Understanding the molecular actions of bile acid receptor activation for treating human liver disease

Purpose and aims

The interplay between the liver, the gastrointestinal tract and lipid metabolism is complex and not well understood, but bile acids are key players in these interactions. Bile acids are synthesized in the liver, used for lipid absorption in the small intestine, and then reabsorbed and returned to the liver via the portal vein (enterohepatic circulation). By activating the nuclear receptor farnesoid X receptor (FXR), bile acids also function as signaling molecules that regulate not only their own synthesis but also key pathways in lipid and glucose metabolism.

FXR activation has emerged as a very promising treatment option for chronic cholestatic liver diseases such as primary biliary cirrhosis and complications of the metabolic syndrome such as type 2 diabetes, atherosclerosis and fatty liver disease. However, it is not known how activation of FXR affects bile acid, glucose and lipid metabolism at the molecular level in humans. FXR activation studies have almost exclusively been conducted in mouse models of liver disease and metabolic disorders. These studies are of limited human relevance since bile acid profiles differ profoundly between humans and mice.

We now will perform two pharmacodynamic studies with the FXR agonist obeticholic acid (OCA) in humans: (1) a prospective placebo-controlled trial in patients with gallstone disease who will undergo laparoscopic cholecystectomy or patients who are morbidly obese and will undergo gastric bypass surgery, and (2) a prospective placebo-controlled trial in morbidly obese patients and healthy volunteers. We therefore have access to a unique source of human material in which we can decipher the molecular pathways of FXR activation and to define global DNA binding sites of FXR. Importantly, these studies will provide us with the opportunity to investigate several interacting metabolic active tissues and compartments (i.e. liver, adipose tissue, jejunum, gallbladder, bile and serum, and feces for gut microbiota) using cutting-edge technology.

Specific Aim 1:
To determine how FXR activation affects bile acid metabolism in humans.
We will investigate how FXR activation affects hepatobiliary detoxification pathways and expression and secretion of a recently identified biliary factor, fibroblast growth factor 19 (FGF19).

Specific Aim 2:
To determine how FXR activation affects lipid metabolism in humans.
We will test the hypothesis that FXR profoundly affects lipid metabolism by controlling key regulators of lipid synthesis in the liver and lipid storage in white adipose tissue.

Specific Aim 3:
To use an unbiased approach to define FXR-regulated pathways in human liver and jejunum.
We will use chromatin immunoprecipitation coupled with high-throughput DNA sequencing (ChIP-seq) to identify global DNA binding sites of FXR in the human liver and jejunum genome.
Background

**Bile acids are signaling molecules and endocrine metabolic regulators**

In addition to their detergent properties in lipid digestion, bile acids have recently been recognized as signaling molecules and endocrine metabolic regulators (1). Bile acids regulate their own hepatic synthesis, biliary secretion and intestinal uptake through the nuclear receptor FXR in the liver and ileum (Fig. 1). Bile acid-activated FXR pathways also modify hepatic inflammation, regeneration, fibrosis, and tumor formation as well as glucose homeostasis, hepatic lipid synthesis and secretion and lipid storage in adipose tissue (1, 2). Thus, intensive research efforts are focused on studying bile acid-induced FXR pathways with the aim of developing targeted treatments of not only cholestatic liver diseases such as primary biliary cirrhosis and primary sclerosing cholangitis but also obesity-associated metabolic disorders such as fatty liver disease, type 2 diabetes and cardiovascular disease (3).

Fig. 1. FXR regulates bile acid turnover.

**Bile acids (BAs) are synthesized from cholesterol by cholesterol 7α-hydroxylase (CYP7A1) and actively excreted into bile, which drains into the duodenum. In the distal ileum, ~99% of BAs are actively taken up and transported back to the liver via the portal vein. FXR regulates BA synthesis in a dual way: in the liver via short heterodimer partner (SHP) and in distal ileum via FGF19, which is secreted into portal blood and triggers hepatic signaling pathways that downregulate CYP7A1. FXR also regulates hepatic BA uptake and excretion via sodium-dependent taurocholate cotransporting polypeptide (NTCP) and bile salt export pump (BSEP), respectively.**

**Bile acid treatment improves cholestatic and fatty liver disease**

Chenodeoxycholic acid (CDCA) is the most potent naturally occurring FXR agonist and it is metabolized by gut microbiota to ursodeoxycholic acid (UDCA), a weak FXR agonist (4-6). UDCA is at present the first-line therapy of intrahepatic cholestasis of pregnancy and primary biliary cirrhosis (7). However, clinical trials of UDCA in fatty liver disease have been disappointing.

The applicant has participated in several placebo-controlled, double-blind clinical trials of obeticholic acid (OCA), a 6-ethyl derivative of CDCA with 100-times higher affinity to FXR. Phase II and III trials in patients with primary biliary cirrhosis showed that OCA resulted in highly significant decreases of serum alkaline phosphatase, a well-established predictor of outcome (manuscripts submitted). A phase II trial in patients with type 2 diabetes and nonalcoholic fatty liver disease (NAFLD) showed that OCA promoted dose-dependent improvements of insulin sensitivity and reductions in markers of liver inflammation and fibrosis (8). The subsequent NIH-sponsored phase IIb FLINT trial for non-alcoholic steatohepatitis (NASH), was stopped prematurely in January 2014 because the interim analysis revealed that OCA (25 mg/day) resulted in a decrease in the NAFLD Activity Score of at least 2 points with no worsening of fibrosis in liver histology as compared to placebo (p=0.0023). The outcome for the patients treated with OCA was thus better than expected, emphasizing the need to decipher the molecular actions of FXR activation in humans. The optimal approach to achieve this is by analysis of human liver.
Project description

The applicant has recently gained approval from national and international (EU, USA) authorities to perform an investigator-initiated *pharmacodynamic* study with the FXR agonist OCA. Intercept Pharmaceuticals (San Diego, CA, USA) provides OCA 25 mg tablets and matching placebo and arranges drug safety monitoring committee meetings but is otherwise not sponsoring this study.

![Diagram](image_url)

**Fig 2. Synopsis of the OCABSGS study. ClinicalTrials.gov NCT01625026**

The **OCA** in **Bariatric Surgery and Gallstone Surgery** (OCABSGS) study is a double-blind, placebo-controlled, parallel-group trial (Fig. 2). The study population consists of 20 patients with symptomatic, ultrasound-verified gallstone disease awaiting laparoscopic cholecystectomy and 20 morbidly obese patients [body mass index (BMI) ≥35 kg/m²] awaiting Roux-en-Y gastric bypass surgery. Patients of either gender between 20 and 65 years of age will be randomized to placebo or three weeks’ treatment with OCA (25 mg/day) until the day before surgery (day 21).

Exclusion criteria are chronic liver disease other than NAFLD, previous gastric or small bowel surgery, inflammatory bowel disease, uncontrolled diabetes mellitus (fasting blood glucose >6.7 mmol/L), uncontrolled hypothyroidism or hyperthyroidism, other significant endocrine disease, other serious disease, including depressive disorders, and any medication known to induce P450 enzymes such as CYP3A4.

The unique feature of this study is access to biopsy material from the liver, subcutaneous and visceral adipose tissue in all patients and, in addition, the gallbladder and bile from gallstone patients; biopsies will be analyzed by established cutting-edge molecular biology techniques.

*Insulin resistance and glucose tolerance may be affected by FXR activation*

FXR activation may improve glucose tolerance and insulin sensitivity dependent on BMI at inclusion. Thus, we will measure HOMA-IR (homeostatic model assessment of insulin resistance) and perform an oral glucose tolerance test at inclusion and day 21 in all patients, and in bariatric surgery patients also after 6 months.
Lipid profiles and adipokines/incretins may be affected by FXR activation
FXR activation may affect serum lipid profiles dependent on BMI at inclusion, in particular HDL-cholesterol and triglycerides, as well as adipokines (adiponectin, leptin, resistin) and incretins (gastric inhibitory peptide, glucagon-like peptide-1, cholecystokinin). Thus, we will measure these parameters at inclusion and day 21 in all patients, and in bariatric surgery patients also after 6 months.

Power
The sample size of 20 patients in obesity and gallstone surgery groups, respectively, is the same as in the applicant’s previous studies of UDCA where a number of significant changes were found (4-6); as UDCA has much lower affinity for FXR than OCA, we should have sufficient power. To conclude the study, 40 patients must complete the whole trial. This requires full subject compliance to the medication without major missing data. Thus, patients who drop out are replaced on a “per protocol” basis. We will not use “intention to treat” analyses and this will clearly be pointed out in publications. Statistical advice was given by the Swedish Medical Products Agency.

Amount of biopsy materials
At least 1 cm² of subcutaneous and visceral adipose tissue, respectively, will be excised from each patient, both in the bariatric and gallstone surgery groups. The whole gallbladder will be available from gallstone surgery patients. In the applicant’s previous studies, one single liver biopsy was sufficient for all mRNA and protein expression studies as well as for histology for the estimation of the degree of fatty liver disease. In this trial, we have approval to take two biopsies of 30x2 mm, one of which is reserved for the FXR ChIP-seq study (Aim 3).

Specific Aim 1:
To determine how FXR activation affects bile acid metabolism in humans.

Aim 1A: To determine how FXR activation affects hepatobiliary detoxification in humans.

In cholestatic liver disease, bile acids and bilirubin increase within the liver cell to cytotoxic levels. It has been shown that FXR activation is beneficial in the treatment of primary biliary cirrhosis, but the mechanisms involved are not clear. FXR activation may detoxify and enhance excretion of hazardous compounds (Fig. 3) and also improve excretion of cholesterol and phospholipids.

Fig 3. Hepatobiliary bile acid detoxification.

In cholestasis, bile secretion is reduced and bile acids (BAs) increase in the liver cell to toxic levels. The liver aims to protect itself by FXR-mediated downregulation of BA synthesis and uptake (via NTCP) and upregulation of BA export (via BSEP).

Activated FXR cross-talks with other nuclear receptors such as CAR and PXR, which enhance phase 1 (hydroxylation, CYPs), phase 2 (conjugation, UGTs and SULTs) and phase 3 detoxification (excretion, by induction of MRP2 and alternative export pumps, e.g. MRP3, 4).
**Study plan:** Here we will analyze liver tissue, gallbladder bile and serum from the gallstone surgery population in the OCABSGS study. We will compare the effect of treatment with the FXR agonist OCA versus placebo on the following FXR-activated pathways:

1) **Bile acid synthesis.** We will measure hepatic mRNA expression levels of enzymes catalyzing bile acid synthesis (CYP7A1, CYP7B1, CYP8B1, CYP27) using quantitative real-time PCR (qPCR). In addition, we will use ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) to measure the bile acid synthesis marker C4 (7α-hydroxy-4-cholestene-3-one) and to determine comprehensive bile acid profiles in plasma.

2) **Bile acid and bilirubin detoxification.** We will measure hepatic mRNA expression levels of enzymes catalyzing hydroxylation (CYP2B6, CYP3A4), conjugation with glycine or taurine (BACS, BAAT), sulfation (SULT2A1), and glucuronidation (UGT2B/7).

3) **Uptake and excretion of bile acids, bilirubin, cholesterol, and phospholipids.** We will measure hepatic mRNA and protein expression levels of hepatobiliary transporters (for bile acids, BSEP/ABCB11, MRP3/ABCC3, MRP4/ABCC4; for phospholipids, MDR3/ABCB4; for cholesterol, ABCG5/8; for bilirubin and xenobiotics, MRP2/ABCC2). Suitable primers and conditions for qPCR of all genes listed here and above as well as antibodies and conditions for protein expression analyses by western blotting have already been successfully tested.

4) **Biliary lithogenicity.** By reducing bile acid synthesis, FXR activation may result in insufficient levels of biliary bile acids to keep cholesterol in solution, thus enhancing the probability of gallstone formation (lithogenicity). To test this hypothesis, we will measure bile acids, cholesterol and phospholipids in gallbladder bile and calculate the cholesterol saturation index.

**Aim 1B: To determine how FXR activation affects gene expression and secretion of the recently identified biliary FGF19 in humans.**

Ileal human FGF19 and its mouse homolog FGF15 decrease bile acid synthesis from cholesterol via downregulation of hepatic CYP7A1 (Fig. 4). Recently, production of FGF19 in the human biliary tree has been described (9). The role of human biliary FGF19 is unknown (Fig. 4) and cannot be related to findings in mice since mice do not express biliary FGF15.

**Fig 4. Ileal and biliary FGF19 in humans.**

The function of ileal FGF19 in humans seems to reflect that of its murine homolog FGF15, i.e., decreased BA synthesis from cholesterol via downregulation of CYP7A1.

Recently, FGF19 mRNA was found to be abundantly expressed in the human biliary tree, and the gallbladder also contains high levels of FGF19 protein. FGF19 was found to activate signaling cascades in enterobiliary cell lines. Thus it has been hypothesized that gallbladder FGF19 has a signaling function in the biliary tract that differs from its established signaling function in the portal circulation.
**Study plan:** To define whether FXR regulates the expression of biliary FGF19, we will analyze gallbladder tissue and bile from the gallstone surgery population in the OCABS/S study.

1) We will measure **FGF19 mRNA expression levels** in the gallbladder by qPCR. We will also measure mRNA expression of *SLC10A2*, which encodes for the apical sodium-dependent bile salt transporter (ASBT), a prerequisite for bile acids to enter cholangiocytes and ileal enterocytes. In addition, we will measure the expression of FGF19 and ASBT protein in gallbladder sections by staining with commercially available specific anti-FGF19 and anti-ASBT antibodies.

2) We will measure **biliary FGF19 protein** by ELISA in bile and compare with serum FGF19 levels, which are known to be regulated by FXR.

**Preliminary results related to Aim 1**

The applicant has participated in the recently concluded double-blind, placebo-controlled phase II and phase III trials of OCA in patients with primary biliary cirrhosis, without (INT-747/201, manuscript in preparation) and with concomitant UDCA treatment (INT-747/202, INT-747/301: POISE, both submitted). As mentioned in Background, they all showed that OCA dramatically reduced the level of serum alkaline phosphatase, which is the major surrogate marker of cholestasis and a strong predictor of clinical outcome. Enhanced FGF19 production and associated reduced levels of the bile acid precursor C4 and endogenous bile acids confirmed FXR activation.

**Specific Aim 2:**
To determine how FXR activation affects lipid metabolism in humans.

NAFLD can progress to NASH with fibrosis, cirrhosis and liver cancer. Recent clinical trials have shown that FXR agonists can reverse this process. Evidence from animal models indicates that FXR activation results in decreased hepatic lipid synthesis and increased adipose tissue storage of lipids (Fig. 5). In our patients with morbid obesity, we can expect NAFLD and NASH to be prevalent in >90% and 50%, respectively (10). Thus, here we will analyze liver, white adipose tissue and serum from both the bariatric and gallstone surgery populations and thus include biopsies from patients with healthy liver as well as with NAFLD and NASH.

**Fig. 5. FXR regulates lipid synthesis in liver and storage in adipose tissue**

*In the liver, activation of FXR induces SHP, which downregulates sterol regulatory element-binding protein 1 (SREBP1), the major regulator of fatty acid (FA) synthesis. SREBP2 is a major regulator of cholesterol synthesis and may be indirectly regulated by FXR, via decreased activity of CYP7A1, and may thus increase the cholesterol levels in the liver.*

*In white adipose tissue (WAT), FXR activation leads to increased storage capacity for both FA and triglycerides (TG). Fatty acid composition in the liver and WAT is regulated by SCD (stearoyl-CoA desaturase); the FXR regulation of SCD is unknown. The overall effect of FXR activation is decreased TG in the circulation.*
**Study plan:** We will compare the effect of FXR activation in relation to different degrees of histologically defined fatty liver disease on the mRNA expression levels of the following genes:

1) **Hepatic lipid synthesis:** Diglyceride acyltransferases (*DGAT1, DGAT2*), fatty acid elongase 6 (*ELOVL6*), fatty acid synthetase (*FAS*), 3-hydroxy-3-methylglutaryl-CoA reductase (*HMGCR*), *SCD*, *SRBP1C* and *SRBP2*.

2) **Hepatic lipid uptake and export:** Apolipoproteins (*APOA4, APOB*), fatty acid translocase (*CD36*), low-density lipoprotein receptor (*LDLR*), LDLR-related protein (*LRP1*) and microsomal triglyceride transfer protein (*MTTP*).

2) **Hepatic lipolysis:** Adipose triglyceride lipase (*ATGL*), hormone sensitive lipase (*HSL*) and monoglyceride lipase (*MGL*).

3) **Lipid synthesis in white adipose tissue:** *DGAT2, ELOVL6, FAS, HMGCR, SCD, SRBP1C* and *SRBP2* both in subcutaneous and visceral adipose tissue. Suitable primers and conditions for qPCR of all genes listed here and above have been successfully tested.

4) **Lipid composition of white adipose tissue** by high-throughput lipidomics.

5) **Serum lipids** by measuring triglycerides, cholesterol fractions and apolipoproteins.

**Preliminary results related to Aim 2**

The applicant recently concluded an investigator-initiated study in 20 morbidly obese patients who were randomized to UDCA (20 mg/kg/day three weeks before surgery) or no treatment (controls) ([ClinicalTrials.gov NCT01548079](https://clinicaltrials.gov/ct2/show/NCT01548079)). UDCA increased bile acid synthesis and hepatic mRNA expression levels of key regulators of lipid turnover, which was reflected by significantly decreased serum LDL-cholesterol and increased triglycerides. In addition, UDCA altered lipid partitioning in white adipose tissue and liver, counteracting lipotoxicity in morbid obesity (manuscript submitted).

**Specific Aim 3:**

**To use an unbiased approach to define FXR-regulated pathways in human liver and jejunum.**

The potential of FXR ligands as novel therapeutics for various metabolic diseases highlights the importance of obtaining global and complete profiles of FXR regulation, which will provide a unique set of information on expected effects and potential unexpected side effects (e.g. tumor promotion) of FXR ligands.

Several studies using microarray gene profiling of mRNA have generated transcriptional profiles of FXR in diverse metabolic mouse systems with activated or deleted FXR. However, these techniques cannot explore direct FXR binding and the transcriptional results obtained represent a combination of both direct FXR effects and secondary effects that could be either FXR independent or indirectly dependent. To override this drawback and to extend the number of individually characterized FXR target genes and identify putative new direct FXR targets, DNA binding of FXR on a genome-wide scale using chromatin immunoprecipitation and DNA sequencing (ChIP-seq, Fig. 6) has been applied in a few studies conducted in mice ([11, 12]). However, transfer of these results to humans is limited, in particular due to substantial differences in murine and human bile acid profiles and accordingly, affinities to FXR and downstream metabolic signaling. Therefore, we will now for the first time perform ChIP-seq experiments in human tissues.
Fig 6. FXR ChiP-seq

Principal steps of chromatin immunoprecipitation coupled with high-throughput DNA-sequencing (ChIP-seq) experiments.

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.

**Aim 3A: To identify genes in human liver that are potentially activated by FXR.**

**Study plan:** To define the genes in the human liver that can potentially be activated by FXR, we will use ChIP-seq to identify FXR binding sites in the genome of liver biopsies obtained in the OCABSGS study (Fig. 2). Since expertise for ChIP-seq in human liver, particularly in diseased tissue, is very limited, these experiments will be performed in collaboration with Martin Wagner at the Medical University of Graz, Austria, who has developed this technology under the supervision of David Moore at the Baylor College of Medicine, Houston, TX, USA (13, 14).

The goal of this large-scale systems biology effort will be to integrate and interpret the genome-wide FXR DNA binding sites (cistromics) with the global gene expression profile (transcriptomics). For comparative analysis, we will use published and publicly available datasets for the FXR mouse cistrome. Cistromic data will be matched with transcriptomic gene array profiling and metabolomics from our Specific Aims 1 and 2 (i.e. global integromic approach). We predict that we will identify new sets of genes that are transcriptionally activated by FXR only in humans (or *vice versa* only in mice). These human genes might then emerge as novel targets for bile acid-based therapies of cholestatic and fatty liver diseases.

Preliminary studies with mouse liver have shown that it is possible to reduce the material needed for ChIP-seq to needle biopsy amounts. The optimal conditions for immunoprecipitation of human liver obtained from the OCABSGS study have been successfully tested using waste resection material from patients undergoing surgery for liver cancer. We managed to reduce the amount of tissue needed to that usually obtained in single needle biopsies.

**Aim 3B: To identify genes in human jejunum that are potentially activated by FXR.**

FXR is expressed in human jejunum but its regulation is unknown. Based on our preliminary results with human liver, we now will extend our ChIP-seq experiments to human jejunum. Since gut microbiota and bile acids strongly interact with each other in part via FXR (15), we will also study how FXR stimulation impacts on gut microbial ecology and intestinal bile acid composition.
Fig 7. Synopsis of the OCAPUSH study. EudraCT 2014-002313-33

The OCAPUSH study (Fig. 7) has a design that is very similar to that of the OCABSGS study (Fig. 2). The design was chosen to facilitate approval from regulatory agencies and to ensure sufficient statistical power. It has the same inclusion and exclusion criteria for bariatric surgery patients. However, instead of gallstone patients, we study healthy volunteers. Furthermore, instead of sampling tissue during laparoscopic surgery, we take 12 biopsies from the jejunum with a pediatric colonoscope (push enteroscopy) for ChIP-seq experiments including gene expression array profiling. Before and after treatment with OCA or placebo, we will collect feces for gut microbiota and bile acid composition analyses. Enteroscopy and feces sampling are repeated after 6 months in bariatric surgery patients. Blood samples are analyzed for routine biochemistry and for bile acids to verify compliance.

Significance
Our studies aim to characterize FXR activation pathways in human hepatobiliary and adipose tissues, to define global DNA binding sites of FXR in human liver and jejunum, and to study the effect of FXR activation on gut microbiota. Our studies will be the first to explore the global effects of a ligand-activated transcription factor in gallstone patients (who are otherwise healthy) and patients with morbid obesity, and therefore will be regarded as landmark studies. Ultimately, our studies aim to develop new treatment options for human cholestatic liver diseases (by improving detoxification) and fatty liver diseases (by reversal of NAFLD progression).

Local cooperation
Patients will undergo surgery at Sahlgrenska University Hospital, Gothenburg. The applicant has established close collaboration with gastrointestinal surgeons for a number of bariatric surgery projects. All the equipment required for the project plan is available within the infrastructure of the Wallenberg Laboratory, University of Gothenburg. Bile acid analysis and lipidomics by UPLC-MS/MS and gut microbiota composition analyses by pyrosequencing of the bacterial 16S rRNA gene will be performed in close collaboration with Professors Jan Borén and Fredrik Bäckhed, leading experts in lipidomics and gut microbiota, respectively.

Ethical considerations
All studies are conducted according to the Helsinki Declaration following approval from the regional Ethic’s Committees and the European and Swedish Drug Agencies. The handling of personal data is carried out according to their and the Swedish legal instructions.
International cooperation

FXR ChIP-seq experiments will be performed in collaboration with David Moore and Martin Wagner, as described. The applicant has ongoing close collaboration with Prof. Peter Fickert’s group and Prof. Michael Trauner’s group at the Medical Universities of Graz and Vienna, respectively.

Further international cooperation of the applicant in related fields
- with Prof. Catherine Williamson’s group at King’s College in London: genetics, molecular pathogenesis and treatment of intrahepatic cholestasis of pregnancy.
- with Prof. Frank Lammert’s group at the Dept. of Internal Medicine II, Saarland University Hospital, Homburg, Germany: genetics of liver disease and gallstone susceptibility.

References