CHAPTER 1

GENERAL INTRODUCTION
1) THE CLINICAL PERSPECTIVE

CYSTIC FIBROSIS DISEASE

Cystic Fibrosis (CF) is a severe, lifespan limiting, disease. Cystic fibrosis is one of the most frequent autosomal inherited diseases in the world. The incidences differ globally according to regional genetic variations (1). In the Netherlands around 1:1500-6000 inhabitants suffer from CF (2, 3). The disease is usually already manifest at birth and progresses with age. To date the median survival of CF patients is around 40 years (4). Most patients die from end stage lung disease. Although severe lung disease dominates the clinical picture, CF is a multi-organ disease. In particular diseases of intestine, pancreas and liver can be serious and potentially life threatening (figure 1.).

In 1989, the CF disease causing gene was identified and named “cystic fibrosis transport regulator” (CFTR). The CFTR gene encodes for the CFTR protein (5, 6). The CFTR gene is localized on chromosome 7(7). Cystic fibrosis can be caused by a variety of mutations in the CFTR gene. To date, over 1900 different mutations in the CFTR gene, are identified (8). The CFTR gene mutation can be divided into separate groups. A mutation in one copy of the CFTR gene that always causes CF, as long as it is paired with another CF-causing mutation in the other copy of the CFTR gene, is a CF-causing mutation. A mutation in one copy of the CFTR gene that does NOT cause CF, even when it is paired with a CF-causing mutation in the other copy of the CFTR gene, is a non CF-causing mutation. A mutation that may cause CF, when paired with CF-causing mutation in the other copy of the CFTR gene, is a mutation of varying clinical consequence. A mutation for which we do not have enough information to determine whether or not it falls into the other three categories is a mutation of unknown significance (9).

The disease causing mutations can be divided into 5 different mutations classes according to the type of malfunction of the CFTR protein (11). Based on this classification, difference in clinical disease presentation and severity can be recognized (figure 2). The various mutation classes offer different potential therapeutic targets for the treatment of Cystic fibrosis (12).

CF can develop if a person carries a disease causing mutations in each CFTR allele. The most common CFTR mutation in humans is the 508del gene variation (13). In the 508del mutation one nucleotide T, on the 508 position of the gene, is replaced by a G nucleotide. 508del is a, so called, class 2 mutation. These class 2 mutations cause an almost complete failure (less than 5% of normal) of the CFTR function, leading to the typical severe phenotype with
progressive pulmonary disease, complete exocrine pancreatic insufficiency, diabetes and cirrhosis (14).

CFTR is a cell membrane protein localized in various cell and in particularly in all epithelial tissues. The protein can, for instance, be found in the alveolar cells of the airways, the ductular cells of the pancreas, the enterocytes of the intestine, the cholangiocytes of the bile ducts but also in the sweat glands of the skin (15). Mutations in the CFTR gene are directly responsible for the symptomatology and disease development in these organs.

**Manifestations of Cystic fibrosis**

- **Liver**
  - Hepatic steatosis
  - Portal hypertension
- **Gallbladder**
  - Biliary cirrhosis
  - Neonatal cholestasis
  - Choledolithiasis
- **Intestine**
  - Meconium ileus
  - Meconium peritonitis
  - Rectal prolaps
  - Intussusceptions
  - Volvulus
  - Fibrosing colonopathy
  - Appendicitis
  - Intestinal atresia
  - Distal intestinal obstruction syndrome
  - Inguinal hernia
- **Bone**
  - Hypertrophic osteoarthropathy (Clubbing)
  - Arthritis
  - Osteoporosis
- **Nose and sinuses**
  - Nasal polyps
  - Sinusitis
- **Lungs**
  - Bronchiectasis
  - Bronchitis
  - Bronchiolitis
  - Pneumonia
  - Atelectasis
  - Hemoptysis
  - Pneumothorax
  - Reactive airway disease
  - Cor pulmonale
  - Respiratory failure
  - Mucoid impaction of the bronchi
  - Allergic bronchopulmonary aspergillosis
- **Heart**
  - Right ventricular hypertrophy
  - Pulmonary artery dilatation
- **Spleen**
  - Hypersplenism
- **Stomach**
  - GERD
- **Pancreas**
  - Exocrine pancreatic insufficiency
  - Pancreatitis
  - Insulin deficiency
  - Symptomatic hypoglycemia
  - Diabetes
- **Reproductive**
  - Infertility (aspermia, absence of vas deference)
  - Amenorrhea
  - Delayed puberty

**Figure 1.** Cystic fibrosis is a multi-organ disease. Because so many bodily functions rely on normal water flow, a disruption in water flow can cause a number of devastating effects, as shown in the "Manifestations of Cystic Fibrosis" image above (illustration adapted from: Wikimedia Commons, 2011) (10). In particular diseases of intestine, pancreas and liver can be serious and potentially life threatening.

In epithelial cells, CFTR functions as a chloride ion (Cl⁻), trans-membrane transporter protein (17). CFTR actively pumps Cl⁻ across the cellular membrane by concomitant with ATP hydrolysis. The Cl⁻ transport serves various roles in the different epithelia. In the lungs, the Cl⁻ transport induces a Cl⁻ ion gradient across the cell membrane (18). Based on this gradient,
bicarbonate (HCO₃⁻) is passively exchanged against Cl⁻ and transported out of the alveolar cell in to alveolar lumen. Here HCO₃⁻ here serves a crucial role in maintaining viscosity and fluidity the alveolar fluid layer. Disturbance of this HCO₃⁻ transport function causes a thick, highly viscous fluid layer in the alveoli. The sticky secretion impairs lung mucus clearance and thereby an increased susceptibility for bacterial pulmonary infections in CF disease. CFTR functions as a Cl⁻ channel in different epithelia (19). However, the contribution of CFTR channel dysfunction, in disease development in different organs, for example, intestine and liver, is not clear.

**Molecular consequences of CFTR mutations**

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<tr>
<th>Class</th>
<th>Description</th>
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<tr>
<td>I</td>
<td>No synthesis</td>
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<td>II</td>
<td>Block in processing</td>
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<tr>
<td>III</td>
<td>Block in regulation</td>
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<tr>
<td>IV</td>
<td>Altered conductance</td>
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<td>V</td>
<td>Reduced synthesis</td>
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**Figure 2.** Molecular consequences of CFTR mutations. a, CFTR correctly positioned at the apical membrane of an epithelial cell, functioning as a chloride channel. b, Class I. No CFTR messenger ribonucleic acid or no CFTR protein formed (e.g., nonsense, frameshift, or splice site mutation). c, Class II. Trafficking defect. CFTR messenger ribonucleic acid formed, but protein fails to traffic to cell membrane. d, Class III. Regulation defect. CFTR reaches the cell membrane but fails to respond to cAMP stimulation. e, Class IV. Channel defect. CFTR functions as altered chloride channel. f, Class V. Synthesis defect. Reduced synthesis or defective processing of normal CFTR. Chloride channel properties are normal (Illustration from The Journal of Pediatrics, 127, 5, 1995) (16)
GASTRO-INTESTINAL AND HEPATIC DISEASE IN CYSTIC FIBROSIS

CF presents with clinical symptomatology in various abdominal organs like pancreas, intestine and liver (20). The clinical phenotype and contribution of different organ systems varies in CF patients (21). In cystic fibrosis the clinical, organ specific, presentation and phenotypical penetration depend on gene modifiers. Gene modifiers are genetic or environmental factors that determine the actual organ specific phenotype. Different disease presentations in CF are in greater or lesser extent dependent on the influence of gene modifiers (figure 3). The most important and relevant gastro-intestinal disease presentations in CF are exocrine pancreatic insufficiency and cirrhosis (22, 23). However, also specific intestinal diseases, like neonatal meconium ileus, the distal intestinal obstruction syndrome (DIOS) and intestinal fat- and bile salt malabsorption, are frequently found CF patients (24-26).


EXOCRINE PANCREATIC INSUFFICIENCY AND FAT MALABSORPTION IN CYSTIC FIBROSIS

Intestinal fat malabsorption is a serious clinical manifestation of CF. The decreased absorption of dietary fats impairs the development and maintenance of a healthy nutritional status in particular in growing children (27). In CF nutritional status relates to the prognosis and
The leading cause of the intestinal fat malabsorption in CF is exocrine pancreatic insufficiency (PI). CF causes fibrotic degeneration of the acinar tissue of the pancreas secondary to destruction of the ductular structures due to loss of CFTR function. The fibrotic pancreas is no longer able to excrete pancreatic enzymes including lipases and proteases essential for intestinal fat and protein absorption. The pancreatic destruction, partly based on auto-digestion, starts already in utero and, in most patients with a severe genotype, this develops into complete PI already during infancy.

PI is treated with pancreas enzyme replacement therapy (PERT). These products contain pancreatic enzymes mostly of animal origin. However, bioengineered products are currently developed and coming to the market. PERT is individually dosed based on the dietary fat intake and its effects on intestinal fat absorption. Despite optimizing and maximizing PERT many patients a degree of fat malabsorption persists.

LIVER DISEASE IN CYSTIC FIBROSIS

The earliest form of liver involvement in CF is neonatal cholestasis. Infants can present with prolonged jaundice and vitamin K dependent coagulopathy. Liver histology displays signs of biliary obstruction, portal fibrosis and inflammation with bile duct proliferation. Mucous plugs in bile ducts are described. The pathogenesis of CF related neonatal cholestasis is not known, but it might be related to temporary biliary obstruction in the neonatal period. The disease is mostly self-limiting and does not seem to be related to the development of severe liver CF related liver disease later in life. To date CF related neonatal cholestasis is probably earlier recognized in countries with a neonatal screenings program for CF.

CF patients frequently show signs of hepatic steatosis or fatty liver. Steatosis is often diagnosed based during routine ultrasonography of the liver in the clinical follow up of CF patients. An elevated fat fraction has also been observed in over 80% of subjects with cystic fibrosis on MRI scanning. Histologically proven steatosis can be found in up to 35% of CF patients. There is no direct correlation between steatosis and the later development of cirrhotic liver disease. Steatosis in infants with CF is often suggested being related to poor nutritional status in particular early or late recognized disease. It has further been suggested that steatosis in CF has to do with to essential fatty acid deficiency due to the CF related intestinal fat malabsorption.

Cholelithiasis or bile stone disease is frequent in cystic fibrosis patients. Asymptomatic stones are often seen on routine ultrasonography studies. However, CF patients do
regularly present with symptomatic cholelithiasis that necessitate cholecystectomy (46). Cystic fibrosis related cholelithiasis does not usually respond to non-lithogenic treatment like for instance ursodeoxycholic acid (UDCA) probably because cholesterol is not the main component of stone or sludge (49). It is hypothesized that cholelithiasis in CF is related to CFTR dependent alterations in the biliary bile composition. However, the exact pathogenesis of the susceptibility of CF patients for bile stone disease has remained unknown (50).

During routine laboratory checkups in CF patients often elevations of liver enzymes (AST, ALT and GGT) are found (51). If these laboratories abnormalities persist, in repeated measurements, they are sometime classified as signs of CF related liver disease (52). Persistent elevation of liver enzymes above two times the upper limit of normal are suggested as an indication to start UDCA as potential treatment option (53). However, the predictive value and the relation of elevation of liver enzymes to the presence of relevant liver disease has remained a subject of controversy (54).

### CIRRHOSIS IN CYSTIC FIBROSIS

The most severe hepatic complication in CF is the development of hepatic fibrosis into cirrhosis. This potentially life threatening liver disease develops in about 10% of the CF patients (40). The clinical presentation is dominated by symptoms of portal hypertension such as splenomegaly, hypersplenism, gastrointestinal variceal disease and bleeding (42, 53). Less frequent are ascites and hepatopulmonary syndrome found in CF related cirrhosis (CCFLD) (55). The liver parenchymal functions, including protein synthesis and detoxification, are usually spared (4, 56).

The disease is not yet clinically present at birth and develops during childhood. The majority of patients have developed clinically manifest cirrhosis before adulthood with a peak incidence around the age of 10 years (57). Although CF related cirrhosis is rapidly progressive during childhood, the disease tends to stabilize into adulthood. Cirrhotic decomposition is a rare event and liver transplantation while feasible in CF patients, is relatively rarely indicated (56).

The clinical diagnosis of CCFLD is generally made on the basis of multi nodular irregular aspect of the liver on ultrasound in combination with the presence of splenomegaly and signs of hypersplenism including thrombocytopenia (58). Liver biopsy can be used for histological diagnosis of cirrhosis (59). Since CCFLD can be localized in a segmental manner, liver biopsy poses a risk for sampling error. Therefore, it has been advised to perform multiple biopsies in patients suspected of CCFLD (60). For the clinical situation, it is not necessary to perform liver biopsy in the situation of established cirrhosis. However to evaluate developing fibrosis for diagnostic of therapeutic studies biopsy is probably needed to serve as the gold standard.
To date ursodeoxycholic acid (UDCA) is the only medical treatment used in CF related liver disease. UDCA is an endogenous, relatively hydrophilic bile salt with choleretic and antifibrotic properties (61). UDCA is given to CF patients with persistently increased liver enzymes during routine laboratory follow up and/or hepatomegaly (53). It is proven that UDCA is capable of reversing liver enzyme elevation (62). However, the benefit of UDCA in the treatment or prevention of CCFLD is not known (63). Therefore, the use of UDCA in CF remains subject of discussion (64).

THE ROLE OF CFTR IN CYSTIC FIBROSIS RELATED LIVER DISEASES.

Despite the fact that, in recent years, there has been a rapid increase in the knowledge on CF and CFTR protein function, the pathogenesis of CCFLD remains unknown. To improve the treatment and clinical outcome of CCFLD a more fundamental understanding of the mechanisms causing the disease is crucial.

Proliferation and destruction of the bile ducts is are prominent histological features of CCFLD (65, 66). In the past, these observations lead to the assumption that biliary obstruction lays at the basis of the disease (67). In CF lung disease, due to CFTR malfunction, the mucus in the alveoli is thick and sticky. In the liver, CFTR is exclusively expressed in the cholangiocytes or bile duct cells. In parallel to the lung disease, it was assumed that the observed bile duct obstruction was caused by thick and sticky bile obstructing the bile ducts (68). This hypothesis formed one of the substantiations to try the choleretic bile acid UDCA as a treatment in CFLD (69). It was believed that UDCA would make the bile more fluid and increase the bile flow, thereby preventing the bile duct obstruction. However, it was never been shown that indeed the increased viscosity of bile in CF is the primary cause of the disease.

It is not known why only up to 10% of CF patients develop CCFLD. It has been demonstrated that only patients with a severe phenotype, including pancreatic insufficiency, can develop CCFLD (70). However, within the group of CF patients with a severe phenotype the risk for developing CCFLD is not “further” genotype related. It is assumed that additional genetic risk factors or external factors have co-responsibility for the development of CCFLD (71). Extensive genetic modifiers studies in patients with CCFLD have shown for instance that carrier ship of the Pi allele for alpha-1 antitrypsin deficiency adds to risk for CCFLD (57).

Another striking and yet unexplained clinical phenomenon of CCFLD is the young and distinct age of presentation of the disease. CCFLD is not present at birth. In most patients, cirrhosis develops before the age of 18 years with a peak incidence of about 10 years (40). In adulthood, hardly any new case of cirrhosis develops. It is known from historical autopsy studies that liver fibrosis and biliary obstruction are relatively common in adult CF patients (72). However not all patients did eventually develop clinical manifest cirrhosis. To date we do
not know what the risks factors are that trigger the disease to start development in children. Most CCFLD patients have a severe form of portal hypertension with splenomegaly. Gastrointestinal variceal disease is common and in particular young patients are at high risk for hemorrhages (58, 73). This risk for variceal bleeding diminishes with age and is less common in adult CF patients (74).

2) THE ROLE OF BILE SALT IN CYTOTOXICITY AND BILE FLOW

A potential mechanism for the biliary liver disease in CF involves the concept of bile salt related cytotoxicity. Before discussing the contribution of bile salts in pathology, the physiological roles of bile salts will be addressed.

THE ROLE OF BILE SALT IN DIETARY FAT DIGESTION

Bile salts are polarized steroids that play a vital role in intestinal fat absorption and biliary disposal of endogenous and exogenous compounds (75). In the intestine bile salts function as essential surfactants used to solubilize dietary fats in the hydrophilic milieu of gut (76). However, based on the same properties, bile salts can also act as detergents to liver tissue, for example, in the situation of bile salt accumulation (cholestasis) (77). Corresponding with their potentially toxic effects to cells, the bile salt synthesis and their concentrations are tightly regulated (78).

Bile salts are synthesized in the hepatocytes from cholesterol. Bile salts are excreted into the bile and transported, to the intestine, via the intra- and extrahepatic bile ducts. In the bile and the gut, bile salts form water-solvable aggregates, so called micelles, together with the fatty acids originating from the dietary fats. The formation of micelles is essential to transport the dietary fats towards the enterocytes across the aqueous intestinal lumen. Absence of bile salts in the gut results in severe intestinal fat malabsorption. In recent years, it has become clear that bile salts are not only involved in dietary food digestion. It has been shown that bile salts also play vital roles a variety of systemic metabolic regulatory processes, which are, however, outside the scope of this thesis (79).
THE ENTEROHEPATIC CIRCULATION OF BILE SALTS

Bile salts are efficiently recycled via the portal system back to the liver in the so called enterohepatic circulation (80). Bile salts are to a large extent (>95%), absorbed in the terminal ileum, the final section of the small intestine. The total amount of bile salts in the body is balanced and is kept in a tight, steady state, (81). Under steady state conditions, the fecal loss of bile salts is entirely compensated by de novo bile salt synthesis of primary bile salts in the liver. The primary bile salts in humans are cholate and chenodeoxycholate. The primary bile salts are excreted via de bile into the intestine. In the intestinal lumen, the bile salts can be metabolized by the gut flora. Bacteria are capable of deconjugating bile salts and transforming them into a variety of different secondary bile salts. Bile salt species are amphipathic molecules with a hydrophilic and a hydrophobic domain. Bile salts differ in their water solubility and their hydrophobic-hydrophilic balance.

Hydrophobic bile salts have a high capability for solubilizing fats and lipids (82). As a result, hydrophobic bile salts also have the ability to solubilize the lipid structures of cell membranes. Therefore, the more hydrophobic a bile salt, the higher their detergent cytotoxicity for cells and tissues exposed to them.

Under physiological conditions, the hydrophobic cytotoxicity of biliary bile salts is limited by co-secretion of phospholipids (and cholesterol), leading to the formation of mixed micelles (83). Phospholipids are excreted into bile by the membrane transporter enzyme MDR3 (Mdr2 in mice). A genetic incapacity to excrete phospholipids into the bile results in severe bile duct destruction and biliary liver disease. In humans, an inactivating MDR3 mutation is the basis for the disease “progressive familiar cholestatic disease type 3 (PFIC 3)” (84). The disease presents with severe biliary cirrhosis and cholestasis, usually at child age. A genetic mouse model without functional Mdr 2 protein expression (Mdr2 knockout mice) spontaneously develops biliary disease, similar to human PFIC 3 patients (85). The bile duct destruction in Mdr2 knockout mice can be even augmented by increasing the hydrophobicity of the bile salt pool via administration of the hydrophobic bile salt cholate to the mice (86).

BILE PRODUCTION AND BILE FLOW

The magnitude of bile flow is determined at two levels in the biliary tract. The first level is the so called canalicular lumen, the smallest intercellular biliary domain between hepatocytes. Canaliculi form the start of the bile ducts and are surrounded by hepatocytes. When hepatocytes actively transport bile salt into the canalicular lumen, water passively follows as a result of the osmotic activity of the bile salts. The amount of water transport that is generated via bile salt osmosis is called the bile salt dependent bile flow (87).
Various bile salt species differ in their choleretic capacity, i.e., the capability to induce bile flow (88). The choleretic capacity of bile salt depends on the molecular structure and their hydrophobicity. Several hydrophilic bile salts are capable of inducing an exceptionally high bile salt dependent bile flow. For this choleretic capacity some bile salts, like for instance UDCA, are used in clinical situations where decreased bile flow or bile duct obstruction is hypothesized to be contributing to the biliary disease (89).

The second level at which bile flow is determined are the bile ducts. Cholangiocytes form the lining of the bile ducts. Cholangiocytes are capable of secreting water and e.g., bicarbonate into the bile, thereby increasing bile flow and diluting the (canalicular) bile content (90). This portion of the bile production is called the ductular bile flow. Different ion and anion membrane transporter proteins are involved in the complex mechanism of water secretion over the apical membrane by cholangiocytes (91).

The actual driving force behind this process of water secretion of cholangiocytes is the active Cl⁻ transport into the bile duct lumen (92). The most important Cl⁻ transporter is CFTR, but other Cl⁻ channels have also been described (93-95). The active Cl⁻ transport creates a Cl⁻ gradient over the apical membrane. The luminal Cl⁻ is then exchanged for bicarbonate (HCO₃⁻) via a protein called Anion exchanger-2. In this way, the Cl⁻ gradient is replaced by a HCO₃⁻ osmotic gradient. Water leaves the cholangiocytes, via water channels or so called aquaporins on the basis of this osmotic HCO₃⁻ gradient. In this manner, the water secretion by cholangiocytes is directly related to the Cl⁻ transport capacity and CFTR function (96).

The chloride transport of CFTR is an active ATP consuming, process (97). CFTR belongs to the extensive family of ATP binding cassette membrane proteins (98). The CFTR chloride channel only opens after binding and hydrolysis of the intracellular energy source ATP (99). The ATP binding necessary for CFTR activation is regulated via the protein cycle AMP (c-AMP). In the cholangiocytes, c-AMP activity is controlled via different pathways. The most important stimulating pathway of c-AMP is the hormone secretin (100). This gastro-intestinal hormone is released during feeding and in this manner influences the bile flow rate. c-AMP can also be induced via intraluminal factors like bile salts or intraluminal ATP (94, 101).

**BILE SALT SYNTHESIS**

Bile salts are synthesized in the hepatocytes from cholesterol (figure 4). The synthesis requires several sequential enzymatic steps (78). The synthesis of the bile acids is quantitatively the predominant pathway of cholesterol catabolism in mammals. The major pathway for the synthesis of the bile acids is initiated via hydroxylation of cholesterol at the 7 position via the action of cholesterol 7α-hydroxylase (CYP7A1), an ER localized enzyme. CYP7A1 is a member of the cytochrome P450 family of metabolic enzymes. This pathway
initiated by CYP7A1 is referred to as the "classic" pathway of bile acid synthesis. There is an alternative pathway via the mitochondrial enzyme sterol 27-hydroxylase (CYP27A1). Although, in rodents the alternative pathway can account for up to 25% of total bile acid synthesis, in humans it has been suggested to account for no more than 6% (102).

Figure 4. Schematic representation of the bile salt synthesis pathways. (Illustration from Lipid research 2009;50:s120-s125)
Bile salts have an important physiological contribution to the intestinal fat digestion, besides elimination of cholesterol and other endogenic and exogenic catabolytes and metabolites from the body. Bile salt synthesis is under the control of at least two different negative feedback pathways (figure 5.). In the first pathway, the sensing of bile salt takes place in the hepatocytes (103). In the other pathway, bile salt sensing takes place at the level of the enterocytes of the gut. In both pathways binding of bile salt to Farnesoid X receptor (FXR) plays a pivotal role in the initiation of the negative feedback regulation. FXR is a nuclear receptor. Nuclear receptors are transcription factors, whose ligands can determine their DNA binding and transcription modulating activity. The ligands for the nuclear receptors are, among others, steroids. In response, these nuclear receptors work with other proteins to regulate expression of specific genes, thereby controlling and regulating homeostasis in an organism. In the case of the nuclear receptor FXR bile salt can function as ligands.

Figure 5. Schematic representation of the enterohepatic circulation of bile salts in the context of CFTR. Several bile salt involving feedback loops regulated bile salt synthesis and liver growth. The bile salt induced nuclear receptor FXR plays a pivotal role in intestine and in the liver.
However different bile salt species vary in their efficacy as FXR ligands (104). In general hydrophobic bile salts are stronger ligands for FXR compared to hydrophilic bile salts.

Bile salt/FXR interaction results in different physiological responses, in the different cell types, for example, hepatocytes and intestinal cells. In hepatocytes FXR activation down regulates the expression of a protein called small heterodimer protein or SHP. SHP can directly down regulates the expression of CYP7A the rate limiting step in the bile salt synthesis from cholesterol (105).

The intestinal regulation of bile salt synthesis is more indirect: FXR stimulates the expression of FGF19 (fibroblast growth factor 19, equivalent to  Fgf15 in rodents) that is subsequently released into the portal blood (106). At the basolateral membrane of the hepatocytes FGF19 can bind to the FGFR4 receptor (107). This binding leads to activation of an intracellular pathway that, via the so called ERK system, down regulates CYP7A expression and subsequently reduces bile salt synthesis (108). The hepatic and intestinal sensing of bile salts by FXR and their respectively negative feedback pathways are functionally independent.

3) THE ROLE OF CFTR IN GASTROINTESTINAL DISEASE.

Different from other organs like lungs and sweat gland, the contribution of CFTR in the intestine to the clinical phenotype is not entirely clear. In the intestine CFTR is expressed on the apical membrane of the enterocytes (109). Also in the gut, CFTR functions as a Cl⁻ channel. The Cl⁻ channel activity of the gut can actually be quantified in ex vivo electrophysiological studies of gut tissue (110). It is hypothesized that CFTR in the intestine plays a direct role in the lubrication of the gut contents. Reduced water content of intestinal content may lead to thickened stools (111). This principle of decreased water content probably lies at the basis of intestinal pathology in CF like meconium ileus and constipation. Based on studies in mice we know that in the intestine bile salts are capable of stimulating  Cftr via the intestinal membrane receptor protein Asbt (apical sodium binding protein) (112). The latter indicating that in Cystic fibrosis intestinal disease, besides the intrinsic malfunction of the CFTR protein, additionally disease related alternation in bile salt metabolism can be responsible for decreasing lubrication of the gut contents

FAT MALABSORPTION

One of the most striking features of the gastrointestinal phenotypes in CF is the intestinal fat malabsorption (113). The fatty stools or steatorrhea is a clinical sign of fat malabsorption. Fat malabsorption in patients causes several severe problems. Due to the high energy content of
fat in general, fecal fat malabsorption causes malnutrition and poor growth. A secondary effect of the intestinal fat malabsorption is the reduced absorption of the fat soluble vitamins A, D, E and K. Vitamin malabsorption can lead to hypovitaminosis and vitamin K dependent coagulopathy (114-116). Therefore, CF patients with intestinal fat malabsorption are usually dependent on oral vitamin ADEK supplementation.

The main reason the intestinal malabsorption is exocrine pancreatic dysfunction (113). As a result of the fibrotic destruction, already during pregnancy and infancy, the pancreas loses the capacity to excrete sufficient amounts of digestive enzymes into the intestine. Pancreatic enzymes form the bulk of the available intestinal digestive enzymes. The pancreas excretes lipases for digestion of nutritional fat and proteases for the digestion of nutritional proteins (117).

The majority of dietary fats consist of triglycerides. These triglycerides are hydrolyzed, by lipases, into smaller and more polar fatty acids. To overcome the intestinal fat malabsorption, CF patients with pancreatic insufficiency, are prescribed pancreas enzyme replacement therapy (PERT) (33). This supplementation is administered simultaneously with every dietary fat containing meal. PERT dosing is primarily related to the amount of fat calculated from the diet and adjusted based on clinical effects on symptoms of steatorrhea and growth (118).

INTESTINAL BILE SALT MALABSORPTION

CF patients have a persistently elevated fecal BS excretion compared to healthy controls (26). The origin of the increased bile salt excretion is not known. First it was suggested to be related to the intestinal fat malabsorption (119). However increased fecal bile salt excretion is still present during adequate PERT treatment. Furthermore, increased fecal bile salt excretion is present in patients with still sufficient exocrine pancreas function, i.e. without fat malabsorption (120). The latter observation was confirmed in experimental CF mice models. Also in “mild” CF mouse models, with normal fat absorption, fecal bile salt loss is increased (121).

Based on the observation that increased fecal bile salt excretion is independent of fecal fat malabsorption it is assumed, that increased fecal bile excretion in CF is related to the dysfunctional Cftr, located in the enterocytes of the intestine itself (122, 123). The majority of bile salts are re-absorbed in the distal part of the small intestine, the terminal ileum. Bile salt re-absorption into the enterocytes is facilitated by a membrane protein called apical sodium dependent bile salt transporter (ASBT). In CF mouse models, it has been shown that the absence or Cftr function in the enterocytes down regulates the transport capacity of bile salt by the co-located membrane protein ASBT (112). This observation is suggesting that the
intestinal re-absorption of bile salt maybe hindered by dysfunctional CFTR. This may in turn contribute to the observed increased fecal bile salt loss.

Finally, there are suggestions that the increased fecal bile salt excretion could be associated with CFTR dependent differences in the intestinal bacterial flora (124). Small intestinal bacterial overgrowth (SIBO) is common in CF. SIBO can result in steatorrhea, abdominal pain, bloating, flatulence, nausea, and anorexia (125). Also based on results in CF mice models, it has become known that absence of Cftr function causes significant changes in the intestinal bacterial composition (126). In mice, differences in intestinal bacterial composition are associated with changes in bile salt metabolism and with increased fecal bile salt loss (127). Therefore, it could well be that the reported changes in intestinal bacterial flora in CF condition contribute to the increased fecal bile salt excretion.

INTESTINAL INFLAMMATION

A more recently recognized GI condition in CF is intestinal inflammation. Based on fecal calprotectin levels (a marker for intestinal inflammation), intestinal inflammation is a frequent event in CF patients (128). Evaluation via capsule endoscopy has indicated that macroscopic inflammatory lesions are frequently present in CF patients (129). It is not known whether the inflammation is a direct effect of dysfunctional CFTR in the gut or otherwise. Another hypothesis is that Cftr function interferes with inflammatory regulatory mechanism and proteins like the peroxisome proliferator activated receptors (PPARs) (130, 131). Finally, it is also possible that the intestinal inflammation is related to the combination complex interactions of the bacterial flora of the gut, bile salts metabolism and the immunological inflammatory response in the context of CFTR dysfunction.

4) CURRENT HEPATIC AND GASTROINTESTINAL ISSUES OF CYSTIC FIBROSIS DISEASE

Recent developments in the pathophysiology of CF have elucidated many aspects of the hepatic and gastrointestinal consequences and pathology of cystic fibrosis. However, many questions have remained unanswered in this research field. To optimize the care for and prognosis of CF patients in the future, we aimed to gain more insights in the role of CFTR in the pathology of hepatic and gastrointestinal CF. It is to be expected that insights in the pathophysiology of CF in the GI tract will also provide knowledge concerning the physiological roles of CFTR in other tissues. Furthermore, we are facing a new era of therapeutic options in CF, when CFTR correctors and potentiators are currently coming to the market (132). These
promising new treatments have to be monitored for their efficacy. New insights in the role of CF and CFTR in the liver and gastrointestinal tract can offer growing opportunities to test and evaluated these new therapeutic options (133).

CURRENT ISSUES FOR CFLD

To improve prevention and treatment possibilities of CFLD, a better knowledge of the etiology and pathophysiology of liver disease in CF is essential. Several potential leads for the etiology of CFLD have been reported in the literature (134-136). One of the suggestions for the pathogenesis of CFLD has been the involvement of increased viscosity of bile, due to CFTR dependent changes in bile composition. Bile duct obstruction then, sequentially, leads to the development of an obstructive biliary cirrhosis (137). However, no firm experimental evidence is available to support this hypothesis.

Another potentially contributing factor is bile salt cytotoxicity. In the liver CFTR is located at the apical membrane of the cholangiocytes lining the bile ducts. In cholangiocytes, CFTR functions, as a chloride channel, indirectly in water and bicarbonate secretion into the bile (91). Therefore, CFTR plays an important role in the magnitude of bile production and bile composition (96). Bile salt cytotoxicity can theoretically be enhanced in CF conditions. The overall bile salt concentration can be increased due to reduced water secretion and reduced dilution of the bile.

Furthermore, bile cytotoxicity can be increased due to changes in bile composition. Normally the detergent effects bile salts are reduced by the biliary phospholipids (75, 83). CFTR related changes in bile composition of the biliary lipids and/or bile salts could play a role in enhanced susceptibility to bile salt cytotoxicity and the development of biliary liver disease in cystic fibrosis.

Bile salt cytotoxicity could be enhanced by increasing the contribution of hydrophobic bile salts and/or by decreasing the biliary secretion of “protective” phospholipids. The biliary bile salt profile is determined by de novo synthesis of bile salts and by the secondary bile salt conversion by the intestinal bacterial flora. A change towards a more cytotoxic bile salt composition could be the result of either altered primary bile salt synthesis or secondary bile salt conversion.

The treatment of CFLD remains a controversy in the care for CF patients. The only medication currently used is ursodeoxycholate (UDCA) (64). This relatively hydrophilic bile salt is administered orally. Its working mechanism is supposed to be related to its choleretic and anti-inflammatory/anti-fibrotic capacities. In several clinical trials, it has been shown that UDCA is capable to recover elevated liver functions tests like AST, ALT and GGT to normal
values (138, 139). However, there are no long-term follow up studies of the therapeutic effects of UDCA with respect to clinical endpoints, such as mortality, need for liver transplantation, or fibrosis/cirrhosis complications. It has never been shown that UDCA is able to prevent cirrhosis, nor its complications. One of the problems for clinically studies is that we are not able to identify the subgroup of the CF patients (~10%) that are at risk for cirrhotic disease.

It has been assumed that the therapeutic effect of UDCA functions via its capacity to increase bile flow (61). However, based on fundamental studies, it has not been demonstrated that UDCA indeed is actually capable of increasing bile flow in CF conditions and if so, to what extent. Measuring bile flow and bile production in humans is invasive and difficult. Therefore, there is a need for experimental support that clarifies this issue.

Recent studies on cirrhosis by other etiologies indicate that, even in progressive fibrotic liver diseases, the cirrhosis can be stopped or even reverse on removal of the causative agent and/or on treatment of the underlying disease (140). In particular antiviral treatment has been shown to be able to reverse the severity of Hepatitis B virus cirrhosis (141, 142). New developments are evolving concerning the use of anti-fibrotic therapies in liver fibrosis and cirrhosis. Although theoretically promising, to date there not yet anti-fibrotic therapies available in humans (143). However, the scope of these positive developments indicates the rising opportunity and potential profit for preemptive treatment in CCFLD.

These promising scientific advances indicate the need for reliable and relevant markers to identify patients at risk for CCFLD. Therefore, one of the most urgent issues in CFLD is the possibility of early detection of patients at risk for, or in an early phase of, the disease. Progress in this diagnostic field of CF would offer opportunities for evaluation of current or new interventional therapies to prevent or treat CFLD. As stated above, no tools for early detection or recognition are available. As a consequence, CFLD is frequently only recognized clinically in an advanced stage of severe fibrosis or even cirrhosis. However, cirrhosis typically becomes manifest around the age of 10 years and it is not present at birth. This observation indicates that cirrhosis in CFLD develops progressively over a period of years during childhood.

At this time, cirrhosis is usually diagnosed based on physical examination and abdominal ultrasound studies of the liver and spleen (53, 144). On physical examination, the most prominent clinical feature of cirrhosis is splenomegaly. This is often accompanied by hematological signs of hypersplenism, like thrombo- and leucocytopenia. Ultrasound of the liver, in case of cirrhosis, can show irregular liver edges and an inhomogeneous, nodular, pattern of the parenchyma (43, 145, 146). The spleen span can be enlarged for age. Since portal hypertension is often a characteristic symptom in CF related cirrhosis, abdominal ultrasound may reveal signs vascular collaterals originating from the portal system. Upper GI
endoscopy may reveal evidence for esophageal or gastric variceal disease based on the often severe portal hypertension (147).

Liver function test (LFTs), like AST, ALT and GGT, are often used to diagnose CFLD. However, their clinical value for diagnosing (development of) liver fibrosis or even cirrhosis has not been established. CF patients are routinely checked for elevation LFTs. Frequently patients have (even persistent) elevations of LFTs above the upper limit of normal (148). By some authors, this elevation of LFTs has been defined as one of the characteristics of CFLD (52). There is no proven correlation, however, between elevation of LFTs and development of liver fibrosis or cirrhosis (60). The current way of evaluating LFTs elevation is thus not validated and useable to identify patient at risk or actually developing CFLD.

The gold standard for validation of the stage of liver fibrosis in general is histology, normally obtained via percutaneous liver biopsy. Several studies have shown that liver histology can classify milder forms of fibrosis in CF (149). However, liver biopsy has several practical and important drawbacks. Based on autopsy studies in CF patients it has been reported that fibrosis and cirrhosis may not be evenly spread throughout the liver. This observation highlights the risk of a sampling error in the case of liver biopsy (60, 150). Another problem with liver biopsy in CF is the patient selection. It is known that ("only") around 10% of CF patients with severe mutations will develop cirrhosis. Since liver biopsy is an invasive procedure, it is not ethical to use it, with a low threshold, in CF patients. Also in the case of a liver biopsy, the reliable identification of patients seriously at risk for cirrhosis, could justify the use of this invasive diagnostic procedure.

Several studies have reported on the possibilities to use ultrasound of the liver as a screening tool for staging liver fibrosis and cirrhosis in CF patients (43, 145, 150). Ultrasound of the liver seems promising as a screening measure. It is non-invasive, widely available and already frequently used in standard clinical CF care. Apart from the inhomogeneity and nodular abnormalities consistent with cirrhosis in general, several other ultrasound particularities can be found in CF (146). Often on ultrasound the echogenicity of liver parenchyma is increased. Increased echogenicity of the liver, however, is not specific for the development of cirrhosis: rather, it may be related to an increased fat content or steatosis of the liver. There are no indications that steatosis is preceding the development of fibrosis in CFLD. In general, it is concluded that ultrasound studies of the liver are a reliable tool for the diagnosis of cirrhosis in CF, i.e. the end-stage, but are not sensitive enough to recognize or classify CFLD in an earlier phase.

A more recent development in the diagnosis for liver fibrosis in CF is the use of transient elastography (151, 152). This technology is based on a method to determine tissue stiffness by measuring sound wave reflection produced by a special probe. The method is non-invasive and could potentially be used as a screening tool in all pediatric CF patients. Problem with this method is that it has not been satisfactorily validated for the pediatric (CF) population (153,
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Another difficulty with elastography is that, in CF, it remains to be validated against the gold standard for liver fibrosis; histology from liver biopsy.

CURRENT ISSUES FOR INTESTINAL CF

Better nutritional status is related to a better survival of CF patients (28). Therefore, improvement and maintenance of the nutritional status is one of the cornerstones in the care for CF patients (118). To achieve this goal optimizing dietary intake and improving intestinal absorption of nutrients are priorities in the research agenda and treatment of CF. Since dietary fats provide the largest caloric share in the normal dietary energy intake, optimizing dietary fat intake and absorption provides the greatest potential benefit for improvement of the nutritional status.

Despite adequate and optimal pancreatic enzyme replacement therapy for exocrine pancreatic insufficiency, in many CF patients, a persistent fat malabsorption remains (35). This PERT resistant fat malabsorption is probably associated with other, non-pancreatic, CF related factors involved in the intestinal fat absorption (36). To further optimize the diagnosis and the treatment possibilities of PERT resistant fat malabsorption we need more insight in the factors involved.

Basically, intestinal fat absorption proceeds in two sequential phases. First lipolysis is facilitated by the digestive enzymes mainly produced by the pancreas. Secondly there are the processes involved in post-lipolytic fat absorption like the solubilization of fats by bile salts. To date there is no clear answer to the exact cause of the malfunction of the postlipolytic phase in CF conditions. However, several factors are associated with PERT resistant fat malabsorption like changes in bile salt metabolism and the enterohepatic circulation and differences in bacterial flora. It could also possible that the PERT resistant fat malabsorption in CF is a direct consequence of the dysfunctional CFTR protein in the apical membrane of the enterocytes.

5) OUTLINE OF METHODOLOGY USED FOR THIS THESIS

For the studies in this thesis, we applied a variety of methods to obtain more pathophysiological insights in CF disease in the liver and gastrointestinal tract. Broadly, our methods could be divided into 3 major groups. First, we aimed to carry out a literature review study concerning the reported and theoretical background of PERT resistant intestinal fat malabsorption in the context of CF and CFTR malfunction. Secondly, we performed a retrospective study in CF patients, to determine the potential value of specific LFTs in
identifying CF patients at risk for cirrhosis. Thirdly, we performed fundamental studies in a variety of CF mice models to determine the role of bile salt metabolism in CF conditions and the role of bile salts in the development of CFLD. In the CF mice models, we performed experiments to assess the role of bile salts, their metabolism and enterohepatic circulation. To aid the reader with limited knowledge on CF mice models, the background of these models and the methodologies used will first be discussed in more detail.

**CYSTIC FIBROSIS MOUSE MODELS**

Cystic fibrosis was the first monogenic genetic disease in which the disease causing gene mutation was identified (5, 6). As a result of this breakthrough in 1989, the genetic and molecular insights into the functions of the CFTR protein increased rapidly. Based on this knowledge, several genetically engineered experimental mouse models were developed, in which the genotype of human CF was mimicked (155, 156). These mice models have been used successfully to understand CF pathophysiology in different organs. Also in the present thesis, we used specific CF mouse models to delineate the role of Cftr in the pathophysiology of cystic fibrosis.

Globally the CF mice models can be divided into two categories. In the first category the Cftr gene is no longer functionally expressed, the so called complete Cftr knockout mice or \( \text{Cftr}\)-/ mice. The knockout mice are not able to produce any functional Cftr protein. The second category of CF mouse models is engineered in a way that they express a mutated form of the Cftr gene, in accordance with the most frequent CFTR gene mutations in human CF patients. Theoretically these Cftr mutated mice resemble the genetic and functional situation of CF patients more closely than the knock-out mouse models. For example, in parallel to human CF, mouse models have been constructed that carry the homozygous 508del mutations in their (murine) CF gene. The delta F508 mutation consists of a deletion of the three nucleotides that comprise the codon for phenylalanine (F) at position 508. Having two copies of this mutation (one inherited from each parent) is the leading cause of CF (157).

The phenotype of CF mouse models does not resemble the human CF disease in every aspect. Some CF disease traits are found similarly in CF mice and humans, including increased fecal bile salt excretion (121). Others, however, like for instance lung disease, are hardly or not present in CF mice. Furthermore, strong mouse strain background effects are present among the different CF gene modifications, irrespective of complete or incomplete Cftr inactivation. The strain effects are probably related to the presence of disease modifying genes in the genome of the different genetic strain backgrounds.
HEPATIC PHENOTYPE OF CF MOUSE MODELS

CF mouse models display varying types and degrees of hepatic histological phenotypes. Most mouse models show a normal liver histology. However, there are several reports of CF mice models with spontaneous developed histopathology. The most severe hepatic phenotype is described in the University of North Carolina (UNC) Cftr\textsuperscript{unc/unc} knockout mice. Even without a further challenge, these Cftr\textsuperscript{−/−} animals develop focal and progressive hepatobiliary disease (158). By 3 months of age, they have varying degrees of periportal and bridging fibrosis, which then progresses with age. Freudenberg et Al. describe histopathology in the homozygous del508 (Cftr\textsuperscript{508/508}) mice (135). Liver histopathology of CF and controls livers displays variable, mild, patchy cholangiopathy characterized by reactive changes in the biliary epithelium, bile ductular proliferation, and mild portal fibrosis. These findings were only rarely present in WT mice. Notwithstanding the hepatic phenotype, none of these mice manifested any advanced degree of liver fibrosis or cirrhosis. The mice did not exhibit any bile duct lesions even though some animals were more than 12 month old.

Besides spontaneous development of liver histopathology, there are also CF mouse models with increased susceptibility to an exogenous challenge to induce a liver phenotype. There is an existing clinical and experimental relationship between colitis and another biliary disease namely primary sclerosing cholangitis. In experimental animals, a chemical colitis can be induced via oral dextran sulfate sodium (DSS) administration (136). Based on this concept it was hypothesized that loss of Cftr function in the setting of a DSS induced colitis can lead to (enhanced) bile duct injury. Blanco et al. demonstrated that mice homozygous for Cftr mutations developed bile duct injury following the DSS induction of colitis. In an additional study from the same research group, the bile duct susceptibility of the Cftr\textsuperscript{−/−} mouse could be related to decrease Ppar α expression in Cftr\textsuperscript{−/−} mice (159). These results seem to suggest that changes in the systemic inflammatory regulation could be involved in the etiology of CFLD.

THE INTESTINAL PHENOTYPE OF CF MICE MODELS

The most marked phenotypical symptomatology of CF mice is intestinal obstruction (160). This phenotypical trait resembles in some aspects the clinical picture of intestinal obstruction in meconium ileus or DIOS in CF patients. In mice, it typically presents when mouse pups are weaned from breast feeding to solid foods (usually at age ~18-19 days). The obstruction can be severe and even lethal for the mice. Some mice models have to be treated with oral laxatives to restore normal bowel movements and/or to prevent mortality. Other mice with an even more severe intestinal phenotype need persistent liquid feeding to prevent (re)occurrence of intestinal obstruction (161).
Histology of the pancreas does show some preliminary signs of pancreatic fibrosis. However, based on evaluation of pancreatic function tests, CF mice are pancreatic sufficient. Despite this normal exocrine pancreatic function, CF mice models do present an increased fecal fat excretion compared to non CF normal control mice. The latter, indicating other, non-lipolytic, causes for intestinal fat malabsorption in CF conditions (158).

Another intestinal phenotype of the CF mouse models is intestinal inflammation in combination with small intestine bacterial overgrowth (SIBO). Oxana et al. describe the novel finding that a specific innate immune response in the CF mouse small intestine has a role in inflammation and contributes to the failure to thrive in this mouse model of CF (162). When then treated with broad-spectrum antibiotics for 3 weeks, the Cfr−/− mice increased in body weight when compared with controls (163). Wouthuyzen et al. reported that this positive effect of antibiotics on body weight was not mediated by increasing the absorption of long-chain triglycerides (127). Antibiotic treatment of homozygous ΔF508 mice without SIBO neither augmented body weight nor increased fat absorption. Collectively, the data indicate that the positive effect of antibiotics on body weight in CFTR-knockout mice may be attributable to the treatment of SIBO and not necessarily to enhanced absorption of long-chain triglycerides.

Like human CF patients, CF mice also have an increased fecal bile salt excretion compared to control mice. To date there is no conclusive explanation for this observation. Since CF mouse models display normal exocrine pancreatic function, the increased bile salt excretion seems not related to the pancreatic enzyme dependent lipolysis of the dietary fats (164). Another possibility is a decreased bile salt uptake in the ileum by the apical sodium dependent bile salt transporter protein (ASBT). Bijvelds et al. demonstrated that ileal TC uptake was reduced by 17% in Cfr-null mice (112). Because the distal ileum is the discrete site of active BS uptake and has a pivotal role in the near-complete recovery (~95%) of the intestinal BS load, this reduction may well explain the high level of fecal BS excretion reported previously for our Cfr-null mice (165). However, for homozygous F508del mice, we did not find a reduction in ileal BS uptake, despite the fact that fecal BS loss is increased to a similar extent as in null mice. Another explanation may involve interaction between intestinal bile salt metabolism and Cfr related changes in intestinal bacterial flora. Even under physiological conditions there is an active and complex interaction between bacterial flora and bile salt metabolism. For instance, mice with a deficiency in microbiota (germ free mice or mice treated with antibiotics), display decreased fecal bile acid excretion and an increased bile acid pool size (166, 167). These studies indicate that bacteria of the gut microflora are interrelated with the modulation of host bile salts. Since bacterial flora is altered in CF conditions, this could lead changes in fecal bile salt excretion.
There are several methods available to study the hepatic and intestinal consequences of Cftr function in experimental mice models. There are anthropometry methods like measuring body weight, liver weight and the determining the liver weight to body weight ratio. These methods provided the possibility to assess nutritional status and possible trophic factors of liver growth.

Fecal sample collection can be used for different purposes. In combination with 72 hour registration of the oral dietary intake, we used 72 hours stools collection to estimate the percentage of fat absorption. Fecal fat content was further evaluated to determine the differential absorbability of the various fatty acids species. Fecal samples were also used to measure the total bile salt excretion and determine the profile of the different bile salts species.

We performed bile duct cannulation to determine bile production and bile composition. In this method, the mice are operated via microsurgery. The abdomen is explored and the gallbladder identified. The choledochal duct is identified and ligated, thereby blocking the bile flow to the intestine. A hole is punctured in the gallbladder with a needle, after which it is canulated and fixated. Subsequently all the bile produced by the liver flow freely via the tube and can be collected in a tube for gravimetric analysis to measure bile production. The bile is used for biochemical analysis like bile salt concentration and composition, and biliary lipids (phospholipids and cholesterol).

Additionally we applied the determination of bile salt kinetic parameters, i.e., pool size (amount of bile salts in the body), fractional turnover rate (the portion of the pool that is newly synthesized per day), and synthesis rate. These parameters reflect hepatocellular function, the metabolism of bile salts and the efficiency of enterohepatic cycling. To measure
these kinetic parameters we used a previously developed and validated stable isotope dilution technique without the need to interrupt the enterohepatic circulation. This method allows simultaneous determination of kinetic parameters. We used the stable isotope technique with $[^{2}H_4]$-cholate as labeled bile salt. Cholate is a major primary bile salt species and comprises 50 to 80% (rodents) of the total bile salt pool. Therefore, cholate pool size, fractional turnover rate (FTR) and synthesis rate are kinetic parameters that allow description of bile salt kinetics of the quantitatively most important bile salt.

**EXPERIMENTAL BILE SALT SUPPLEMENTED DIETS**

For several of our mouse experiments, we used bile salt supplemented diets. The bile salt were added and mixed to regular chow mice feeding. We used ursodeoxycholate (UDCA) supplemented diets to mimic the clinical situation of UDCA treatment in CF patients. In other experiments, we used supplementation of the hydrophobic bile salt cholate to mimic the human hydrophobic bile composition (humans have a higher hydrophobicity than mice). The purpose of the latter approach was to evaluate if a higher hydrophobicity would contribute to the cytotoxic effect of bile on the development of CFLD like pathology in the CF mice models.

**6) SCOPE OF THIS THESIS:**

Despite great progress in the care and treatment of CF, the disease still is severe, lifelong and lifespan limiting. Besides the clinically progressive pulmonary disease, the CF phenotype includes a variety of gastro-intestinal and hepatic manifestations. Some of these CF manifestations, like intestinal fat malabsorption and the development of cirrhosis, give rise to severe and life threatening complications. Better understanding, concerning the pathogenesis, the mutual interrelationships and the potential treatment options, of the gastro-intestinal and hepatic manifestation of CF will have profound positive effects on the prognosis of CF patients.

The enterohepatic circulation of bile salts connects the physiology of the liver with the physiology of the intestine. Bile salts are crucial functional and regulatory molecules in a variety of essential physiological processes of the hepatobiliary and gastrointestinal tract. For example, bile salt secretion is the driving force behind bile formation and bile salts are indispensable for intestinal fat, and thus energy, absorption. On the other hand, bile salts are strong detergents, capable of inflicting serious and permanent cell and tissue injury. The balance between the essential physiologic functions and the destructive forces of bile salts requires a precise regulation of this delicate system. Disturbances of the equilibrium of the enterohepatic circulation can potently lead to physiological malfunction and disease.
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CF is a multi-organ disease including, among others the hepatobiliary and gastrointestinal tract. Therefore, it is plausible that primary or secondary consequences of CFTR protein dysfunction lead to alteration or disturbances in the enterohepatic circulation of bile salts. The specific aim of the research described in this thesis was to determine the role of bile salts and their enterohepatic circulation in the hepatic and intestinal phenotype in CF.

Many patients with exocrine pancreatic insufficiency suffer from persistent intestinal fat malabsorption despite adequate PERT therapy. In chapter 2, we describe, in a comprehensive literature review, all factors potentially involved in PERT resistant fat absorption including the enterohepatic circulation of bile salts. In the review, we focus in particularly on the role of bile salts and enterohepatic circulation.

In the following chapters we switch gear towards CF related liver disease. First we turn to the CF mouse models to answer basic questions concerning the pathogenesis and development of CFLD. In chapter 3, we test the hypothesis that biliary bile salt cytotoxicity lays at the basis of the development of CFLD in CF a specific mice model with spontaneous liver disease. This initial experimental chapter is followed by chapter 4 in which we study if CFLD can be induced, by feeding a hydrophobic bile salt-containing diet to CF mice without spontaneous liver disease. Since CFLD is often treated with UDCA, in chapter 5, we tested the effects of UDCA on bile production and bile composition in a CF mouse model.

To prevent or treat CFLD, it is essential to be able to recognize CF patient at risk or in an early phase of the disease. To address this issue, in chapter 6, we return to the clinical aspects of CCFLD. In this chapter, we aim to identify CF patients at risk for cirrhosis in an early clinical phase. In a retrospective approach, we tested the hypothesis that the development of cirrhotic CFLD can be predicted on the basis of follow up of biochemical liver function tests. In chapter 7, we discuss our overall results, place these in a clinical and experimental framework, and discuss future perspectives.
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