CHAPTER 5

BILE SALT METABOLISM IN A CF MICE MODEL WITH SPONTANEOUS LIVER DISEASE

Submitted

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Chapter 5

ABSTRACT

Introduction:

Long-living congenic C57BL/6J cystic fibrosis transmembrane regulator (Cftr)\textsuperscript{−/−} mice are reported to spontaneously develop liver pathology in an age-dependent fashion. Alterations in bile production and composition have been postulated to be involved in the development of CFLD. To address this hypothesis, we determined bile production and composition in this CF mouse model and controls.

Methods:

C57BL/6J Cftr\textsuperscript{−/−} mice and control littermates (N=10 per genotype), mean age 74±25 days, were fed a liquid diet (Peptamen Nestle) ad libitum from the time of weaning. We determined bile production and composition after collection via gall bladder cannulation and sampled stools for bile salt secretion and bile salt composition.

Results:

Cftr\textsuperscript{−/−} and control mice did not differ in bile production rate, biliary bile salt and phospholipid concentration and secretion rate, and phospholipid to bile salt ratio. Bile salt secretion— bile flow graphs indicated that Cftr\textsuperscript{−/−} mice had a significantly higher bile salt dependent bile flow compared with controls (Cftr\textsuperscript{+/+}, +130%, p=0.01). The biliary bile salt profile had a similar bile hydrophobicity index in Cftr\textsuperscript{−/−} and control mice. However, the proportion of secondary bile salts was significantly higher in Cftr\textsuperscript{−/−} mice than in controls (17±3 vs. 29±10%, resp.; p<0.001). In particular, the secondary, hydrophilic bile salt ursodeoxycholate was ~7 fold higher in Cftr\textsuperscript{−/−} mice than in controls. (25% vs. 3% of total bile salts resp.; p=0.004).

Conclusion:

The reported development of liver disease in the C57BL/6J Cftr\textsuperscript{−/−} mice is not related to alterations in bile production or biliary lipid composition. Cftr\textsuperscript{−/−} mice, on a liquid diet, have increased amounts of secondary bile salts, which may be due to specific changes in bile salt metabolism in the intestinal lumen of this CF mouse model.
INTRODUCTION

Cystic fibrosis can be accompanied by liver disease (CFLD), which in ~10% of all patients can result in cirrhosis (1). Liver histology of these patients shows a severe biliary type of fibrosis and cirrhosis (2).

To this day the pathogenesis and developmental pathways of CFLD remains obscure. It has long been suggested that, in parallel to the suggested development of the obstructive CF lung disease, local biliary obstruction by thickened, sticky bile formed the pathological basis for the liver disease (3). However, this hypothesis is not well supported by experimental evidence. Most information concerning the pathology of CFLD in humans has been derived from post mortem evaluations (4). Prospective histological studies in humans are scarce, mainly because of the need for invasive diagnostic procedures in asymptomatic patients (5).

Different CF mice models, with a wide variation in genetic background, are available (6). Most CF mice models do not show liver pathology. Durie et al., however, described, age related, histological abnormalities in the liver of C578L/6J Cftr−/− mice (7). Additionally Freudenberg et al. reported that older ΔF508 CF mice (100-200 days) had more liver fibrosis than their controls (8).

Bile salts play an important role in intestinal lipid absorption and in biliary lipid excretion. Bile salts can act as detergents and have cytotoxic properties. Bile salt composition and in particular biliary bile hydrophobicity is related to development of liver disease in general (9). The primary bile salts are synthesized in the liver and secreted into the intestine via the bile ducts. In the intestine they serve mainly as detergents to solubilise water insoluble components such as cholesterol and fats. In the intestine primary bile salts are subject to biotransformation by the intestinal flora. In this way a variety of different secondary bile salts are formed. All bile acids are absorbed in the terminal ileum and return to the liver, thus undergoing enterohepatic circulation (10).

We studies the potential role of bile salt metabolism and biliary bile salt cytotoxicity in the pathogenesis of the liver pathology the C578L/6J Cftr−/− mice model. To address this question we measured bile production and determined biliary and fecal bile salt excretion and composition.
**METHODS**

**Animals.** All experimental protocols were conducted in the Hospital for Sick children, the Research Institute, Toronto, Canada, after approval by the institutional Animal Care Committee. Mice were bred to wild-type C57BL/6J mice, obtained from the Jackson Laboratories (Bar Harbor, ME). Mice were genotyped at 14 days of age using polymerase chain reaction analysis of tail clip DNA. To minimize bowel obstruction and optimize long-term viability, 20- to 23-day-old congenic C57BL/6J Cftr<sup>−/−</sup>tm1Unc mice and their Cftr<sup>+/+</sup> littermates were weaned to a liquid diet (Peptamen, Nestlé Nutrition, Canada) using glass liquid mouse feeders, prepared in sterile water according to the manufacturer’s instructions. Fresh diet and feeders, sterilized by autoclave, were replaced daily. Mice were housed in a non-sterile conventional housing unit in micro-isolators cages, with corncob bedding changed daily, and provided with sterile water in addition to the liquid diet. The colony was maintained at a pathogen-free status by serological screening at a commercial laboratory. Mice were kept in a 12-hour light-dark cycle. The animals used for the experiments were approximately 1.5-4 months old.

**Experimental procedures.** The total feces of 3 consecutive days was collected, dried and homogenized. Bile was collected after surgical ligation of the common bile duct and gall bladder cannulation using silicone tubing (size 0.020" x 0.037", Degania Silicone Ltd.) under anesthesia. The anesthetic mixture consisted of: 0.75 ml ketamine (100 mg/ ml), 0.25 ml xylazine (20 mg/ ml) and 4 ml saline. The mixture (0.1 ml per 10 grams of body weight) was intraperitoneal administered. Body temperature was maintained by placement of the animal in a temperature and humidity controlled incubator. Bile secretions were collected in 30 min. fractions, after a 10 equilibration period. Bile flow rate was assessed gravimetrically, assuming that 1 g of secretion corresponds to 1 ml.

**Analytical techniques.** Biliary BS concentrations in bile and feces were determined by an enzymatic fluorimetric assay (11). Lipids were extracted from the bile (12). The phospholipids and cholesterol concentrations were determined using a spectrophotometric assay. Biliary BS composition in bile and feces was determined by capillary gas chromatography (13). The hydrophobicity of BS in bile was calculated according to the Heuman index based on the fractional contribution of the mayor BS species (14).

**Liver histology.** Hemotoxylin and eosin (H&E) sections of the liver were assessed blindly by an expert hepato-pathologist (JP) at The Hospital for Sick Children (Toronto, Canada) for the degree of biliary duct obstruction and inflammation. We applied a previously published numerical scoring system for each parameter, using an arbitrary 0 to 4 scale, in which zero represents normal histology and four represents the most severe pathology for each parameter (7). A minimum of five portal tracts was assessed for each mouse.
RNA isolation from whole livers. Quantitative real time polymerase chain reactions (qRT-PCR, combined for in vivo and in vitro experiments PCR was performed on a 7900HT Fast Real-Time PCR system (Applied Biosystems).

Statistical analysis. Analyses were performed using SPSS version 18.0 for Windows (SPSS Inc., Chicago, IL). All nominal results are reported as means ± SEM. Differences between study groups were evaluated using the Mann-Whitney U test. Bile flow was analyzed by correlation and regression analysis. The categorized histology results were analyzed using cross tabs and Fischer’s exact test. The level of significance was set at a P value of less than 0.05.

RESULTS

Body and liver weights. Figure 1 shows body weight and liver weight of CF and control mice. C57BL/6J Cfr+/+ mice bodyweight was significantly lower compared to control littermates. The liver weight of the Cfr+/+ was significantly less compared to controls, but the difference was less pronounced than for the body weight. Accordingly, the relative liver weight compared to the total bodyweight was significantly higher in the Cfr−/− mice.

Bile production and bile salt secretion. To determine if Cfr plays a role in the magnitude of the bile production in C57BL/6J CF mice in vivo we measured bile production after interruption of the enterohepatic circulation via gall bladder cannulation (Figure 2). Interestingly, we found that the total bile production, was ~20-30% higher in Cfr−/− mice compared to control animals (P<0.05 in the last time period, Figure 2A)
Bile production is largely determined by the bile salt concentration and bile salt secretion rate. The total bile biliary salt concentration was not different between Cftr\textsuperscript{-/-} versus control mice (Figure 2B). The total biliary bile salt secretion rate was not significantly different between Cftr\textsuperscript{-/-} and control mice (Figure 2C), but a consistent tendency towards higher values was observed in the former. To evaluate the magnitude by which bile salts determine bile production in CF conditions we related the bile flow to the bile salt secretion rate. (Figure 2D) In this figure the BA-dependent flow (slope) and BA independent flow (intercept y-axis) can be distinguished. Regression analysis showed a similar BA-independent bile flow between both groups (P= 0.193). In contrast, the BA-dependent flow on the contrary, was higher in Cftr\textsuperscript{-/-} vs. Cftr\textsuperscript{+/+} mice (P= 0.014).

**Figure 2.** Biliary parameters of Cftr\textsuperscript{+/+} and Cftr\textsuperscript{-/-} mice. Biliary bile flow (A), bile salt flow (B) bile salt secretion rate (C) and bile secretion vs. bile flow (D) in Cftr knockout mice (Cftr\textsuperscript{-/-}) and control littermates (Cftr\textsuperscript{+/+}). In panel D there is a significant correlation between BASR and bile flow in Cftr\textsuperscript{+/+} (Spearman’s Rho= 0.394, P=0.042), as well as in Cftr\textsuperscript{-/-} mice (Spearman’s Rho=0.762, P=0.000). Data are presented as means ± SEM of N=9-9 mice per group. *P-value<0.05.
Biliary bile salt composition. Bile salt composition and in particular biliary bile hydrophobicity is related to development of liver disease in general (9). To address the hypothesis that bile salt hydrophobicity plays a role in CFLD we evaluated biliary and fecal bile salt composition. Significant differences in bile salt composition were observed between the genotypes (Figure 3A). In Cfr−/− compared to controls there were significant lowered biliary proportion of CA (40.3±8.5 vs. 53.0±7.5% respectively; p<0.05), DCA (1.6±2.0 vs. 5.3±2.7% respectively; p<0.05) and ω-MCA (0.9±0.5 vs. 6.1± 2.6% respectively; p<0.05) content in bile. Primary BA (produced in the liver: CA, CDCA, α- and β-MCA) content was decreased in Cfr−/− bile compared to Cfr+/+ (70% vs. 84% respectively; p<0.05) with a replacement by secondary BA (HDCA, UDCA, DCA, ω-MCA). (Figure 3C) Gas chromatographic analysis of bile showed an unexpectedly, ~7-fold higher, UCA content in Cfr−/− mice compared to controls (24.6 vs. 3.3% respectively; p<0.01). Mass spectrometry confirmed the identification and abundance of UCA in CF mice. As a result of increased proportion of the hydrophilic UCA the biliary bile salt hydrophobicity index of Cfr−/− mice compared to Cfr+/+ mice was significantly lower vs. (-0.2.5±0.09 vs. -0.13±0.05 respectively; P<0.05; Figure 3C).

Fecal bile salt composition. Increased total fecal bile salt loss has been frequently observed in CF conditions, including by ourselves. In the C57BL/ 6J CF mice model we found that fecal bile salt loss was ~80% higher compared to control animals. (Figure 4A) Corresponding with the biliary bile salt composition results, Cfr−/− mice had a profoundly higher UCA content (27%) in feces than the Cfr+/+ mice (1%, mass spectrometry analysis; Figure 4B) Cfr−/− mice had a
significantly lower DCA (11.4 ± 10.3 vs 42.0 ± 10.7% respectively; P<0.01), HDCA (0.3 ± 0.2 vs. 2.2 ± 0.9% respectively; P<0.01) and ω-MCA (1.2 ± 0.9 vs. 18.1 ± 8.3 respectively; P<0.01) content in their feces. *Cftr<sup>−/−</sup> mice had significantly more CA in their feces, compared to controls (26.5 ± 9.1% vs. 8.4 ± 7.4% respectively; P<0.01).

To address whether the observes differences were genotype or (microbial) environment related, we performed fecal bile salt analysis in mice of the same CF and control genotypes (N=5/5) from a different colony at another institution (courtesy of Dr R.C. De Lisle University of Kansas, Kansas City, Missouri). The pattern and of the bile salt composition, in particular the presence of UCA, was virtually identical, supporting a genotype related effect. (Data not shown)

**Figure 4.** Fecal bile salt excretion and fecal bile salt composition of Cftr<sup>+/+</sup> and Cftr<sup>−/−</sup> mice. Total bile salt excretion (A), presented as means ± SEM. BA profiles (B), data are presented as percentage of different BA species present in bile. Abbreviations: CA = cholic acid, DCA = deoxycholic acid, CDCA = chenodeoxycholic acid, HDCA = hyodeoxycholic acid, UDCA= ursodeoxycholic acid, UCA= ursocholic acid, MCA= muricholic acid. N=9-9 mice per group. *P-value<0.05.
Biliary lipids composition. Apart from bile salt hydrophobicity the biliary cytotoxicity and detergent activity is determined by the biliary lipid composition (15). We found that the phospholipid secretion was not significantly different between Cftr−/− mice and controls (Figure 5A). In both Cftr−/− and controls the phospholipid secretion rate was linearly related to the bile salt secretion rate, despite the significant differences in biliary bile salt hydrophobicity. (Figure 5B). We found no difference in the biliary phospholipid to bile salt ratio between Cftr−/− and control mice (Figure 5C). Additionally we found no difference in biliary cholesterol concentration and cholesterol secretion rate between Cftr−/− and Cftr+/+ mice (data not shown).

Bile salt synthesis genes. Different bile salts have distinct influence in the induction of bile salt synthesis. In particular DCA and CDCA are regarded as strong ligands for Fxr. We found a significantly difference in CA and DCA proportions (Figure 6). This could imply difference in bile synthesis activation between genotypes. To address the issue of potential difference we determined the expression of different bile salt synthesis genes. (Figure 6) We found no difference in expression of Fxr between Cftr−/− and control mice, but the expression of the orphan receptor Shp was significantly lower in the former. We did not observe significant differences in the hepatic expression of Cyp 7a, encoding the rate limiting protein in the bile salt synthesis process.
Chapter 5

**Figure 6.** Gene expression in isolated livers in Cftr\(^{+/+}\) and Cftr\(^{-/-}\) mice. mRNA levels were normalized to a housekeeping gene (β-actin) in Cftr knockout mice (Cftr\(^{-/-}\)) and control littermates (Cftr\(^{+/+}\)). Data are presented as means ± SEM of N=9-9 mice per group. *P-value<0.05.

**Histology.** Durie et al described in their initial report concerning liver pathology in Unc mice that all Cftr\(^{-/-}\) animals showed focal and progressive hepatobiliary disease (7). The mean age of the mice in the current experiment was 75±25 days in both the Cftr\(^{-/-}\) and control group. Although, in general the current histo-pathological findings are consistent with the previous report they are less pronounced and the global duct proliferation score, in this cohort, revealed no significant difference between Cftr\(^{-/-}\) and control mice. (Figure 7)

**Figure 7.** Histological bile duct proliferation score. Bile duct proliferation score base on the observation of 10 portal tracts per mouse graded: normal: 1 point, mild proliferation: 2 points, severe proliferation: 3 points. The bile duct proliferation represents the mean of al scored points per mouse. Data are presented as means ± SEM of N=10-10 mice per group.

**DISCUSSION**

We evaluated bile salt metabolism and bile salt composition and in the C57BL/ 6J CF mice model that has been reported to spontaneously develop CFLD like liver disease (7). We
hypothesized that this $Cftr^{-/-}$ mice develop liver disease in the context of a cytotoxic biliary bile salt profile. However in contrast of what could be expected we found that in $Cftr^{-/-}$ mice on a liquid diet, the biliary bile salt profile is substantially more hydrophilic and the bile flow is increased compared to control mice. This increase in bile production is explained by the increased presence of the secondary hydrophilic and highly choleretic bile salt ursodeoxycholate in this mice model. Since UCA metabolism is converted form cholate by intestinal microbiota our results emphasize the critical role of intestinal microflora in modifying the enterohepatic circulation of bile salt in CF conditions.

Based on hydrophobicity and PI content we did not find an increased cytotoxic profile of the biliary bile of $Cftr^{-/-}$ mice that could explain the reported spontaneous development of biliary liver disease in this model. Others have suggested different potential mechanism for the development of liver disease in the CF mouse models. Blanco et al. reported that chemical induction of colitis, by dextran, in $Cftr^{-/-}$ Unc-mice results in a increase in bile duct injury (16). In an additional report from the same group they describe that decreased peroxisome proliferator activated receptor alpha is associated with bile duct Injury in $Cftr^{-/-}$ mice report (17). These findings point in the direction of a $Cftr$ related difference in immune response to development of liver disease in CF mice. Beharry et al. reported a highly significant protective effect of long-term docosahexaenoic acid therapy on the progression of CF-like hepatobiliary disease in C57BL/6J CF $Cftr^{-/-}$ mice (18). They suggested that the inhibition of inflammation by DHA may account for the anti-inflammatory effects in the liver. The latter report again endorsing a potentially immunologic/inflammatory genesis of CF related liver disease.

UCA, the 7α-hydroxy epimer of cholic acid, is a natural bile acid characterized by sixfold and threefold greater hydrophilicity than chenodeoxycholic and ursodeoxycholic acid, respectively (19). The contribution of UCA to the bile salt pool is usually rather low, due to rapid biotransformation of UCA during enterohepatic circulation into DCA by intestinal microflora. In our $Cftr^{-/-}$ mice, however, DCA contribution was significantly lower than in control mice, suggesting a decreased biotransformation of UCA into DCA in the CF intestine. Due to it high choleretic potency the enrichment of UCA in our model leads to a significant increased bile salt dependent flow and low hydrophobicity index in $Cftr^{-/-}$ mice.

It cannot be excluded that the exclusively liquid feeding could have influenced biliary bile salt composition in our model. The C57BL/6J CF $Cftr^{-/-}$ mice are dependent on liquid feeding after weaning to prevent lethal intestinal obstruction (20). Therefore Peptamen liquid feeding is used. Peptamen is a commercial infant formula use for instance for patients with short bowel syndrome, intractable malabsorption, patients with proven inflammatory bowel disease and bowel fistulae. Peptamen is a semi-elementary formula with a high proportion of protein and medium chain triglycerides. Different from regular chow, Peptamen does not contain dietary fibre or grain products. Since $Cftr^{-/-}$ mice and controls were both fed the liquid Peptamen, diet effects cannot explain the differences in bile salt composition between the genotypes.
In our model we found a distinct increased fecal bile salt excretion. Increased fecal bile salt loss has reported before in CF patients and animals models (21, 22). It has been suggested that the underlying mechanism involves increased fecal fat excretion in CF conditions (23), but previously we reported that increased fecal bile salt loss was also observed in CF mice without fat malabsorption. Increased hepatic bile salt synthesis compensates for the higher bile salt loss in Cftr⁻/⁻ mice (24). However, in the current study, the proportion of primary bile salt in the biliary bile was actually lower in Cftr⁻/⁻ mice, acutely indicating a decreased synthesis of bile salt in these CF mice.

Several publications in recent years have revealed the complex regulatory interaction of bile salt metabolism between intestine and liver (25, 26). Bile salt synthesis in rodents is regulated in the intestine via the Fxr-Fgf15 axes as well as more directly in the hepatocyte, via the Fxr-Shp axis. Both routes eventually target the expression of Cyp7a, the rate limiting enzyme in the bile salt synthesis. UCA is reported to have no or limited influence on bile salt synthesis, in contrast to the hydrophobic DCA, which is one of the most potent FXR ligands to suppress bile salt synthesis (27). The reduction of intestinal transformation of UCA to DCA may have lead to a reduced bile salt synthesis as represented by the reduction in the proportion of primary biliary bile salt. However this finding did not correspond to the Cyp7a expression levels that were not different in Cftr⁻/⁻ mice compared to controls. On the other hand the reported reduction in Shp expression would imply a partial down regulation of bile salt synthesis could be induced via the alternative intestinal Fxr-Fgf15 pathway.

We found that the reported biliary disease in the Unc Cftr⁻/⁻ mice is not related to increased biliary cytotoxicity. However Cftr⁻/⁻ mice shows distinctive changes in the metabolism and enterohepatic circulation of the bile salts. Recent discoveries in the intestinal regulation of bile salt synthesis shed a new light on the importance and role of the intestinal bile salt composition. The reported difference in bile salt metabolism could be origin of the intestinal bile salt loss and in CF condition. However additional research is needed to further determine the role of the enterohepatic circulation in CF.

REFERENCE LIST


Chapter 5

17. Pall H, Zaman MM, Andersson C, Freedman SD. Decreased peroxisome proliferator
activated receptor [alpha] is associated with bile duct injury in cystic fibrosis transmembrane

docosahexaenoic acid therapy in a congenic murine model of cystic fibrosis. American Journal

19. Roda A, Hofmann AF, Mysels KJ. The influence of bile salt structure on self-association in

1992;257(5073):1083.


CFTR by ASBT-mediated bile salt absorption. American Journal of Physiology-Gastrointestinal

23. Weber AM. Relationship between bile acid malabsorption and pancreatic insufficiency in

absorption in cystic fibrosis mice is impeded by defective lipolysis and post-lipolytic events. Am

antibacterial defense in the small intestine by the nuclear bile acid receptor. Proc Natl Acad Sci
U S A. 2006;103(10):3920.

26. Lundåsen T, Gälman C, Angelin B, Rudling M. Circulating intestinal fibroblast growth factor
19 has a pronounced diurnal variation and modulates hepatic bile acid synthesis in man. J

27. Heuman DM, Hylemon PB, Vlahcevic ZR. Regulation of bile acid synthesis. III. correlation
between biliary bile salt hydrophobicity index and the activities of enzymes regulating
cholesterol and bile acid synthesis in the rat. Journal of Lipid Research. 1989 August
01;30(8):1161-71.