TITLE

Ursodeoxycholate modulates bile flow and bile salt pool independently from the cystic fibrosis transmembrane regulator (Cftr) in mice.

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RUNNING HEAD:

UDCA: bile flow and bile salt metabolism in Cftr\(^{-/-}\) mice

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ABSTRACT

Introduction. Cystic fibrosis liver disease (CFLD) is treated with ursodeoxycholate (UDCA). Our aim was to evaluate, in \textit{Cftr}^{-/-} mice and wild type controls, if the supposed therapeutic action of UDCA is mediated via choleretic activity or effects on bile salt metabolism. Material and methods. \textit{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 \textit{Cftr}^{-/-} and controls IV TUDCA stimulated bile flow by \textasciitilde 250\% and dietary UDCA by \textasciitilde 500\%, compared with untreated animals (p<0.05). In non-UDCA treated \textit{Cftr}^{-/-} 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 mol/hour/100gramBW resp.; p<0.05) and CA pool size (28\%\pm 3\% vs. 19\%\pm 1\% \mu mol/100gramBW, resp.; p<0.05). In both \textit{Cftr}^{-/-} 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 mol/day/100gramBW, resp.; NS) and CA pool size (3.6\%\pm 0.6\% vs. 1.5\%\pm 0.3\% \mu mol/100gramsBW, resp.; p<0.05). Conclusion. Acute TUDCA infusion and chronic UDCA treatment both stimulate bile flow in CF conditions independently from \textit{Cftr} function. Chronic UDCA treatment reduces the hydrophobicity of the bile salt pool in \textit{Cftr}^{-/-} mice. These results support a potential beneficial effect of UDCA on bile flow and bile salt metabolism in CF conditions.

KEYWORDS
1. Ursodeoxycholic acid, UDCA
2. Cystic fibrosis liver disease
3. CFTR, cystic fibrosis transmembrane conductance regulator
4. Mouse model
5. Bile flow, bile salt kinetics, bile salt metabolism
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 (7, 23). CFTR is a cAMP activated chloride channel and is present in the apical membrane of various epithelial and non-epithelial tissues (26, 27, 46). Cirrhotic Cystic Fibrosis Liver Disease (CFLD) develops in ~5-10% of CF patients and is the second leading cause of mortality in CF patients (10, 28, 36). 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 (14).

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 (21). Indeed, it has been shown that UDCA stimulates biliary secretion of bile acids in patients with primary biliary cirrhosis and primary sclerosing cholangitis (29, 30). 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 (5).

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 (40). However, Fiorotto et al. suggested that UDCA induced bile flow in a Cftr dependent manner (13). 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 Cfr dependent and that, in CF conditions, UDCA induced cholangiocyotic 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 (18). 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 (1). Freudenberg et. al reported an increased biliary hydrophobicity index in Cfr<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 (15). In contrast, Beuers et al. described that UDCA did not decrease the bile salt pool size in patients with cholestatic liver disease (2). 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 (9). 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 (22, 39). We determined the effect of UDCA on bile flow and bile salt metabolism in Cfr 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 Cfr<sup>Cfr<sup>−/−</sup> tm1CAM</sup> mouse model we use does not exhibit CF related gallbladder or liver abnormalities (12, 16, 38). The lack of hepatobiliary abnormalities allows us to exclusively investigate the action UDCA under complete Cfr null conditions, without the secondary interference of cholestasis on biliary parameters.
MATERIAL AND METHODS

Animals and diets

C57Bl/6;129 Cfr⁻/⁻ tm1CAM mice and Cfr⁺/⁻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 (38). In accordance with previous studies, the Cfr⁻/⁻ 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 (25). 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 Cfr⁻/⁻ 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(47). 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 (8). 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
production 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 (32). Biliary bile
salt composition was determined by capillary gas chromatography (24). 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 (17). 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. (44). 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 (34). 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$_{2-}$/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, 34, 43).

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\textsuperscript{-/-} 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 Fig. 1A-C). We found that bile flow (4.5±0.6 vs. 4.0±0.4 \textmu l/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\textsuperscript{-/-} and control mice respectively, indicating no quantitative differences in the choleretic capacity between Cftr\textsuperscript{-/-} mice and their controls at baseline.

TUDCA administration increased bile flow equally in Cftr\textsuperscript{-/-} and control mice in a dose-dependent manner. Infusions with TUDCA increased bile flow to a similar extend in Cftr\textsuperscript{-/-} mice and controls (+ ~250%; Fig. 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 (panel 1A) and concentration (panel 1B), increased in equal pace with an increased TUDCA IV dosage and was not different between Cftr\textsuperscript{-/-} and control mice (Fig. 1C). We performed Mann–Whitney U tests for all individual time point during the TUDCA infusion experiments. There was no significant difference between Cftr\textsuperscript{-/-} and Cftr\textsuperscript{+/+} 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\textsuperscript{-/-} and control mice we related the bile flow to the bile salt secretion rate (Fig. 1D) The choleretic capacity of TUDCA was similar in Cftr\textsuperscript{-/-} and control mice, as was the estimated bile salt dependent bile flow, based on the Y-intercepts (4.6 vs. 5.3 \textmu l/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 \textmu mol/nmol; Cftr\textsuperscript{-/-}: \(R^2\ 0.9\), slope 0.003 \textmu mol/nmol).

UDCA treatment increased bile flow equally in Cftr\textsuperscript{-/-} 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\textsuperscript{-/-} and control mice (29.0±2.6 vs. 31.0±1.9 \textmu l/min/100gram BW, resp.; NS; Fig. 2A). Chronic UDCA treatment decreased the total biliary bile salt concentration in both genotypes, but to a
lower extent in $C_{ftr}^{-/-}$ mice ($C_{ftr}^{+/+}$ -51% vs. $C_{ftr}^{-/-}$ mice -37%), resulting in significantly higher BS concentration in $C_{ftr}^{+/+}$ mice compared to controls (42±3 vs. 26±3 mM, resp., p<0.05, Fig. 2B). The bile salt output was significantly higher in $C_{ftr}^{-/-}$ mice compared with controls during chronic UDCA treatment ($C_{ftr}^{-/-}$, 1232±147 vs. control, 827±113 μmol/min/100gram BW, resp.; p<0.05, Fig. 2C). A significant reduced growth of the Cambridge $C_{ftr}^{-/-}$ mice compared to wild type animals has been reported (38). Since the findings of the differences in the bile composition on treatment with UDCA may we affected by the nutritional status we measured body weight of the treated and untreated mice (Fig. 2D). In our current experiment the phenotype of the $C_{ftr}^{-/-}$ mice includes a decreased body weight compared to controls, however this is not affected by UDCA treatment.

*Increased hydrophobic bile salt composition of $C_{ftr}^{-/-}$ mice compared to control mice.* In non-UDCA treated $C_{ftr}^{-/-}$ 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 $C_{ftr}^{-/-}$ mice compared with controls (2.7±0.4 vs. 6.0±0.5% resp.; p<0.05; Fig. 2A). In non UDCA treated mice $\beta$-muricholate is the major hydrophilic biliary bile salt in both $C_{ftr}^{-/-}$ 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 $C_{ftr}^{-/-}$ mice was significantly more hydrophobic than the control mice (0.05±0.002 vs. -0.07±0.005 resp.; p<0.01; Fig. 3B).

*UDCA treatment decreased the biliary hydrophobicity of $C_{ftr}^{-/-}$ 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 $C_{ftr}^{-/-}$ mice and controls (Figure 3A). UDCA treatment drastically reduced the fraction of cholate in the bile to below 5% in both $C_{ftr}^{-/-}$ mice and controls. However, the fraction of $\beta$-muricholate was also reduced in $C_{ftr}^{-/-}$ 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 $C_{ftr}^{-/-}$ mice to wild type levels (-0.08±0.003 vs. -0.08±0.002 resp.; NS; Fig. 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 (Fig. 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 Cfr⁻/⁻ mice compared to Cfr⁺/⁺ on control diet (15±1 vs. 60±13 U/l resp.; p<0.05).

UDCA treatment decreased cholate synthesis and pool size in Cfr⁻/⁻ and control mice. Non-UDCA treated Cfr⁻/⁻ 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 (Fig. 4).

Chronic UDCA treatment reduced the cholate pool size by ~90% in both Cfr⁻/⁻ and control mice (p<0.05). Nevertheless, the pool size of Cfr⁻/⁻ 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 Cfr⁻/⁻ mice and controls compared to untreated mice (p<0.05; Fig. 4B). Interestingly, UDCA treatment straightened out the difference in synthesis rate between Cfr⁻/⁻ and control mice (2.3±0.3 vs. 2.2±0.4 μmol/hour/100 gram BW, resp.; NS).
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 (37). In vitro and ex vivo studies indicated that the stimulatory of UDCA on cholangiocyte secretion depends on the presence of CFTR (13, 40). In the present study, we demonstrated that UDCA, in vivo, either during acute or chronic administration, induced a significant Cfr independent increase of bile flow in mice. Therefore, our results 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 Cfr-/- 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 (13, 40). 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 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 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 (33). CaCCs have been suggested to play a more prominent role in epithelial fluid secretion in mice than in other species, including pigs (6). This might therefore explain the relatively mild phenotype in CF mouse models (31). 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(3). 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<sup>-/-</sup> mice (4). Although UDCA
could potentially influence intestinal fat malabsorption we could not find an effect of UDCA treatment
on the body weight in Cftr<sup>-/-</sup> or control mice. Furthermore we recently reported in rats that UDCA does
not influence fecal fat excretion(11). 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 (20), 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 (45). 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 (42). 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 (35). 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% (41). In addition, a reduction in cholate pool size and cholate synthesis is also described in gallstone patients treated with 750 mg UDCA (19). 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.

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 provide a firm experimental basis to explain the beneficial effects of UDCA observed in CF patients.
Reference list


FIGURE CAPTIONS

Figure 1A  Effects of acute TUDCA infusion on bile production
Figure 1B  Effects of acute TUDCA infusion on biliary bile salt concentration
Figure 1C  Effects of acute TUDCA infusion on biliary bile salt secretion rate
Figure 1D  Relation between bile production and biliary bile salt secretion rate during TUDCA infusion

Figure 2A  Effects of chronic UDCA treatment on bile production
Figure 2B  Effects of chronic UDCA treatment on biliary bile salt concentration
Figure 2C  Effects of chronic UDCA treatment on biliary bile salt secretion rate
Figure 2D  Effects of chronic UDCA treatment on body weight

Figure 3A  Effects of chronic UDCA treatment on biliary bile salt composition profile
Figure 3B  Effects of chronic UDCA treatment on biliary hydrophobicity (Heuman index)
Figure 3C  Effects of chronic UDCA treatment on liver biochemistry e.g. alanine aminotransferase (ALT)

Figure 4A  Effects of UDCA treatment on cholate pool size
Figure 4B  Effects of UDCA treatment on the cholate synthesis rate

Figure 5  Schematic representations of the effects of chronic UDCA treatment on cholate kinetics
Figure legends

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.

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.

Figure 3. Bile salt composition after chronic dietary UDCA administration. (A) Percent contribution of the bile salts cholate, ursodeoxycholate and others (chenodeoxycholate, deoxycholate, α-muri cholate and β-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−/− 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 2H₄-cholate after a normal or 0.5%-UDCA chow diet for 3 weeks Enrichment of the administered 2H₄-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.
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*<sup>−/−</sup>) 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.

Table 1. Biliary bile salt profile: proportional contribution of the major murine bile salt species.

| Biliary Bile salt composition of the major bile salt species (cholate, chenodeoxycholate, deoxycholate, ursodeoxycholate, α-muri cholate and β-muricholate) 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. N= 4-6 mice per group, Means ± SEM, * P<0.05 difference between genotype (*Cftr*<sup>+/+</sup> vs. *Cftr*<sup>−/−</sup>) and † P<0.05 difference between treatment group (non-UDCA vs. UDCA).
Table 1. Biliary bile salt profile

<table>
<thead>
<tr>
<th></th>
<th>Non-UDCA treatment</th>
<th>UDCA treatment</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Cfr&lt;sup&gt;+/+&lt;/sup&gt;</td>
<td>Cfr&lt;sup&gt;−/−&lt;/sup&gt;</td>
</tr>
<tr>
<td>CA</td>
<td>46.5 ± 3.8</td>
<td>61.5 ± 1.5&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>CDC</td>
<td>3.4 ± 0.4</td>
<td>2.1 ± 0.3</td>
</tr>
<tr>
<td>DC</td>
<td>1.7 ± 0.4</td>
<td>0.7 ± 0.4</td>
</tr>
<tr>
<td>UDCA</td>
<td>6.0 ± 0.5</td>
<td>2.7 ± 0.4&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>α-M</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>β-M</td>
<td>42.0 ± 3.2</td>
<td>33.0 ± 1.6</td>
</tr>
</tbody>
</table>

N = 4-6 mice per group, Means ± SEM, nd: not detectable

Mann–Whitney U test

* P<0.05 difference between genotype (Cfr<sup>+/+</sup> vs. Cfr<sup>−/−</sup>)

† P<0.05 difference between treatment group (non-UDCA vs. UDCA)