



Postprandial bile acid levels in intestine and plasma reveal altered biliary circulation in chronic pancreatitis patients^S

Lydie Humbert,* Dominique Rainteau,^{1,*†} Noshine Tuvignon,^{S,**} Claude Wolf,^{††}
Philippe Seksik,^{*,§§} René Laugier,^{S,**} and Frédéric Carrière^{1,§}

Sorbonne Universités,* UPMC, INSERM ERL1157, CNRS UMR 7203 LBM, CHU Saint Antoine, Paris, France; Assistance Publique-Hôpitaux de Paris, PM2 Peptidomique and Métabolomique[†] and Service d'Hépatogastroentérologie,^{§§} Hôpital Saint Antoine, Paris, France; CNRS,[§] Aix-Marseille Université, UMR 7281 Bioénergétique et Ingénierie des Protéines, Marseille, France; Assistance Publique-Hôpitaux de Marseille,** Service d'Hépatogastroentérologie, Hôpital de la Timone, Marseille, France; and APLIPID[®],^{††} Paris, France

ORCID ID: 0000-0003-4848-9418 (F.C.)

Abstract Bile acid (BA) secretion and circulation in chronic pancreatitis (CP) patients with exocrine pancreatic insufficiency (EPI) were investigated by simultaneously measuring postprandial levels of individual BAs in duodenal contents and blood plasma using LC-MS/MS. CP patients and healthy volunteers (HVs) were intubated with gastric and duodenal tubes prior to the administration of a test meal and continuous aspiration of duodenal contents. Pancreatic lipase outputs in CP patients were very low (0.7 ± 0.2 mg) versus HVs (116.7 ± 68.1 mg; $P < 0.005$), thus confirming the severity of EPI. Duodenal BA outputs were reduced in CP patients (1.00 ± 0.89 mmol; 0.47 ± 0.42 g) versus HVs (5.52 ± 4.53 mmol; 2.62 ± 2.14 g; $P < 0.15$). Primary to secondary BA ratio was considerably higher in CP patients (38.09 ± 48.1) than HVs (4.15 ± 2.37 ; $P < 0.15$), indicating an impaired transformation of BAs by gut microbiota. BA concentrations were found below the critical micellar concentration in CP patients, while a high BA concentration peak corresponding to gallbladder emptying was evidenced in HVs. Conversely, BA plasma concentration was increased in CP patients versus HVs suggesting a cholangiohepatic shunt of BA secretion. Alterations of BA circulation and levels may result from the main biliary duct stenosis observed in these CP patients and may aggravate the consequences of EPI on lipid malabsorption.—Humbert, L., D. Rainteau, N. Tuvignon, C. Wolf, P. Seksik, R. Laugier, and F. Carrière. **Postprandial bile acid levels in intestine and plasma reveal altered biliary circulation in chronic pancreatitis patients.** *J. Lipid Res.* 2018. 59: 2202–2213.

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Chronic pancreatitis (CP) is an ongoing inflammatory disorder associated with the loss of exocrine and endocrine

pancreatic parenchyma and its replacement by fibrotic tissue. It results in various nutritional deficits linked to maldigestion subsequent to exocrine pancreatic insufficiency (EPI) and diabetes mellitus subsequent to pancreatic endocrine insufficiency. CP is mainly induced by alcohol abuse in Western countries (1). Other toxic and/or metabolic agents and gene mutations regulating the pancreatic enzyme-activating cascades and their inhibitors are also factors associated with a hereditary form of the disease (2, 3). The first symptom is commonly the abdominal pain. Over the first 5 years of the course of the disease, complications such as pseudocysts or bile duct stenosis can occur. Occurrence of pancreatic calcifications is a lately observed diagnostic key. Because the clinical symptoms remain vague and nonspecific in many CP patients, biological tests are required (4) for the diagnosis as well as the follow up.

In CP patients, fat digestion is more severely impaired than carbohydrate and protein digestion, and steatorrhea is usually a major symptom. The decrease in fat absorption is explained by a decrease in human pancreatic lipase

Abbreviations: AUC, area under the curve; BA, bile acid; CA, cholic acid; CDCA, chenodeoxycholic acid; CMC, critical micellar concentration; CP, chronic pancreatitis; DA, discriminant analysis; DCA, deoxycholic acid; EPI, exocrine pancreatic insufficiency; GCA, glycocholic acid; GCDCA, glycochenodeoxycholic acid; GDCA, glycodeoxycholic acid; GHDCa, glycohyodeoxycholic acid; GLCA-3S, glycolithocholic acid 3-sulfate; GUDCA, glyoursodeoxycholic acid; GUDCA-3S, glyoursodeoxycholic acid 3-sulfate; HCA, hyocholic acid; HPL, human pancreatic lipase; HV, healthy volunteer; LCA, lithocholic acid; O-PLS, orthogonal-partial least square; PEG, polyethylene glycol; TCA, taurocholic acid; TCDCA, taurochenodeoxycholic acid; TDCA, taurodeoxycholic acid; TLCA, tauroolithocholic acid; TLCA-3S, tauroolithocholic acid 3-sulfate; UDCA, ursodeoxycholic acid; UDCA-3S, ursodeoxycholic acid 3-sulfate.

¹To whom correspondence should be addressed.

e-mail: carriere@imm.cnrs.fr (F.C.)

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(HPL) secretion and duodenal pH due to the reduced pancreatic bicarbonate secretion (5, 6). The decrease in duodenal pH impairs the activity and stability of residual HPL. It also promotes the precipitation of bile acids (BAs) and, thus, a further deterioration of intestinal lipid absorption (7, 8). It was shown that human gastric lipase secretion and contribution to lipolysis are increased in CP patients, but not sufficiently to compensate for the loss of the prominent pancreatic lipase (5).

The current treatment of EPI is enzyme replacement with porcine pancreatic extracts. Fat absorption can be improved with enteric-coated formulations of these exogenous pancreatic enzymes, associated also with proton pump inhibitors to increase the duodenal pH and enhance enzyme stability and activity (9–11). In some patients with severe EPI, pancreatic enzyme replacement therapy may not lead to the reduction of steatorrhea, suggesting that other mechanisms interplay with the reduced lipase secretion. Although exogenous lipases act correctly in the small intestine, it can be suspected that other factors affecting the absorption of lipolysis products are limiting. BA levels, for instance, were rarely addressed in CP patients (12). The BAs are critical for the digestion and absorption of fats (13, 14). Due to their amphiphilic properties, they are found adsorbed at the surface of oil-in-water emulsions where they regulate the lipolytic activity of the pancreatic lipase-colipase complex. BAs are also found in the form of mixed micelles with other bile lipids (cholesterol, phosphatidylcholine) and lipolysis products (free fatty acids, 2-monoglycerides). The micellar cosolubilization of lipolysis products by BA is required for removing these products from the lipid-water interface and for ensuring their diffusion in the aqueous milieu of the gut toward the enterocytes. In the absence of bile secretion, fat absorption is impaired (15).

Apart from their function in fat absorption and regulation of cholesterol homeostasis, BAs are increasingly being appreciated as complex metabolic integrators and signaling factors. They have become attractive therapeutic targets for metabolic disorders and useful markers of hepatobiliary and intestinal diseases (16). Individual BA molecular species from various biological samples (blood plasma, feces, urine) can now be separated and quantified in the clinical laboratory using LC-MS/MS. The BA profiles are used for a sensitive diagnosis of cholestasis (17). BA levels and profiles in patients with EPI have not been fully investigated with the LC-MS/MS profiling method, whereas BA malabsorption in patients with CP was fully evidenced by the radiolabeled synthetic BA standard test (18). In the present study, we have detailed the postprandial individual BA level variations in both the duodenum and plasma of CP patients and healthy volunteers (HVs) after a standard test meal.

MATERIALS AND METHODS

Ethical approval

The duodenal content and blood samples used in this study were collected in the framework of two clinical studies (mrtm02-01 for HVs and mrtm03-01 for CP patients; Principal Investigator:

Prof. René Laugier, MD, PhD) that were not initially designed for the analysis reported here. The clinical study protocols were accepted on January 17, 2003 by the institutional board of the ethics committee (Comité Consultatif de Protection des Personnes dans la Recherche Biomédicale, Hôpital d'Adultes de la Timone, Marseille). Test meal experiments and sample collection were performed from March to July 2003 at "Centre de Pharmacologie Clinique et d'Etudes Thérapeutiques (Hôpital d'adultes de la Timone, Marseille, France)" after written informed consent was obtained from all CP patients and HVs. The study conformed to the standards set by the Declaration of Helsinki, except for registration in a database.

CP patients and HVs

Two groups of six HVs (mean age: 30 ± 3 years) with no history of pancreatic disease and six patients with severe CP (mean age: 53 ± 8 years) were selected for this study. The severity of each patient's pancreatic insufficiency was estimated from the medical record and steatorrhea level in the absence of treatment by pancreatic extracts (see supplemental Table S1). All selected CP patients had high levels of steatorrhea (8–45 g/day). All were documented with pancreatic calcification. Patients with common biliary duct stenosis had been treated by endoscopy within the 5 years preceding the study. Some patients were also treated for stenosis after the present study. Only one patient had highly elevated liver enzymes, presumably due to severe hepatic disease.

Test meal

The mixed solid/liquid meal used for the in vivo experiments contained 80 g string beans, 90 g beef meat, 70 g fried potatoes, 10 g butter, 15 ml olive oil, and water for a total volume of 700 ml. The string beans, beef meat, and fried potatoes were passed individually into a mincer with 2 mm holes before they were mixed. The total amount of neutral fat given as triglycerides in the meal was measured to 31.8 ± 2.9 g after extraction of total lipids with chloroform-methanol and triglyceride quantification using thin-layer chromatography coupled to flame ionization detection (19). A nonabsorbable marker was added to the meal [5 g polyethylene glycol (PEG) 4000] to measure the gastric emptying rate and correct the duodenal volumes based on the PEG recovery rates (20).

Experimental device for collecting samples

After fasting overnight, the patients and volunteers were intubated with a double-lumen duodenal tube (outside diameter 5 mm) and a separate single-lumen gastric tube (outside diameter, 3 mm). The tubes were placed under fluoroscopy and their positions were checked by analyzing the pH of their contents. The distal end of the duodenal tube with the occluding balloon was located at the ligament of Treitz (see supplemental Fig. S1). During the sampling period, the subjects stayed in bed with continuous duodenal aspiration by connection of the duodenal tube to a vacuum (around -10 mbar). Aspiration was started a few minutes before the meal and the occluding balloon was inflated (10 ml air). After collecting basal gastric and duodenal samples, the test meal was introduced into the stomach via the gastric tube using a 50 ml syringe during a period of 5 min.

The duodenal fluid was collected continuously for 15 min periods by aspiration upstream of the occluding balloon. The parameters measured in each sample were: *i*) volume and pH (to the nearest 0.5 ml and 0.1 pH unit, respectively); *ii*) PEG 4000 concentration; *iii*) pancreatic lipase activity; and *iv*) individual BA concentrations. In order to prevent the proteolytic inactivation of lipase before the assay, 1 ml of glycerol and 40 μ l of protease inhibitors were added immediately to 1 ml of duodenal sample

before storage at -20°C . The solution of protease inhibitors was prepared by dissolving a pellet of Complete™ protease inhibitor mix (Roche) in 2 ml of distilled water. All samples were kept at -80°C before they were analyzed.

Blood sample collection

Blood samples were collected before and after the meal for a total duration of 240 min. All samples were kept at -80°C before they were analyzed.

Measurement of PEG 4000 in gastric and duodenal samples

The PEG 4000 concentration was measured using the turbidimetric method developed by Hydén (21) as modified by Malawer and Powell (22).

Assay of pancreatic lipase activity

The enzyme activity of HPL in duodenal contents was measured by the pHstat technique as previously reported (20, 23). Lipase levels were expressed in international units per milliliter (1 international unit = 1 μmol of fatty acid released per minute from the standard substrate, tributyrin). Lipase outputs were expressed either in total units or milligrams of active lipase based on the known specific activity (8,000 U/mg) of a pure HPL acting on tributyrin under similar conditions.

Use of PEG for correcting the volume of duodenal aspirates

A correction factor accounting for the partial recovery in duodenal aspirates of the nonabsorbable marker, PEG 4000, was calculated for the whole period of measurement by dividing the total amount of marker initially added in the meal by the recovered amount at the end of the experiment.

Estimation of HPL duodenal outputs

The duodenal output of HPL was estimated from the assay of enzyme activity in duodenal samples, the volume of duodenal aspirates, and the correction factor based on the recovery of PEG.

Preparation of BA standards for LC-MS/MS analysis

Concentrated BA calibration solutions were prepared in methanol (1 mg/ml) and stored in a sealed container at -20°C . These stock solutions were diluted to obtain calibration solutions ranging from 31.3 ng/ml to 31.3 $\mu\text{g}/\text{ml}$. Cholic acid (CA), deoxycholic acid (DCA), chenodeoxycholic acid (CDCA), ursodeoxycholic acid (UDCA), lithocholic acid (LCA), hyocholic acid (HCA), and the corresponding glyco- and tauro-conjugates were obtained from Sigma-Aldrich (Saint Quentin Fallavier, France). The 3-sulfate derivatives of the BAs were a generous gift from Dr. J. Goto (Niigata University of Pharmacy and Applied Life Science, Japan). The 23-nor-5 β -cholanoic acid-3 α ,12 β diol, muricholic acid derivatives, and glyco- and tauro-derivatives were purchased from Steraloids Inc. (Newport, RI).

BA analysis by LC-MS/MS

An internal standard solution (2 μl of 23-nor-5 β -cholanoic acid-3 α ,12 β diol at 1 mg/ml) was added to plasma samples (500–1,000 μl) and duodenal aspirates (100–200 μl). Proteins were precipitated by addition of ammonium carbonate 0.4 M for 30 min at 60°C (17). The clean-up procedure was achieved by centrifugation (4,000 g for 10 min) followed by a solid-phase extraction. Reverse phase Chromabond C₁₈ cartridges (100 mg; Macherey-Nagel, Düren, Germany) were prewashed with 5 ml of methanol and 5 ml of water, successively, before the sample was loaded onto

the cartridge. The subsequent steps were processed on a vacuum manifold designed for solid-phase extraction. The cartridge was rinsed successively with 20 ml of water, 10 ml of hexane to discard neutral lipids, and again with 20 ml water. BAs were finally eluted by methanol. The eluates were dried under a nitrogen stream at 50°C and the residue dissolved in 150 μl of methanol. Five microliters were injected into the HPLC-MS/MS equipment.

The chromatographic separation of BAs (see supplemental Fig. S2) was carried out on a reverse phase column [Restek C18 Pinnacle II (250 \times 3.2 mm, 5 μm); Restek, Lisses, France] thermostated at 35°C (Agilent 1100 HPLC; Agilent Massy, France). The column was initially equilibrated with a 65/35 (v/v) mixture of solvent A (15 mM of aqueous ammonium acetate, pH 5.3) and solvent B (methanol). BA elution was achieved by increasing the proportion of solvent B from 65/35 to 95/5 (v/v). Simultaneously, the flow rate was increased from 0.3 to 0.5 ml/min for 30 min.

The HPLC column eluates were infused into the ESI source of a triple quadrupole mass spectrometer (QTRAP 2000; Applied Biosystems-SCIEX, Concord, Ontario, Canada). Electrospray ionization was set in the negative mode. Nebulizer, curtain, and heater nitrogen gases were set at 40, 20, and 40 (arbitrary units), respectively. The temperature for the evaporation gas (nitrogen) was set at 400°C . The ion spray, declustering, and entrance potentials were set at $-4,500$, -60 , and -10 V, respectively. The MS/MS detection was operated with a unit resolution in the multiple reaction monitoring mode. The dwell time for each transition was set at 70 ms. Multiple reaction monitoring was performed by examination of the transition reactions from precursor conjugated BA to product fragment-ions after collision-induced dissociation: the sulfite (m/z 80, SO_3 fragment-anion cleaved from taurine) and glycine moieties (m/z 74), respectively, for tauroconjugated and glycoconjugated BAs. Sulfo-conjugates were identified by the sulfuric anion (m/z 97, HSO_4). No specific fragment-ions were observed for unconjugated BAs, except those corresponding to loss of water, and those fragments were not reliable for quantification. Therefore, we used selected ion monitoring mode for quantifying unconjugated BAs, with mono-, di-, and tri-hydroxylated BAs scanned at m/z 375, 391, and 407, respectively. The 23-nor-5 β -cholanoic acid-3 α ,12 β diol (m/z 377) was used as the internal standard for normalization. The method for BA analysis was validated according to Humbert et al. (17), who previously checked that similar values were obtained with three different internal standards (23-nor-5 β -cholanoic acid-3 α ,12 α diol, ursodeoxycholic-2,2,4,4-d₄ acid, and lithocholic-2,2,4,4-d₄ acid) with distinct hydrophobicity and retention times.

Statistics

All the data are expressed as mean \pm SD unless stated otherwise. The data obtained in the various series of experiments were compared using paired and unpaired Student's *t*-tests. The data obtained at time 30 min after meal administration were also analyzed using principal component analysis and discriminant analysis (DA) using an orthogonal-partial least square (O-PLS) statistical model [SIMCA14 software package (Umetrics, Umeå, Sweden)].

RESULTS

Variations in the duodenal pH

In HVs, the duodenal pH values remained in the pH 4.1–7.4 range during the meal digestion, with a mean value of 6.10 ± 0.8 (Fig. 1A). In CP patients, the duodenal pH

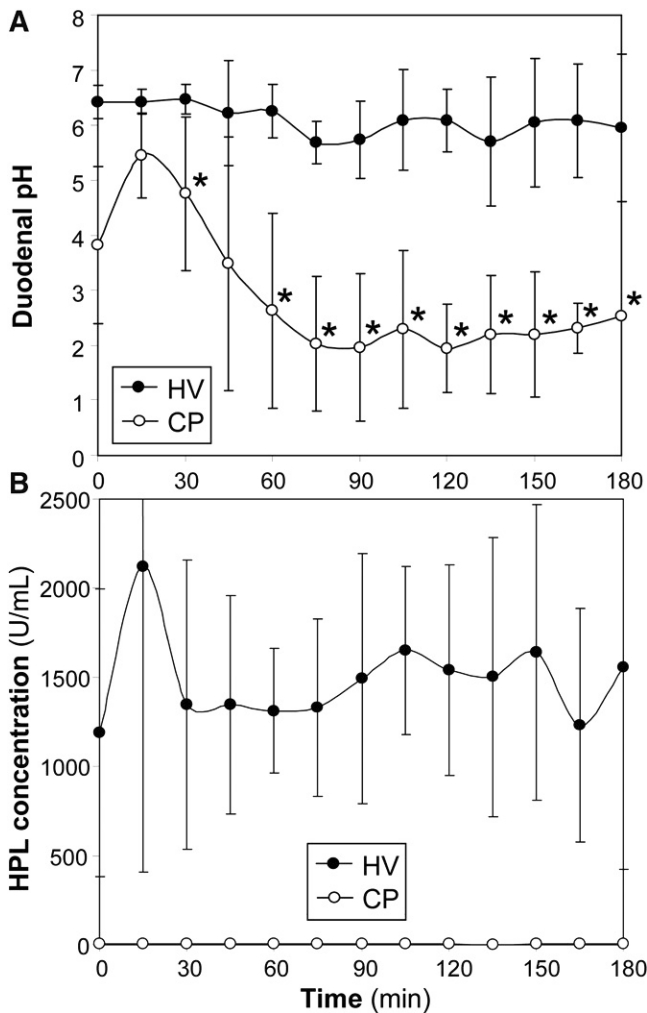


Fig. 1. Variations with time in duodenal pH (A) and HPL concentration (B). Values are mean \pm SD (severe CP patients: $n = 6$; HVs: $n = 6$). In panel A, asterisks indicate significant differences ($P < 0.05$) between severe CP patients and HVs. In panel B, lipase concentration is expressed in units per milliliter and values for CP patients were always significantly different ($P < 0.005$) from those obtained with HVs.

decreased rapidly after 30 min, and then remained very low with mean pH values ranging between pH 2 and pH 3.5 (Fig. 1A). The pH differences between CP patients and HVs were significant ($P < 0.05$) from 30 to 180 min.

HPL duodenal concentration and outputs

Pancreatic lipase outputs during the whole digestion period were estimated from the recovery of duodenal contents. The HPL activities recorded in HVs were in the 320–5,250 U/ml range (i.e., 40–656 $\mu\text{g}/\text{ml}$ of active HPL), whereas HPL activity in CP patients was extremely low and never greater than 12 U/ml (1.5 $\mu\text{g}/\text{ml}$; Fig. 2B). From the cumulated outputs of HPL, it was observed that pancreatic lipase secretion was almost abolished in CP patients (0.7 \pm 0.2 mg vs. 116.7 \pm 68.1 mg in HVs; $P < 0.005$).

Total BA concentrations in duodenal contents

In HVs, the BA concentration in duodenal contents was increased immediately after the meal intake and reached a maximum value (14.8 \pm 12.7 mM) after 30 min (Fig. 2A). It

then decreased and leveled off around 3–4 mM after 120 min. Except during the 75–90 min period, mean BA concentration found in human duodenal contents was above the average critical micellar concentration (CMC) of the main BAs found in human bile (24) (around 4 mM; see supplemental Table S2). In CP patients, the BA concentration in the duodenum was much lower than in HVs. A maximum value of 3.9 \pm 5.6 mM was observed at 45 min (Fig. 2A). Except at 30 and 45 min, the total BA concentration remained below the CMC.

The ratio of primary BA (7 α -hydroxylated in the liver) over secondary BA (produced from primary BA by the intestinal microbiota after 7 α -dehydroxylation) was considerably higher in CP patients (38.09 \pm 48.1) than in HVs (4.15 \pm 2.37; $P < 0.15$) (Fig. 2B). The ratio ranged from 19.3 to 72.8 in CP patients as compared with 4.1 to 7.1 in HVs.

BA duodenal outputs

Total BA outputs in duodenal contents over the postprandial period (Table 1) were much lower in CP patients (1.00 \pm 0.89 mmol; 0.47 \pm 0.42 g) versus HVs (5.52 \pm 4.53 mmol; 2.62 \pm 2.14 g). The ratio of primary to secondary BA was also much higher in CP patients (38.1 \pm 39.4) than in HVs (4.2 \pm 1.9). The examination of the detailed BA composition (expressed as total BA mole percent) revealed a significant decrease in taurochenodeoxycholic acid (TCDCA) ($P < 0.015$), taurodeoxycholic acid (TDCA) ($P < 0.025$), glycolithocholic acid 3-sulfate (GLCA-3S) ($P < 0.02$), tauroolithocholic acid 3-sulfate (TLCA-3S) ($P < 0.01$), UDCA 3-sulfate (UDCA-3S) ($P < 0.035$), LCA 3-sulfate ($P < 0.05$), glyoursodeoxycholic acid 3-sulfate (GUDCA-3S) ($P < 0.03$), glyoursodeoxycholic acid (GUDCA) ($P < 0.035$), glyohyodeoxycholic acid (GHDCA) ($P < 0.005$), glycocholic acid (GCA) ($P < 0.015$), and tauroolithocholic acid (TLCA) ($P < 0.05$) in CP patients versus HVs (Table 1). Only traces of glucuronated conjugates (<0.01% of total BAs) were detected and were not taken into account for comparison of BA profiles between CP patients and HVs. For comparison, total sulfated BAs accounted for around 0.04% (CP patients) to 0.3% (HV) of total BAs in duodenal contents.

BA concentrations in blood plasma

The BA concentration in blood plasma was in the micromolar range that is three orders of magnitude lower than duodenal BA concentration (millimolar range; Fig. 2). After meal intake, the total BA concentration in the plasma of CP patients showed a peak (3.88 \pm 2.32 μM ; Fig. 3C) at 30 min. The peak contrasted with the decreased BA concentration in HVs (1.92 \pm 1.56 μM ; Fig. 2C). In HV plasma, BA concentration increased only lately to 2.32 \pm 1.55 μM at 60 min (Fig. 2C) before decreasing. BA concentrations in HVs and CP patients reached similar values at 120–180 min.

The analysis of individual circulating BAs revealed a higher primary to secondary BA ratio in CP patients. The largest difference in the ratio was observed at 90 min (Fig. 2D). The comparison of the area under the plasma concentration curve (AUC; $\mu\text{mol}\cdot\text{l}^{-1}\cdot\text{min}$) for individual BAs was not statistically different between the HV and CP groups (Table 2), except for ursodeoxycholic acid (UDCA

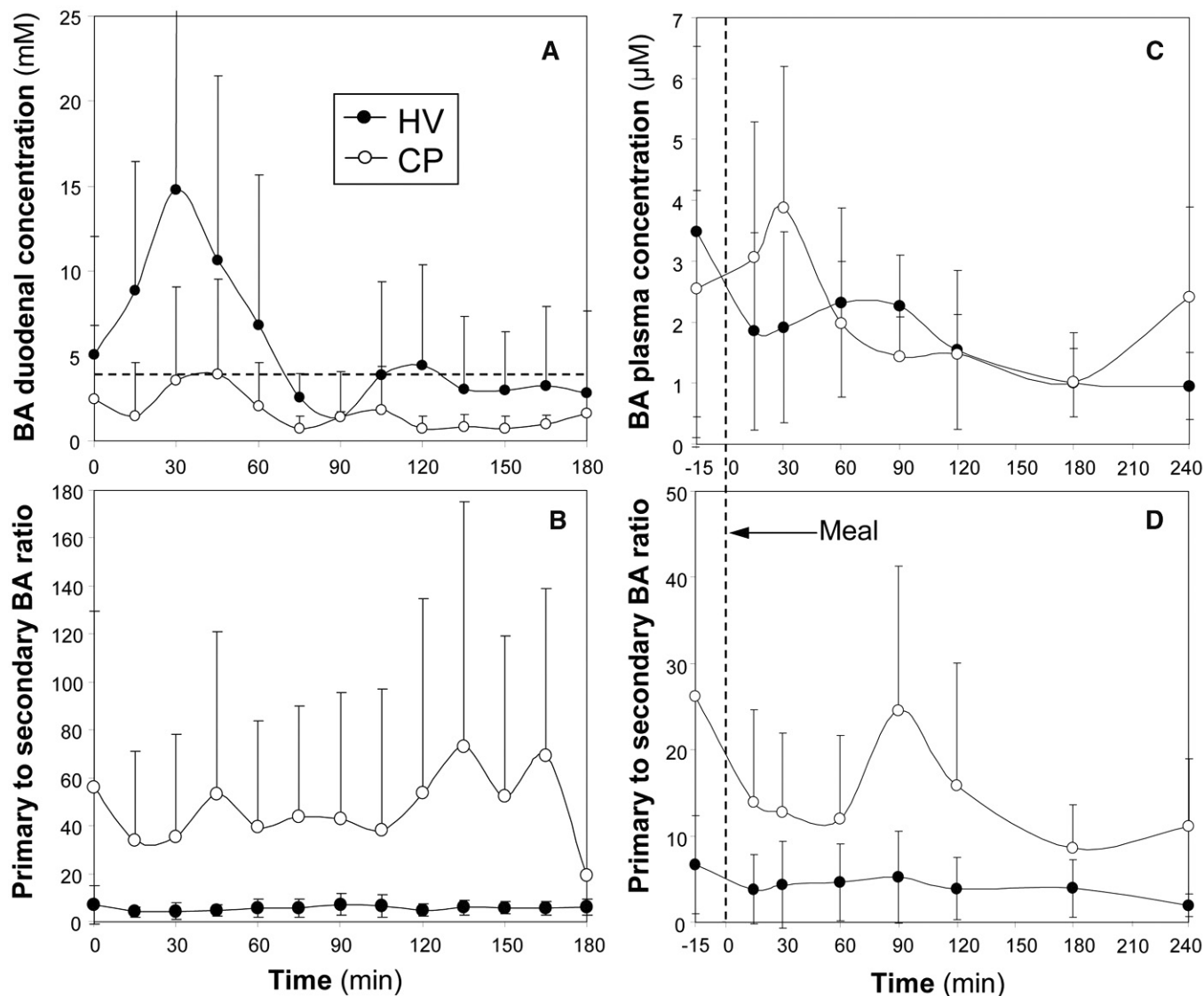


Fig. 2. BA levels in duodenal contents and blood plasma. A: Variations with time in total BA concentration in duodenal contents. The horizontal dashed line indicates the mean CMC of the main BAs found in human bile [around 4 mM according to (24)]. B: Primary to secondary BA ratio in duodenal contents as a function of time. C: Variations with time in total BA concentration in blood plasma. D: Primary to secondary BA ratio in blood plasma as a function of time. The vertical dashed line in panels C and D indicates the time at which the test meal was given. Values are mean \pm SD ($n = 6$ for CP patients and $n = 6$ for HVs).

and GUDCA), which was significantly lower in CP patients ($P < 0.05$). The decreased ursodeoxycholic conjugates were unexpected regarding a commonly found increase in prolonged cholestasis (25). Analysis of AUCs showed a significantly higher ($P < 0.05$) proportion of primary BA in CP patients (87.1 ± 6.5 mol% of total BAs) as compared with HVs (67.1 ± 17.0 mol% of total BAs). Only traces of glucuronated conjugates ($<0.01\%$ of total BAs) were detected in the plasma of CP patients and HVs, while total sulfated BAs accounted for around 0.7% (HV) to 1.5% (CP patients) of total BAs in plasma.

Simultaneous changes in individual BA concentrations in duodenal contents and blood plasma

The variations with time in individual BA duodenal concentrations were similar to those observed with total BAs (Fig. 2A), as illustrated by the kinetics of GCA (Fig. 3A), a

major BA contained in human bile. Variations in other BAs are reported in supplemental Fig. S3. The main deviation between HVs and CP patients was observed 30 min after the meal with mean GCA duodenal concentrations of 2,012 and 616 μ M, respectively (Fig. 3A). Then, the mean GCA duodenal concentration in HVs decreased to values similar to CP patients after 60 min. In CP patients, only weak changes in the GCA duodenal concentration were observed (Fig. 3A).

Interestingly, opposite variations in GCA concentration were observed in blood plasma (Fig. 3B). The plasma concentration of GCA increased clearly in CP patients and peaked at 30 min (mean concentration of 0.72 μ M); but in HVs, the mean GCA concentration remained low (mean 0.15 μ M). Plasma GCA concentration of CP patients returned to low HV values after 60 min.

Because the main differences between CP patients and HVs were observed at 30 min, we focused on duodenal

TABLE 1. BA outputs and distribution in duodenal contents of HVs and patients with severe CP after intake of a liquid-solid test meal

BAs	HVs (Controls) (n = 6)		CP Patients (n = 6)		
	Output (μmol)	Total BA Percent (mol%)	Output (μmol)	Total BA Percent (mol%)	Percent of Controls
TUDCA	53.02 ± 58.93	0.78 ± 0.79	0.67 ± 0.53	0.07 ± 0.03	1.27
THDCA	10.04 ± 14.32	0.08 ± 0.12	0.54 ± 0.75	0.02 ± 0.04	5.42
TCDCa	777.22 ± 618.97	14.56 ± 2.99	101.68 ± 94.82	8.78 ± 3.15	13.08
TDCA	522.95 ± 549.81	7.80 ± 4.93	27.33 ± 32.81	1.71 ± 1.37	5.23
THCA	2.30 ± 1.79	0.05 ± 0.02	1.36 ± 1.37	0.16 ± 0.21	59.35
TCA	827.88 ± 570.98	19.13 ± 8.82	229.80 ± 214.30	22.76 ± 12.92	27.76
GLCA-3S	4.43 ± 3.01	0.11 ± 0.06	0.43 ± 0.56	0.02 ± 0.03	9.62
TLCA-3S	5.81 ± 4.63	0.14 ± 0.08	0.27 ± 0.37	0.01 ± 0.02	4.62
UDCA-3S	0.03 ± 0.03	<0.01	0.00	0.00	0.00
CDCA-3S	0.05 ± 0.04	<0.01	0.01 ± 0.01	<0.01	15.33
DCA-3S	0.23 ± 0.24	<0.01	0.02 ± 0.02	<0.01	10.16
LCA-3S	0.21 ± 0.10	<0.01	0.02 ± 0.02	<0.01	8.12
CA-3S	0.00	—	0.00	—	—
TUDCA-3S	0.42 ± 0.49	0.01 ± 0.01	0.01 ± 0.02	<0.01	3.22
GUDCA-3S	1.92 ± 1.65	0.03 ± 0.02	0.09 ± 0.12	0.01 ± 0.01	4.86
GLCA	20.37 ± 13.31	0.43 ± 0.30	2.39 ± 3.06	0.15 ± 0.18	11.76
GUDCA	181.35 ± 193.46	2.33 ± 1.52	5.38 ± 5.51	0.61 ± 0.45	2.97
GHCA	29.32 ± 28.11	0.42 ± 0.15	1.40 ± 1.74	0.10 ± 0.08	4.77
GCDCA	1438.15 ± 1126.45	26.19 ± 8.74	278.11 ± 277.77	29.35 ± 11.58	19.34
GDCA	889.17 ± 867.84	12.55 ± 5.13	79.39 ± 99.12	5.71 ± 4.87	8.93
GHCA	1.74 ± 1.41	0.03 ± 0.02	0.90 ± 0.73	0.13 ± 0.16	51.63
GCA	736.51 ± 559.03	15.02 ± 4.34	242.89 ± 184.29	28.23 ± 8.82	32.98
TLCA	13.83 ± 11.07	0.29 ± 0.22	1.02 ± 1.22	0.06 ± 0.05	7.40
LCA	0.02 ± 0.03	<0.01	0.06 ± 0.10	<0.01	315.21
UDCA	0.11 ± 0.10	<0.01	0.04 ± 0.07	<0.01	36.24
HDCA	0.00	—	0.00	—	—
CDCA	0.84 ± 0.66	0.02 ± 0.01	5.71 ± 9.10	0.41 ± 0.77	680.48
DCA	0.28 ± 0.21	<0.01	2.27 ± 3.72	0.15 ± 0.32	804.00
MCA	0.01 ± 0.01	<0.01	0.03 ± 0.05	<0.01	312.19
HCA	0.00	—	0.00	—	—
CA	1.77 ± 1.50	0.04 ± 0.03	16.62 ± 22.53	1.54 ± 1.87	937.62
Total BA	5,519.97 ± 4,534.78	100.00	998.46 ± 889.62	100.00	18.09

BA outputs are expressed in micromoles and BA distributions are expressed as the mole percent of total BA. CA-3S, CA 3-sulfate; CDCA-3S, CDCA 3-sulfate; DCA-3S, DCA 3-sulfate; GHCA, glycohyocholic acid; GLCA, glycolithocholic acid; HDCA, hyodeoxycholic acid; LCA-3S, LCA 3-sulfate; MCA, muricholic acid; THCA, taurohyocholic acid; THDCA, taurohyodeoxycholic acid; TUDCA, taoursodeoxycholic acid; TUDCA-3S, taoursodeoxycholic acid 3-sulfate.

(Fig. 3C) and plasma (Fig. 3D) concentrations of individual BAs in duodenal and blood samples at 30 min [TCDCA, TDCA, taurocholic acid (TCA), GUDCA, glycochenodeoxycholic acid (GCDCA), glycodeoxycholic acid (GDCA), GCA]. For each individual BA, the duodenal concentration measured in CP patients was significantly lower ($P < 0.05$) than in HVs (Fig. 3C). Conversely, plasma concentrations in CP patients were higher with significant differences ($P < 0.05$) for the abundant BAs, GCDCA and GCA (Fig. 3D).

Conjugated BA levels

Both glyco- and tauro-conjugated BA levels were much lower in CP patients (0.6 ± 0.7 mmol and 0.4 ± 0.5 mmol, respectively) than in HVs (3.3 ± 3.7 mmol and 2.2 ± 2.7 mmol, respectively). However, the glyco- to tauro-conjugated BA ratios in CP patients (2.5 ± 1.7) and HVs (1.5 ± 0.7) were not significantly different.

Unconjugated BA levels

Unconjugated BA levels (UDCA, CDCA, DCA, CA) were measured at a low level ($<25 \mu\text{M}$) in the duodenal contents of both HVs and CP patients (Table 1; 0.01–1.5% of total BA) except for a CP patient with particularly high concentrations of CDCA, DCA, and CA (646, 293, and 124 μM , respectively, at 30 min). This outlier patient had an impact on the statistical significance of the difference with the control HV group

(Table 1). Unconjugated BA showed higher contributions in the plasma than in the bile for both HVs ($28.5 \pm 24.2\%$ of total BAs) and CP patients ($18.2 \pm 15.4\%$ of total BAs). However, no significant difference between the two groups was found, including for the CP patient showing the particularly high duodenal concentrations of unconjugated BAs.

BA levels in patient with alcoholic liver disease

Only one CP patient out of six had highly elevated liver enzymes, presumably due to alcoholic liver disease [patient 4 (G.R.); see supplemental Table S1]. Total BA outputs (0.66 mmol) in this patient were not significantly different from those found in the five other patients with severe CP and were lower than those observed in HVs. Primary to secondary BA molar ratio in this patient was the highest found among all CP patients, in both duodenal contents (55–190) and blood plasma (10–110), but this finding did not change the fact that all CP patients showed a higher primary to secondary BA ratio than the HV group.

DISCUSSION

The availability of LC-MS/MS analytical methods for BA in the clinical laboratory allows the investigation of BA secretion and circulation. The comparison of HVs and CP patients,

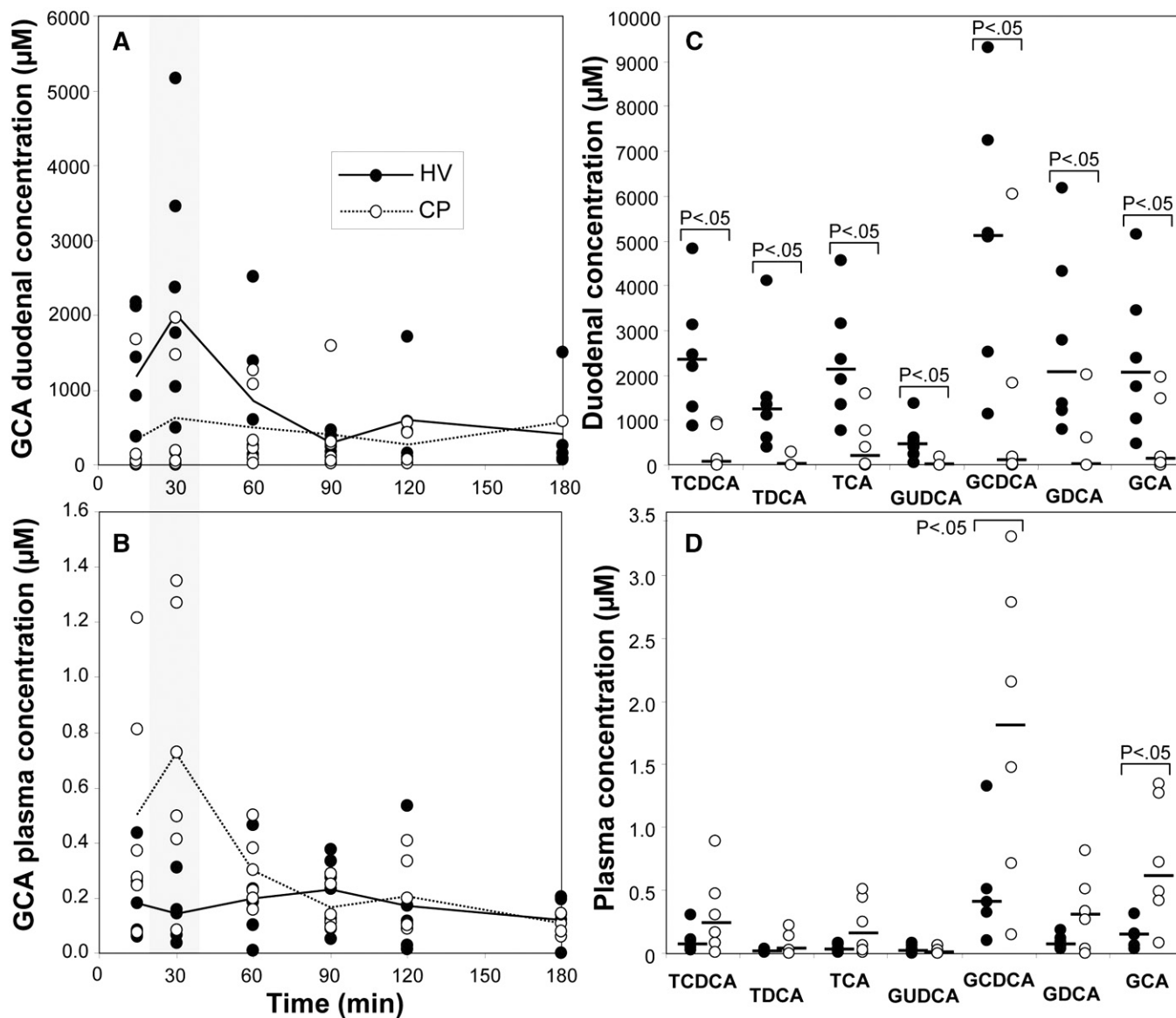


Fig. 3. Comparison of main BA concentrations in duodenal contents and blood plasma of HVs and severe CP patients. Panels A and B show the variations with time of the concentration (micromoles) of a representative BA (GCA) in duodenal contents and blood plasma, respectively. Individual data points for CP patients ($n = 6$) and HVs ($n = 6$) are displayed at times for which both the duodenal and blood plasma samples were collected (15, 30, 60, 90, 120, and 180 min). The solid and dotted lines represent the variations of mean values for HVs and CP patients, respectively. Panels C and D show the concentrations (micromoles) in the main BAs measured 30 min after meal ingestion in duodenal contents and blood plasma, respectively. All individual data points are displayed and the horizontal bars indicate median values for each group.

using samples of duodenal contents and blood plasma collected during test meal experiments, contributes to a detailed understanding of the condition. We selected CP patients with a severe pancreatic exocrine insufficiency authenticated by a low duodenal pH value (Fig. 1A) resulting from weak bicarbonate secretion and extremely low pancreatic lipase (HPL) levels (Fig. 1B). On average, HPL secretion in CP patients (0.7 ± 0.2 mg) represented only 0.6% of control outputs (116.7 ± 68.1 mg in HVs). The HPL outputs were found in the range previously reported for severe CP patients (5) and healthy subjects (5, 26), respectively.

BAs in HVs

The data on BA analysis with HVs confirmed concentrations and outputs previously reported in healthy humans

under fed conditions. Total output (2.62 g; 5.52 mmol) during the duration of the meal (3 h) was close to the 3 g BA amount estimated for the pool of BAs circulating 4–12 times per day in the human gastrointestinal tract (27). Furthermore, the peak in duodenal concentration of BAs observed 30 min after meal ingestion was typical of normal gallbladder emptying (27, 28). While highly variable intestinal concentrations of BAs under fed conditions have been reported in the literature, ranging from 0.5 (29) to 37 mM (30), there is an agreement for duodenal concentrations recorded 30–60 min after a meal and following gallbladder emptying: 14.5 ± 8.8 mM (mean \pm SD, $n = 5$) reported by Hernell, Staggers, and Carey (30), 14.7 ± 8.0 mM (mean \pm SD, $n = 16$) by Tangerman, Schaik, and van der Hoek (31), 15.8 ± 5.6 mM (mean \pm SD, $n = 5$) by Ladas

TABLE 2. Kinetics and composition of BAs in serum of HVs and patients with severe CP after intake of a liquid-solid test meal

BAs	HVs (Controls) (n = 6)		CP Patients (n = 6)		Percent of Controls
	AUC ($\mu\text{mol}\cdot\text{l}^{-1}\cdot\text{min}$)	Total BA Percent (mol%)	AUC ($\mu\text{mol}\cdot\text{l}^{-1}\cdot\text{min}$)	Total BA Percent (mol%)	
TUDCA	0.00	—	0.00	—	—
THDCA	0.00	—	0.00	—	—
TCDCa	17.29 ± 12.50	5.93 ± 1.56	18.99 ± 12.38	6.70 ± 4.47	109.81
TDCA	4.19 ± 3.66	1.54 ± 0.67	3.57 ± 3.30	1.11 ± 1.05	85.19
THCA	0.00	—	0.00	—	—
TCA	6.51 ± 5.84	1.98 ± 0.78	12.92 ± 12.44	5.27 ± 5.63	198.41
GLCA-3S	0.73 ± 1.34	0.24 ± 0.34	1.42 ± 0.96	0.44 ± 0.26	193.32
TLCA-3S	0.09 ± 0.13	0.03 ± 0.04	0.80 ± 1.05	0.27 ± 0.32	891.11
UDCA-3S	0.00	—	0.00	—	—
CDCA-3S	0.00	—	0.00	—	—
DCA-3S	0.10 ± 0.19	0.03 ± 0.04	0.18 ± 0.27	0.05 ± 0.07	182.25
LCA-3S	0.00	—	0.00	—	—
CA-3S	0.02 ± 0.04	0.01 ± 0.02	0.00	—	0.00
TUDCA-3S	0.00	—	0.05 ± 0.08	0.03 ± 0.04	—
GUDCA-3S	0.72 ± 1.22	0.36 ± 0.47	1.58 ± 1.89	0.73 ± 1.02	218.94
GLCA	0.36 ± 0.72	0.10 ± 0.19	0.02 ± 0.06	0.01 ± 0.01	6.66
GUDCA	8.81 ± 8.30	3.77 ± 2.21	0.82 ± 1.37	0.21 ± 0.34	9.27
GHDCA	0.00	—	0.00	—	—
GCDCA	109.08 ± 72.62	38.93 ± 9.02	140.99 ± 62.57	45.15 ± 6.32	129.26
GDCA	24.82 ± 23.00	8.58 ± 5.18	19.39 ± 13.71	5.64 ± 4.01	78.12
GHCA	0.00	—	0.00	—	—
GCA	27.79 ± 21.80	9.51 ± 3.52	45.60 ± 17.61	16.20 ± 6.61	164.09
TLCA	0.00	—	0.00	—	—
LCA	0.05 ± 0.12	0.02 ± 0.05	0.00	—	0.00
UDCA	8.14 ± 9.76	3.52 ± 3.68	0.26 ± 0.49	0.07 ± 0.14	3.21
HDCA	0.27 ± 0.4	0.08 ± 0.10	0.00	—	—
CDCA	20.03 ± 30.15	9.67 ± 11.80	21.69 ± 26.06	7.04 ± 7.32	108.26
DCA	21.82 ± 18.98	7.85 ± 2.09	14.85 ± 15.71	4.35 ± 4.06	68.05
MCA	0.56 ± 1.24	0.22 ± 0.50	0.00	—	0.00
HCA	0.41 ± 0.91	0.11 ± 0.25	0.00	—	—
CA	19.89 ± 32.67	7.48 ± 6.68	21.04 ± 16.13	6.75 ± 3.91	105.79
Total BA	271.69 ± 185.62	100.00	304.18 ± 104.84	100.00	111.96

See the legend to Table 1 for abbreviations.

et al. (29), 16.2 ± 1.5 mM (mean \pm SE, $n = 13$) by Ratureau, Bisalli, and Rambaud (32), 14.5 ± 9.4 (mean \pm SD, $n = 12$) by Fausa (33), and 8.3 mM (normal meal) to 11.9 mM (high-fat meal) reported by Clarysse et al. (28). The mean duodenal BA concentration measured presently after 30 min in HVs (14.8 ± 12.7 mM, $n = 6$; Fig. 2A) was therefore consistent with previous data. BA profiles were also in the normal range with conjugated BAs >90%, but low levels of unconjugated and sulfated BAs (Tables 1, 2). The primary to secondary BA ratio in duodenal contents of HVs (approximately 5.5) was close to previously reported values for healthy subjects under fasting (5.5) and fed (8.2) conditions (28).

BAs were found at a low concentration (micromolar range) in the peripheral blood circulation as a result of the normal highly efficient uptake by the liver (34). Plasma BA composition was also similar to previous reports with healthy subjects (17, 35). It was noticeable that the contribution of unconjugated BA to total BAs found in plasma was much higher ($28.5 \pm 24.2\%$; Table 2) than in duodenal contents (around 0.06%; Table 1). These higher plasma concentrations of unconjugated BAs can be explained by their slower first-pass hepatic clearance, which disproportionately increases their concentration in plasma versus conjugated BAs. Therefore, the measurement of the relative amounts of conjugated versus unconjugated BAs in plasma is not a good indicator of

their relative proportions in the whole-body BA pool or in other compartments.

BAs in CP patients

In addition to the deficit in pancreatic bicarbonate and enzyme secretions, total BA concentration in duodenal contents was decreased approximately 5-fold in CP patients as compared with HVs (Fig. 2A). No peak of BA concentration in duodenal contents that could reflect gallbladder emptying was observed at 30 min. The maximum concentration was observed with a delay of 15 min compared with HVs. Overall, the total BA output in the duodenum was severely reduced in CP patients (0.47 g; 1.00 mmol). It amounted to only 18% of BA output in HVs (2.62 g; 5.52 mmol; Table 1). As a result, BA duodenal concentration was often found below the average CMC of the main bile salts found in human bile and duodenal contents (Table 3). It is worth noticing that we analyzed complete duodenal samples and, therefore, low BA levels in CP patients could not be explained by some BA precipitation at low pH, as reported in previous studies (12, 36). This point will be further discussed.

As in healthy subjects, conjugated BAs represented >90% of total BAs and very low levels of unconjugated BAs and sulfated BAs were measured (Table 1). The glyco-tauro-conjugated BA ratio was not changed compared with HVs, indicating that no selective precipitation of

TABLE 3. CMCs of the main BAs found in duodenal contents

BAs	Occurrence in Duodenal Contents (mol% of Total BAs)	CMC (mM)
GCA	15.02	10
GDCA	12.55	2
GCDCa	26.19	1.8
GUDCA	2.33	4
TCA	19.13	6
TDCA	7.8	2.4
TCDCa	14.56	3
TUDCA	0.78	2.2
Weighted average		4.1

CMC values are from Roda et al. (24) and were determined by surface tension measurements, using 0.15 M Na⁺ solution for bile salt dispersion. Occurrences in duodenal contents (mole percent of total BAs) are based on data obtained with HVs.

glyco-conjugated BA at low pH occurred in CP patients compared with previous reports (12, 36). We therefore re-analyzed these previous studies by comparing BA concentrations and pH levels with those we measured here. In the article by Go et al. (36), which reports BA precipitation at low jejunal pH in one patient with gastrin-producing islet cell tumor, gastric hypersecretion, and steatorrhea, BA precipitation occurred at very low pH values (≤ 2.5), which could be reproduced in vitro. Only 48% of BAs were present in the micellar phase. This finding is probably the first one that led to the explanation of low BA levels in CP patients by acid precipitation. However, the sum of BA levels in all phases was only 23% of that recorded in normal subjects, which shows a reduced secretion of BAs independently from a loss by acid precipitation and is in agreement with our own observations. In this study, it was shown that the precipitated BAs were largely the glycine dihydroxy conjugates (around 70%). In the article by Regan et al. (12) on patients with advanced acquired EPI, the lowest pH values (between pH 3 and 4) and BA concentrations were not as low as in Go's study (36) and in our work. Indeed, at the BA secretion peak, the concentration in the micellar phase was 8.1 ± 0.6 mM in EPI patients versus 9.3 ± 0.4 mM in normal subjects, which indicates that BA secretion was not drastically reduced in these patients compared with the CP patients with severe EPI enrolled in our study. Although a pH-dependent lowering of BA concentration in the micellar phase was observed, its impact on BA levels was limited and the patients involved in this study had clearly much higher BA levels than those we included in our study. Nevertheless, this publication and its conclusions have certainly led to an overestimation of the impact of acid precipitation on BA levels in EPI patients, and have delayed the recognition that low BA in these patients could also be explained by an altered BA circulation.

On a relative basis, primary BAs (CA and CDCA conjugates) increased, but secondary BAs (DCA derivatives) decreased, in CP patients, which is consistent with a reduced bacterial conversion of primary to secondary BA in the intestine or fecal loss of BA due to intestinal BA malabsorption. In any case, this finding suggests changes in the enterohepatic circulation of BAs (27), and this is supported by the fact that the increased primary to secondary BA ratio observed in duodenal contents (Fig. 2B) was also observed

in the peripheral blood circulation (Fig. 2D). Intestinal BA malabsorption is one hypothesis to explain these findings: it would lead to the fecal loss of the total BA pool and depletion of secondary BA formed in the intestine, and would derepress hepatic production of newly synthesized primary BAs. We have no information, however, on the fecal loss of BA in these CP patients and cannot confirm whether it could account for the 5-fold depletion in BA pool observed in CP patients versus HVs.

Another hypothesis could be a bypassed BA circulation via the cholehepatic shunt, a pathway that is minored under the normal physiological conditions (37) by the extensive enterohepatic recycling of BAs from the intestine. Indeed, a remarkable particularity in CP patients was a sharp increase in plasma BA concentration 30 min after meal ingestion (Fig. 2C) and the absence of a BA concentration peak in duodenal contents corresponding to gallbladder emptying, which is consistent with a shunt of BA circulation. The cholehepatic shunt represents the fraction of BAs absorbed by cholangiocytes lining the biliary ducts and gallbladder, which is recycled back to hepatocytes before reaching the intestinal lumen (38). BA absorption by cholangiocytes involves specific BA transporters, like the apical sodium BA transporter and heteromeric organic solute transporter (38–40). The cholehepatic shunt pathway might be favored in severe CP patients due to the drastic acidification of intestinal contents. Indeed, using a mouse model of cystic fibrosis (*Cftr*^{-/-}), Debray et al. (41) have shown that increased duodenal acidity results in attenuation of gallbladder contraction through the overexpression of vasoactive intestinal peptide, which leads to an increase in cholecystohepatic shunting of BAs and reduced levels of secondary BAs. Cholecystectomy reversed those changes, which provided evidence that the gallbladder, rather than the bile duct epithelia, may be the major site of BA reabsorption in the cholehepatic shunt. In our study, the rise in plasma BAs observed at the 30 min time point in the CP patient group suggests that the gallbladder contraction after meal intake occurs properly, but BAs may be reabsorbed before reaching the duodenum. The cholehepatic shunt has also been proposed as a protective mechanism for the liver (41, 42), particularly in response to bile duct obstruction. Indeed, it has been shown that this pathway provides an alternative route for continuation of hepato-cholangiocyte flux of BAs in bile duct-ligated rats (43, 44).

Based on these findings, we hypothesized that the low BA levels observed in the duodenal contents of CP patients (Fig. 2A) might be explained by bile duct obstruction, which could eventually lead to a cholehepatic shunt of BA secretion, rather than by BA precipitation at low pH or intestinal BA malabsorption. We checked the medical history of the CP patients selected for this study (supplemental Table S1). They all had alcohol abuse-related CP. Four of them were diagnosed for stenosis of the main bile duct before they were involved in this study and underwent an endoscopic treatment in the years preceding the study (1998–2002). The two other CP patients were diagnosed for main bile duct stenosis after the study (2005–2006). None of them had cholecystectomy. A cholehepatic shunt

of bile secretion in these patients could therefore be explained by the main bile duct stenosis encountered in CP (45).

Because the cholehepatic shunt is usually considered to be a minor pathway, it might not be sufficient to explain the extensive duodenal depletion in BAs in CP patients. As indicated before, there is no evidence that the precipitation of BAs at low pH (46) could explain this depletion. Alternatively, BA secretion by the liver might be reduced in CP patients by a feedback control of the liver BA synthesis and transport (47). Measurements of the plasma levels of 7 α -hydroxy-4-cholesten-3-one and fibroblast growth factor 19 would have helped in distinguishing between cholehepatic shunting of BAs and intestinal BA malabsorption [elevated 7 α -hydroxy-4-cholesten-3-one, reduced fibroblast growth factor 19 (48)] as an explanation for the BA phenotype in CP patients. Unfortunately, we did not have enough plasma samples to perform these assays.

Test meal and plasma BA analysis as a potential diagnostic tool for the follow up of EPI and biliary duct stenosis

Using a test meal and BA analysis in both duodenal contents and blood plasma, we found a clear anti-correlation between duodenal and plasma BA concentrations in the time frame (30 min after meal ingestion) corresponding to gallbladder emptying (well seen in HVs). In HVs, high BA concentrations in duodenal contents corresponded to low BA concentrations in plasma, while in CP patients, low BA concentrations in duodenal contents corresponded to high BA concentrations in plasma (Fig. 4A). In CP patients, the primary to secondary BA ratio also increased in duodenal contents and blood plasma. A DA performed with the O-PLS statistical model has established an S-plot for the circulating blood plasma BAs assayed at 30 min after meal intake. The S-plot revealed the most discriminative biomarkers for CP patients (DCA, UDCA, CDCA) out of 17 plasma BAs (Fig. 4B; additional analysis can be found in supplemental Figs. S4–S8). Therefore, the levels of these three plasma BAs may be used as a quantitative appreciation of EPI complications related to the altered circulation of BAs. BA profiling could also be used in the follow up after endoscopic treatment of main bile duct stenosis in parallel with the assay of hepatic enzyme levels in cholestatic plasma. It should be noted that the plasma BA biomarkers in this study (Fig. 4, supplemental Figs. S4–S8) were identified following a test meal in patients that were intubated with a duodenal occluding balloon near the ligament of Treitz (supplemental Fig. S1). Additional studies are required to determine whether these plasma BA biomarkers would be predictive in patients without the duodenal occluding balloon because the balloon restricts the movement of luminal contents toward the distal intestine and may distort the pattern (post-prandial rise) in BAs present in plasma.

In conclusion, the data presented here show that duodenal BA levels can be drastically reduced in patients with severe CP. Low levels of BAs in intestinal contents have already been described in cases of pancreatic insufficiency. This was usually attributed to BA precipitation at low pH,

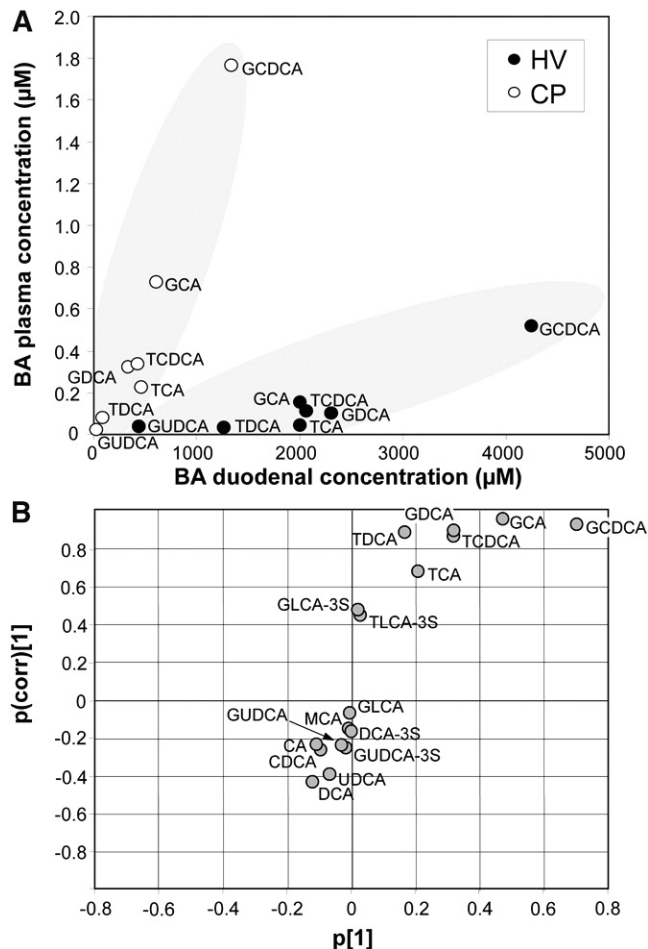



Fig. 4. Discrimination of severe CP patients HVs based on BA analysis. A: Correlation between BA duodenal and blood plasma concentrations measured 30 min after meal ingestion. Only the main conjugated BAs found in both duodenal contents and blood plasma are displayed. Values are means ($n = 6$ for CP patients and $n = 6$ for HVs). B: S-plot of the circulating blood plasma BAs 30 min after ingestion of the test meal. The plot illustrates the selection of the most significant biomarkers among of the various plasma BAs ($n = 17$) in an O-PLS model calculated to discriminate CP patients and HVs. The BA levels measured in plasma by LC-MS/MS are “Pareto” scaled. The vertical axis in the S-plot [$p(\text{corr})$] represents the correlation between BAs and the score of CP patients and HVs. The horizontal axis ($p[1]$) is the contribution of the distinct BAs estimated by the O-PLS-DA model. The S-plot identifies BAs with the best prediction capability and the largest variation between the CP group (high unconjugated DCA, UDCA, CDCA) and the control group (high GDCDA, GCA, TCDCA). $R^2X[1] = 0.617$, i.e., 61.7% of the BA total variability.

particularly that of glyco-conjugated BAs (7, 8, 12, 46), which was not observed here. Our results strongly suggest that low duodenal BA levels in the CP patients involved in our clinical study results from the bile duct stenosis that may lead to a cholehepatic shunt of BA. Enzyme replacement therapy based on the oral administration of pancreatic extracts might not be sufficient to restore fat absorption in these patients, because BAs are essential for the micellar solubilization of lipolysis products and absorption by enterocytes (49). BA duodenal concentrations were often below CMC (Fig. 2A) and formation of mixed BA-lipolysis

product micelles could be seriously impaired. Also, because BAs have antimicrobial effects, their deficiency in the small intestine of CP patients can promote bacterial growth and changes of the intestinal microbiota (50).

The analytical method described here for measuring BAs in plasma samples can be used easily to obtain additional information on EPI patients and identify whether their EPI is coupled with changes in BA secretion and enterohepatic circulation. The “cholehepatic shunt” signature obtained from plasma BA analysis might also be useful for orienting the diagnosis toward intra-hepatic or extra-hepatic cholestasis, with more specific markers than those currently used in clinics, such as levels in hepatic enzymes (γ -glutamyl transpeptidase, alkaline phosphatase, transaminases). The increase in primary to secondary BA ratio could also be used as a secondary marker. In connection with this potential diagnosis tool, it is now essential to develop new treatments for improving fat absorption in EPI patients, and one can think about a coadministration of pancreatic enzymes and BAs. BA replacement therapy has already been used for improving fat absorption in patients using desiccated ox bile or the synthetic conjugated BA, cholylsarcosine (51–53). Other approaches are using the formulation of pancreatin with a semi-solid self-microemulsifying excipient (Gelucire® 44/14) to improve pancreatic lipase activity and intestinal absorption (54, 55). These studies could serve as a basis for further improvement of EPI treatment. 

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