupation of FXR binding sites is determined predominantly by the metabolic background. Principal component analyses showed that obese patients are clearly separated from non-obese patients independent of their treatment with OCA or placebo. OCA did not significantly increase FXR binding to additional binding sites. In general, FXR binding appeared to be weaker and less frequent in obese conditions. We also showed that alkaline phosphatase, at least in the liver, is transcriptionally activated by FXR. This finding is counterintuitive given that OCA promotes reductions in GGT, the other marker of cholestasis.

Biochemistry - Lipids - FGF19 – C4 – Bile Acids

	Bariatric Surgery						Gallstone Surgery					
	OCA			Placebo			OCA			Placebo		
	Day 1	Day 21	Day 180	Day 1	Day 21	Day 180	Day 1	Day 21	Day 180	Day 1	Day 21	Day 180
Demographics												
Age [years]		38.2 ± 8.9			38.1 ± 9.9			48.8 ± 8.9			50.8 ± 13.3	
Gender [F/M]		80% F			80% F			80% F			80% F	
BMI [kg/m2]	40.6 ± 2.3	40.8 ± 2.2	30.1 ± 3.1	40.8 ± 2.0	41.0 ± 2.3	30.7 ± 2.1	28.8 ± 6.0	28.7 ± 6.0	28.2 ± 4.6	27.5 ± 9.0	27.6 ± 8.9	28.8 ± 5.8
HOMA-IR	4.4 ± 3.1	4.5 ± 2.6	1.7 ± 0.7	3.5 ± 2.6	3.4 ± 2.1	1.4 ± 1.0	1.6 ±1.0	1.7 ± 1.0	2.7 ± 1.7	2.9 ± 2.6	3.0 ± 2.8	3.2 ± 2.2
Liver Enzymes												
ALT [U/L]	32.8 ± 11.5	29.9 ± 17.5	21.7 ± 7.5	31.5 ± 15.8	35.3 ± 25.8	20.4 ± 7.7	25.6 ± 4.9	27.1 ± 3.5	25.0 ± 16.2	22.7 ± 11.7	45.9 ± 33.4	17.6 ± 8.6
AST [U/L]	30.7 ± 21.0	29.6 ± 22.8	19.1 ± 7.3	27.0 ± 6.2	26.1 ± 9.7	20.6 ± 5.6	20.6 ± 3.9	23.7 ± 2.9	24.5 ± 7.1	21.8 ± 5.7	35.3 ± 19.1	20.4 ± 4.9
ALP [U/L]	78.5 ± 28.1	87.8 ± 35.1	84.3 ± 23.3	76.8 ± 27.7	76.8 ± 26.7	74.7 ±8.5	78.0 ± 40.4	85.5 ± 40.6	74.0 ± 8.2	64.0 ± 32.5	59.9 ± 32.5	63.6 ± 16.3
GGT [U/L]	40.8 ± 29.0	22.3 ± 11.3	19.3 ± 12.8	30.8 ± 21.9	34.3 ± 21.7	15.1 ± 8.6	39.4 ± 40.8	29.5 ± 31.3	29.4 ± 27.3	23.9 ± 10.9	35.3 ± 19.1	26.9 ± 13.2
Serum lipids												
Total Cholesterol [mmol/L]	5.0 ± 1.8	5.2 ± 2.0	3.9 ± 1.0	5.5 ± 1.8	5.3 ± 1.8	4.2 ± 0.6	5.1 ± 2.4	5.2 ± 2.6	4.3 ± 1.6	4.8 ± 2.17	5.4 ± 2.6	4.9 ± 0.7
LDL-Cholesterol [mmol/L]	3.1 ± 1.2	3.6 ± 1.5	2.3 ± 0.9	3.5 ± 1.2	3.4 ± 1.2	2.6 ± 0.5	2.1 ± 1.2	2.3 ± 1.2	2.5 ± 1.1	3.1 ± 1.6	3.3 ± 1.7	2.9 ± 0.7
HDL-Cholesterol [mmol/L]	1.2 ± 0.4	1.1 ± 0.4	1.2 ± 0.1	1.4 ± 0.6	1.3 ± 0.5	1.3 ± 0.2	1.7 ± 0.8	1.5 ± 0.8	1.4 ± 0.5	1.5 ± 0.7	1.7 ± 0.9	1.5 ± 0.3
Triglycerides [mmol/L]	1.9 ± 1.4	1.2 ± 0.6	1.1 ± 0.3	1.6 ± 1.4	3.4 ± 2.1	1.0 ± 0.3	0.8 ± 0.4	0.6 ± 0.3	1.0 ± 0.5	1.6 ± 1.4	3.4 ± 2.1	1.3 ± 0.5
Bile acid synthesis												
FGF19 [ng/L]	64.4 ± 30.4	180.3 ±128.1	123.8 ± 73.8	97.1 ± 53.1	90.6 ± 35.1	212.2 ± 47.1	95.0 ± 8.5	234.4 ± 35.6	179.8 ± 44.3	99.2 ± 23.9	126.1 ± 24.5	191.9 ± 34..8
C4 [nmol/L]	26.5 ± 25.4	2.9 ± 3.4	9.2 ± 2.5	34.4 ± 30.3	21.4 ± 18.4	18.3 ± 5.3	31.4 ± 22.8	2.8 ± 4.0	56.1 ± 16.7	29.1 ± 20.1	25.1 ± 19.1	61.0 ± 7.3
Total Bile Acids [nmol/L]	1245 ± 95	652 ± 45	959 ± 394	1132 ± 56	1243 ± 84	1858 ± 881	1312 ± 236	930 ± 260	1809 ± 215	1394 ± 24	1181 ± 126	2734 ± 943
Endogenous BAs [nmol/L]	1245 ± 95	416 ± 35	959 ± 394	1132 ± 56	1243 ± 84	1858 ± 881	1312 ± 236	518 ± 179	1809 ± 215	1394 ± 24	1181 ± 126	2734 ± 943
Obeticholic Acid [nmol/L]	n.d.	236 ± 70	n.d.				n.d.	412 ± 103	n.d.			
Paired t-test	down p<0.05 D 21 vs. D 1			up <0.05 D 21 vs. D 1			down p<0.05 D 180 vs. D 21			up p<0.05 D 180 vs. D 21		

Table 2. Patients demographics in OCABSGS showing the dynamic changes in serum biochemistry and bile acid synthesis at day 1, 21 and 180. Unit conversion: 1µkat/L = 60 IU/L

Abbreviations: BMI, Body Mass Index; HOMA-IR, Homeostatic Model Assessment of Insulin Resistance; ALT, Alanine Aminotransferase; AST, Aspartate Aminotransferase; AP, Alkaline Phosphatase; GGT, Gamma-Glutamyl Transferase; LDL, Low-density Lipoprotein; HDL, High-density Lipoprotein; FGF19, Fibroblast Growth Factor 19; C4, 7 alpha-hydroxy-4-cholestene-3-one; BAs, Bile Acids.

5 DISCUSSION

In this Thesis, I investigated two different aspects of BA receptor activation; indirectly by blocking FXR in the intestine through the use of an IBAT inhibitor, or directly by using an FXR agonist in the liver and intestine. To that end, we performed two clinical studies with drugs that target BA turnover. The first was a clinical pilot study in patients with PBC investigating the potential benefit of the inhibition of BA reabsorption in the terminal ileum as an approach to treat cholestatic pruritus. The primary objective of this study was to assess the safety and tolerability of the IBAT inhibitor A4250, as determined by the occurrence of treatment-emergent adverse events. The idea behind blocking IBAT as a potential therapeutic modality of cholestatic itch stemmed from the theory that BAs are responsible for cholestatic pruritus and that by lowering their circulating levels, we might achieve subjective improvement in pruritus severity. From a theoretical point of view, this concept is intriguing, however, we found out that the therapeutic benefit may be very limited. We had to stop our pilot study prematurely as five of the first 10 patients discontinued treatment after short-time exposure to study medication due to abdominal side effects (cramps and diarrhea). As we only had approval to recruit twelve patients it was considered unethical to recruit two more study subjects, because at best only questionable scientific results could have been obtained. We are not the only ones who had investigated this concept. Indeed, clinical trials with IBAT inhibitors in adults with cholestatic liver disease had similar outcomes, with a large proportion of patients reporting abdominal side effects, regardless of the type of IBAT inhibitor administered (94, 134, 135). At least those industrial-sponsored trials were sufficiently powered to show the expected biochemical changes, i.e. lowering of circulating BAs and increases in C4 as a response to decreased FGF19 levels (134, 135). To our knowledge, there are no ongoing clinical trials with IBAT inhibitors in adults with cholestatic liver disease. Surprisingly, pediatric patients with cholestatic pruritus seem to tolerate a variety of IBAT inhibitors much better, which is why clinical trials in children and adolescents with biliary atresia, Alagille syndrome and progressive familial intrahepatic cholestasis are still ongoing and showing promising results (136) (see appendix). There are some speculations as to why this is the case, e.g. lower intestinal load of BAs in inborn cholestasis but also a predisposed

abdominal hypersensitivity due to pretreatment with BA sequestrants, notoriously causing constipation, in our adult PBC patients might be a plausible cause.

Our IBAT study had critical limitations. It was a pilot study with a small sample size and the design was non-randomized, without a placebo control, which makes it difficult to perform statistics or draw major conclusions. This is especially important since pruritus is a subjective symptom that is perceived differently by various patients where the placebo effect may be as high as 30% (137). Also, we might have had a too optimistic approach in our study by starting treatment with a dose of 1.5 mg/day initially, then decreasing to 0.75 mg/day in a similar fashion as our previous study with healthy volunteers on 1.5 or 3 mg daily (138). Of note, in that particular phase 1 study, a 20% incidence of abdominal pain was reported during 7 days administration at the lowest dose of 1.5 mg daily. Similar and dose-dependent incidence of abdominal side effects was reported in phase 1 studies with other IBAT inhibitors in healthy volunteers and ranged from 25–75% of participants (NCT01416324, NCT01607385) (139). It might have been prudent to have tested A4250 in lower doses or split regiments to minimize adverse events. It would also have been of academical interest to sample feces for BA analysis, but this was not included in the study protocol as BAs in human feces are supposed to be analysed in completely collected 24h samples, preferentially during three consecutive days, which is in general considered not feasible, particularly in patients suffering from diarrhea.

In addition to the potential role of IBAT inhibitors in alleviating cholestatic itch, they are also tested as a treatment option for non-alcoholic fatty liver disease/steatohepatitis (NAFLD/NASH). By interruption of the enterohepatic circulation with decreased intestinal FXR signalling they could deplete the BA pool from potentially toxic compounds which might result in decreased liver fat and improved liver function in NAFLD/NASH (140). Preclinical studies with the IBAT inhibitor SC-435 in high-fat diet fed mice showed that this compound prevented hepatic accumulation of triglycerides and restored whole body insulin sensitivity. Blocking BA reuptake prevented hepatic cholesterol accumulation by inducing BA biosynthesis from cholesterol stores and shifting the liver BA pool composition toward one enriched

in hydrophobic species that are more FXR agonistic in nature, while reducing the hydrophilic, FXR antagonistic BAs (141). However, this treatment approach did not affect intestinal fat absorption and was not protective against hepatic fibrosis in a choline-deficient mouse model of NASH (142). Given the interspecies differences in BAs, it is mandatory to test IBAT inhibitors in humans and indeed, phase I trials with the IBAT inhibitor SHP626 (Volixibat) showed that it could significantly decrease total and LDL cholesterol levels and increase fecal BA excretion and serum C4 levels (143-145) thus providing a rationale for exploring IBAT inhibition in NASH. Based on these findings, Volixibat entered in a phase II study in patients with NASH (NCT02787304). NASH is a chronic illness of still insufficiently defined pathogenesis where long-term management most likely will require combination treatments, and IBAT inhibitors could find their places in this strategy, in addition to the metabolism modifying drugs, such as FXR agonists (140). However, developing IBAT inhibitors for NASH is challenging, because of the complex interplay between BA metabolism, EHC transport and NASH. NASH is associated with increased systemic inflammation which not only down-regulates IBAT, but also hepatic BA transporting proteins which can affect FXR activation in the liver. NASH patients have higher levels of serum total primary BAs and an altered circulating BA composition with decreased EHC as well as down-regulated NTCP, up-regulated OSTα/β and dysbiosis of gut microbiome (146, 147). As a result, IBAT inhibition might have unintended consequences if there is an increase in hepatic BA synthesis conflicting with decreased biliary secretion due to down-regulation of BSEP. Furthermore, the increase in serum conjugated BA could activate the sphingosine-1-phosphate receptor 2 which can worsen NASH by promoting inflammation and fibrosis in the liver (148).

In the other study with morbidly obese or gallstone patients awaiting laparoscopic Roux-enY or gallbladder surgery, we primarily did not investigate a clinical outcome, but performed a pharmacodynamic study aiming to decipher the mechanisms initiated by FXR activation on a molecular level. The rationale behind this is that a large number of clinical trials is currently ongoing with drugs that affect the regulation of BA synthesis either by targeting the nuclear receptor FXR with a ligand (steroidal or non-steroidal), or the target of intestinal FXR activation (FGF19 analogues). Various clinical endpoints were tested in

phase IIa trials, including surrogate markers of outcome in cholestatic liver disease such as PBC (82, 83), and indicators of the metabolic syndrome such as weight loss and improvement of insulin resistance in NAFLD/NASH (84), followed by liver histology improvements as primary end points in phase IIb/III NAFLD/NASH trials (FLINT, REGENERATE) (70, 85) and one ongoing phase IV trial in PBC (COBALT). All these trials provide only very little indirect information about the molecular mechanisms of FXR activation. We took the opportunity to in detail study what happens on a molecular level by investigating a large number of tissues from the liver, gallbladder and adipose tissue in two separate population groups. Our data show that FXR activation with the BA-derived FXR agonist OCA profoundly affects the biochemistry and composition of gallbladder bile, making the individual more prone to gallstone disease. This finding has substantial clinical relevance as the interim analysis of the ongoing REGENERATE trial reported a significant incidence of gallstones and cholangitis, occurring in 3% of NASH patients treated with 25 mg OCA (85). Hence, the sponsor of that trial has substantially amended ongoing clinical trial protocols, specifically addressing the problem regarding the development of gallstones and cholecystitis/cholangitis, and most recently, pancreatitis, a condition that is often caused by gallstones (149). Pruritus is a major and often bothersome side effect experienced by almost half of the patients treated with OCA, which can potentially restrict patients' compliance (70, 82). One of the molecular mechanisms underlying the pruritus observed with OCA administration could be imputable to OCA's residual activity toward TGR5 (150, 151). Whether or not this effect is solely FXR- or OCA-related is still unknown because final data from ongoing studies with non-steroidal FXR agonists is not available yet. One might speculate that the side effect profile might not be as severe since non-steroidal FXR agonists do not undergo EHC. In particular, they may not impact on gallstone formation as much as BA-derived FXR agonists as they are not secreted into bile in amounts to significantly alter the hydrophobicity of gallbladder bile. However, this remains a speculation since data from ongoing phase II trials with non-steroidal FXR agonists is missing. However, these compounds seem to generate a similar incidence of pruritus and undesirable shifts in cholesterol profiles as seen with OCA.

Several non-steroidal FXR agonists have been developed. The first promising candidate was the isoxazole derivative GW4064, which is endowed with high potency and efficacy, and an enormous fundamental FXR research has and is still being done with this compound. However, its clinical application was hampered by its hepatocellular toxicity and poor pharmacokinetic properties (152). Structurally related compounds such as WAY-362450 did not enter into clinical trials either (153-155). In contrast, this was possible for Px-104, currently named GS9674 or Cilofexor. Px-104 and the corresponding trans-isomer Px-102 have been tested in animal models of NASH and fibrosis with promising results (156). Cilofexor has similar FXR affinities as GW4064, but with improved aqueous solubility and metabolic stability (157). GS9674 has progressed into phase II clinical trials in NAFLD/NASH. Results from the first phase II trial have very recently shown Cilofexor to be well tolerated and provided reductions in hepatic steatosis, liver biochemistry, and serum BAs after 24 weeks of therapy, without improvements in liver stiffness measurements (158). A 12-week treatment with Cilofexor also led to significant improvements in liver biochemistry and markers of cholestasis in a phase II trial in patients with PSC (159). Other non-steroidal FXR agonists like LJN452 (Tropifexor) (160) have proved to be efficacious and have progressed into phase II clinical trials for the treatment of PBC and NASH, or as in the case of EDP305, for NASH.

In addition to BA-derived and non-steroidal FXR agonists, also down-stream targets of FXR activation, such as FGF19, are tested for the treatment of cholestasis and NASH. The rationale is given by findings in mice that administration of human recombinant FGF19 protected against cholestasis and reduced liver inflammation and necrosis by reducing the total BA pool (161). However, safety concerns have been raised due to the mitogenic properties of FGF19 (162, 163). FGF19 is overexpressed in patients with hepatocellular carcinoma and correlates with worse prognosis (164). Of note, the rodent ortholog FGF15 has no mitogenic properties, thus an animal model is lacking to adequately assess the carcinogenicity of FGF15/19 administration (165). In humans, the tumorigenic activity of FGF19 has been attributed to a cross-talk between FGFR4 and β-catenin (166). In line with this, it was speculated that the removal of the domain responsible for FGFR4 binding would allow to preserve the FGF19 beneficial effects, while

suppressing its tumorigenic activity (167). This led to the discovery of M70 (NGM282), which fully retains BA regulatory activity but is devoid of pro-tumoral activity in mouse models (168). After successful preclinical studies with NGM282 in mouse models of cholestasis and NASH (169, 170), it was evaluated in a phase II trial for the treatment of NASH. In these patients, the administration of NGM282 was well tolerated and reduced liver fat content as well as liver inflammation and fibrosis (171). NGM282 increased serum LDL levels in patients, however, concurrent treatment with a statin brought LDL levels back to baseline (172). NGM282 has also been evaluated in PSC (173), PBC (174), and type 2 diabetes (165).

Taken together, a judgment whether non-steroidal FXR agonists or FGF19 analogues are superior to BA-derived FXR activators is currently impossible. Indeed, a definitive answer will probably never be given as independent head-to head comparisons are very unlikely to be done. It will be interesting to see whether FXR agonists will become approved for the treatment of NASH at all.

In the time given for this Thesis we were not able to comprehensively decipher the molecular mechanisms as intended from the very beginning. The unique comparisons of cistromics (ChIP-seq) and transcriptomics (RNA-seq) to rodent in vivo or human in vitro data is still ongoing. Only very recently a "consensus" comprehensive FXR signalling atlas was derived from pooled ChIP-seq Data (PMID: 31118325) allowing a meaningful biostatistical evaluation of our human in vivo data. To our great surprise the induction of FXR binding sites (cistromics) was clearly related to the major difference in the phenotype of our bariatric or gallstone surgery patients, namely the prevalence of (morbid) obesity, while the expression of FXR target genes (transcriptomics) followed the randomization to treatment with OCA or placebo in both surgical groups. The reason why the induction of FXR binding sites relates to the obese phenotype is under extensive ongoing evaluation, and an answer might be given after imputing the proteomics in a systems biology approach. Of note, experiments in mice have shown a higher degree of FXR acetylation in obese conditions which inhibits DNA binding of FXR/RXRα heterodimer, thus hampering its ability to exert effects on downstream genes (175).

We had a few critical limitations in our second study. Firstly, liver biopsies were taken only after treatment with OCA/placebo and therefore, we do not have proper information as to the initial degree of NAFLD/NASH in both our study cohorts. Secondly, in our gallstone surgery group, we did not study the role of FXR activation / increased hepatobiliary FGF19 on gallbladder filling and relaxation by performing ultrasound-based kinetic studies. However, since all our patients had established symptomatic gallstone disease, any conclusion of these studies would have been questionable, in particular, as adjustments for number and size of stones within the gallbladder would have been necessary.

6 CONCLUSION

In our investigator-initiated studies we were able to study two different compounds and reached the following conclusions:

1) From a clinical point of view, it is unlikely that IBAT inhibitors will be established as a treatment modality for cholestatic itch in the adult population.
2) Steroidal FXR agonists that undergo EHC, specifically OCA increase the risk of gallstone formation by altering the physiochemical composition of gallbladder bile. Increased vigilance is therefore warranted.
3) Deterioration of plasma lipid profile can be demonstrated after short-term treatment with OCA and we believe that the regulatory mechanisms are complex and further results from our multi-omics approach are awaited. Also, phenotype differences in humans (obesity vs non-obesity) seem to significantly impact FXR signature patterns, which necessitates taking this into account when performing studies with FXR activators.

7 APPENDIX

Ileal bile acid transporter inhibitor for the treatment of chronic constipation, cholestatic pruritus and, NASH.
Review article.

8 ACKNOWLEDGMENTS

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