Research Article

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Ursodeoxycholic acid inhibits hepatic cystogenesis in experimental models of polycystic liver disease

Patricia Munoz-Garrido[1,2], José J.G. Marin[2,3], María J. Perugorria[1,2,4], Aura D. Urribarri[5], Oihane Erice[1], Elena Sáez[5], Miriam Úriz[5], Sarai Sarvide[5], Ainhoa Portu[2,5], Axel R. Concepcion[5], Marta R. Romero[2,3], María J. Monte[2,3], Álvaro Santos-Laso[1], Elizabeth Hijona[1,2], Raúl Jimenez-Agüero[1], Marco Marzioni[6], Ulrich Beuers[7], Tatyana V. Masyuk[8], Nicholas F. LaRusso[8], Jesús Prieto[2,5], Luis Bujanda[1,2,†], Joost P.H. Drenth[9,†], Jesús M. Banales[1,2,4,5,*]

[1]*Department of Liver and Gastrointestinal Diseases, Biodonostia Health Research Institute – Donostia University Hospital, University of the Basque Country (UPV/EHU), San Sebastián, Spain;* [2]*National Institute for the Study of Liver and Gastrointestinal Diseases (CIBERehd, Instituto de Salud Carlos III), Spain;* [3]*Experimental Hepatology and Drug Targeting (HEVEFARM), Biomedical Research Institute of Salamanca (IBSAL), University of Salamanca, Salamanca, Spain;* [4]*IKERBASQUE, Basque Foundation for Science, Spain;* [5]*Division of Gene Therapy and Hepatology, CIMA of the University of Navarra, Pamplona, Spain;* [6]*Department of Gastroenterology, "Università Politecnica delle Marche", Ancona, Italy;* [7]*Department of Gastroenterology and Hepatology, Tytgat Institute for Liver and Intestinal Research, Academic Medical Centre, University of Amsterdam, Amsterdam, The Netherlands;* [8]*Division of Gastroenterology and Hepatology, Mayo Clinic, Rochester, MN, USA;* [9]*Department of Gastroenterology & Hepatology, Radboud University Nijmegen Medical Center, The Netherlands*

Background & Aims: Polycystic liver diseases (PLDs) are genetic disorders characterized by progressive biliary cystogenesis. Current therapies show short-term and/or modest beneficial effects. Cystic cholangiocytes hyperproliferate as a consequence of diminished intracellular calcium levels ($[Ca^{2+}]_i$). Here, the therapeutic value of ursodeoxycholic acid (UDCA) was investigated.

Methods: Effect of UDCA was examined *in vitro* and in polycystic (PCK) rats. Hepatic cystogenesis and fibrosis, and the bile acid (BA) content were evaluated from the liver, bile, serum, and kidneys by HPLC-MS/MS.

Results: Chronic treatment of PCK rats with UDCA inhibits hepatic cystogenesis and fibrosis, and improves their motor behaviour. As compared to wild-type animals, PCK rats show increased BA concentration ([BA]) in liver, similar hepatic *Cyp7a1* mRNA levels, and diminished [BA] in bile. Likewise, [BA] is increased in cystic fluid of PLD patients compared to their matched serum levels. In PCK rats, UDCA decreases the intrahepatic accumulation of cytotoxic BA, normalizes their diminished [BA] in bile, increases the BA secretion in bile and diminishes the increased [BA] in kidneys. *In vitro*, UDCA inhibits the hyperproliferation of polycystic human cholangiocytes via a PI3K/AKT/MEK/ERK1/2-dependent mechanism without affecting apoptosis. Finally, the presence of glycodeoxycholic acid promotes the proliferation of polycystic human cholangiocytes, which is inhibited by both UDCA and tauro-UDCA.

Conclusions: UDCA was able to halt the liver disease of a rat model of PLD through inhibiting cystic cholangiocyte hyperproliferation and decreasing the levels of cytotoxic BA species in the liver, which suggests the use of UDCA as a potential therapeutic tool for PLD patients.

Keywords: Polycystic liver diseases (PLDs); Ursodeoxycholic acid (UDCA); Cystogenesis; Cholangiocyte; Intracellular calcium; Therapy.
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* Corresponding author. Address: Department of Liver and Gastrointestinal Diseases, Biodonostia Health Research Institute – Donostia University Hospital, Paseo del Dr. Begiristain s/n, E-20014 San Sebastian, Spain. Tel.: +34 943006067.
E-mail address: jesus.banales@biodonostia.org (J.M. Banales).
† Both authors contributed equally to this work.

Abbreviations: ADPKD, autosomal dominant polycystic kidney disease; ADPLD, autosomal dominant polycystic liver disease; AKT, v-akt murine thymoma viral oncogene homolog 1; ALP, alkaline phosphatase; α-Sma, alpha-smooth muscle actin; ALT, alanine aminotransferase; ARPKD, autosomal recessive polycystic kidney disease; AST, aspartate aminotransferase; BA, bile acid; BDL, bile-duct ligation; $[Ca^{2+}]_i$, intracellular calcium concentration; CA, cholic acid; cAMP, 3′-5′-cyclic adenosine monophosphate; CDCA, chenodeoxycholic acid; CK19, cytokeratin 19; Col1a1, collagen type 1 alpha 1; Ctgf, connective tissue growth factor; Cxcl1, C-X-C motif ligand 1 [interleukin 8 (IL8) homolog]; Cyp7a1, cytochrome P450 7A1; DCA, deoxycholic acid; DMEM/F-12, Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12; ERK1/2, extracellular signal-regulated kinases 1/2; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GCDCA, glycochenodeoxycholic acid; GDCA, glycodeoxycholic acid; IL6, interleukin 6; LCA, lithocholic acid; MAP kinases, mitogen-activated protein kinases; MEK, mitogen-activated protein kinase kinase; mRNA, messenger RNA; pAKT, phosphorylated AKT; PCK rat, polycystic kidney rat; PLDs, polycystic liver diseases; PCNA, proliferating cell nuclear antigen; pERK, phosphorylated ERK; PKA, protein kinase A; PKDs, polycystic kidney diseases; PKHD1, polycystic kidney and hepatic disease 1; qPCR, quantitative polymerase chain reaction; RT-PCR, reverse transcription polymerase chain reaction; TCDCA, taurochenodeoxycholic acid; TDCA, taurodeoxycholic acid; Tgfβ1, transforming growth factor beta 1; TUDCA, tauroursodeoxycholic acid; UDCA, ursodeoxycholic acid.

Introduction

Polycystic liver diseases (PLDs) are a group of genetic disorders characterized by bile-duct dilation and/or development of biliary cysts, which progressively grow and are the main cause of morbidity [1]. The large volume of hepatic cysts may cause different symptoms and complications such as severe hepatomegaly, abdominal distension with local pressure, back pain, hypertension, gastro-esophageal reflux, dyspnea, as well as bleeding, infection and/or rupture of the cysts [1]. PLDs are inherited in dominant or recessive form and developed alone or in association with polycystic kidney diseases (PKDs). Autosomal dominant polycystic liver disease (ADPLD) develops cysts preferentially in the liver, whereas the kidney is also markedly affected in autosomal dominant (ADPKD) and recessive (ARPKD) forms of PKDs [1]. Local surgery of symptomatic cysts is commonly employed and partially prevents and/or ameliorates the disease progression. However, this therapy show short-term and/or modest beneficial effects, liver transplantation being the only curative option [1].

Current research is focused on elucidating the molecular mechanisms involved in the pathogenesis of PLDs with the aim of identifying new potential targets for pharmacological therapy [2]. Novel evidence suggests that PLDs share several pathological mechanisms that may provide a key for treatment [2]. In this regard, cAMP-mediated hyperproliferation of cystic cholangiocytes is considered a central event in PLDs [2]. Different experimental and clinical studies have previously demonstrated the potential therapeutic value of somatostatin analogues in partially reducing the increased intracellular cAMP concentration $[cAMP]_i$ and the concomitant cAMP-mediated hyperproliferation in cystic cholangiocytes [3–10], indicating that drugs specifically directed to inhibit this pathway could have key therapeutic value. In this regard, we have previously reported that cholangiocytes from PCK rats, an animal model of ARPKD, are characterized by increased $[cAMP]_i$ and diminished intracellular calcium concentration $[Ca^{2+}]_i$ [11]. Restoration of $[Ca^{2+}]_i$ with a calcium ionophore inhibited both basal and cAMP/PKA/MEK/ERK1/2-stimulated proliferation of PCK rat cholangiocytes via activation of the PI3K/AKT pathway [11]. These data indicate that normalization of $[Ca^{2+}]_i$ in cystic cholangiocytes may have a translational impact. Different molecules and mechanisms are able to upregulate the $[Ca^{2+}]_i$ in cholangiocytes. In particular, ursodeoxycholic acid (UDCA; 3α, 7β-dihydroxy-5β-cholanoic acid) is a minor endogenous hydrophilic bile acid (BA) used for the treatment of several cholestatic disorders [12,13]. Experimental evidence suggests that UDCA stimulates the hepatobiliary secretion of bicarbonate and protects cholangiocytes against the cytotoxicity of hydrophobic BAs, and that these effects are mediated in part by increasing the $[Ca^{2+}]_i$ [12,13]. In an experimental model of obstructive cholestasis, such as bile-duct ligation (BDL) in rats, cholangiocytes hyperproliferate as a consequence of increased $[cAMP]_i$ and decreased $[Ca^{2+}]_i$ resulting in bile-duct proliferation. Interestingly, treatment of this animal model with UDCA for 1 week inhibits the aforementioned cholangiocyte hyperproliferation by normalizing the $[Ca^{2+}]_i$ [14,15].

In the present study, we explored the potential therapeutic value of UDCA in inhibiting the hepatic cystogenesis of experimental models of PLD and the molecular mechanisms of action.

Materials and methods

Treatment of PCK rats with UDCA

The PCK rat (Charles River Laboratory) is a well characterized animal model of ARPKD that presents a spontaneous mutation in the *PKHD1* orthologous gene [16,17]. Hepatic cystogenesis and fibrosis, as well as serum biochemical markers were analysed in non-treated wild-type (n = 12) and PCK (n = 10) rats, as well as in PCK rats (n = 10) orally-treated with UDCA (25 mg/kg/day) for 5 months. The details are described in Supplementary data.

Determination of messenger RNA expression

Total RNA was obtained from rat liver tissue with TriReagent (Sigma). Detection and quantification of messenger RNAs (mRNAs) were performed via reverse transcription polymerase chain reaction (RT-PCR) and real-time quantification (qPCR) as we previously described [18] (cf. Supplementary Table 1 for specific primers). The *Gapdh* gene expression was employed as a normalizing control.

Western blot analysis

Changes in protein expression and/or phosphorylation were detected through immunoblotting using cell extracts or PCK rat liver tissue as detailed in Supplementary data.

Bile acid measurement

BAs were analysed in total liver, bile collected before the sacrifice, peripheral blood and total kidney of rats by high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) after C18-based extraction as we previously reported [19,20]. Similarly, BA concentration ([BA]) was determined in cystic fluid of patients with PLD and compared with their paired samples of peripheral blood. All human procedures were approved by the Medical Ethical Committee of the Radboudumc, Nijmegen, The Netherlands.

Animal motor behaviour

The degree of motor activity in PCK rats was analysed using the *Open field* test [21]. One day prior to sacrifice, both untreated and UDCA-treated PCK rats were individually placed in the centre of a quadrangular box (511 cm × 511 cm) and the free trajectory was monitored for 5 min using an infrared X-ray camera. The distance and the average speed were measured. Each animal performed one trial.

Isolation and culture of normal and polycystic human cholangiocytes

Normal and polycystic human cholangiocytes were isolated and characterized as we previously described [18].

Measurement of intracellular Ca^{2+} level in normal and polycystic human cholangiocytes in culture

Intracellular Ca^{2+} level was determined as we previously described [11]. Briefly, normal and polycystic human cholangiocyte cultures were loaded with 4 μM Fura-2/AM (Biotium) for 30 min in HCO_3^- -Ringer's solution (with 2 mM $CaCl_2$) at 37 °C and washed twice. Then, the fluorescence intensities (dual excitation at 340/380 nm and emission at 510 nm) were measured overtime in the presence or absence of 100 μM UDCA using an inverted Leica DMIRB fluorescent microscope equipped with a software to acquire and analyse the images.

Proliferation assays in normal and polycystic human cholangiocytes in culture

Proliferation of both normal and polycystic human cholangiocytes was assessed in the presence or absence of different doses of UDCA, TUDCA (tauroursodeoxycholic acid) or GDCA (glycodeoxycholic acid) (i.e. 100, 200 and/or 500 μM) and/or inhibitors (MEK inhibitor U0126: 15 μM; Calbiochem) in quiescent medium (DMEM-Ham's-F12 with 3% fetal bovine serum and 1% penicillin/streptomycin) for 48 h at 37 °C using the *CellTrace™ CFSE Cell Proliferation Kit* (Invitrogen) for flow cytometry (*FC 500 MCL System*, Beckman Coulter).

Evaluation of apoptosis in polycystic human cholangiocytes in culture

The rate of apoptosis was determined in both normal and polycystic human cholangiocytes using the *FITC Annexin V Apoptosis Detection Kit II* (BD Pharmingen) for flow cytometry. Briefly, normal and polycystic human cholangiocytes were cultured in the presence or absence of different doses of UDCA (i.e. 100, 200 and 500 μM) in quiescent medium for 48 h at 37 °C. Then, cells were incubated with the aforementioned kit following the manufacturer guidelines and apoptotic rates were measured in a *FC 500 MCL* fluorometer.

Statistical analysis

Data are shown as mean ± SEM. Once normality was assessed with D'Agostino-Pearson or Shapiro–Wilks tests, we used the Student *t* test for statistical comparisons between two groups of normally distributed variables, and one-way analysis of variance (ANOVA) and subsequent Bonferroni post hoc test for comparisons between more than two groups. When non-parametric methods were required, Mann–Whitney tests were used. Analyses were carried out with the *GraphPad Prism 5* statistical software.

Results

Beneficial effect of UDCA treatment in PCK rats

To evaluate the potential therapeutic value of UDCA for the treatment of PLDs, it was administered to 8-week-old PCK rats for 5 months. Treatment of PCK rats with UDCA decreased hepatic cystogenesis (Fig. 1A, B); this event was associated with decreased protein levels of the cholangiocyte-marker Ck19 in the liver (Fig. 1C, D). Moreover, PCK rats treated with UDCA showed lower levels of fibrosis i.e. less areas stained for collagen with Sirius Red (Fig. 1E) and decreased liver expression of both pro-fibrotic [collagen-1a1 (*Col1a1*) and connective tissue growth factor (*Ctgf*)] and pro-inflammatory [IL8-homolog (*Cxcl1*)] genes at mRNA level (Fig. 1F). On the other hand, no statistical differences in alpha-smooth muscle actin (*α-SMA*), transforming growth factor beta 1 (*Tgfβ1*) and interleukin 6 (*IL6*) were found at mRNA level (Supplementary Fig. 1). By contrast, UDCA treatment induced no significant change in the renal cystogenesis also developed in PCK rats (Supplementary Fig. 2).

As compared with wild-type animals, PCK rats showed increased liver weight, bile flow and serological alkaline phosphatase (ALP) levels, as well as decreased body weight and serological albumin and protein levels (Table 1). In PCK rats, UDCA administration was not accompanied by variation in body, liver or kidney weights, or changes in the serum levels of biochemical markers, such as ALP, aspartate aminotransferase (AST), alanine aminotransferase (ALT), total bilirubin, urea, and albumin (Table 1). The serum levels of total protein were slightly decreased in PCK rats treated with UDCA compared with controls and the liver weight mean value had a tendency to decrease after UDCA treatment. On the other hand, UDCA treatment induced an almost statistically significant increase in bile flow of PCK rats compared to non-treated PCK controls (Table 1).

PCK rats tend to be less mobile with the disease progression, possibly because of increasing discomfort and deterioration of their physical condition overtime. Since these animals were apparently less lethargic under UDCA treatment, their motor behaviour was evaluated using the open field test, which is accepted as an indicator of the animal physical state [21]. The results indicated that PCK rats treated with UDCA for 5 months walked more distance than untreated PCK rats without affecting their average speed (Fig. 1G). These data suggest that UDCA treatment improves the physical state of PCK rats.

Effect of UDCA treatment on the BA levels in liver, bile, peripheral blood and kidney of PCK rats

To investigate whether the beneficial effect induced by UDCA could be mediated in part by changes in the exposure of biliary tissue to the more toxic BA species, we analysed the liver BA levels and composition of 7-month-old PCK (untreated and treated with UDCA for 5 months) and untreated wild-type rats. We found that PCK rats showed ~16-fold higher intrahepatic total [BA] compared to matched wild-type control rats (Fig. 2A). The levels of major BAs, both primary [i.e. cholic acid (CA) and chenodeoxycholic acid (CDCA)] and secondary [i.e. deoxycholic acid (DCA) and lithocholic acid (LCA)] species, were higher in the liver of PCK than wild-type rats (Fig. 2B). Moreover, the intrahepatic levels of the most abundant and toxic dihydroxylated BAs [i.e. DCA and CDCA conjugated with either taurine (TDCA and TCDCA) or glycine (GDCA and GCDCA)] were markedly higher in PCK than in wild-type rats (Fig. 2C). Chronic treatment of PCK rats with UDCA did not alter the liver content of total BAs (Fig. 2A). However, UDCA treatment resulted in a trend that decreased intrahepatic concentrations of major BAs (Fig. 2B). When the more toxic species were analysed separately, it was notable that UDCA treatment decreased the hepatic concentration of TDCA and GDCA and almost the concentration of TCDCA and GCDCA (Fig. 2C). However, the proportions of unconjugated and tauro- and glyco-conjugated BAs were similar in wild-type and PCK rats and were not substantially affected by UDCA treatment (Fig. 2D). In this regard, the percentage of UDCA family (unconjugated + glyco-conjugated + tauro-conjugated) in the hepatic BA pool was very low and similar in normal (1.33%) and untreated PCK (1.40%) rats, and increased (to 3.26%) after treatment of PCK rats with UDCA (Fig. 2D). Therefore, the increased UDCA concentration observed in the liver of untreated PCK rats compared to wild-type rats is linked to the general accumulation of BAs within the liver of PCK rats (Fig. 2A).

To investigate whether changes in BA synthesis could account for the increased intrahepatic accumulation of BAs, the expression level of *Cyp7a1*, a key enzyme in BA synthesis, was determined. The abundance of *Cyp7a1* mRNA was not different between wild-type and PCK rats, and was not affected by the treatment with UDCA (Fig. 2E), suggesting that changes in BA synthesis was probably not the cause of enhanced accumulation of these compounds in liver tissue. In addition, we measured the bile flow (Table 1) and collected bile samples from the common bile-duct before the animal's sacrifice. The analysis revealed that total [BA] is lower in bile samples collected from PCK than wild-type rats (Fig. 3A, B); interestingly, UDCA treatment almost completely prevented this decreased [BA] (Fig. 3A, B) and stimulated the BA secretion to bile in PCK rats (Fig. 3A, C; Supplementary Fig. 3A, B).

To evaluate where the BA accumulation takes place in polycystic livers, BA concentration was directly determined in the cystic fluid of PLD patients and compared with BA concentration in their paired samples of peripheral blood. Results show that BAs are significantly concentrated in cystic fluid compared with peripheral blood, although not as much as in bile. BA concentration in the cystic fluid is in "μM range" whereas in bile it is in "mM range" (Fig. 4).

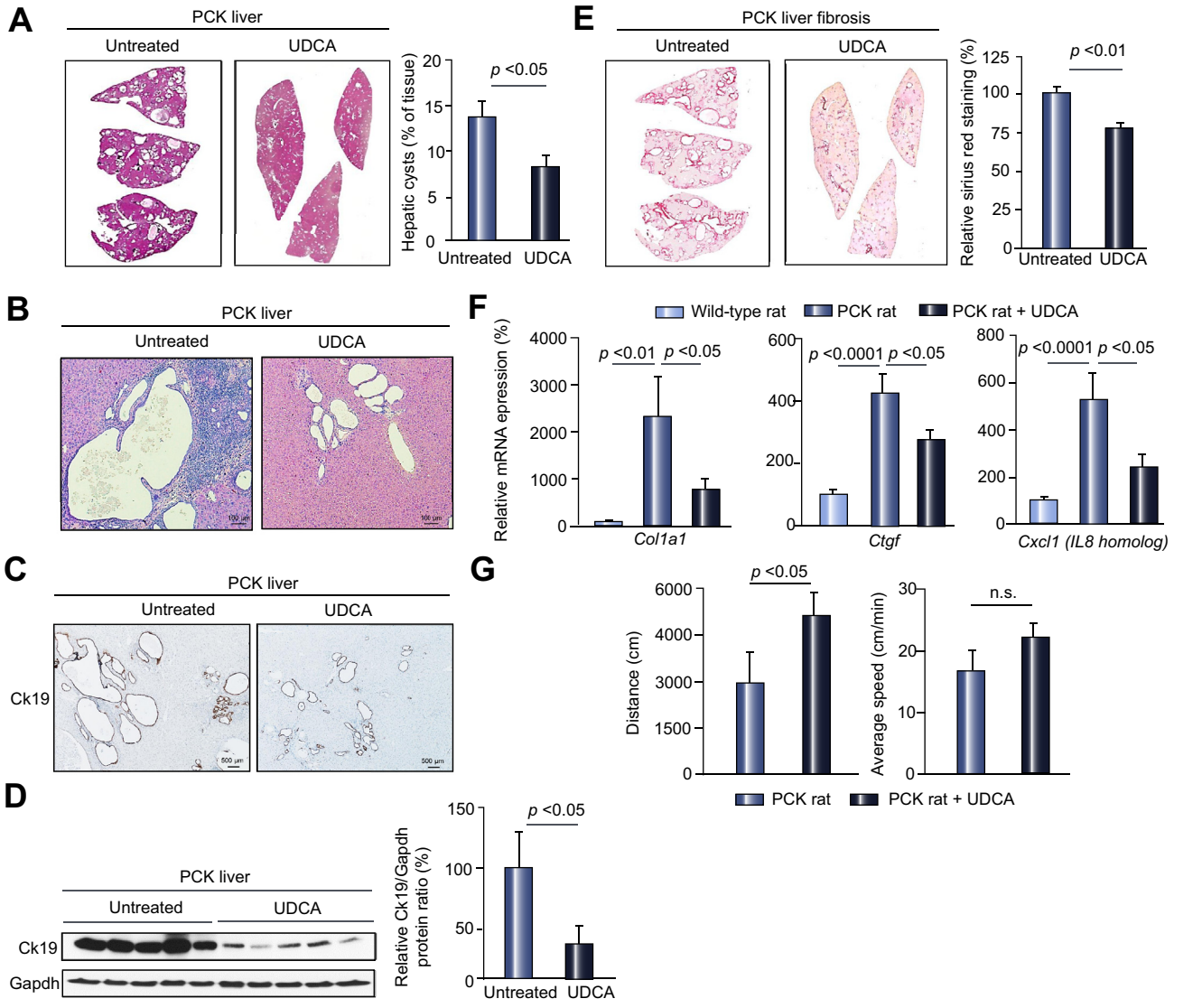


Fig. 1. Treatment of PCK rats with UDCA halts hepatic cystogenesis and fibrosis, and improves their motor behaviour. (A and B) Representative images (hematoxylin-eosin staining; 5× magnification in Fig. 1A) and bar graph showing hepatic cysts in untreated and UDCA-treated PCK rats. (C and D) Hepatic expression of the cholangiocyte-marker Ck19 at protein level. Representative images of Ck19 immunohistochemistry (C) and Western blots (D) of 5 representative untreated or UDCA-treated PCK rats. Bar graph shows Ck19 quantification (n = 10 and n = 9 in PCK and PCK + UDCA groups, respectively). (E) Representative images (Sirius Red staining; 5× magnification) and bar graph showing the hepatic collagen deposition in untreated and UDCA-treated PCK rats. (F) Expression levels (mRNA) of pro-fibrotic (*Col1a1* and *Ctgf*) and pro-inflammatory (*Cxcl1*) genes in liver of wild-type and PCK (untreated and UDCA-treated) rats. (G) Open field test (distance and average speed) in untreated and UDCA-treated PCK rats. N = 12 in wild-type (untreated) and n = 10 in PCK (untreated and UDCA-treated) groups unless specified.

Additionally, BA concentration is increased in peripheral blood of untreated PCK rats compared to wild-type rats, whereas UDCA family concentration is found to be decreased (Fig. 5; Supplementary Fig. 4A–C). UDCA treatment upregulated UDCA family concentration in peripheral blood of PCK rats but level is still lower than that observed in wild-type rats (Fig. 5).

Moreover, BA concentration is increased in kidneys of non-treated PCK rats compared to wild-type rats (Fig. 6A–C; Supplementary Fig. 5). Treatment with UDCA diminished the concentration of different families of BAs in kidney, particularly the most cytotoxic (Fig. 6A–C; Supplementary Fig. 5).

UDCA inhibits the proliferation of polycystic human cholangiocytes in culture

To investigate the potential direct effect of UDCA on biliary epithelia, several *in vitro* assays were carried out using polycystic human cholangiocytes. We first analysed the basal proliferation rates of normal and polycystic human cholangiocyte cultures. Confirming previous results of our group in PCK rat cholangiocytes [11,22], in the present study polycystic human cholangiocytes showed increased proliferation rates compared to normal human cholangiocytes in culture (Fig. 7A). In addition, the presence of UDCA in the culture medium inhibited such

Table 1. Characteristics of the animal groups at the moment of the sacrifice.

Parameters	A	B	C		
	Wild - type rats	PCK Rats (non - treated)	PCK Rats (UDCA - treated)	*p* value (A *vs* B)	*p* value (B *vs* C)
Body weight (g)	675.9 ± 15.72	575.6 ± 12.42	573.8 ± 9.61	0.0001 *	0.91
Liver weight (g)	20.69 ± 0.95	32.68 ± 3.99	26.2 ± 2.28	0.0045 *	0.17
Liver/body weight (%)	3.09 ± 0.10	5.79 ± 0.82	4.598 ± 0.43	0.003 *	0.21
Kidney weight (g)	----------	2.71 ± 0.16	3.2 ± 0.64	-----	0.46
Kidney/body weight (%)	----------	0.9 ± 0.1	1.0 ± 0.1	-----	0.37
Bile flow (μl/min/g)	0.337 ± 0.03	0.76 ± 0.13	1.033 ± 0.10	0.007 *	013
Alkaline phosphatase (U/L)	89.83 ± 7.2	122.7 ± 13.96	117.9 ± 10.95	0.04 *	0.79
Aspartate aminotransferase (U/L)	149.1 ± 32.64	176.7 ± 36.32	159.3 ± 17.85	0.57	0.67
Alanine aminotransferase (U/L)	59.0 ± 6.68	48.6 ± 3.89	53.7 ± 5.02	0.20	0.43
Albumin (g/L)	3.8 ± 0.05	2.04 ± 0.09	1.99 ± 0.06	0.0001 *	0.66
Protein total (g/dl)	5.65 ± 0.08	4.13 ± 0.08	3.88 ± 0.05	0.0001 *	0.02*
Bilirubin total (mg/dl)	----------	0.16 ± 0.0	0.17 ± 0.0	-----	0.67
Blood urea (mg/dl)	33.6 ± 1.3	36.27 ± 2.5	33.6 ± 2.8	0.35	0.49

"Dashed line" indicate non/analysed values.

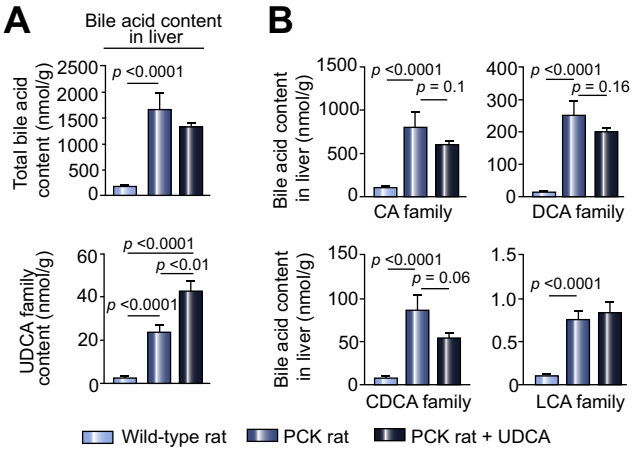
hyperproliferation in a dose-dependent manner (100, 200, or 500 μM) (Fig. 7A); this effect was associated with decreased protein levels of proliferating antigen PCNA (Fig. 7B) and with no changes in apoptosis (Fig. 7C). Moreover, the presence of a MEK inhibitor in the culture medium decreased the basal hyperproliferation of polycystic human cholangiocytes; interestingly, UDCA did not show an additive inhibitory effect with the MEK inhibitor indicating that its effect on proliferation is mediated, at least in part, by blocking the MEK pathway (Fig. 7D). Since UDCA is mainly conjugated with taurine in rat liver, the effect of TUDCA was evaluated on the basal hyperproliferation of polycystic human cholangiocytes *in vitro*. Our data indicate that TUDCA (100, 200, or 500 μM), in contrast to UDCA, does not alter the proliferation of polycystic human cholangiocytes (Fig. 7E).

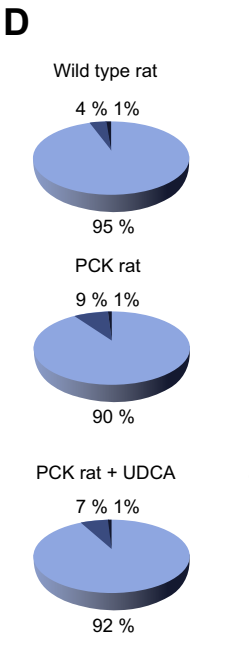
We previously reported that PCK rat cholangiocytes are characterized by decreased $[Ca^{2+}]_i$, which are responsible for both basal and cAMP/PKA/MEK/ERK1/2-dependent hyperproliferation through PI3K/AKT regulation [11]. Using microfluorimetric assays we have now observed that intracellular Ca^{2+} levels of polycystic human cholangiocytes are lower than those found in normal human cholangiocytes (Fig. 7F). Importantly, the presence of 100 μM UDCA in the culture medium normalizes the diminished Ca^{2+} levels of cystic cholangiocytes (Fig. 7F) and stimulates AKT phosphorylation via a PI3K-dependent mechanism (Fig. 7G). In agreement with our previous observation on proliferation (Fig. 7C), UDCA decreases the ERK1/2 phosphorylation levels, and this effect is prevented by PI3K inhibition (Fig. 7H). Thus, UDCA-induced inhibition of cystic cholangiocyte proliferation is dependent on the rise of intracellular Ca^{2+} levels that via a PI3K/AKT attenuates the MEK-dependent ERK1/2 phosphorylation and the subsequent proliferation.

Finally, the role of GDCA (a major secondary BA found concentrated in the liver of PCK rats) was evaluated on the proliferation of polycystic human cholangiocytes *in vitro*. The results indicate that GDCA stimulates the proliferation of these cells and this effect is inhibited by both UDCA and TUDCA (Fig. 7I).

Discussion

The key findings reported here suggest that UDCA may be a valuable therapeutic tool for the treatment of PLDs. Our data indicate that: i) treatment of PCK rats (8-week-old) with UDCA for 5 months decreases hepatic cystogenesis and fibrosis; ii) their motor behaviour is also improved following UDCA treatment, suggesting a better physical fitness; iii) as compared to wild-type animals, PCK rats have increased [BA] in liver, similar hepatic *Cyp7a1* mRNA levels, diminished [BA] in bile, and increased [BA] in peripheral blood and kidneys; iv) the cystic fluid of patients with PLD concentrates BAs compared to their matched serum levels; v) in PCK rats, UDCA decreases the intrahepatic accumulation of the most cytotoxic BAs, normalizes their diminished [BA] in bile, increases the BA secretion in bile and diminishes the increased [BA] in kidneys; vi) polycystic human cholangiocytes hyperproliferate as a consequence of diminished intracellular Ca^{2+} levels compared to normal human cholangiocyte cultures; vii) UDCA normalizes the intracellular Ca^{2+} levels and inhibits the hyperproliferation of polycystic human cholangiocytes via a PI3K/AKT/MEK/ERK1/2-dependent mechanism without affecting apoptosis; viii) GDCA promotes the proliferation of polycystic human cholangiocytes, which is inhibited by both UDCA and TUDCA. These results are consistent with the





Liver

	Bile acid (%)	Wild type rat	PCK rat	PCK rat + UDCA
Tauro-conjugated	TLCS	0.00	0.00	0.06
	TαMCA	4.56	3.65	2.87
	TβMCA	16.99	25.28	29.33
	TUDCA	1.18	1.20	2.88
	THCA	0.04	0.8	0.07
	THDCA	4.84	10.13	12.01
	TCA	60.20	41.81	39.23
	TCDCA	4.40	4.31	3.42
	TDCA	2.27	3.87	2.41
	TLCA	0.07	0.03	0.11
Glyco-conjugated	GUDCA	0.09	0.19	0.29
	GCA	3.56	6.85	5.56
	GCDCA	0.29	0.78	0.62
	GDCA	0.19	0.82	0.39
	GLCA	0.00	0.00	0.00
Unconjugated	UDCA	0.06	0.01	0.09
	αMCA	0.13	0.09	0.05
	βMCA	0.12	0.09	0.10
	CA	0.66	0.59	0.29
	CDCA	0.16	0.02	0.01
	DCA	0.02	0.05	0.03
	HDCA	0.15	0.15	0.16

Fig. 2. PCK rats show increased intrahepatic bile acid concentration compared to wild-type rats, and UDCA decreases the intrahepatic accumulation of cytotoxic bile acids in PCK rats. (A) Total bile acid concentration ([BA]) in liver of wild-type and PCK rats (untreated and UDCA-treated). (B) Levels of both primary (CA and CDCA) and secondary (DCA and LCA) major species of BAs in liver of wild-type and PCK (untreated and UDCA-treated) rats. (C) Intrahepatic levels of the toxic dihydroxylated BAs (TDCA, TCDCA, GDCA and GCDCA) in wild-type and PCK (untreated and UDCA-treated) rats. (D) Proportion of unconjugated and, tauro- and glyco-conjugated BAs in wild-type and PCK (untreated and UDCA-treated) rats. (E) Expression level (mRNA) of *Cyp7a1* in liver of wild-type and PCK (untreated and UDCA-treated) rats. N = 12 in wild-type (untreated) and n = 10 in PCK (untreated and UDCA-treated) groups.

notion that polycystic cholangiocytes hyperproliferate as a consequence of decreased intracellular Ca^{2+} concentration and its restoration with UDCA may halt the hepatic cystogenesis of PLDs, representing a potential therapeutic tool.

We have previously reported that hepatic cystogenesis in PLDs may be a consequence of hyperproliferation [11], altered secretion [23], matrix-metalloprotease hyperactivity [18], centrosomal [24], and ciliary [22] abnormalities and global deregulation of microRNAs [25] in cholangiocytes, and that these cellular alterations may represent potential targets for therapy [2]. In this regard, we focused our attention on the characteristic hyperproliferation of cystic cholangiocytes and determined that this altered event is mediated by increased $[cAMP]_i$ in cholangiocytes [6]. Our previous published *in vitro* and *in vivo* data indicate that $[cAMP]_i$ is increased in PCK rat cholangiocytes compared to normal rat cholangiocytes, which stimulate their proliferation via a PKA/MEK/EKR1/2-dependent mechanism [6,11]. Treatment of PCK rats with the somatostatin analogue octreotide inhibited the hepatic cystogenesis through the downregulation of $[cAMP]_i$ in cholangiocytes and the concomitant hyperproliferation [6]. Those data led us and others to conduct clinical trials employing the somatostatin analogues octreotide [3–6] and lanreotide [7–9] with positive results in terms of reduced hepatic cystogenesis and improved life quality for patients. However, the inhibition of hepatic cystogenesis was modest (~5%). Thus, alternative approaches to guarantee a more pronounce and persistent inhibition of $[cAMP]_i$ in polycystic cholangiocytes are still needed.

We previously reported that PCK cholangiocytes are also characterized by decreased intracellular Ca^{2+} level, and that its restoration with a Ca^{2+} ionophore was able to block both basal and cAMP/PKA/MEK/ERK1/2-dependent hyperproliferation via PI3K/AKT activation [11]. As a consequence, the normalization of the $[Ca^{2+}]_i$ in polycystic cholangiocytes may represent a valuable therapeutic approach for PLDs.

UDCA is an endogenous hydrophilic BA with hepatoprotective features present in low concentration in humans [12,13]. This BA stimulates the hepatobiliary secretion and protects both hepatocytes and cholangiocytes against the cytotoxicity of hydrophobic BAs through the biliary secretion of bicarbonate [12,13]. At the molecular level, UDCA mediates these effects in part by increasing the intracellular Ca^{2+} levels [12,13]. Interestingly, administration of UDCA to BDL rats (experimental model of cholestasis) resulted in inhibition of the characteristic cAMP-mediated cholangiocyte hyperproliferation via upregulation of the $[Ca^{2+}]_i$, which is abnormally diminished in BDL-cholangiocytes [14,15]. Since hepatic cystogenesis in PLDs is also characterized by increased $[cAMP]_i$ and decreased $[Ca^{2+}]_i$ in cystic cholangiocytes [11,26], we hypothesized that UDCA could have therapeutic value for these genetic disorders. To test this theory, we conducted *in vivo* and *in vitro* studies using different experimental models of PLD. Chronic treatment of PCK rats with UDCA halted the development and progression of the liver disease compared to untreated PCK rats. Thus, inhibition of hepatic cystogenesis and fibrosis was observed in PCK rats treated with UDCA compared to controls. In addition, we found that PCK rats have increased liver [BA]. Treatment of PCK rats with UDCA decreased the elevated levels of the most cytotoxic BAs. Since hepatic mRNA expression levels of *Cyp7a1* are not altered between PCK and wild-type rats, and no elevation of liver transaminases was found in PCK *vs.* wild-type rats, we hypothesize that BAs are not hyperproduced and/or concentrated in hepatocytes of PCK rats. On the other hand, BAs may be concentrated in the bile of those cysts that are still connected to the

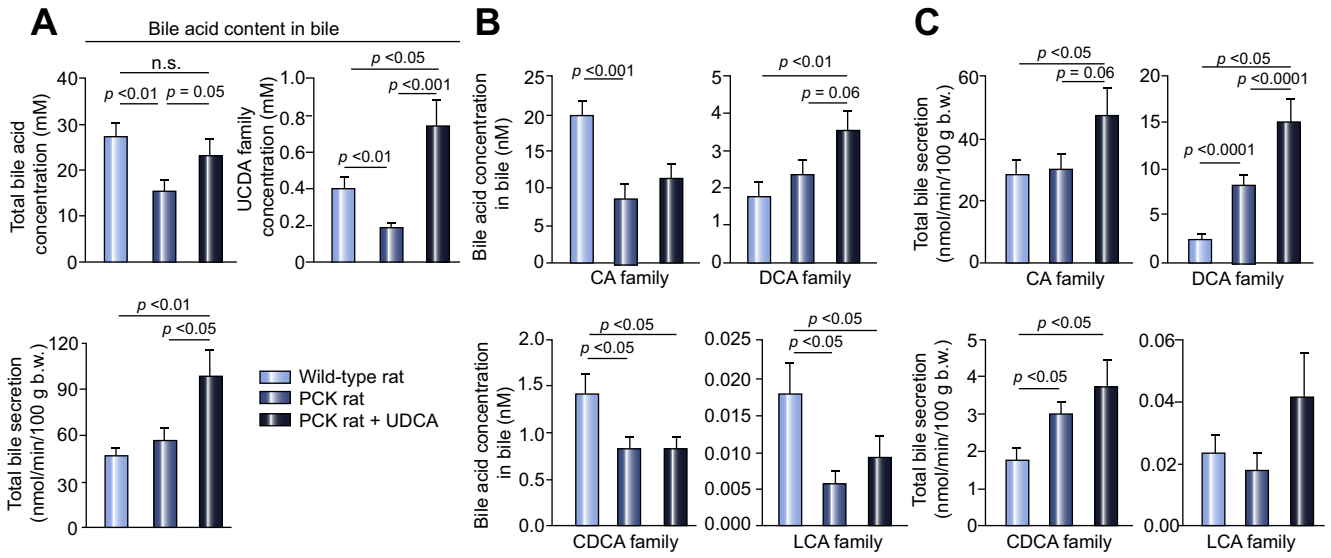


Fig. 3. PCK rats show decreased [BA] in bile, and UDCA normalizes it and increases the BA secretion to bile. (A, B) [BA] in bile of wild-type and PCK (untreated and UDCA-treated) rats. (A, C) BA secretion to bile in wild-type and PCK (untreated and UDCA-treated) rats. n = 12 in wild-type (untreated) and n = 10 in PCK (untreated and UDCA-treated) groups.

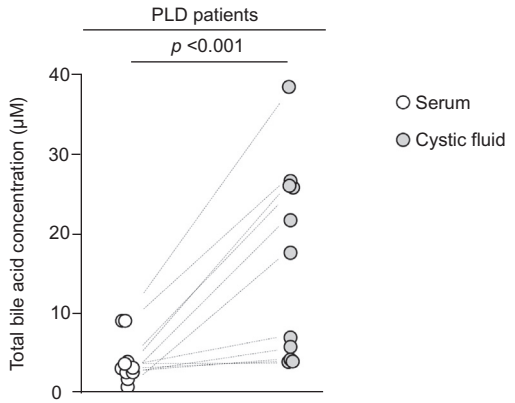


Fig. 4. The cystic fluid of PLD patients presents increased [BA] compared to their matched serum levels. Dots represent each patient (n = 11).

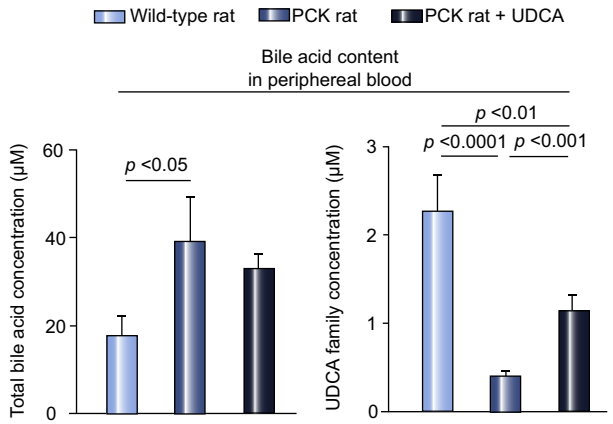


Fig. 5. PCK rats show increased total [BA] in peripheral blood compared to wild-type rats, as well as decreased UDCA family levels that are upregulated by UDCA treatment. n = 12 in wild-type (untreated) and n = 10 in PCK (untreated and UDCA-treated) rats.

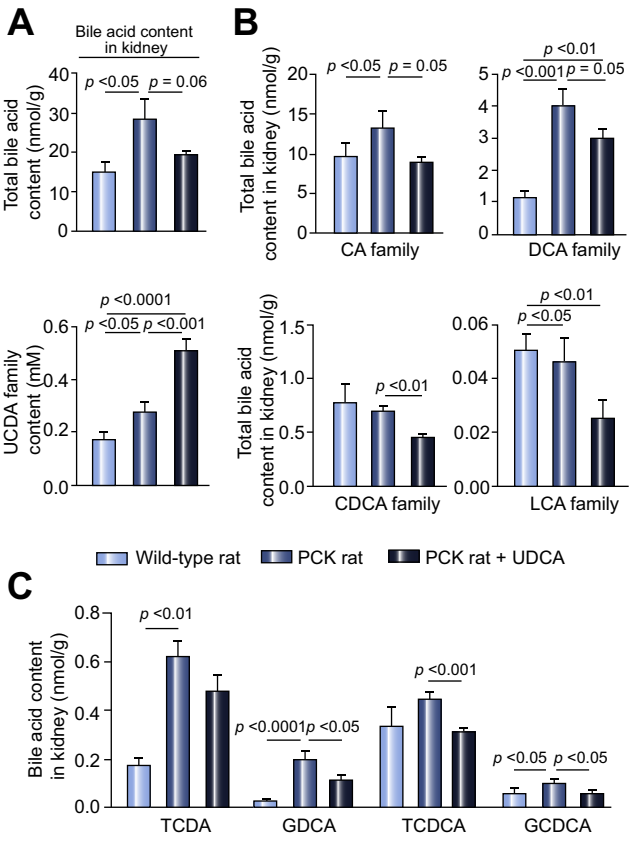


Fig. 6. The [BA] is increased in kidneys of PCK rats compared to wild-type rats and is diminished by UDCA treatment (A, B, C). n = 12 in wild-type (untreated) and n = 10 in PCK (untreated and UDCA-treated) groups.

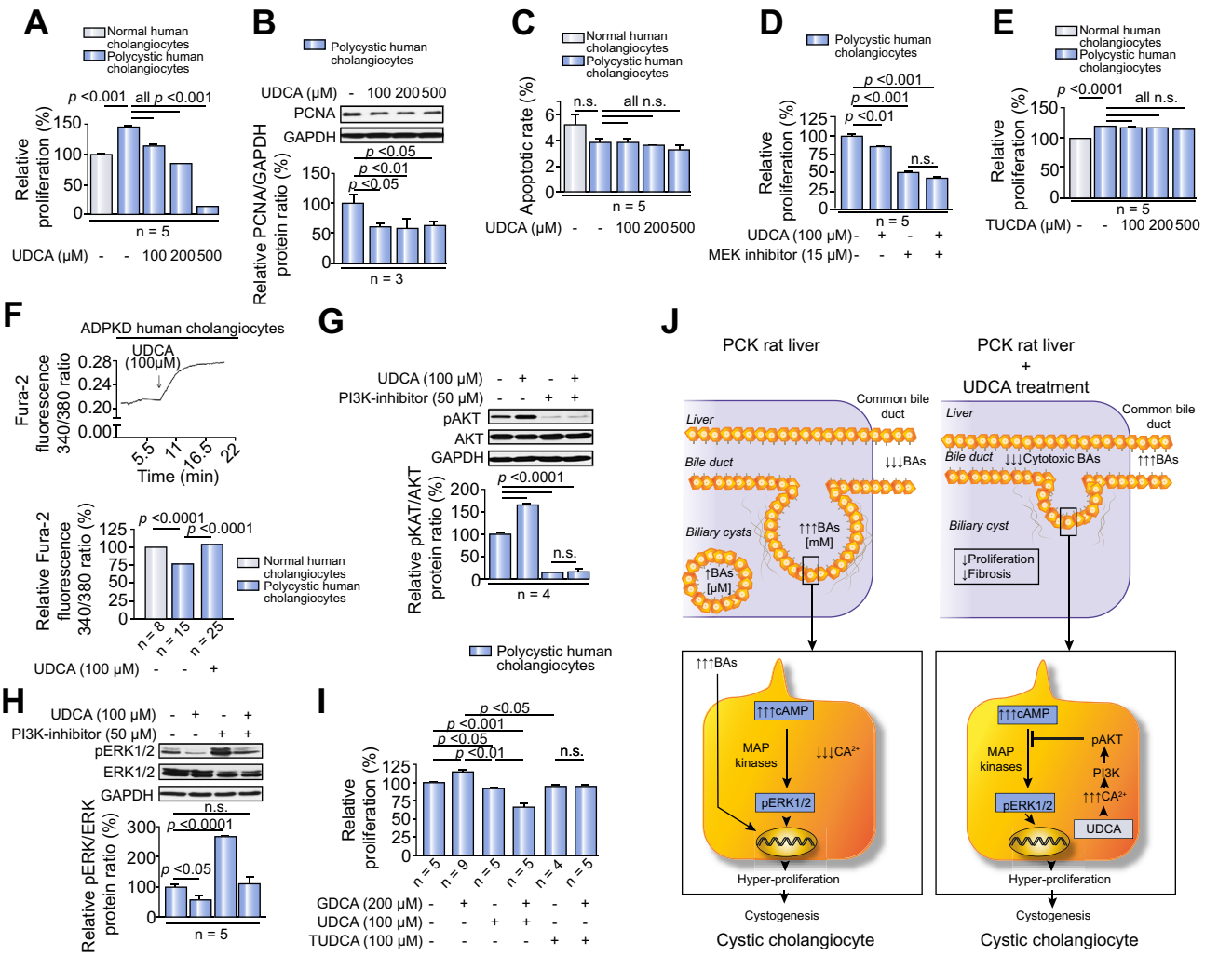


Fig. 7. UDCA inhibits the proliferation of polycystic human cholangiocytes by raising the intracellular Ca^{2+} levels and via a PI3K/AKT/MEK/ERK1/2-dependent mechanism. (A) Proliferation of normal and polycystic human cholangiocytes in the presence or absence of UDCA in the culture medium. (B) Protein expression of the pro-mitotic marker PCNA in polycystic human cholangiocytes in the presence or absence of UDCA in the culture medium (C) Basal apoptotic rates in normal and polycystic human cholangiocytes in the presence or absence of UDCA. (D) Role of MEK in basal and UDCA-inhibited proliferation of polycystic human cholangiocytes. (E) Role of TUDCA in the basal proliferation of polycystic human cholangiocytes. (F) Representative experiment and bar graph showing the intracellular Ca^{2+} levels in normal and polycystic human cholangiocytes (n = number of cell groups analysed). (G, H) Representative Western blots and bar graphs showing AKT (G) and ERK1/2 (H) phosphorylation levels in the presence or absence of UDCA and/or PI3K-inhibitor. (I) Role of GDCA, UDCA and TUDCA in the proliferation of proliferation of polycystic human cholangiocytes. (J) Working model: hepatic cystogenesis in PLDs is characterized by cAMP/PKA/MEK/ERK1/2-dependent cholangiocyte hyperproliferation associated to decreased intracellular Ca^{2+} level. The [BA] is increased in the liver of PCK rats and may promote the proliferation of polycystic cholangiocytes. UDCA inhibits the MEK/ERK1/2-dependent proliferation of polycystic cholangiocytes via Ca^{2+}/PI3K/AKT mechanism, resulting in decreased hepatic cystogenesis and fibrosis. UDCA, through its choleretic features, may also flow the increased concentration of cytotoxic bile acids in the liver preventing their biliary pathogenic effects.

biliary tree, and that UDCA, through its choleretic effect, may reduce this intrahepatic concentration of BAs. In this regard, cysts in the PCK rat usually appear in clusters connected to the biliary tree, which may partially separate and become isolated when the disease progresses. However, even in advanced stages of the liver disease, a certain proportion of cysts are still connected to the biliary tree [17]. In addition, our data show that BAs are significantly concentrated in cystic fluid of isolated cysts from PLD patients compared with their paired samples of peripheral blood. However, [BA] in the cystic fluid is in "μM range" whereas in bile it is "mM range". Therefore, we believe that BA accumulation in the liver of PCK rats may mainly take place in two compartments: i) a proportion in isolated cysts with [BA] in "μM range"; and ii) a certain proportion in cysts still connected to the biliary tree that contain [BA] in "mM range" and hence quantitatively play a major role in total amount of BAs retained in this organ. In addition, it is reasonable to hypothesize that those cysts that are still connected to the biliary tree may display slower drainage due to the architecture of the system and the presence of cellular and secretory debris that may partially block the exit of the cyst and hence the lavage of the content by the bile flow. The high [BA] within the cystic fluid may also be responsible of the hyperproliferation of cystic cholangiocytes *in vivo*, since we found that the major secondary bile acid GDCA stimulate the proliferation of polycystic human cholangiocytes *in vitro*. Based on these results, we further analysed the potential direct beneficial effect of UDCA

in cystic cholangiocytes and the molecular mechanisms involved. We found that polycystic human cholangiocytes, similarly to a previous report using cholangiocytes from PCK rats [11], have decreased intracellular Ca^{2+} levels. UDCA normalizes the intracellular Ca^{2+} levels and inhibits the proliferation of polycystic human cholangiocytes via a PI3K/AKT/MEK/ERK1/2-dependent mechanism without affecting apoptosis. In addition, the GDCA-stimulated proliferation of polycystic human cholangiocytes is inhibited by both UDCA and TUDCA. Therefore, UDCA might halt hepatic cystogenesis *in vivo* through both direct inhibition of cholangiocyte proliferation and by decreasing the intrahepatic concentration of certain pro-mitotic and cytotoxic BAs. It can be postulated that the benefits of UDCA on PCK rat physical status can be ascribed to the above described favourable effect on animal liver injury. Indeed, treatment with UDCA restored their mobility indicating better physical fitness.

In contrast, and as expected, chronic treatment of PCK rats with UDCA did not affect the renal cystogenesis that is also apparent in PCK rats [16]. However, UDCA diminished the increased [BA] in the kidneys of PCK rats, particularly the levels of the most cytotoxic, suggesting that UDCA may facilitate the exit of BAs in the kidney through the urine. Although kidneys of PCK rats treated with UDCA showed increased levels of UDCA compared to both non-treated PCK rats and wild-type rats, UDCA family levels are much lower than those found in the liver of treated animals. This may be the reason why UDCA shows no effect on renal cystogenesis, or perhaps UDCA does not regulate in the same way the proliferation of polycystic cholangiocytes and polycystic renal epithelial cells.

Altogether, these data strongly support the concept that UDCA may be a promising agent for the treatment of PLD patients. Oral administration of UDCA is well tolerated, safe (range between 13–25 mg/kg/day) and the only effective therapy approved by the U.S. Food and Drug Administration (FDA) for the treatment of chronic cholestatic disorders such as primary biliary cirrhosis [27–29]. Among other disorders, UDCA is also recommended for cholesterol gallstone dissolution and hepatobiliary disorders associated with cystic fibrosis [30].

In summary (Fig. 7J), this mechanistic study provides pre-clinical evidence of the potential therapeutic role of UDCA for the treatment of PLD patients. Consequently, we have initiated an international multicentre phase II clinical trial (http://clinicaltrials.gov/show/NCT02021110) to evaluate this hypothesis. Future results will elucidate its potential use as a monotherapy or in combination with somatostatin analogues or other new drugs for the treatment of PLDs.

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Conflict of interest

The authors who have taken part in this study declared that they do not have any conflict of interest with respect to this manuscript.

Author's contributions

PMG, JJGM, MJP, ADU, OE, ES, MU, SS, AP, ARC, MRR, MJM, ASL, EH, RJA, TVM, MM, UB, NFL, JP, LB, JPHD, JMB: study concept and design, analysis and interpretation of data, drafting of the manuscript. PMG, JJGM, MJP, ADU, OE, ES, MU, SS, AP, ARC, MRR, MJM, ASL, EH, RJA, JMB: acquisition of data. PMG, JJGM, MJP, OE, ADU, ES, MU, MRR, MJM, ASL, JMB: statistical analysis. JJGM, NFL, TVM, JP, JPHD, LB, JMB: obtained funding.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jhep.2015.05.023.

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