Summary
Introduction
The liver is the second largest organ of the body and plays an important role in the metabolism of carbohydrates, fats, and proteins. It is the main producer of plasma proteins, like albumin and coagulation factors. It is also the organ involved in cleaning the body from toxic and waste products, eliminating a wide variety of these compounds predominantly via bile and feces. Bile is not only an escape route, but is also necessary for efficient digestion and absorption of dietary fats and fat-soluble vitamins. The driving force for bile formation is the hepatic synthesis and secretion of bile salts. After delivery into the intestine, bile salts are efficiently reabsorbed and returned to the liver. This shuttle of bile salts between the liver and intestine is called the enterohepatic circulation of bile salts. Every cycle, a small portion (~ 5%) of bile salts is lost via feces. De novo bile salt synthesis from cholesterol takes place in the liver parenchymal cells (hepatocytes) and substitutes for the fecal loss. Improper functioning of the enterohepatic circulation may lead to cholestasis, i.e. high serum levels of bile salts, and liver disease.

The hepatocytes form a physical barrier between blood and bile. Bile salts and most other compounds cannot pass this barrier without the help of specialized transport systems. The hepatocyte houses various specific transport proteins located at the cell membrane, mediating the vectorial transport of compounds from blood to bile. For example, reabsorbed bile salts are taken up from the blood by members of the “solute carriers” superfamily. For secretion into bile, a steep bile salt concentration gradient must be overcome. This transport requires ATP hydrolysis and is accomplished by the Bile Salt Export Pump (also called ABCB11).

Classically, bile salts are considered a detergent that is required to keep hydrophobic compounds, such as cholesterol or vitamins, in solution to aid in their excretion or absorption. Recent research has, however, shown that bile salts are also important signaling molecules. For instance, bile salts have been shown to regulate their own synthesis rate, as well as the transporters active in the enterohepatic circulation. They can bind to, and activate the transcription factor Farnesoid X Receptor (FXR), which in its turn regulates transcription of the genes that produce rate-limiting enzymes in the bile salt biosynthetic pathway, as well as the bile salt transporters. The Bile Salt Export Pump, bile salts and FXR are the central topics of this thesis. In chapter 1, an overview is given of the current knowledge of hepatic transport proteins, their roles in inherited and acquired liver disease, and their function and regulation under normal and pathophysiological conditions.

The D482G mutation results in a functional but unstable BSEP protein
The inherited liver disease, Progressive Familial Intrahepatic Cholestasis type 2 (PFIC2), is characterized by jaundice, itching, low biliary bile salt level, high serum bile salts level, but normal serum γ-glutamyltransferase (γ-GT) levels. These features imply that defective transport of bile salts from the hepatocyte into bile is the basis for this type of cholestasis. It has been shown that PFIC2 patients have mutations in the
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*ABCB11* gene, which encodes for the hepatocanaliculare bile salt export pump (BSEP). One of these PFIC2-causing mutations is studied in detail in chapter 2. The mutant protein harbors a glycine (G) residue at amino acid position 482 in stead of an aspartate (D), which is present at this position in the normal (wild type) protein. The rest of the 1,320 amino acids of both proteins are identical. We examined what the effect this specific mutation (D482G) is on bile salt transport activity, canalicular sorting and protein expression of BSEP. To study the effect on protein activity we expressed the murine Bsep protein in insect cells and performed ATPase and transport assays. Surprisingly, the mutation, which is located in one of the two ATP-binding domains, did not abolish the ATPase nor the transport of the bile salt, taurocholate. To study the effect on protein sorting and expression, we expressed murine Bsep tagged with the Green Fluorescent Protein (GFP) in human hepatoma cells (HepG2). We observed that at physiological temperatures (37°C) the sorting as well as the protein expression were strongly effected. The GFP-Bsep\[^{D482G}\] was not fully glycosylated and significant amounts of the protein were detected in the cytoplasm. In addition, the amount of GFP-Bsep\[^{D482G}\] protein present was significantly lower compared to GFP-Bsep\[^{WT}\] whereas the corresponding mRNA levels were 4 to 5-fold higher for GFP-Bsep\[^{D482G}\] in the obtained stably transfected HepG2 cell lines. Lowering the culture temperature to 30°C increased the GFP-Bsep\[^{D482G}\] protein level to 4 to 5-fold compared to GFP-Bsep\[^{WT}\], and resulted in efficiently targeted and fully glycosylated protein. These data demonstrated that the D482G mutation results in a still functional, but highly unstable BSEP protein. Therefore, PFIC2 patients, carrying this specific mutation, may benefit from therapies aimed at stabilization of the protein.

**Bile salts and FXR regulate BSEP transcription**

In chapter 3 the relationship between bile salts, FXR and the expression of the human *ABCB11* gene (encoding BSEP) is described. HepG2 cells were incubated with the bile salt chenodeoxycholic acid (CDCA), which resulted in a 2-3 fold increased expression of *ABCB11*. The promoters of human and mouse *ABCB11* were analyzed for regulatory elements and a candidate responsive element for the transcription factor FXR was found. We cloned the promoter region of the human *ABCB11* gene containing this element and demonstrated that bile salts and FXR positively regulate *ABCB11* promoter activity, leading to increased *ABCB11* mRNA levels. Mutation of the FXR responsive element confirmed that this region in the *ABCB11* promoter is responsible for bile salt-induced transcription of the *ABCB11* gene. In addition, we showed that bile salts were unable to increase *ABCB11* transcription, when the amount of endogenous FXR was reduced by RNA interference. We conclude that *ABCB11* gene transcription is positively regulated by bile salts through activation of FXR. The level of *ABCB11* transcription activation is dependent of the cellular amount of this transcription factor.

**Vitamin A is important for BSEP expression**

Bile salts facilitate the uptake of fat-soluble vitamins, including vitamin A, D, E and K. The liver is a rich source of vitamin A, where it is stored in the hepatic stellate cells.
Vitamin A may be converted into 9-cis retinoic acid (9cRA) which is activating ligand for the transcription factor retinoid X receptor (RXR). RXR is another member of the superfamily of nuclear hormone receptors (NHRs) and functions as a key-regulator of gene transcription. It is a central dimerization partner for several NHRs, including FXR. 9cRA-activated RXR is considered to be a permissive partner of FXR, since both NHRs and both ligands are required for maximal induction of transcription of target genes, such as I-BABP and PLTP. In chapter 4 we investigate whether this is also true for ABCB11 gene transcription. Surprisingly, 9cRA reduced the bile salt-induced ABCB11 mRNA expression in human hepatoma cells almost to control level. This effect of 9cRA is exerted via a decrease in binding of the FXR/RXR heterodimer to the FXR responsive element of the ABCB11 promoter. To determine whether the inhibition of ABCB11 expression by 9cRA plays a role in vivo, we generated vitamin A-deficient (VAD) mice and determine the effect of bile salt feeding compared to control (normal vitamin A) mice. In VAD mice, Bsep (Abcb11) mRNA and protein expression was significantly more increased by bile salts (cholate) compare to bile salt-fed control mice. We conclude that the vitamin A derivative 9cRA indeed plays an active role in the bile salt-dependent regulation of human and mouse ABCB11/Abcb11 transcription. The physiological rational behind this mechanism may be that at low vitamin A levels, bile salt secretion is increased to aid in the intestinal absorption of this vitamin. The treatment of cholestatic patients with vitamin A-containing supplements, however, may need further exploration, since this may result in extra inhibition of BSEP expression, whereas the therapeutic goal would be the opposite.

**FXR is involved in transcription of non-bile salt related genes**

In the past few years, several genes have been shown to be transcriptionally regulated by FXR and bile salts. Most of them are involved in bile salt, cholesterol, and lipoprotein metabolism. FXR-target genes are however not restricted to these metabolic pathways, exemplified by the identification of Kininogen as a FXR-target. To get insight in genome-wide effects of bile salt-activated FXR, we performed a transcriptome analysis. This search for novel FXR and bile salt-regulated genes is described in chapter 5. We generated a human hepatoma cell line, which stably overexpresses rat Fxr (HepG2-rFxr). These and the native cells were treated with the bile salt, chenodeoxycholic acid and mRNA expression of over 13,000 human genes were analyzed using microarrays. A novel candidate FXR-target gene was identified from this analysis, namely fibrinogen beta. Expression of this gene was induced in HepG2 cells by CDCA alone and superinduced in CDCA-treated HepG2-rFxr cells. Together with fibrinogen alpha and gamma, this protein forms fibrinogen, a plasma protein. It is synthesized in the liver and is involved in blood coagulation and possibly in the formation of liver fibrosis. Screening the conserved promoter regions of human, rat and murine fibrinogen beta for possible regulatory elements revealed a candidate FXR responsive element. Additional studies are necessary to firmly establish that this element is indeed responsible for the FXR and bile salt-dependent regulation of fibrinogen beta gene transcription.
Conclusions and perspectives

During the past decade, important progress has been made in our understanding of the pathophysiology of cholestasis. Inherited disorders have been explained at the molecular level and were shown to be the result of mutations in enzymes involved in bile salt biosynthesis or transmembrane transporters involved in bile formation. Acquired cholestasis, for instance due to inflammation, is linked to disregulation of these proteins. The challenge of future research is to use this knowledge to develop successful therapies for cholestatic patients. The studies described in this thesis initiate such studies. We showed that a mutation in BSEP may cause PFIC-2 even though the protein product is a normally active bile salt export pump. The cause of the disease is due to a specific mutation in the BSEP gene that strongly reduces the BSEP protein level, most likely due to increased protein degradation. This phenomenon has also been described for several other inherited diseases. An important focus of research is therefore to define conditions or drug therapies that will maintain or increase the protein level of the mutant, but active protein in these patients.

Novel targets to treat cholestasis are the transcription factors that regulate the levels of enzymes and transporters involved in the synthesis and enterohepatic circulation of bile salts. RXR and FXR are key players in this process. Endogenous and synthetic ligands for these transcription factors have been identified. Recent research, however, shows that the activation of FXR-target genes is ligand-dependent. In other words, a specific target gene may be activated by the endogenous ligand, CDCA, but not by the synthetic ligand GW4064. In addition, our own research shows that the ligand for RXR, 9-cis retinoic acid, in combination with bile salt-activated FXR, may simultaneously stimulate and inhibit expression of a subset of FXR-target genes. Therapies to treat cholestatic disease aimed at modulating the activity of these transcription factors therefore requires an in depth study of the effect of the (combination of the) individual ligands on human gene transcription.

In the meantime, it should be noted that in a significant number of PFIC patients the genetic defect still has not been elucidated. Therefore, novel genes/proteins that are essential for bile salt homeostasis may be identified in the near future.

Altogether, many challenges await us still to further understand the (molecular) causes of cholestasis and to develop successful therapies for this disease.