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## Cystic fibrosis liver disease and the enterohepatic circulation of bile acids

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# CHAPTER 6

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

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## ABSTRACT

## Introduction

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

## Methods

*Cftr*<sup>-/-</sup> 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*<sup>-/-</sup> 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*<sup>-/-</sup> mice, the proportion of CA in bile was higher compared to controls ( $61 \pm 4\%$  vs.  $46 \pm 4\%$ , resp.;  $p < 0.05$ ), accompanied by an increased CA synthesis ( $16 \pm 1$  vs.  $10 \pm 2$   $\mu\text{mol}/\text{hour}/100\text{gramBW}$  resp.;  $p < 0.05$ ) and CA pool size ( $28 \pm 3$  vs.  $19 \pm 1$   $\mu\text{mol}/100\text{gramBW}$ , resp.;  $p < 0.05$ ). In both *Cftr*<sup>-/-</sup> and controls UDCA treatment drastically reduced the proportion of CA in bile below 5% and diminished CA synthesis ( $2.3 \pm 0.3$  vs.  $2.2 \pm 0.4$   $\mu\text{mol}/\text{day}/100\text{gramBW}$ , resp.; NS) and CA pool size ( $3.6 \pm 0.6$  vs.  $1.5 \pm 0.3$   $\mu\text{mol}/100\text{gramsBW}$ , 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*<sup>-/-</sup> 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 choleric 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 cholangiocyte 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 cholangiocyte 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*<sup>508/508</sup> 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*<sup>-/-tm1CAM</sup> 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

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**Animals and diets.** C57Bl/6;129 *Cftr*<sup>-/-tm1CAM</sup> mice and *Cftr*<sup>+/+tm1CAM</sup> 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*<sup>-/-</sup> 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°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

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

*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*<sup>-/-</sup> and control mice. Acute TUDCA administration provides the possibility to measure the direct choleric 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*<sup>-/-</sup> 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 [<sup>2</sup>H<sub>4</sub>]-cholate in a solution of 0.5% NaHCO<sub>3</sub> 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  $\mu\text{l}$ ) 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.

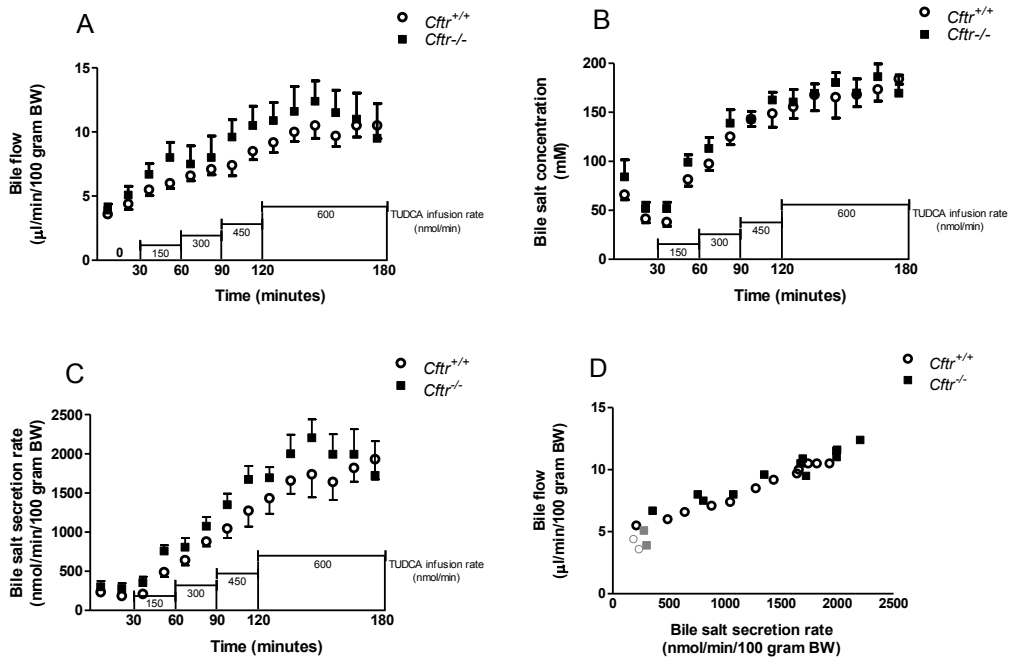
*Analytic procedures.* Biliary bile salt concentrations were determined by an enzymatic fluorimetric assay (29). Biliary bile salt composition was determined by capillary gas chromatography (30). The hydrophobicity of bile salts in bile was calculated according to the Heuman index based on the fractional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

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  $^{14}\text{C}$ -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  $^2\text{H}_4$ -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 ( $[\text{administered amount of label} \times \text{isotopic purity} \times 100] / \text{intercept of the y-axis of the ln APE curve}$ ) 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 $\pm$ 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 Cfr<sup>-/-</sup> 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 ( $4.5 \pm 0.6$  vs.  $4.0 \pm 0.4$   $\mu\text{l}/\text{min}/100\text{gram BW}$ , NS), biliary bile salt concentration ( $67 \pm 12$  vs.  $54 \pm 5$  mM, NS) and biliary bile salt output ( $313 \pm 72$  vs.  $208 \pm 25$  nmol/min/100gram BW, NS) were similar in *Cfr<sup>-/-</sup>* and control mice respectively, indicating no quantitative differences in the choleretic capacity between *Cfr<sup>-/-</sup>* mice and their controls at baseline.

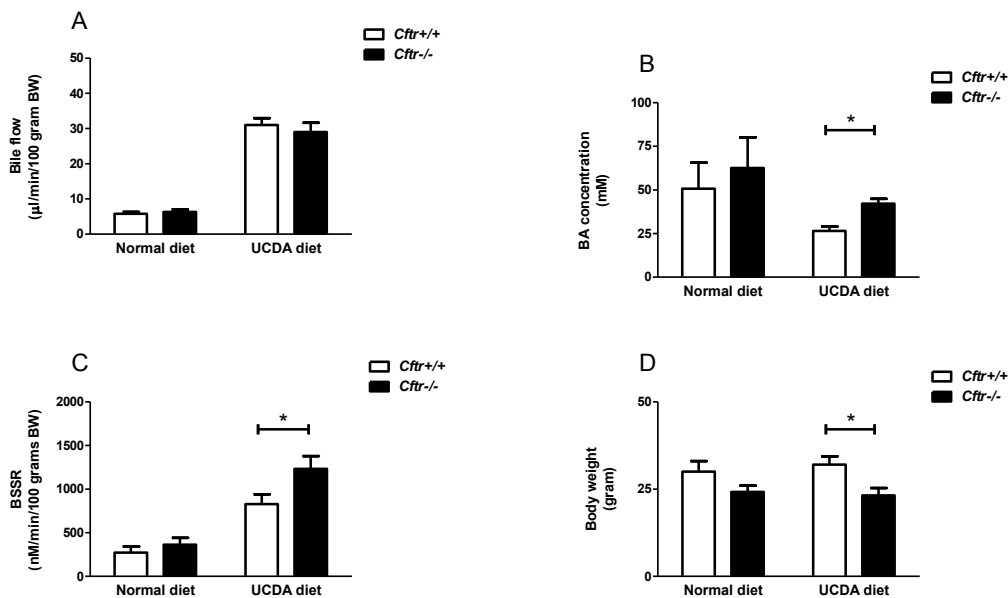


**Figure 1** Biliary parameters during acute intravenous TUDCA administration. Biliary bile flow (A), bile salt concentration (B), bile salt secretion rate (C) and relationship between bile salt secretion rate and bile flow (D) in *Cfr* knockout mice (*Cfr<sup>-/-</sup>*) and control littermates (*Cfr<sup>+/+</sup>*) 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  $\pm$  SEM of  $N=5$  mice per group. There was no significant difference between *Cfr<sup>-/-</sup>* and *Cfr<sup>+/+</sup>* 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 *Cfr<sup>-/-</sup>* and control mice in a dose-dependent manner. Infusions with TUDCA increased bile flow to a similar extent in *Cfr<sup>-/-</sup>* 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*<sup>-/-</sup> 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*<sup>-/-</sup> and *Cftr*<sup>+/+</sup> mice at any of the individual time points for bile flow, bile salt concentration and bile salt secretion rate. To determine the choleric capacity of TUDCA in both *Cftr*<sup>-/-</sup> and control mice we related the bile flow to the bile salt secretion rate (Figure 1D) The choleric capacity of TUDCA was similar in *Cftr*<sup>-/-</sup> and control mice, as was the estimated bile salt dependent bile flow, based on the Y-intercepts (4.6 vs. 5.3  $\mu\text{l}/\text{min}/100\text{gram BW}$ , respectively, NS). The increase in bile flow was linearly related to the bile salt output (control:  $R^2$  0.9, slope 0.003  $\mu\text{mol}/\text{nmol}$ ; *Cftr*<sup>-/-</sup>  $R^2$  0.9, slope 0.003  $\mu\text{mol}/\text{nmol}$ ).



**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*<sup>-/-</sup>) and control littermates (*Cftr*<sup>+/+</sup>) after a normal or 0.5%-UDCA chow diet for 3 weeks. Data are presented as means  $\pm$  SEM of  $N=5-6$  mice per group. \* $P$ -value $<0.05$ .

UDCA treatment increased bile flow equally in *Cftr*<sup>-/-</sup> 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*<sup>-/-</sup> and control mice ( $29.0\pm 2.6$  vs.  $31.0\pm 1.9$

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$\mu\text{l}/\text{min}/100\text{gram BW}$ , resp.; NS; Figure 2A). Chronic UDCA treatment decreased the total biliary bile salt concentration in both genotypes, but to a lower extent in *Cftr*<sup>-/-</sup> mice (*Cftr*<sup>+/+</sup> - 51% vs. *Cftr*<sup>-/-</sup> mice -37%), resulting in significantly higher BS concentration in *Cftr*<sup>-/-</sup> mice compared to controls ( $42\pm 3$  vs.  $26\pm 3$  mM, resp.,  $p<0.05$ , Figure 2B). The bile salt output was significantly higher in *Cftr*<sup>-/-</sup> mice compared with controls during chronic UDCA treatment (*Cftr*<sup>-/-</sup>,  $1232\pm 147$  vs. control,  $827\pm 113$   $\mu\text{mol}/\text{min}/100\text{gram BW}$ , resp.;  $p<0.05$ , Figure 2C). A significant reduced growth of the Cambridge *Cftr*<sup>-/-</sup> 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*<sup>-/-</sup> mice includes a decreased body weight compared to controls, however this is not affected by UDCA treatment.

*Increased hydrophobic bile salt composition of Cftr<sup>-/-</sup> mice compared to control mice.* In non-UDCA treated *Cftr*<sup>-/-</sup> mice, the fractional biliary cholate content was higher compared with control mice ( $61.4\pm 1.5\%$  vs.  $46.5\pm 3.8\%$ , resp.;  $p<0.05$ ; Table 1.) The natural biliary UDCA enrichment was ~50% lower in *Cftr*<sup>-/-</sup> mice compared with controls ( $2.7\pm 0.4$  vs.  $6.0\pm 0.5\%$  resp.;  $p<0.05$ ; Fig. 2A). In non UDCA treated mice  $\beta$ -muricholate is the major hydrophilic biliary bile salt in both *Cftr*<sup>-/-</sup> and control mice ( $42\pm 3,2$  vs.  $33\pm 1,6\%$  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*<sup>-/-</sup> mice was significantly more hydrophobic than the control mice ( $0.05\pm 0.002$  vs.  $-0.07\pm 0.005$  resp.;  $p<0.01$ ; Figure 3B).

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

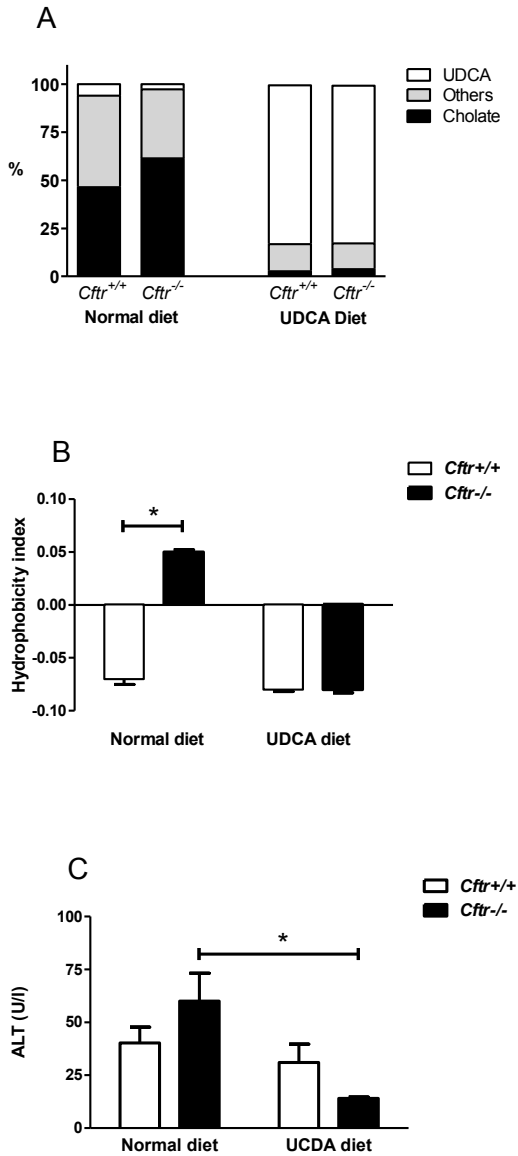
	Non-UDCA treatment		UDCA treatment	
	Cftr <sup>+/+</sup>	Cftr <sup>-/-</sup>	Cftr <sup>+/+</sup>	Cftr <sup>-/-</sup>
CA	46.5 ± 3.8	61.5 ± 1.5*	2.7 ± 0.9 <sup>†</sup>	4.0 ± 1.3 <sup>†</sup>
CDC	3.4 ± 0.4	2.1 ± 0.3	2.8 ± 0.5	1.3 ± 0.2*
DC	1.7 ± 0.4	0.7 ± 0.4	ND	ND
UDCA	6.0 ± 0.5	2.7 ± 0.4*	83.0 ± 2.7 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

**Table 1.** Values are means ±SE in percent; N 4–6 mice per group. Biliary bile salt composition is shown of the major bile salt species [cholate (CA), chenodeoxycholate (CDC), deoxycholate (DC), ursodeoxycholate (UDCA), α-muricholate (α-M), and β-muricholate (β-M)] in cystic fibrosis transmembrane regulator (Cftr) knockout mice (Cftr<sup>-/-</sup>) and control littermates (Cftr<sup>+/+</sup>) 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<sup>-/-</sup> vs. Cftr<sup>+/+</sup>); <sup>†</sup>P < 0.05, difference between treatment group (non-UDCA vs. UDCA).

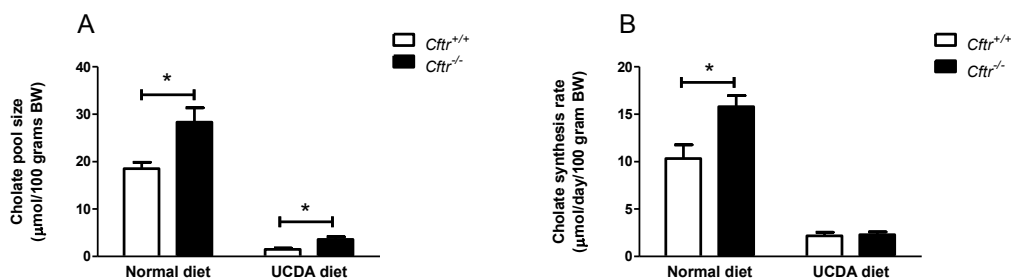
UDCA treatment decreased the biliary hydrophobicity of Cftr<sup>-/-</sup> mice. After UDCA treatment, the biliary bile salt composition changes extensively (Table 1.) After treatment the bile salt composition consisted for more than ~80% of UDCA in both Cftr<sup>-/-</sup> mice and controls (Figure 3A). UDCA treatment drastically reduced the fraction of cholate in the bile to below 5% in both Cftr<sup>-/-</sup> mice and controls. However, the fraction of β-muricholate was also reduced in Cftr<sup>-/-</sup> 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<sup>-/-</sup> 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.

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15±1 U/l resp.; NS). UDCA treatment significantly lowered plasma ALT levels in *Cftr*<sup>-/-</sup> mice compared to *Cftr*<sup>-/-</sup> 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, ursodeoxycholate and others (chenodeoxycholate, deoxycholate,  $\alpha$ -muri cholate and  $\beta$ -muricholate) 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*<sup>-/-</sup> and control littermates (*Cftr*<sup>+/+</sup>) after a normal or 0.5%-UDCA chow diet for 3 weeks. Data are presented as means  $\pm$  SEM of 4-9 mice per group. \*P-value<0.05.



**Figure 4** Cholocate pool size and cholocate synthesis after chronic dietary UDCA administration. *Cftr* knockout mice (*Cftr*<sup>-/-</sup>) and control littermates (*Cftr*<sup>+/+</sup>) were intravenously injected with <sup>2</sup>H<sub>4</sub>-cholocate after a normal or 0.5%-UDCA chow diet for 3 weeks. Enrichment of the administered <sup>2</sup>H<sub>4</sub>-cholocate was determined in plasma until 72 hours after the administered label. From the plasma decay curve, cholocate pool size (A) and cholocate 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 cholocate synthesis and pool size in *Cftr*<sup>-/-</sup> and control mice. Non-UDCA treated *Cftr*<sup>-/-</sup> mice had a higher cholocate synthesis rate (16 ± 1 vs. 10 ± 2 μmol/day/100 gram BW resp.; p < 0.05) and larger cholocate 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 cholocate pool size by ~90% in both *Cftr*<sup>-/-</sup> and control mice (p < 0.05). Nevertheless, the pool size of *Cftr*<sup>-/-</sup> 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 cholocate synthesis rate by ~85% in both *Cftr*<sup>-/-</sup> mice and controls compared to untreated mice (p < 0.05; Fig. 4B). Interestingly, UDCA treatment straightened out the difference in synthesis rate between *Cftr*<sup>-/-</sup> 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 choleric effect of UDCA can also to be expected in CF conditions. UDCA treatment reduced the relative

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hydrophobic biliary bile salt composition in *Cfr*<sup>-/-</sup> 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 *Cfr* 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 *Cfr* 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 choleric 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 *Cfr* dependent secretion effect of UDCA at the level of the cholangiocytes. Second, the activation of *Cfr* 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 *Cfr* 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).

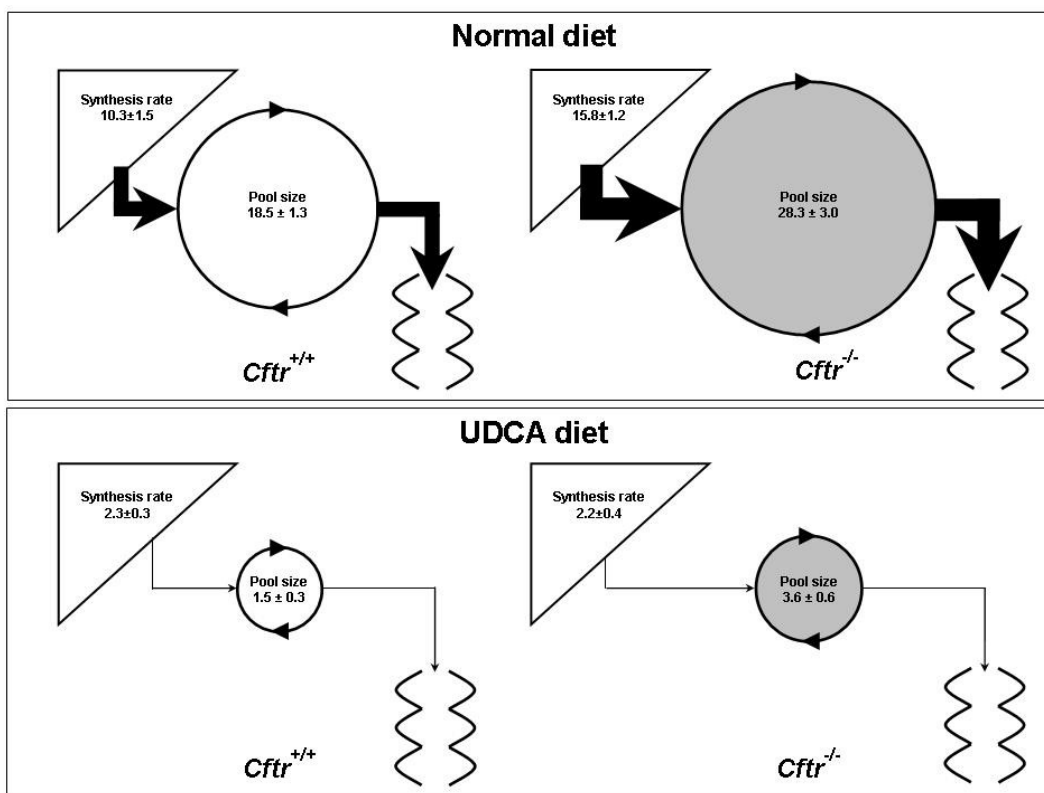
During bile salt treatment, however, CF and control mice were administered the same, supraphysiological dosages of TUDCA and UDCA in the acute and chronic experiment, respectively. We previously reported an increased fecal loss of bile salts in the *Cfr*<sup>-/-</sup> mice (40). Although UDCA could potentially influence intestinal fat malabsorption we could not find an effect of UDCA treatment on the body weight in *Cfr*<sup>-/-</sup> 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*<sup>-/-</sup> mice in bile composition. Untreated *Cftr*<sup>-/-</sup> 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*<sup>508/508</sup> 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*<sup>-/-</sup> 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% of 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 cholates in *Cftr* knockout mice (*Cftr*<sup>-/-</sup>) and control littermates, either untreated (top) or treated for 3 weeks with a 0.5%-UDCA chow diet for 3 weeks. Cholates undergo enterohepatic cycling (dotted lines). In the liver bile salts 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 cholates loss is compensated for by de novo cholates synthesis in the liver as represented by the width of the black arrows coming from the liver ( $\mu\text{mol}/\text{day}/100$  gram BW), maintaining the total cholates pool size in equilibrium. The diameters of the circles represent the magnitude of the pool size ( $\mu\text{mol}/100$  gram BW). Cholates synthesis rate and pool size are determined as detailed in the Materials and Methods. Data are presented as means  $\pm$  SEM of 4-6 mice per group.

In conclusion, our results in mice *in vivo* indicate that UDCA exerts a choleric 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 provide a firm experimental basis to explain the beneficial effects of UDCA observed in CF patients



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