CHAPTER 7

SUMMARY AND GENERAL DISCUSSION
In this thesis, we studied clinical and basic aspects of the hepatic and gastrointestinal involvement in cystic fibrosis. We focused on the physiological and pathological role of bile salts and the enterohepatic circulation in Cystic fibrosis conditions. From our clinical study, we learned that, based on the involvement of gamma-GT (GGT), cirrhotic Cystic fibrosis liver disease (CCFLD) is primarily a biliary disease since GGT elevation is a predictor for the development of CCFLD. From our experiments in CF mice models we conclude that cystic fibrosis related liver disease is not related to biliary bile salt cytotoxicity. Nevertheless, in different CF mice models we could establish a CFTR dependent and bile salt specific, biliary phenotype. We provided proof that this biliary phenotype can, partially, be corrected by the hydrophilic bile salt UDCA. Finally, we demonstrated that the CF phenotype in mice includes alterations in the metabolism and enterohepatic circulation of bile salts. These changes are due to interaction between the CFTR protein and the bacterial flora of the gut. Our findings provide new experimental evidence for the significant role of CFTR in the equilibrium of the enterohepatic circulation. Impaired CFTR function, like in Cystic fibrosis disease conditions, can therefore give rise to negative alterations in the enterohepatic circulation and bile salt metabolism. Our results provide new understanding in the gastro-intestinal and hepatic role of CFTR that provides new insights for development of future treatment options in Cystic fibrosis.

CCFLD is a life-threatening, hepatic complication of CF (1, 2). The clinical presentation of CCFLD is often characterized by severe portal hypertension and gastrointestinal variceal bleeding (3). In patients with end stage CCFLD liver transplantation might be indicated (4, 5). Therefore patients at risk for or still in early stages of the disease could benefit from preventive treatment for CCFLD. However, to date no predictors to identify patients at risk for CCFLD were available. In chapter 2, we report that, in the time period 2 years prior to the diagnosis of CCFLD, an increase of GGT, even if the level remains within the normal range, indicates a significantly increased risk for the development CCFLD. This finding provides new opportunities for identifying high risk patient for CCFLD and more targeted preventive or preemptive therapy strategies like UDCA.

Within a few years after the CFTR gene was identified in humans, the first genetically engineered CF mouse models were developed (6-9). These models have been of great importance for increasing the understanding Cftr physiology and pathology in various organ systems. However, the phenotype of the developed CF mice models differs in several aspects from the human CF disease equivalent. For example lung disease, the most prominent CF disease feature in CF patients, is barely found in mice models (10). On the other hand, CF mouse models exhibit a wide range of gastrointestinal phenotypes that closely resemble the human CF condition (11). In chapter 3, we reviewed current knowledge on the hepatic and
gastrointestinal CF phenotypes in mouse models and in human patients. We focused on chronic intestinal fat malabsorption, present in many CF patients. We indentified useful similarities in the phenotype between mice and man. For example, the comparable phenotype of increased fecal bile salt loss and alterations in bile salt composition in both CF mice and CF patients (12). Also, the intestinal mucosal abnormalities like small intestinal bacterial overgrowth (SIBO), intestinal mucus accumulation, intestinal inflammation and increased intestinal permeability have corresponding phenotypes in CF humans and mice (13, 14). From these findings we concluded that the CF mouse, despite differences in phenotypic expression among different genetic mice backgrounds, can well serve as useful experimental model for gastrointestinal CF phenomena. Based on this analysis, our studies in CF mice models could provide an educated basis for future translational research.

We investigated to what extent CF mouse models can provide pathophysiological and mechanistic understanding in the development of CF related hepatic disease (chapter 4-6). Our main focus was to determine whether biliary bile salt cytotoxicity is involved in the development of CFLD like disease in CF mice. To answer this question we analyzed the liver involvement in various CF mouse models. In the Cftr\(^{-/-}\)tm1Unc CF mice, a model characterized by spontaneous development of CFLD like disease, we could not establish a contribution of biliary bile salt cytotoxicity to its hepatic histo-pathologic phenotype. On the other hand, in other CF mouse models we did find a significant increase in the hydrophobic profile of the biliary bile. However, even in these mice models, this increased biliary cytotoxicity could not be related to CFLD like disease. Finally, we could not prove that CF mice, when challenged with and prolonged hydrophobic bile salt exposure, were more susceptible to develop CFLD like disease. Based on these combined results we conclude that the development of CFLD like disease, at least in mice, is not related to Cft related changes in biliary cytotoxicity. We postulate that this conclusion can be extrapolated to the development of liver involvement in humans with CF.

Although we could not establish a specific histo-pathological phenotype in mice that was corresponding with CCFLD in humans, we did find a unique hepatic and biliary phenotype. (chapter 4). Cftr\(^{-/-}\) mice, when exposed to the hydrophobic bile salt cholate, over an extended period of time, display an increased hydrophobicity of bile and a decreased capacity to increase bile flow. Cftr\(^{-/-}\) mice are apparently less able to dilute the bile during chronic cholate administration. This is clearly illustrated by a significantly increased biliary BS concentration but unaffected BS secretion rates. We observed a distinct Cft dependent liver growth and proliferation response, on prolonged hydrophobic BS exposure: in wild type mice the liver weight increased by ~50% upon a chronic BS administration, whereas liver weight was stable in different CF mouse models. These observations point to a, hitherto unrecognized, role of CFTR in the hepatic adaptive response to prolonged hydrophobic BS exposure. We speculate that, this impaired regenerative capability of the CF phenotype, could be related to an increased hepatic vulnerability, for example in cholestatic conditions. This deficiency in regenerative hepatic capacity may play a role in liver disease development.
Our results in $Cfr^{-/-\text{tm1Unc}}$ mice (chapter 5) demonstrated Cftr dependent differences in intestinal bile salt metabolism. We found, Cftr specific, alterations in the bacterial formation of the secondary bile salts and accordingly in the fecal and biliary bile salt composition. As a result we found that in $Cfr^{-/-\text{tm1Unc}}$ mice, exclusively fed a liquid diet, the biliary bile salt profile was considerably more hydrophilic and the bile flow was increased compared to wild type mice. The increase in bile production, in the $Cfr^{-/-\text{tm1Unc}}$, could be explained by increased concentrations of ursodeoxycholate (UDCA). The contribution of UCA to the bile salt pool is usually relatively small, due to its rapid biotransformation into DCA by the intestinal bacterial flora. Therefore, the reported increased UCA enrichment of the $Cfr^{-/-\text{tm1Unc}}$ mice can only be explained by, specific Cftr related, changes in the functioning of intestinal bacterial flora. UCA is a very hydrophilic and highly choleretic bile salt. Therefore, UCA enrichment in the $Cfr^{-/-\text{tm1Unc}}$ mice, results in a significantly increased bile salt dependent bile flow and in a significantly decreased biliary bile hydrophobicity. Our results provide a clear example of the way in which alterations in the interaction between intestinal flora and bile salt formation can significantly affect the enterohepatic circulation of bile salt. In CF conditions these changes, in bile salt metabolism, could be related to its phenotypical characteristics or even result in induction or prevention of liver disease development. Several intestinal mucosal abnormalities are described in CF patients and CF mice models that could influence the composition of the bacterial micro flora. These abnormalities include accumulation of viscous and sticky mucus, small intestinal bacterial overgrowth, increased intestinal permeability and inflammation of the small intestine (15-24).

The importance of the intestinal bacterial flora for the CF gastrointestinal phenotype was also illustrated by other experiments. We treated control and CF mice with either the hydrophilic bile salt UDCA or the hydrophobic bile salt cholate (CA). We found that control mice, on CA diet, displayed a slightly more hydrophobic bile composition compared to the $Cfr^{-/-}$ mice. This effect, in wild type mice, can, in its entirety, be explained by the presence of the secondary bile salt deoxycholate (DCA), not found in bile of $Cfr^{-/-}$ mice. DCA is produced exclusively, via biotransformation, by the intestinal micro flora. Recently, we and others have reported that intestinal micro flora and the intestinal mucosal permeability are changed in CF (25-28). Our results emphasize the critical role of the intestinal micro flora, for CF conditions, in modifying the enterohepatic circulation of bile salts. We believe that, Cftr specific, genotypic changes in intestinal flora are primary responsible for a majority of the, CF related, alterations in bile salt composition and production. These observations open the perspective for targeted manipulation of the intestinal micro flora in CF mice and patients.

As stated, the Cftr dependent changes in bile salt composition and metabolism can have profound effects on the enterohepatic circulation in CF conditions. Yet, to some extent, the observed changes in bile salt formation can also result from, Cftr related, alterations in the enterohepatic circulation itself. The reported difference in bacterial BS biotransformation, in particular the conversion CA to DCA, can indirectly affect hepatic bile salt synthesis. For example DCA is a strong activating ligand of the farnesoid X receptor (FXR) (29). This nuclear
receptor is highly expressed in the liver and ileum (30). In the liver, an important function of FXR activation is the suppression of bile acid synthesis from cholesterol (31). Activation of intestinal FXR induces the expression of FGF19 (fibroblast growth factor 19, equivalent to Fgf15 in rodents) by the enterocytes (32). FGF19 is excreted and transported via the portal circulation to the liver. Here it can induce the cascade of bile salt synthesis from cholesterol.

Additionally, recent publications describe the central role of FGF15/19 in hepatocyte proliferation (33, 34). Normally FGF15/19 is only expressed in the terminal ileum and not in normal liver. However, hepatic expression of FGF15/19 can occur in cholestatic conditions and is associated with hepatic adaptation aimed at protecting against BS injury (35).

In our chronic hydrophobic bile salt (cholate) exposure model, expression of Fgf15 could potentially be induced both in the intestine and in the liver. The increased biliary fraction of DCA, found in the controls after chronic CA diet, can induce Fgf15 expression via FXR in the enterocytes or directly by inducing Fgf15 expression in the liver. In either situation, induction of Fgf15 could induce liver proliferation and liver growth (36). There could also be a primary role for the intestinal flora. A recent study in germ free mice showed that gut microbiota do regulate bile salt metabolism by influencing naturally occurring FXR antagonists like tauro-beta-muricholic acid (37). On the other hand cholate induced liver growth in mice, similar to our current findings in controls, was described previously. Huang et al. reported that CA feeding (0.2%) for 5 days stimulated normal liver growth and increased the relative liver weight by approximately 30% in wild type mice (38). These investigators also observed that CA supplementation enhanced liver regeneration after partial hepatectomy. They concluded that normal liver regeneration depends on BS activation via nuclear receptor-dependent signaling pathways, in particular FXR.

The potential role of FXR is underlined by our finding that liver growth is not observed during chronic UDCA exposure. UDCA does not lead to increased presence of strong FXR ligands like DCA. Current findings offer opportunities for future research. To differentiate the flora effect from the bile salt effect in regulating FXR it would be of value to develop germ free CF mice to repeat the experiments. Furthermore it would be of great interest to perform a cholate diet experiment in FXR intestinal and liver tissue specific knockout mice models. These models provide the opportunity to separate the intestinal FXR stimulation from the hepatic FXR effects on liver growth.

UDCA as a preventive treatment option for cirrhotic CFLD remains controversial (39). An important factor in this argument is the lack of support by basic experimental evidence. Additionally there or no well-designed clinical study showing unequivocally a long term clinical benefit on relevant clinical outcome (40). A major physiological effect of UDCA is its capacity to increase bile flow. This property has supported the therapeutic use of UDCA in a variety of cholangiopathies, including CFLD (41). In vitro and ex vivo studies indicated, however that the stimulatory role of UDCA on cholangiocyte secretion depends on the presence of CFTR (42, 43). In the present thesis (chapter 6), we studied the effects of UDCA in
vivo in CF mice. We demonstrated that UDCA, in vivo, either during acute or chronic administration, induced a significant, Cfr independent, increase of bile flow in mice. Therefore, our results suggest that a positive choleretic effect of UDCA can also be expected in human CF conditions. Furthermore, UDCA treatment reduced the relative hydrophobic biliary bile salt composition in Cfr−/− mice. In bile UDCA replaced cholate and reduced the quantitative total pool size of cholate. These properties could contribute to the assumed beneficial effects of UDCA in CFLD. However, we would like to underline that our results do not provide evidence for preventive effects of UDCA on the development of liver fibrosis.

We feel that the results of our studies lead to an improved understanding of the development of Cystic fibrosis liver disease. Our results provide new opportunities for future clinical and basic research. This is especially relevant given the current groundbreaking therapeutic developments in CF. These new therapies are target to directly correct the basic genetic CFTR defect (44). To explore these new therapeutic options further, early intervention in young children, who have not yet developed severe pulmonary disease, would be most favorable (45). Classic lung function measurements, the traditional clinical endpoint in CF clinical research, may not be the most indicative in young patients still without a measurable decrease pulmonary function. To perform future therapeutic studies new clinical endpoints and intermediate endpoints for Cystic fibrosis disease need to be developed (46).

The results of his thesis offer additional options for the development of new gastro-intestinal and hepatic outcome parameters for future clinical trials (47). For example measuring differences in fecal bile secretion or changes in bile salt metabolism might have high potential as clinical outcome variables. Total bile salt secretions, as well as differences in fecal bile salt formation, are relatively easily obtainable results and do not require an invasive approach. These measurements are feasible to achieve even in young children. Another example that open new research opportunities could be the ever growing possible study methods to identify and quantify the intestinal bacterial micro flora. The CFTR specific changes in the interaction of the intestinal bacterial flora with intestinal bile salt metabolism and absorption as reported in this thesis can serve as the basis for further research in this direction.

Moreover, our results proved new opportunities for treatment options for CCFLD. Based on our results, it appears justifiable to evaluate the prognostic value of GGT to identify patients at risk for severe cirrhotic CFLD in a large prospective cohort study. This study can than provide the necessary validation and support for clinical use and interventional therapeutic strategies. Simultaneously, therapeutic interventional and observational (registry) studies could provide additional candidate parameters on gastrointestinal function. These could serve as new parameters to evaluate either the severity of the gastrointestinal phenotype in general or for the development of CCFLD in particular.

In addition to clinical, patient-oriented studies, we feel that continuation of the basic studies in relevant model systems is essential. Concerning the search for the pathogenesis of CCFLD we believe the focus should switch from bile salt cytotoxicity to the liver-intestine-micro flora
interaction. The results presented in this thesis provide strong support for the concept that CFTR dependent changes in the intestinal bacterial flora composition are strongly related to intestinal inflammation and intestinal bile salt metabolism. Focus on pathophysiology of small intestinal bacterial overgrowth (SIBO) and of the intestinal flora in general in CF seems indicated. The role of intestinal paneth cells, secreting anti-bacterial proteins into the intestinal lumen, may also be relevant to delineate in CF conditions.

Another line of logical continuation of our current research involves the hepatic-intestinal axis and the enterohepatic circulation of bile salt in CF conditions. Our results show a distinct CFTR dependent phenotype of bile salt synthesis, intestinal bile salt biotransformation and fecal bile salt loss. This interaction of CFTR function with the enterohepatic circulation of bile salts including the interaction with the intestinal bacterial flora needs further exploration.

Finally there are new developments in possibilities to study different CF animal models. Recently CF ferrets and CF pigs were generated and characterized in experimental studies (48, 49). The firsts result of these studies show that, in particular the CF pigs, demonstrate a hepatic CFLD like phenotype (50). These new models provide opportunities to further study CFLD development and treatment.

In conclusion we believe that our research provides key additional expertise in the field of gastro-intestinal and hepatic consequence of Cystic fibrosis disease. We feel that our present results, in combination with the current promising developments in CF drug development, the expansion of global patient registries and the development of new experimental animal models, add to improve the understanding and the treatment for CF disease in the near future.


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