CHAPTER 6

URSODEOXYCHOLATE MODULATES BILE FLOW AND BILE SALT POOL INDEPENDENTLY FROM CFTR IN MICE

Based on: American Journal of Physiology-Gastrointestinal and Liver Physiology302.9 (2012): G1035-G1042.

Frank A.J.A. Bodewes[1]

Marjan Wouthuyzen-Bakker[1]

Marcel J. Bijvelds[2]

Rick Havinga[1]

Hugo R. de Jonge[2]

Henkjan J. Verkade[1]

[1] Department of Pediatrics, University of Groningen, Beatrix Children's Hospital - University Medical Center, Groningen, The Netherlands

[2] Department of Gastroenterology & Hepatology, Erasmus University Medical Center, Rotterdam, The Netherlands

ABSTRACT

Introduction

Cystic fibrosis liver disease (CFLD) is treated with ursodeoxycholate (UDCA). Our aim was to evaluate, in Cftr-/- mice and wild type controls, if the supposed therapeutic action of UDCA is mediated via choleretic activity or effects on bile salt metabolism.

Methods

Cftr-/- mice and controls, under general anesthesia, were IV infused with TUDCA in increasing dosage or were fed either standard or UDCA enriched chow (0.5%wt/wt) for 3 weeks. Bile flow and bile composition were characterized. In chow fed mice, we analyzed bile salt synthesis and pool size of cholate (CA).

Results

In both Cftr-/- and controls IV TUDCA stimulated bile flow by ~250% and dietary UDCA by ~500%, compared with untreated animals ($p<0.05$). In non-UDCA treated Cftr-/- mice, the proportion of CA in bile was higher compared to controls (61±4% vs. 46±4%, resp.; $p<0.05$), accompanied by an increased CA synthesis (16±1 vs. 10±2 µmol/hour/100gramBW resp.; $p<0.05$) and CA pool size (28±3 vs. 19±1 µmol/100gramBW, resp.; $p<0.05$). In both Cftr-/- and controls UDCA treatment drastically reduced the proportion of CA in bile below 5% and diminished CA synthesis (2.3±0.3 vs. 2.2±0.4 µmol/day/100gramBW, resp.; NS) and CA pool size (3.6±0.6 vs. 1.5±0.3 µmol/100gramsBW, resp.; $p<0.05$).

Conclusion

Acute TUDCA infusion and chronic UDCA treatment both stimulate bile flow in CF conditions independently from Cftr function. Chronic UDCA treatment reduces the hydrophobicity of the bile salt pool in Cftr-/- mice. These results support a potential beneficial effect of UDCA on bile flow and bile salt metabolism in CF conditions.

INTRODUCTION

Cystic Fibrosis (CF) is caused by mutations in the Cystic Fibrosis Transmembrane conductance Regulator (*Cftr)* gene which result in dysfunction of the CFTR protein (1, 2). *CFTR* is a cAMP activated chloride channel and is present in the apical membrane of various epithelial and non-epithelial tissues (3-5). Cirrhotic Cystic Fibrosis Liver Disease (CFLD) develops in ~5-10% of CF patients and is the second leading cause of mortality in CF patients (6-8). Absence of functional CFTR in biliary epithelium is thought to initiate abnormal secretin/cAMP-stimulated chloride and bicarbonate secretion, leading to decreased bile flow and bile duct plugging by thickened secretions. Secondary cholangiocyte and hepatocyte injury can ultimately lead to the development of cirrhosis (9).

Treatment with ursodeoxycholate (UDCA) is applied for different cholangiopathies, including CFLD. Although routinely applied in CF patients with increased levels of liver function parameters in serum, the therapeutic action of UDCA in CF conditions remains unclear. It has been hypothesized that UDCA is beneficial by its choleretic activity and/or its capacity to correct aberrant bile salt metabolism (10). Indeed, it has been shown that UDCA stimulates biliary secretion of bile acids in patients with primary biliary cirrhosis and primary sclerosing cholangitis (11, 12). However, there are only a few trials assessing the efficacy of UDCA for the treatment of CFLD and currently, there is insufficient evidence to justify the routine use of UDCA in CF (13).

Shimokura et al. demonstrated in biliary cells that UDCA, in pharmacological concentrations, increased intracellular calcium and induced chloride efflux. These scientists speculated that UDCA increased bile flow by direct stimulation of ductular secretion, what could be of therapeutic benefit for patients with CF who have impaired cyclic AMP-dependent biliary secretion (14). However, Fiorotto et al. suggested that UDCA induced bile flow in a *Cftr* dependent manner (15). They showed that UDCA stimulated cholangiocytic fluid secretion *in vitro* in bile duct units and in isolated perfused livers from wild type, but not from *Cftr* knockout mice. UDCA induced a *Cftr*-dependent ATP release, which, by activating the purinergic signaling pathway, induced cholangiocyte secretion by stimulation of calcium-activated chloride channels. These *in vitro* and *ex vivo* observations clearly supported the concept that UDCA-induced fluid secretion is *Cftr* dependent and that, in CF conditions, UDCA induced cholangiocytic choleresis can't be achieved.

An alternative beneficial effect of UDCA in CF conditions could be related to changes in bile salt metabolism. It is known, that the size and composition of the bile salt pool is critical for adequate bile formation(16). In addition, a more hydrophobic bile salt composition is suggested to be toxic to the hepatobiliary tract and might contribute to the development of CFLD (17). Freudenberg et. al reported an increased biliary hydrophobicity index in $Cftr^{508/508}$ mice compared to controls. Different effects are published on the influence of UDCA on bile salt metabolism. Frenkiel et al. reported that UDCA reduced the cholate pool size and cholate

synthesis rate in patients with gallstones and decreased the hydrophobicity of bile (18). In contrast, Beuers et al. described that UDCA did not decrease the bile salt pool size in patients with cholestatic liver disease (19). In UDCA treated patients with CFLD, duodenal bile became enriched with the conjugated species of UDCA (accounting for 12% to 3% of the total biliary bile salts), indicating an increased hydrophilic bile composition (20). Thus far, the effect of UDCA on bile salt metabolism under CF conditions has not been conclusive.

We have reasoned that insights in the effects of UDCA on bile production, cholate biosynthesis rate and cholate pool size under controlled *in vivo* CF conditions have been lacking. In the present study we aimed to overcome this knowledge gap by exploiting recently developed techniques allowing the study of these parameters in small experimental animals (21, 22). We determined the effect of UDCA on bile flow and bile salt metabolism in *Cftr* knockout mice and wild type littermates during acute intravenous tauroursodeoxycholate (TUDCA) infusion and after chronic dietary administration of UDCA. Although biliary phenotypes have been described in different CF mouse models the C57Bl/6;129 $Cftr^{-/-\ tm1CAM}$ mouse model we use does not exhibit CF related gallbladder or liver abnormalities (23-25). The lack of hepatobiliary abnormalities allows us to exclusively investigate the action UDCA under complete *Cftr* null conditions, without the secondary interference of cholestasis on biliary parameters.

MATERIAL AND METHODS

Animals and diets. C57Bl/6;129 $Cftr^{-/-\ tm1CAM}$ mice and $Cftr^{+/+tm1CAM}$ littermates were bred and accommodated at the Animal Experimental Center of the Erasmus Medical Center in Rotterdam, The Netherlands. Southern blotting of tail-clip DNA was performed to verify the genotype of individual animals (25). In accordance with previous studies, the $Cftr^{-/-}$ mice did not exhibit CF liver or gallbladder abnormalities (data not shown). Mice were housed in a light-controlled (lights on 6 AM to 6 PM) and temperature-controlled (21^{o}C) facility, and were allowed access to tap water and a semi-synthetic chow diet (SRM-A; Hope Farms BV Woerden, The Netherlands) from the time of weaning. All experiments were performed on female and male animals of 10-20 weeks of age. Group size varied per experiment from 5-9 animals per genotype. Experimental protocols were approved by the Ethical Committee for Animal Experiments of the Erasmus Medical Center.

Experimental procedures. To evaluate the effect of UDCA on bile production and bile composition, we used a mouse bile duct cannulation experimental model. This model provides the option to measure bile production over an extended period of time. The animals were placed in a temperature and humidity controlled incubator. Bile was collected after surgical ligation of the common bile duct and cannulation of the gallbladder using polyethylene tubing under intraperitoneal anesthesia with hypnorm (fentanyl/fluanisone 1 µl/gBW) and diazepam (10 µg/gram BW), as previously described (26). Bile secretions were

collected in 15 minute fractions during the stepwise dose increase phase and in 10 minutes fraction at the highest dose (600 nmol.min^{-1}) for 60 minutes. Bile samples were used for bile salt composition analysis. Bile flow rate was assessed gravimetrically, assuming a density of 1g/ml.

Acute intravenous TUDCA administration. We performed an acute bile salt infusion experiment to evaluate the dose-response effect of acute UDCA supplementation on bile flow and bile salt composition in *Cftr*$^{-/-}$ and control mice. Acute TUDCA administration provides the possibility to measure the direct choleretic effects of bile salts without the possible interfering effects of adaptations to long-term bile salt administration. We infused taurine-conjugated UDCA (TUDCA) for our acute infusion experiment, to closely mimic the physiological condition in which bile salts are secreted into bile almost exclusively in conjugated form. An intravenous line was placed in the jugular vein and the gallbladder was cannulated. After equilibration of the bile flow for 5-10 minutes, spontaneous bile production was assessed for 30 minutes, i.e. without TUDCA infusion. Subsequently, TUDCA solution (43 mM dissolved in phosphate-buffered saline, pH 7.4) was administered using an IV pump(27). The TUDCA dosage was increased every 30 minutes in a stepwise manner (dosage steps 150, 300, 450 and 600 nmol/min). The maximal dosage was given for 60 minutes. During TUDCA infusion, bile was collected in 15 minute- fractions.

Chronic dietary UDCA administration. In CF patients, UDCA supplementation is given chronically via the enteral route (28). Analogous to the human situation, we evaluated the effect of chronic enteral (dietary) UDCA treatment in *Cftr*$^{-/-}$ and control mice compared to untreated animals. Mice were fed either a control diet consisting of standard chow or the same diet enriched with UDCA (0.5% wt/wt) for 3 weeks. Body weight was measured after the diet period. After gallbladder cannulation, spontaneous bile production was determined by bile collection for 30 minutes. We additionally determined cholate synthesis and pool size, using a previously developed and validated stable isotope dilution technique (22). In short, 3.0 mg of [$^2H_4$]-cholate in a solution of 0.5% $NaHCO_3$ in phosphate-buffered saline was slowly injected via the penile vein during isoflurane anesthesia. Blood samples were taken before injection and at 12, 24, 36, 48, 60, and 72 h after injection. Blood samples (100 µl) were collected by tail bleeding. Blood was collected in EDTA tubes and centrifuged to obtain plasma. After centrifuging (3,000 rpm for 10 min at 4°C), plasma was stored at –20°C until analysis. At the last day of the experiment (72 h), mice were anesthetized and equipped with a catheter in the bile duct as described above. Subsequently, bile was collected for 30 min, after an initial equilibration period of 5-10 min. Animals were euthanized by heart puncture.

Analytic procedures. Biliary bile salt concentrations were determined by an enzymatic fluorimetric assay (29). Biliary bile salt composition was determined by capillary gas chromatography (30). The hydrophobicity of bile salts in bile was calculated according to the Heuman index based on the fractional contribution of the major murine bile salt species cholate, chenodeoxycholate, deoxycholate, ursodeoxcholate, α-muricholate and β-

muricholate (31). Alanine transaminase (ALT) was determined in plasma samples. Plasma and bile samples were prepared for GLC-MS analysis on a Finnigan SSQ7000 Quadrupole GC-MS machine as described previously by Stellaard et al. (32). The isotope dilution technique is based on the dilution of a labeled tracer into the pool of the metabolite of interest. It has been demonstrated to result in virtual identical cholate synthesis rates as obtained with a ^{14}C-cholesterol bile salt synthesis measuring methods (33). The tracer is administered as a bolus, which mixes into the pool. Shortly after mixing, the isotopic enrichment is highest. Thereafter, the enrichment decreases due to dilution with unlabelled molecules introduced by de novo synthesis. Enrichment of $^{2}H_4$-cholate in plasma was determined as the increase of the M_4-/M_0-cholate, relative to baseline measurements and was expressed as the natural logarithm of atom percent excess (ln APE). From the decay curve of ln APE (calculated by linear regression analysis), daily fractional turnover rate (FTR; equals the slope of the regression line) and pool size ([administered amount of label x isotopic purity x 100] / intercept of the y-axis of the ln APE curve) of cholate were calculated. Multiplying the pool size with the FTR results in a value for the absolute turnover rate. In the steady-state situation, the absolute turnover rate equals the synthesis rate. (22, 33, 34).

Statistical analysis. Statistical analysis was performed using SPSS version 16.0 for Windows (SPSS Inc., Chicago, IL). All results are reported as means±SEM. Differences between genotypes or diet groups were evaluated using the Mann-Whitney U test. The level of significance was set at a P value of less than 0.05.

RESULTS

The physiological state of the enterohepatic circulation in Cftr$^{-/-}$ mice. We evaluated bile production during the first 30 min after acute interruption of the enterohepatic circulation, i.e. closely reflecting the physiological state (represented by time points 15 and 30 minutes in Figure 1A-C). We found that bile flow (4.5±0.6 vs. 4.0±0.4 µl/min/100gram BW, NS), biliary bile salt concentration (67±12 vs. 54±5 mM, NS) and biliary bile salt output (313±72 vs. 208±25 nmol/min/100gram BW, NS) were similar in *Cftr$^{-/-}$* and control mice respectively, indicating no quantitative differences in te choleretic capacity between *Cftr$^{-/-}$* mice and their controls at baseline.

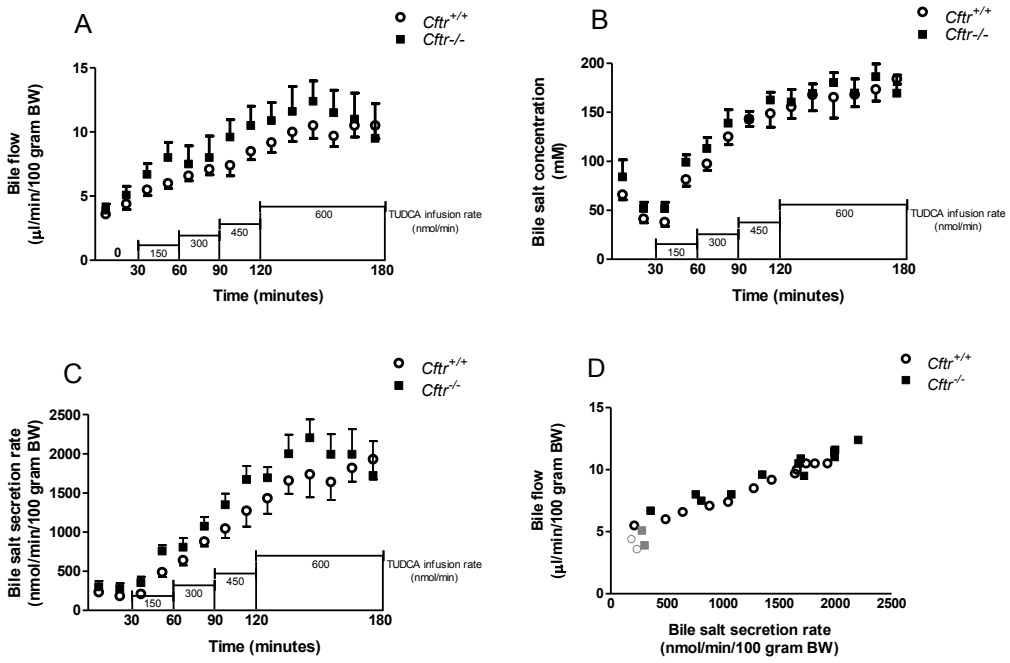

Figure 1 *Biliary parameters during acute intravenous TUDCA administration. Biliary bile flow (A), bile salt concentration (B), bile salt secretion rate (C) and relationship between bile salt secretion rate and bile flow (D) in Cftr knockout mice (Cftr$^{-/-}$) and control littermates (Cftr$^{+/+}$) during acute intravenous infusion with TUDCA. The dosage of TUDCA was increased at indicated intervals. The grey symbols in Fig. D represent baseline values without TUDCA infusion. Data are presented as means ± SEM of N=5 mice per group. There was no significant difference between Cftr$^{-/-}$ and Cftr$^{+/+}$ mice, at any of the individual time points, for bile flow, bile salt concentration and bile salt secretion rate.*

TUDCA administration increased bile flow equally in Cftr$^{-/-}$ and control mice in a dose-dependent manner. Infusions with TUDCA increased bile flow to a similar extend in *Cftr$^{-/-}$* mice

and controls (+ ~250%; Figure 1A). The results indicate that TUDCA is capable of generating a bile salt induced bile flow independent of the expression of *CFTR*. The biliary bile salt concentration increased in parallel to the infused bile salt dosage (Fig. 1B). The bile salt secretion rates, i.e. the product of flow (Figure 1A) and concentration (Figure 1B), increased in equal pace with an increased TUDCA IV dosage and was not different between *Cftr*$^{-/-}$ and control mice (Figure 1C). We performed Mann–Whitney U tests for all individual time point during the TUDCA infusion experiments. There was no significant difference between *Cftr*$^{-/-}$ and *Cftr*$^{+/+}$ mice at any of the individual time points for bile flow, bile salt concentration and bile salt secretion rate. To determine the choleretic capacity of TUDCA in both *Cftr*$^{-/-}$ and control mice we related the bile flow to the bile salt secretion rate (Figure 1D) The choleretic capacity of TUDCA was similar in *Cftr*$^{-/-}$ and control mice, as was the estimated bile salt dependent bile flow, based on the Y-intercepts (4.6 vs. 5.3 µl/min/100gram BW, respectively, NS). The increase in bile flow was linearly related to the bile salt output (control: R^2 0.9, slope 0.003 µmol/nmol; *Cftr*$^{-/-}$ R^2 0.9, slope 0.003 µmol/nmol).

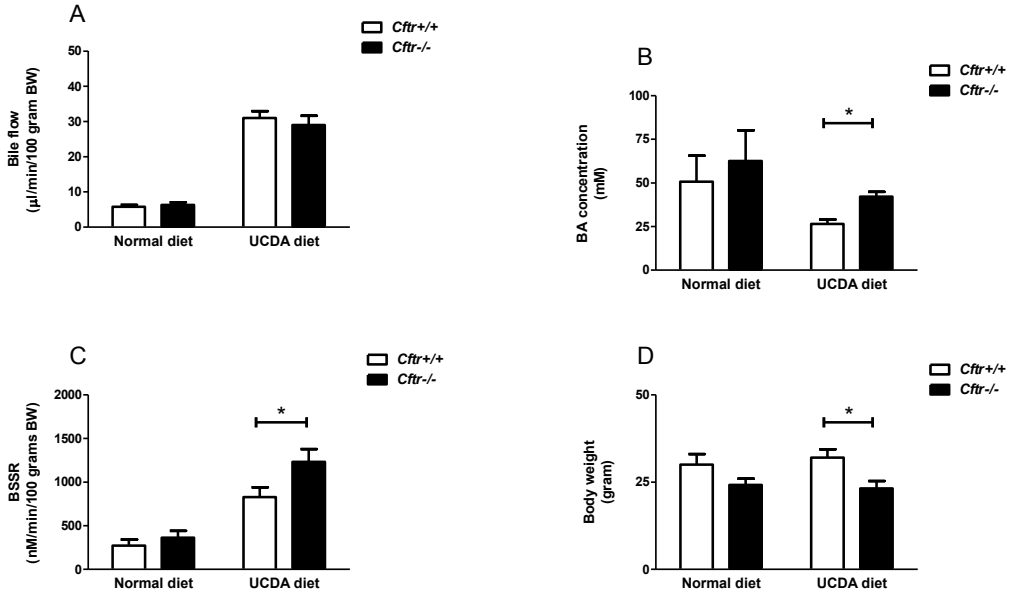

Figure 2 *Biliary parameters after chronic dietary UDCA administration. Biliary bile flow (A), bile salt secretion (B) bile salt secretion rate (C) body weight (D) in Cftr knockout mice (Cftr*$^{-/-}$*) and control littermates (Cftr*$^{+/+}$*) after a normal or 0.5%-UDCA chow diet for 3 weeks. Data are presented as means ± SEM of N=5-6 mice per group. *P-value<0.05.*

UDCA treatment increased bile flow equally in Cftr$^{-/-}$ *and control mice.* Chronic UDCA treatment for 3 weeks increased bile flow with ~500% when compared to untreated mice ($p<0.05$). This increase was comparable for *Cftr*$^{-/-}$ and control mice (29.0±2.6 vs. 31.0±1.9

µl/min/100gram BW, resp.; NS; Figure 2A). Chronic UDCA treatment decreased the total biliary bile salt concentration in both genotypes, but to a lower extent in *Cftr*$^{-/-}$ mice (*Cftr*$^{+/+}$ -51% vs. *Cftr*$^{-/-}$ mice -37%), resulting in significantly higher BS concentration in *Cftr*$^{-/-}$ mice compared to controls (42±3 vs. 26±3 mM, resp., p<0.05, Figure 2B). The bile salt output was significantly higher in *Cftr*$^{-/-}$ mice compared with controls during chronic UDCA treatment (*Cftr*$^{-/-}$, 1232±147 vs. control, 827±113 µmol/min/100gram BW, resp.; p<0.05, Figure 2C). A significant reduced growth of the Cambridge *Cftr*$^{-/-}$ mice compared to wild type animals has been reported (25). Since the findings of the differences in the bile composition on treatment with UDCA may be affected by the nutritional status we measured body weight of the treated and untreated mice (Figure 2D). In our current experiment the phenotype of the *Cftr*$^{-/-}$ mice includes a decreased body weight compared to controls, however this is not affected by UDCA treatment.

Increased hydrophobic bile salt composition of Cftr$^{-/-}$ *mice compared to control mice*. In non-UDCA treated *Cftr*$^{-/-}$ mice, the fractional biliary cholate content was higher compared with control mice (61.4±1.5% vs. 46.5±3.8%, resp.; p<0.05; Table 1.) The natural biliary UDCA enrichment was ~50% lower in *Cftr*$^{-/-}$ mice compared with controls (2.7±0.4 vs. 6.0±0.5% resp.; p<0.05; Fig. 2A). In non UDCA treated mice β-muricholate is the major hydrophilic biliary bile salt in both *Cftr*$^{-/-}$ and control mice (42±3,2 vs. 33±1,6% resp.; NS). Based on the fractional contribution of the bile salt we calculated the biliary hydrophobicity index, according to Heuman et al (14). The bile salt composition of non-UDCA treated *Cftr*$^{-/-}$ mice was significantly more hydrophobic than the control mice (0.05±0.002 vs. -0.07±0.005 resp.; p<0.01; Figure 3B).

Biliary bile salt profile: proportional contribution of the major murine bile salt species

	Non-UDCA treatment		UDCA treatment	
	$Cftr^{+/+}$	$Cftr^{-/-}$	$Cftr^{+/+}$	$Cftr^{-/-}$
CA	46.5 ± 3.8	61.5 ± 1.5*	2.7 ± 0.9†	4.0 ± 1.3†
CDC	3.4 ± 0.4	2.1 ± 0.3	2.8 ± 0.5	1.3 ± 0.2*
DC	1.7 ± 0.4	0.7 ± 0.4	ND	ND
UDCA	6.0 ± 0.5	2.7 ± 0.4*	83.0 ± 2.7†	82.0 ± 1.9†
α-M	ND	ND	3.3 ± 0.3†	2.5 ± 0.3†
β-M	42.0 ± 3.2	33.0 ± 1.6	6.5 ± 1.8†	8.7 ± 1.6†

Table 1. *Values are means ±SE in percent; N 4–6 mice per group. Biliary bile salt composition is shown of the mayor bile salt species [cholate (CA), chenodeoxycholate (CDC), deoxycholate (DC), ursodeoxycholate (UDCA),α-muricholate (α-M), and ß-muricholate (ß-M)] in cystic fibrosis transmembrane regulator (Cftr) knockout mice ($Cftr^{-/-}$) and control littermates ($Cftr^{-/-}$) after a normal or 0.5% UDCA chow diet for 3 wk. ND, not detectable. Mann-Whitney U-test: *P< 0.05, difference between genotype ($Cftr^{-/-}$ vs. $Cftr^{-/-}$); †P <0.05, difference between treatment group (non-UDCA vs. UDCA).*

UDCA treatment decreased the biliary hydrophobicity of $Cftr^{-/-}$ mice. After UDCA treatment, the biliary bile salt composition changes extensively (Table 1.) After treatment the bile salt composition consisted for more than ~80% of UDCA in both *$Cftr^{-/-}$* mice and controls (Figure 3A). UDCA treatment drastically reduced the fraction of cholate in the bile to below 5% in both *$Cftr^{-/-}$* mice and controls. However, the fraction of β-muricholate was also reduced in *$Cftr^{-/-}$* and controls mice compared to the untreated animals (6.5±1.8 vs. 8.7±1.6% resp.; NS). Taken together, UDCA treatment decreased the bile salt hydrophobicity index of *$Cftr^{-/-}$* mice to wild type levels (-0.08±0.003 vs. -0.08±0.002 resp.; NS; Figure 3B). To evaluate the potential hepatotoxic effects of UDCA and differences in biliary bile hydrophobicity we measured plasma ALT levels in during normal diet and in UDCA treated animals (Figure 3C). Under control diet conditions, although not significant, ALT was higher in CF than in control mice (40±8 vs. 60±13 U/l resp.; NS). UDCA decreased ALT both in CF and control mice (31±9 vs.

15±1 U/l resp.; NS). UDCA treatment significantly lowered plasma ALT levels in *Cftr*$^{-/-}$ mice compared to *Cftr*$^{-/-}$ on control diet (15±1 vs. 60±13 U/l resp.; p<0.05).

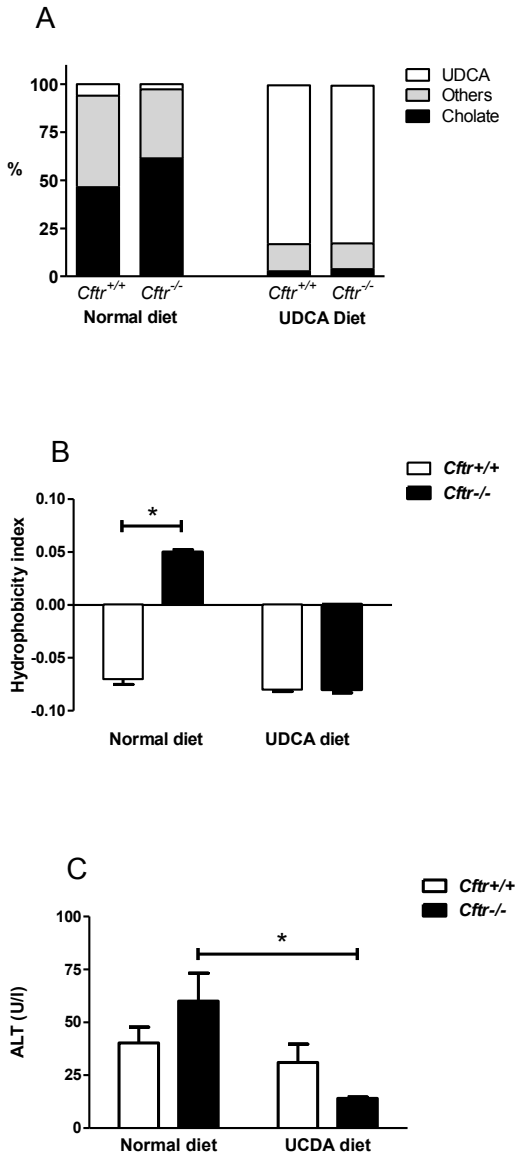

Figure 3 *Bile salt composition after chronic dietary UDCA administration. (A) Percent contribution of the bile salts cholate, ursodeoxcholate and others (chenodeoxycholate, deoxycholate, α-muri cholate and β-murichолate) in bile, (B) Heuman index of total bile salts in bile representing the hydrophobicity of bile salts, (C) Alanine aminotransferase (ALT) in plasma of Cftr*$^{-/-}$ *and control littermates (Cftr*$^{+/+}$*) after a normal or 0.5%-UDCA chow diet for 3 weeks. Data are presented as means ± SEM of 4-9 mice per group. *P-value<0.05.*

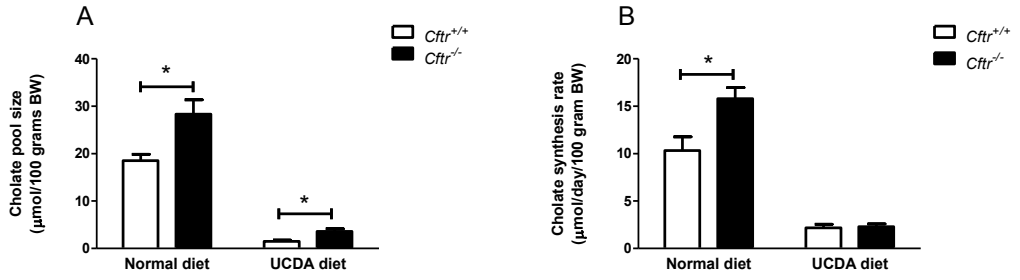

Figure 4 *Cholate pool size and cholate synthesis after chronic dietary UDCA administration. Cftr knockout mice ($Cftr^{-/-}$) and control littermates ($Cftr^{+/+}$) were intravenously injected with $^2H_4$-cholate after a normal or 0.5%-UDCA chow diet for 3 weeks Enrichment of the administered $^2H_4$-cholate was determined in plasma until 72 hours after the administered label. From the plasma decay curve, cholate pool size (A) and cholate synthesis rate (B) were calculated, as detailed in the Materials and Methods. Data are presented as means ± SEM of 4-6 mice per group. *P-value<0.05.*

UDCA treatment decreased cholate synthesis and pool size in $Cftr^{-/-}$ and control mice. Non-UDCA treated *$Cftr^{-/-}$* mice had a higher cholate synthesis rate (16±1 vs. 10±2 µmol/day/100 gram BW resp.; p<0.05) and larger cholate pool size (28±3 vs. 19±1 µmol/100 gram BW, resp.; p<0.05) compared to controls (Figure 4).

Chronic UDCA treatment reduced the cholate pool size by ~90% in both *$Cftr^{-/-}$* and control mice (p<0.05). Nevertheless, the pool size of *$Cftr^{-/-}$* mice remained higher compared with controls (3.6±0.6 vs. 1.5±0.3 µmol/100 grams BW, resp.; p<0.05). Under the assumption that steady state conditions of the bile salt kinetics were obtained after 3 weeks of treatment we found that UDCA decreased the cholate synthesis rate by ~85% in both *$Cftr^{-/-}$* mice and controls compared to untreated mice (p<0.05; Fig. 4B). Interestingly, UDCA treatment straightened out the difference in synthesis rate between *$Cftr^{-/-}$* and control mice (2.3±0.3 vs. 2.2±0.4 µmol/hour/100 gram BW, resp.; NS).

DISCUSSION

The major physiologic effect of UDCA is its capacity to increase bile flow. This property supports the therapeutic use of UDCA in a variety of cholangiopathies, including CFLD (35). *In vitro* and *ex vivo* studies indicated that the stimulatory of UDCA on cholangiocyte secretion depends on the presence of *CFTR* (14, 15). In the present study, we demonstrated that UDCA, *in vivo*, either during acute or chronic administration, induced a significant *Cftr* independent increase of bile flow in mice. Therefore, our results indicate that a positive choleretic effect of UDCA can also to be expected in CF conditions. UDCA treatment reduced the relative

hydrophobic biliary bile salt composition in *Cftr*$^{-/-}$ mice by replacing the high percent contribution of the bile salt cholate by UDCA and by the quantitative reduction of the cholate pool size. These properties could contribute to the assumed beneficial effects of UDCA in CFLD.

Our present *in vivo* results are in apparent contrast with *in vitro* and *ex vivo* studies, which report on the interaction between *Cftr* and bile salt stimulated biliary secretion (14, 15). In these studies, an important role is ascribed to the function of calcium induced chloride channels in UDCA stimulated bile flow, through the induction of purinergic signaling via *Cftr* dependent ATP release. There can be several possibilities underlying the divergence of our *in vivo* results from the *in vitro* and *ex vivo* reported studies. First, the choleretic effect found in our *in vivo* studies probably predominantly reflects an osmotic, bile salt induced canalicular bile flow, rather than a major ductular bile flow. The canalicular bile flow may thereby predominate the *Cftr* dependent secretion effect of UDCA at the level of the cholangiocytes. Second, the activation of *Cftr* independent routes may differ between the *in vitro* and *in vivo* conditions. *In vivo*, UDCA may stimulate hepatocytes to secrete ATP into bile in a *CFTR*-independent manner and subsequently induce bicarbonate secretion via paracrine purinergic pathways linked to calcium-activated chloride channels (CaCC) in the cholangiocytes (36). CaCCs have been suggested to play a more prominent role in epithelial fluid secretion in mice than in other species, including pigs (37). This might therefore explain the relatively mild phenotype in CF mouse models (38). Recently, Beuers et al. postulated the “bicarbonate umbrella hypothesis”, by suggesting that biliary bicarbonate secretion in humans serves to maintain an alkaline pH near the apical surface of hepatocytes and cholangiocytes in order to prevent the uncontrolled membrane permeation of protonated glycine-conjugated bile(39). In this concept, the bile acid receptor TGR5 (GPBAR-1), localized on the tip of the cilia of apical cholangiocyte membranes in mice and humans could trigger a Cftr independent signaling cascade resulting in cholangiocyte secretion.

During chronic UDCA administration, a CF biliary phenotype became apparent: an increased bile salt secretion rate (Fig. 2C, $p<0.05$). The difference in secretion rate is based on the product of two measured parameters. Since the majority of biliary bile salts are derived from enterohepatic circulation, the most plausible explanation is an increased total BS pool size, which during treatment is predominantly accounted for by UDCA (Fig 3A). It seems therefore logical to assume that the total amount of bile salts in the enterohepatic circulation is increased in CF mice. Indeed, this explanation seems to be supported by the ~50% higher cholate pool size in CF mice fed a normal diet (Figure 5).

During bile salt treatment, however, CF and control mice were administered the same, supraphysiological dosages of TUDCA and UDCA in the acute and chronic experiment, respectively. We previously reported an increased fecal loss of bile salts in the *Cftr*$^{-/-}$ mice (40). Although UDCA could potentially influence intestinal fat malabsorption we could not find an effect of UDCA treatment on the body weight in *Cftr*$^{-/-}$ or control mice. Furthermore we

recently reported in rats that UDCA does not influence fecal fat excretion(41). Therefore, our results suggest a different bile salt kinetic steady state in CF mice, in which overcompensation of bile salt synthesis results in an enlargement of the bile salt pool. Since the higher bile salt secretion rate in CF mice was seen most prominently during chronic treatment, we speculate that *CFTR* is involved in adaptation of the enterohepatic circulation to chronic bile salt treatment, either at the level of the intestine, of the liver, or both.

Although differences exist in bile salt metabolism between mice and man (42), we found clear similarities between CF patients and *Cftr*$^{-/-}$ mice in bile composition. Untreated *Cftr*$^{-/-}$ mice (i.e. without UDCA treatment) have a more hydrophobic bile salt pool composition and an increased cholate synthesis rate and pool size compared to control mice. This is in line with the report by Freudenberg et al of the increased biliary hydrophobicity in their *Cftr*$^{508/508}$ mice model. Similar results have been found in CF patients: Strandvik et al. reported an increased proportion of primary bile salts in serum and bile of CF, which has been attributed to an enhanced bile salt biosynthesis in response to increased fecal bile salt disposal (43). The *Cftr*$^{-/-}$ mice used in the present study are therefore comparable to human CF patients in this respect.

The relatively hydrophobic bile salt composition in CF has been implied in the development of liver disease in CF conditions, but definitive proof for this concept is (still) lacking (44). Nevertheless, chronic UDCA treatment is apparently capable to partially correct the hydrophobic bile salt profile in CF mice. Additionally UDCA treatment normalized the initially increased liver function tests in CF mice, in agreement with our hypothesis.

We investigated the effect of UDCA under CF conditions in the absence of CF-related liver disease (CLFD). The advantage of this approach is the ability to exclusively examine UDCA effects on several bile salt parameters during *CFTR* deficient conditions, without possible interference of secondary changes due to liver disease. The major induction of bile flow and alterations in bile salt composition could contribute to a preventive role of UDCA on the development of CLFD. It is unclear whether effects on bile flow and bile salt composition can be found under conditions of CFLD. Nakagawa et. al. evaluated duodenal bile salt composition after a two month UDCA-treatment in nine CF patients with CFLD (45). Similar to our results, the percent contribution of UDCA increased, resulting in a more hydrophilic bile salt pool. The contribution of UDCA was not as high as in our study (25%, compared with ~80% in the present study), possibly due to the relatively low dosage of UDCA that was used (10-15 mg/kg/BW/day). In patients with primary sclerosing cholangitis UDCA treatment dosage of 25-30 mg/kg/day resulted in UDCA comprising 74% or the bile salt pool, with a corresponding cholate reduction from 29% to 6% (46). In addition, a reduction in cholate pool size and cholate synthesis is also described in gallstone patients treated with 750 mg UDCA (47). Therefore, regarding the effect of UDCA on bile composition, we have no indication that the observed effects will be absent during CF-related cholestasis or other signs of CFLD.

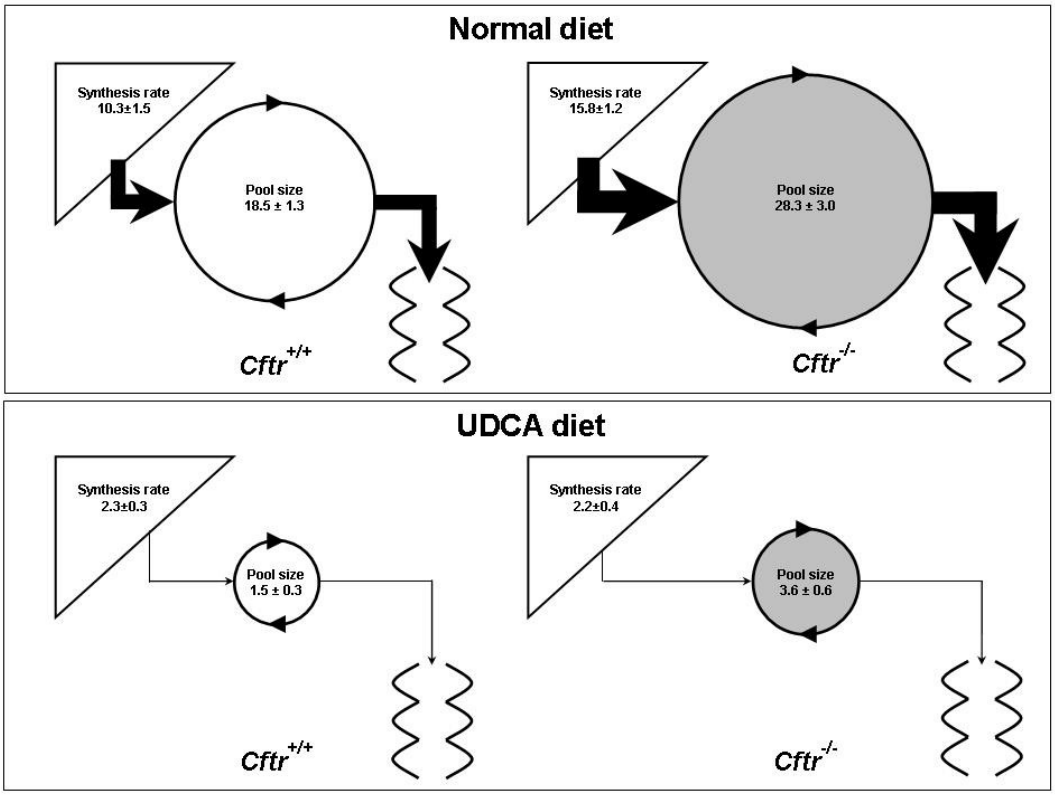

Figure 5 *Schematic representation of Cftr dependent effects of UDCA treatment on bile salt kinetics. Schematic representation of the enterohepatic circulation of the primary bile salt cholate in Cftr knockout mice (Cftr$^{-/-}$) and control littermates, either untreated (top) or treated for 3 weeks with a 0.5%-UDCA chow diet for 3 weeks. Cholate undergoes enterohepatic cycling (dotted lines). In the liver bile salt are excreted via the bile into the intestine, and then almost completely reabsorbed at the level of the terminal ileum. Under steady state conditions the fecal cholate loss is compensated for by de novo cholate synthesis in the liver as represented by the width of the black arrows coming from the liver (µmol/day/100 gram BW), maintaining the total cholate pool size in equilibrium. The diameters of the circles represent the magnitude of the pool size (µmol/100 gram BW). Cholate synthesis rate and pool size are determined as detailed in the Materials and Methods. Data are presented as means ± SEM of 4-6 mice per group.*

In conclusion, our results in mice in vivo indicate that UDCA exerts a choleretic effect and influences the bile salt profile and synthesis, independent of the presence of functional CFTR. When extrapolated to the human situation, this might imply that UDCA treatment results both in CF and in non-CF individuals in increased choleresis, reduced bile salt synthesis and a more hydrophilic bile salt pool. Interpretation of the present results for the human (CF) condition needs to take into account the possibility of species specificity of the observed effects. However the outcome of this study does provides a firm experimental basis to explain the beneficial effects of UDCA observed in CF patients

REFERENCE LIST

1. Collins FS. Cystic fibrosis: Molecular biology and therapeutic implications. Science. 1992 05/08;256(5058):774-9.

2. Kerem B, Rommens JM, Buchanan JA, Markiewicz D, Cox TK, Chakravarti A, et al. Identification of the cystic fibrosis gene: Genetic analysis. Science. 1989 09/08;245(4922):1073-80.

3. Stutts MJ, Canessa CM, Olsen JC, Hamrick M, Cohn JA, Rossier BC, et al. CFTR as a cAMP-dependent regulator of sodium channels. Science. 1995 08/11;269(5225):847-50.

4. Lamhonwah AM, Bear CE, Huan LJ, Chiaw PK, Ackerley CA, Tein I. Cystic fibrosis transmembrane conductance regulator in human muscle: Dysfunction causes abnormal metabolic recovery in exercise. Ann Neurol. 2010;67(6):802-8.

5. Li H, Ganta S, Fong P. Altered ion transport by thyroid epithelia from CFTR−/− pigs suggests mechanisms for hypothyroidism in cystic fibrosis. Exp Physiol. 2010;95(12):1132-44.

6. Lindblad A, Hultcrantz R, Strandvik B. Bile-duct destruction and collagen deposition: A prominent ultrastructural feature of the liver in cystic fibrosis. Hepatology. 1992 08;16(2):372-81.

7. Colombo C, Battezzati PM, Crosignani A, Morabito A, Costantini D, Padoan R, et al. Liver disease in cystic fibrosis: A prospective study on incidence, risk factors, and outcome. Hepatology. 2002 12;36(6):1374-82.

8. Nash KL, Allison ME, McKeon D, Lomas DJ, Haworth CS, Bilton D, et al. A single centre experience of liver disease in adults with cystic fibrosis 1995-2006. J Cyst Fibros. 2008 05;7(3):252-7.

9. Fitz JG, Basavappa S, McGill J, Melhus O, Cohn JA. Regulation of membrane chloride currents in rat bile duct epithelial cells. J Clin Invest. 1993 01;91(1):319-28.

10. Hofmann AF. Biliary secretion and excretion in health and disease: Current concepts. Ann Hepatol. 2007 01;6(1):15-27.

11. Lindor K, Therneau T, Jorgensen R, Malinchoc M, Dickson E. Effects of ursodeoxycholic acid on survival in patients with primary biliary cirrhosis. Gastroenterology. 1996;110(5):1515-8.

12. Lindor K, Jorgensen R, Anderson M, Gores G, Hofmann A, LaRusso N. Ursodeoxycholic acid and methotrexate for primary sclerosing cholangitis: A pilot study. Am J Gastroenterol. 1996;91(3):511-5.

13. Cheng K, Ashby D, Smyth R. Ursodeoxycholic acid for cystic fibrosis-related liver disease. Cochrane Database Syst Rev. 2000(2):CD000222.

14. Shimokura GH, McGill JM, Schlenker T, Fitz JG. Ursodeoxycholate increases cytosolic calcium concentration and activates cl- currents in a biliary cell line. Gastroenterology. 1995 09;109(3):965-72.

15. Fiorotto R, Spirli C, Fabris L, Cadamuro M, Okolicsanyi L, Strazzabosco M. Ursodeoxycholic acid stimulates cholangiocyte fluid secretion in mice via CFTR-dependent ATP secretion. Gastroenterology. 2007 11;133(5):1603-13.

16. Hofmann AF. Chemistry and enterohepatic circulation of bile acids. Hepatology. 1984;4(S2):4S-14S.

17. Benedetti A, Alvaro D, Bassotti C, Gigliozzi A, Ferretti G, La Rosa T, et al. Cytotoxicity of bile salts against biliary epithelium: A study in isolated bile ductule fragments and isolated perfused rat liver. Hepatology. 1997;26(1):9-21.

18. Frenkiel PG, Lee D, Cohen H, Gilmore C, Resser K, Bonorris G, et al. The effect of diet on bile acid kinetics and biliary lipid secretion in gallstone patients treated with ursodeoxycholic acid. Am J Clin Nutr. 1986;43(2):239.

19. Beuers U, Spengler U, Zwiebel FM, Pauletzki J, Fischer S, Paumgartner G. Effect of ursodeoxycholic acid on the kinetics of the major hydrophobic bile acids in health and in chronic cholestatic liver disease. Hepatology. 1992;15(4):603-8.

20. Colombo C, Setchell KDR, Podda M, Crosignani A, Roda A. Effects of ursodeoxycholic acid therapy for liver disease associated with cystic fibrosis. J Pediatr. 1990;117(3):482-9.*

21. Scholte BJ, Davidson DJ, Wilke M, de Jonge HR. Animal models of cystic fibrosis. J Cyst Fibros. 2004 08;3 Suppl 2:183-90.

22. Hulzebos CV, Renfurm L, Bandsma RH, Verkade HJ, Boer T, Boverhof R, et al. Measurement of parameters of cholic acid kinetics in plasma using a microscale stable isotope dilution technique: Application to rodents and humans. J Lipid Res. 2001;42(11):1923.

23. Durie PR, Kent G, Phillips MJ, Ackerley CA. Characteristic multiorgan pathology of cystic fibrosis in a long-living cystic fibrosis transmembrane regulator knockout murine model. Am J Pathol. 2004 04;164(4):1481-93.

24. Freudenberg F, Broderick AL, Yu BB, Leonard MR, Glickman JN, Carey MC. Pathophysiological basis of liver disease in cystic fibrosis employing a DeltaF508 mouse model. Am J Physiol Gastrointest Liver Physiol. 2008 06;294(6):G1411-20.

25. Ratcliff R, Evans MJ, Cuthbert AW, MacVinish LJ, Foster D, Anderson JR, et al. Production of a severe cystic fibrosis mutation in mice by gene targeting. Nat Genet. 1993 05;4(1):35-41.

26. Kuipers F, Van Ree J, Hofker MH, Wolters H, Veld G, Havinga R, et al. Altered lipid metabolism in apolipoprotein e–deficient mice does not affect cholesterol balance across the liver. Hepatology. 1996;24(1):241-7.

27. Verkade HJ, Havinga R, Shields DJ, Wolters H, Bloks VW, Kuipers F, et al. The phosphatidylethanolamine N-methyltransferase pathway is quantitatively not essential for biliary phosphatidylcholine secretion. J Lipid Res. 2007 09;48(9):2058-64.

28. Colombo C, Setchell KDR, Podda M, Crosignani A, Roda A. Effects of ursodeoxycholic acid therapy for liver disease associated with cystic fibrosis. J Pediatr. 1990;117(3):482-9.*

29. Mashige F, Imai K, Osuga T. A simple and sensitive assay of total serum bile acids. Clin Chim Acta. 1976 07/01;70(1):79-86.

30. Kok T, Hulzebos CV, Wolters H, Havinga R, Agellon LB, Stellaard F, et al. Enterohepatic circulation of bile salts in FXR-deficient mice: Efficient intestinal bile salt absorption in the absence of ileal bile acid-binding protein (ibabp). J Biol Chem. 2003;278(43):41930-7.

31. Heuman D, Hylemon P, Vlahcevic Z. 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. J Lipid Res. 1989;30(8):1161-71.

32. Stellaard F, Langelaar S, Kok R, Jakobs C. Determination of plasma bile acids by capillary gas-liquid chromatography-electron capture negative chemical ionization mass fragmentography. J Lipid Res. 1989;30(10):1647.

33. Mitchell JC, Stone BG, Duane WC. Measurement of bile acid synthesis in man by release of 14 CO 2 from [26-14 C] cholesterol: Comparison to isotope dilution and assessment of optimum reference cholesterol specific activity. Lipids. 1992;27(1):68-71.

34. Stellaard F, Brufau G, Boverhof R, Jonkers EZ, Boer T, Kuipers F. Developments in bile acid kinetic measurements using 13C and 2H: 105 times improved sensitivity during the last 40 years†. Isotopes Environ Health Stud. 2009;45(4):275-88.

35. Paumgartner G, Beuers U. Ursodeoxycholic acid in cholestatic liver disease: Mechanisms of action and therapeutic use revisited. Hepatology. 2002 09;36(3):525-31.

36. Minagawa N, Nagata J, Shibao K, Masyuk AI, Gomes DA, Rodrigues MA, et al. Cyclic AMP regulates bicarbonate secretion in cholangiocytes through release of ATP into bile. Gastroenterology. 2007;133(5):1592-602.

37. Clarke LL, Grubb BR, Yankaskas JR, Cotton CU, McKenzie A, Boucher RC. Relationship of a non-cystic fibrosis transmembrane conductance regulator-mediated chloride conductance to organ-level disease in cftr(-/-) mice. Proc Natl Acad Sci U S A. 1994 01/18;91(2):479-83.

38. Liu X, Luo M, Zhang L, Ding W, Yan Z, Engelhardt JF. Bioelectric properties of chloride channels in human, pig, ferret, and mouse airway epithelia. American journal of respiratory cell and molecular biology. 2007;36(3):313.

39. Beuers U, Hohenester S, de Buy Wenniger LJM, Kremer AE, Jansen PLM, Elferink RPJO. The biliary HCO3- umbrella: A unifying hypothesis on pathogenetic and therapeutic aspects of fibrosing cholangiopathies. Hepatology. 2010;52(4):1489-96.

40. Bijvelds MJC, Bronsveld I, Havinga R, Sinaasappel M, de Jonge HR, Verkade HJ. Fat absorption in cystic fibrosis mice is impeded by defective lipolysis and post-lipolytic events. Am J Physiol Gastrointest Liver Physiol. 2005 04;288(4):G646-53.

41. Cuperus FJC, Hafkamp AM, Havinga R, Vitek L, Zelenka J, Tiribelli C, et al. Effective treatment of unconjugated hyperbilirubinemia with oral bile salts in gunn rats. Gastroenterology. 2009;136(2):673,682. e1.

42. Hofmann AF, Hagey LR, Krasowski MD. Bile salts of vertebrates: Structural variation and possible evolutionary significance. J Lipid Res. 2010;51(2):226.

43. Strandvik B, Einarsson K, Lindblad A, Angelin B. Bile acid kinetics and biliary lipid composition in cystic fibrosis. J Hepatol. 1996 07;25(1):43-8.

44. Smith JL, Lewindon PJ, Hoskins AC, Pereira TN, Setchell KDR, O'Connell NC, et al. Endogenous ursodeoxycholic acid and cholic acid in liver disease due to cystic fibrosis. Hepatology. 2004;39(6):1673-82.

45. Nakagawa M, Colombo C, Setchell KDR. Comprehensive study of the biliary bile acid composition of patients with cystic fibrosis and associated liver disease before and after UDCA administration. Hepatology. 1990;12(2):322-34.

46. Sinakos E, Marschall HU, Kowdley KV, Befeler A, Keach J, Lindor K. Bile acid changes after high-dose ursodeoxycholic acid treatment in primary sclerosing cholangitis: Relation to disease progression. Hepatology. 2010;52(1):197-203.

47. Hofmann AF. Medical treatment of cholesterol gallstones by bile desaturating agents. Hepatology. 1984;4(S2):199S-208S.