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Serum bile acids in cystic fibrosis patients – glycodeoxycholic acid as a potential marker of liver disease

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ABSTRACT

Background: Cystic fibrosis (CF) and CF-related liver disease can lead to disturbances in bile acid metabolism.

Aim: This study determined serum bile acid concentrations in CF to define their usefulness in liver disease assessment.

Methods: Primary, secondary and conjugated bile acid levels were measured in three CF groups (25 patients each) exhibiting: liver cirrhosis, other liver disease, no liver disease, and in 25 healthy subjects (HS).

Results: Bile acid levels were higher in CF patients than in HS, except for glycodeoxycholic acid (GDCA). However, bile acid concentrations did not differ between patients with cirrhosis and other liver involvement. GDCA and deoxycholic acid (DCA) differentiated CF patients with non-cirrhotic liver disease from those without liver disease (GDCA-AUC: 0.924, 95%CI 0.822–1.000, $p<0.001$; DCA-AUC: 0.867, 95%CI: 0.731–1.000, $p<0.001$). Principal component analysis revealed that in CF liver disease was related to GDCA, GGTP activity, severe genotype and pancreatic insufficiency.

Conclusions: A CF-specific bile acid profile was defined and shown to relate to liver disease. GDCA differentiates patients with non-cirrhotic liver involvement from those with no detectable liver disease. Hence, GDCA is a candidate for validation as a biomarker of non-cirrhotic progression of liver disease in CF.

1. Introduction

In cystic fibrosis (CF), disease-causing variants in the *CFTR* (CF transmembrane conductance regulator) lead to markedly reduced flow of chloride ions across cellular membranes in the epithelia [1], altering the electrolyte composition of the mucus, increasing its viscosity and acidity throughout the body [2]. The sticky mucus obstructs the glands and their excretory ducts and can be colonised by pathogenic microorganisms [3]. Consequently, CF phenotypes usually comprise recurrent respiratory infections and pancreatic insufficiencies but may also include liver disease and malnutrition [4,5]. CF-related liver disease (CFLD) may already be present early in life, as it affects one in five young children with CF.

Among the crucial liver functions are the synthesis and secretion of bile acids. Their transformation is dependent on inter alia ligands of transcription factors [6,7]. The liver synthesises primary bile acids, mainly cholic acid and chenodeoxycholic acid, whereas the gastrointestinal bacteria (*Bifidobacterium* and *Lactobacillus*) deconjugate and decarboxylate primary acids, thus forming secondary and tertiary acids. Unfortunately, in CF patients, these natural metabolic pathways are disturbed. Cholestasis, one of the CFLDs [8–11], increases the concentration of endogenous bile acids exhibiting hepatotoxicity. Physiologically, the bile acids secreted from the liver accumulate in the gallbladder, then pass into the duodenum. As a result of blockage of the bile ducts, an exces-

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sive concentration of acids is cytotoxic for hepatocytes [12,13]. In CF patients, due to the excessive bile viscosity, a large amount of bile acids accumulates inside the liver cells and once their capacities are surpassed, the bile acids are released into the bloodstream. Since the concentration of bile salts in the small intestine is reduced, emulsification becomes impaired, thus limiting the bioavailability of fat-soluble nutrients. Hence, the negative feedback loop, which would otherwise limit bile acid synthesis in the liver, is interrupted [13]. Furthermore, frequent comorbidities and CF complications, such as pancreatic insufficiency, diabetes, and small intestinal bacterial overgrowth, alter the microbiota and may influence bile acid metabolism [14–16].

Disturbances in the metabolism of bile acids have been reported in CF patients. Previous investigations focused mainly on the secretion of bile acids [17–19], whereas more current studies measured their serum profile. However, these studies only comprised a few CF patients in very good clinical conditions [20] or were designed to evaluate the influence of ursodeoxycholic acid (UDCA) therapy on bile acid composition [21,22]. Despite the previous work, the exact causes of bile acid abnormalities and the independent predictors of their serum concentrations in CF remain unknown, and the available data lack a description of the profile in various stages of CFLD.

For this reason, the aims of the research were: (1) to determine serum bile acids concentrations in CF patients with and without liver involvement, (2) and to determine the potential usefulness of measuring particular bile acids to assess the progression of liver disease.

2. Materials and methods

2.1. Patients

The study comprised 75 CF patients: 25 with diagnosed liver cirrhosis (11 female, 14 male, aged 9 to 33 years), 25 with other non-cirrhotic liver diseases (12 female, 13 male, aged 16 to 44 years) and 25 without liver disease (12 female, 13 male, aged 17 to 36 years). CFLD was diagnosed according to the best practice guidelines for diagnosis and management. The diagnosis of liver cirrhosis was established based on the presence of: splenomegaly and/or esophageal varices, along with either multilobular cirrhosis (biopsy) or firm liver on physical examination and radiologic or ultrasound evidence of cirrhosis [23]. The control group consisted of 25 healthy subjects (HS; 12 female, 13 male, aged 18 to 27 years). The exclusion criteria for CF were pregnancy, lung transplantation, pulmonary exacerbation and any acute infection episode. None of the patients received intravenous or oral antibiotic therapy within six weeks preceding blood collection. The anthropometrical parameters, which included body weight and height, were measured, and BMI (body mass index, kg/m^2) was calculated for all participants. Individual CF characteristics were assessed including serum activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and γ–glutamyltransferase (GGTP), lung function as forced expiratory volume in 1 s (FEV_1 [%]), *Pseudomonas aeruginosa* colonisation (defined as including chronic or intermittent culture-validated colonisation), diabetes (diagnosed according to the International Society for Paediatric and Adolescent Diabetes Clinical Practice Consensus Guidelines 2014) [24] and pancreatic insufficiency by faecal elastase-1 concentrations [25,26]. All CF patients taking part in the present study were supplemented with fat-soluble vitamins, including vitamin *E* [27]. UDCA was used by all CF patients with liver cirrhosis and other liver diseases, and none of the patients from the group without liver disease within two years before the study.

The genotypes of the studied CF patients were as follows:

(a) the group with liver cirrhosis: F508del/F508del (n = 15), F508del/2184insA (n = 1), F508del/1898+1G>A (n = 1), F508del/2143delT (n=1); F508del/- (n = 2), 1717-1G>A/CFTRdel2,3(21kb) (n = 1), 2183AA-G/1717-1G->A (n = 1), N1303K/- (n = 1), -/- (n = 2),
(b) the group with other liver diseases: F508del/F508del (n=11), F508del/1717-1G->A (n = 2), F508del/W1282x (n = 1), F508del/3600+2insT (n = 1), F508del/3272-26A>A (n = 1), F508del/R851X (n = 1), F508del/3171insC (n = 1), F508del/CFTRdel2,3(21kb) (n = 1), F508del/3849+10kbC>T (n = 1), F508del/- (n = 2), 1524+1G>A/3944delGT;406-6T>C (n = 1), N1303K/CFTRdele2,3(21kb) (n = 1), -/- (n = 1),
(c) the group without liver disease: F508del/F508del (n = 8), F508del/3849+10KBC>T (n = 4), F508del/R553X (n = 1), F508del/W1282x (n = 1), F508del/R117H (n = 1), F508del/c.3718-2477C>T (n = 1), F508del/- (n = 2), 3849+10kbC>T/3849+10kbC>T (n = 3), 3659delC/R153i (n = 1), 2184insA/- (n = 1), 1717-1G->A/- (n = 1), -/- (n = 1).

The genotypes were classified by known clinical impact: severe/severe mutations (two class I-III mutations) and other mutations (at least one class IV-VI or unknown mutation).

2.2. Bile acid analysis

Blood samples were collected after overnight fasting. The bile acids precipitated with ice-cold acetonitrile (ACN) [28], 3 mL was added to 300 µL serum spiked with 50 µL of an internal standard. Samples were then vortexed (30 s) and centrifuged at 20,000 g for 15 min at 4°C. The supernatant was evaporated using nitrogen and redissolved in 100 µL of methanol/water (50/50). This method achieved a limit of quantitation (LOQ, S/N = 10) not lower than 0.01 µM and the recovery of bile acids was higher than 90%.

The samples were then subjected to ultra-high-performance liquid chromatography (UHPLC) coupled with electrospray ionisation (ESI) mass spectrometry (MS) analysis. The detailed UHPLC-ESI-MS settings were described previously with regard to Dionex UltiMate 3000 UHPLC (Thermo Fisher Scientific, Sunnyvale, CA, USA) and Bruker maXis impact with ESI operated in the negative-ion mode (Bruker Daltonik, Bremen, Germany) [29]. Gradient chromatographic separation of bile acids was achieved using a Kinetex 1.7 μm C18 100 Å, LC column 100 × 2.1 mm (Phenomenex, Torrance, CA, USA) with ternary eluent mode. The chromatographic separation conditions were developed with DryLab 4 software (Molnár-Institute, Berlin, Germany). All tested bile acids with the same molecular mass and elemental composition were separated after optimisation of the elution process. The mobile phase was composed of methanol (A), acetonitrile (B), and water (C), all containing 0.1% acetic acid. The flow rate was 0.2 mL/min with the elution of 21% A, 29% B, 50% C for 19.5 min, then linearly increased to 40% A and 45% B until 24.5 min and maintained in these conditions for 5 min. The sample injection volume was 10 μL and the column temperature was set to 50°C. Molecular ions [M–H] were extracted from full scan chromatograms and peak areas were integrated. The molecular mass, structural information from the MS detector, and a comparison of retention times of standards and compounds were used for the identification of selected bile acids (cholic acid (CA), chenodeoxycholic acid (CDCA), deoxycholic acid (DCA), lithocholic acid (LCA), glycocholic acid (GCA), glycochenodeoxycholic acid (GCDCA), glycodeoxycholic acid (GDCA), taurodeoxycholic acid (TDCA)) in the samples, with dehydrocholic acid used as an internal standard. The coefficient of determination for all calibration curves was not lower than 0.993.

Table 1
Clinical and demographic data of cystic fibrosis (CF) patients and healthy subjects. Asterisks indicate statistical significance in comparison against: the control group (HS, demographic data) or between CF subgroups (clinical data; * < 0.05; ** < 0.01; *** < 0.001).

Clinical parameters	CF patients with liver cirrhosis(n=25)	CF patients with other liver diseases(n=25)	CF patients without liver disease(n=25)	HS(n=25)
	Median (1st–3rd quartile)			
Age [years]	19.7 (15.7–26.0)	18.9 (17.7–21.7)	20.2 (18.5–25.8)	21.8 (20.2–22.8)
Sex ratio [F/M]	11/14 (44.0%)	12/13 (48.0%)	12/13 (48.0%)	12/13 (48.0%)
Body height [m]	1.63** (1.43–1.72)	1.66 (1.58–1.72)	1.69 (1.59–1.74)	1.75** (1.65–1.82)
Body weight [kg]	47.0*** (32.0–61.0)	55.0** (47.0–57.0)	56.0 (49.6–67.0)	65.0**, *** (60.0–73.0)
BMI [kg/m^2]	18.4*** (15.0–20.5)	19.2 (17.3–21.2)	20.0 (17.8–22.1)	21.2*** (20.0–22.1)
ALT [U/l]	29.5 (16.5–39.5)	21.0 (14.0–30.0)	20.0 (13.0–25.0)	-
AST [U/l]	25.0 (18.8–46.2)	18.0 (16.0–27.0)	20.0 (16.0–26.0)	-
GGTP [U/l]	41.5*, *** (21.7–80.5)	21.5* (12.0–31.0)	18.0*** (13.0–23.0)	-
FEV1 [%]	67.1 (38.0–81.0)	55.1 (47.0–71.6)	82.0 (51.2–94.4)	-
Pseudomonas aeruginosa colonization (positive/negative)	76% (19/5)	76% (19/5)	48% (12/13)	-
Diabetes	32%*	24%	4%*	-
Pancreatic insufficiency	100%***	96%**	60%**, ***	-

BMI - body mass index; ALT - alanine aminotransferase; AST - aspartate aminotransferase; GGTP - γ–glutamyltransferase; FEV1 - forced expiratory volume in 1 s

2.3. Statistical methods

Bile acid concentrations were expressed as μmol/L of serum and descriptive statistics were calculated for all variables. For all parameters, the median and 1st–3rd quartiles are shown unless indicated otherwise. The Shapiro-Wilk test was used to check the normality of the data distribution. The Mann-Whitney U-test, Kruskal-Wallis test, or Fisher's exact test were used to assess differences between groups. Values of $p<0.05$ were considered statistically significant. Statistical analyses were performed using Statistica 12.0 software (StatSoft Inc., Tulsa, USA).

The geometric mean also expressed the central tendency as a better average value for the observed log-normal distribution of bile acid concentrations [30]. Principal component analysis with the extraction of principal components with the NIPALS algorithm (Nonlinear Iterative Partial Least Squares) was performed. The data were transformed (ln) before principal component analysis to achieve a distribution close to Gaussian. The Grubbs test was used to detect outliers (StatSoft Inc., Tulsa, OK, USA).

Areas under receiver operating characteristic (ROC) curves (AUC) were calculated for acids exhibiting pronounced differences between the study groups and visualised using the R v. 3.6.0 with packages *ggplot2* and *ggbeeswarm,* as well as Analyse-it v. 2.30 (Analyse-it Software, Leeds, United Kingdom).

2.4. Ethical considerations

The project was approved by the Bioethical Committee of the Poznań University of Medical Sciences, Poznań, Poland (decision no 1225/16). The study was conducted in accordance with the Declaration of Helsinki. All patients (in the case of minors, patients' parents) provided informed written consent to their participation in the study.

3. Results and discussion

The anthropometric and clinical characteristics of the studied groups are presented in Table 1. The CF patients with liver cirrhosis had a significantly lower body height and BMI than HS. Also, bodyweight was lower in CF patients with liver cirrhosis and other liver diseases than HS. GGTP activity was higher in cirrhotic patients than in other CF subgroups. Diabetes was more frequent in patients with liver cirrhosis than in patients without liver disease, whereas pancreatic insufficiency in patients with liver involvement than without liver disease.

3.1. Bile acid concentrations in CF patients with and without liver involvement

A comparison of selected bile acid concentrations in patients with CF and HS is shown in Table 2 A. In general, levels of bile acids were higher ($p<0.05$) in CF patients, except GDCA.

A detailed analysis of the bile acid concentrations in the defined subgroups of CF patients is shown in Table 2 B. The concentrations of most bile acids were higher in groups of CF patients with liver disease than in subgroups without liver disease with the following exceptions, DCA for liver cirrhosis, LCA and TDCA for other liver diseases. CF patients without liver disease had lower concentrations of CA and LCA than patients with liver cirrhosis or other liver diseases, GCA lower than patients with liver cirrhosis, and lower levels of DCA and GDCA than patients with other liver diseases.

The mutations in *CFTR* alter the content of water, electrolytes, and bile pH. Impaired bile transport and retention of toxic (primary) bile acids promote inflammation, which leads to liver fibrosis, whereas steatorrhea, which is a consequence of pancreatic insufficiency and leads to greater loss of bile salts in the stools, intensifies the formation of hydrophobic bile acids (impairment of reabsorption) and increases the bilirubin bile content [31–33]. It can be assumed that the increased serum concentration of bile acids observed in our study results, among others, from their increased secretion. The serum concentration of almost all measured bile acids was higher in CF patients independently of the presence or absence of liver disease. Likewise, Smith et al. reported that total bile acid concentrations in CF patients (with and without liver disease) were higher than in the non-CF control group. Detailed analyses of the bile acid composition showed increased levels of

Table 2
Serum bile acid concentrations (μmol/l) A–in cystic fibrosis (CF) patients and healthy subjects (HS); B - in subgroups of CF patients (letters indicate statistical significance between CF subgroups: [a] < 0.05, [b] < 0.0,1[c] < 0.001).

A				B			
Bile acid	CF patients(n=75)	HS(n=25)	P	Bile acid	CF patients with liver cirrhosis(n=25)	CF patients with other liver diseases(n=25)	CF patients without liver disease(n=25)
	Median, μmol/l (1st–3rd quartile) Primary bile acids				Median, μmol/l (1st–3r[d] quartile) Primary bile acids		
CA	0.065 (0.021–0.176)	0.015 (0.008–0.060)	0.001	CA	0.066[a] (0.026–0.416)	0.116[b] (0.059–0.204)	0.031[ab] (0.005–0.091)
CDCA	0.393 (0.185–0.703)	0.152 (0.045–0.342)	0.000	CDCA	0.353 (0.154–0.620)	0.582 (0.302–0.831)	0.231 (0.103–0.573)
	Secondary bile acids				Secondary bile acids		
DCA	0.324 (0.130–0.486)	0.138 (0.093–0.263)	0.048	DCA	0.327 (0.139–0.392)	0.457[c] (0.241–0.647)	0.065[c] (0.030–0.178)
LCA	0.013 (0.006–0.022)	0.009 (0.003–0.012)	0.045	LCA	0.029[c] (0.022–0.031)	0.015[b] (0.010–0.020)	0.006 [bc] (0.001–0.012)
	Conjugated bile acids				Conjugated bile acids		
GCA	0.164 (0.043–0.393)	0.019 (0.013–0.034)	0.000	GCA	0.385[c] (0.137–0.703)	0.146 (0.093–0.334)	0.036[c] (0.026–0.190)
GCDCA	0.768 (0.511–1.224)	0.276 (0.209–0.395)	0.000	GCDCA	0.974 (0.588–1.202)	0.929 (0.629–1.406)	0.565 (0.331–0.573)
GDCA	0.175 (0.104–0.406)	0.079 (0.039–0.156)	0.153	GDCA	0.173 (0.110–0.338)	0.373[c] (0.202–0.586)	0.050[c] (0.032–0.135)
TDCA	0.068 (0.038–0.195)	0.048 (0.038–0.072)	0.005	TDCA	0.104 (0.059–0.224)	0.062 (0.039–0.123)	0.052 (0.029–0.124)

CA - Cholic acid, CDCA - Chenodeoxycholic acid, DCA - Deoxycholic acid, LCA - Lithocholic acid, GCA - Glycocholic acid, GCDCA - Glycochenodeoxycholic acid, GDCA - Glycodeoxycholic acid, TDCA - Taurodeoxycholic acid

CA, CDCA and UDCA with a simultaneous lowering of LCA concentration [34].

Liver disease, which is present in a significant proportion of CF patients, results in decreased levels of hydrophilic UDCA and an increase of taurine conjugates of CA (in our study, the highest TDCA concentrations were found in CF patients with liver cirrhosis) [35,36]. High concentrations of hydrophobic bile acids are toxic and cause apoptosis of the hepatocytes [37]. In our study, CF patients with liver disease (liver cirrhosis or other liver diseases) showed significantly higher concentrations of primary, secondary and conjugated bile acids compared to HS. In the CF group without liver disease, only three conjugated bile acids (emerging in the small intestine) were elevated. Smith et al. found that bile acid concentrations in CF patients were higher than in HS, but did not prove the existence of differences in serum bile acid levels in CF patients with and without liver disease, however, only seven patients without liver disease were subject to this measurement [34].

The data regarding changes in bile acid levels in CF patients with liver cirrhosis are scarce. Strandvik et al. documented that serum bile acid concentrations were comparable in CF patients with cirrhosis (n=2), portal fibrosis (n=13) and minor liver changes (n=8). Although no differences were found between individual bile acids, it was emphasised that their metabolism in CF is disturbed. Colombo et al. noted that the bile acid total concentrations and amounts of almost all levels of the measured individual bile acids were higher in CF patients with cirrhosis than in HS [21]. Unfortunately, the GDCA itself was not determined, which could be an interesting reference point for our research. Furthermore, the serum level of CA and glycoconjugate bile acids (GCA, GDCA) in cirrhotic CF patients were higher than in patients with other forms of liver disease. The present study showed that concentrations of almost all bile acids were higher in cirrhotic patients than in HS, but not in CF patients with other liver diseases. DCA and GDCA seem to be particularly interesting, their levels are higher in patients with non-cirrhotic liver involvement compared to those with no detectable liver involvement and liver cirrhosis.

3.2. Exogenous and endogenous determinants of bile acids concentrations

The principal component analysis, due to group overlapping, did not allow to differentiate CF patients with liver cirrhosis from patients with other liver diseases (Fig. 1). This analysis showed that both groups with liver involvement are characterised by severe *CFTR* genotypes, high bile acid levels (GDCA and CA), high GGTP activity and pancreatic insufficiency. Besides, GCA, GCDCA, and TDCA are associated with diabetes. While high LCA and DCA concentrations and higher AST and ALT activities were reported in CFLD patients, patients without liver disease had other genotypes, pancreatic sufficiency and a higher BMI.

It should be stressed that seventeen enzymes are involved in bile acid biosynthesis, some of which (cholesterol 7 alpha-hydroxylase [CYP7A], sterol 27-hydroxylase [CYP27A1] and sterol 12-alpha-hydroxylase [CYP8B1]) are regulated by insulin [38–41], which may explain the elevated level of GCA. Many studies indicated an increased bile acid pool and bile acid excretion in diabetics [42,43], however, diabetes may result in changes in gut microbiota, which can lead to disturbed conjugated bile acid metabolism [15,44]. Our study confirms that higher levels of GCDCA, GCA and TDCA may be found in CF patients with diabetes compared

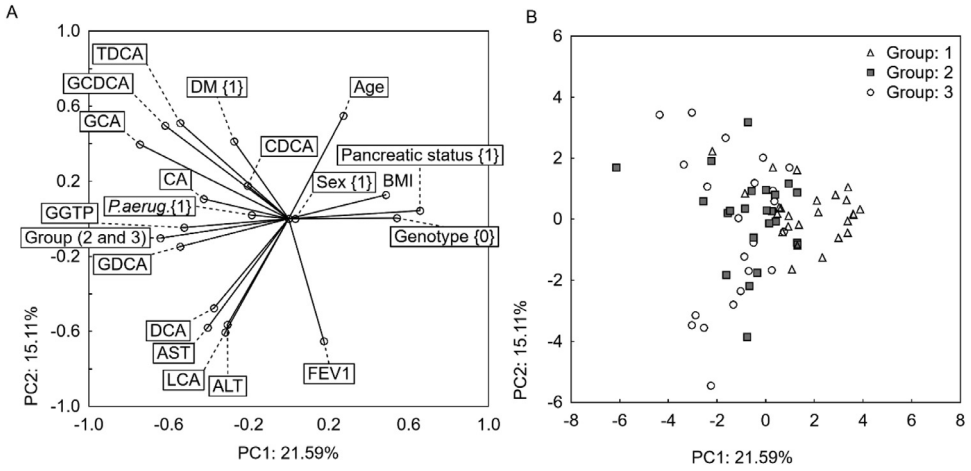

Fig. 1. Principal component analysis - A-loading plot, B-score plot, PCs – principal components
(group: 1 - cystic fibrosis patients without liver disease, 2 - cystic fibrosis patients with other liver diseases, 3 - cystic fibrosis patients with cirrhosis; sex: 0 - men, 1 - women; genotype: 1 - severe/severe, 0 - other; pancreatic status: 0 - pancreatic insufficiency, 1 - pancreatic sufficiency; DM: 0 - lack of diabetes, 1 - diabetes; *P. aerug.* (*Pseudomonas aeruginosa*): 0 - negative, 1 - positive; CA - Cholic acid, CDCA - Chenodeoxycholic acid, DCA - Deoxycholic acid, LCA - Lithocholic acid, GCA - Glycocholic acid, GCDCA - Glycochenodeoxycholic acid, TDCA - Taurodeoxycholic acid, ALT - alanine aminotransferase, AST - aspartate aminotransferase, GGTP - γ–glutamyltransferase, FEV1 - forced expiratory volume in 1 s).

Table 3
Areas under the receiver operating characteristic curves (AUC) for the use of deoxycholic acid and glycodeoxycholic acid in the identification of cystic fibrosis (CF) patient subgroups.

Compared CF subgroups	AUC (95%CI) DCA	*p*	AUC (95%CI) GDCA	*p*
other liver diseases (*n*=25)	0.867	0.000	0.924	0.000
vs no liver disease (*n*=25)	(0.731–1.00)***		(0.822–1.00)***	
liver cirrhosis (*n*=25)	0.758	0.015	0.858	0.004
vs no liver disease (*n*=25)	(0.576–0.941)*		(0.710–1.00)**	
liver cirrhosis (*n*=25)	0.652	0.147	0.650	0.152
vs other liver diseases (*n*=25)	(0.452–0.853)		(0.454–0.847)	

95%CI – 95% confidence interval, CF – cystic fibrosis, DCA - Deoxycholic acid, GDCA - Glycodeoxycholic acid

to those without diabetes (p=0.0109, p=0.0078, p=0.0111, respectively; data not shown).

The relationship between bile acid malabsorption and pancreatic insufficiency in CF was reported by Weber et al [45]. The authors noted that faecal bile acid concentration in CF children with pancreatic insufficiency was higher than in pancreatic sufficient patients. Insufficient bile acid reabsorption in the intestine increases the production of bile in the liver [6] and could explain the higher serum concentration of primary bile acids in CF patients with pancreatic insufficiency in our study. However, malabsorption does not explain the higher concentration of other bile acids (secondary, conjugated), which was earlier noted by Weziman et al. and Colombo et al. [18,46] and which was also found in our study. However, the latest research suggests that not only pancreatic insufficiency but also pancreatic enzyme replacement therapy and changes in intestinal pH can affect intestinal microbiota, thus bile acid metabolism [47,48].

In healthy subjects, CA and CDCA are predominantly synthesized in neutral bile acid biosynthetic pathway. Significant quantities of bile salts reach the small intestine preventing dysbiosis and secondary release of inflammatory markers. Since bile acid 7α-dehydroxylating bacteria appear in normal amounts, the ratio of secondary to primary bile acids in the colon is high. In patients with liver cirrhosis, proinflammatory cytokines repress the neutral pathway due to the downregulation of CYP7A1 [49]. Therefore, the acidic bile acid synthetic pathway dominates with higher production of CDCA than CA, subsequently resulting in lower turnover to DCA. The number of 7α-dehydroxylating bacteria in the colon decreases due to the lower primary bile acids content, which serve as an energy source. Consequently, the secondary/primary bileacids ratio in cirrhosis is low, and it seems to be predominantly related to DCA concentrations.

3.3. Bile acids as potential serum markers of non-cirrhotic liver disease

The principal component analysis evaluated exogenous and endogenous determinants of bile acids levels, however, the strong impact of genotype and comorbidities made it practically impossible to assess the relationship between bile acids concentration and different liver disease stages. For this reason, the AUC of individual serum bile acid concentrations were calculated to differentiate between patients with liver cirrhosis, other liver diseases and normal liver. The highest AUC values for the discrimination between CF patients with other liver diseases and no liver disease were noted for DCA and GDCA. GDCA measurement could be also potentially used to differentiate CF patients with liver cirrhosis and no liver disease (Table 3). DCA and GDCA levels in subgroups of CF patients and HS are presented in Fig. 2.

Cluster analysis and ROC analysis indicated that the GDCA concentration had the strongest diagnostic power to distinguish between non-cirrhotic liver disease and no liver disease (Fig. 3). Analysis of GDCA concentration allowed for reasonable differentiation of patients with other liver diseases from those without liver disease. Importantly, observed ALT and AST differences between CF subgroups did not reach significance. An attempt to measure conjugated primary bile acids to diagnose subjects with hepatic non-CF involvement was described previously by Luo et al. They found that high values of DCA, GDCA, TDCA, and GCDCA were associated with primary liver diseases, such as acetaminophen overdose, liver

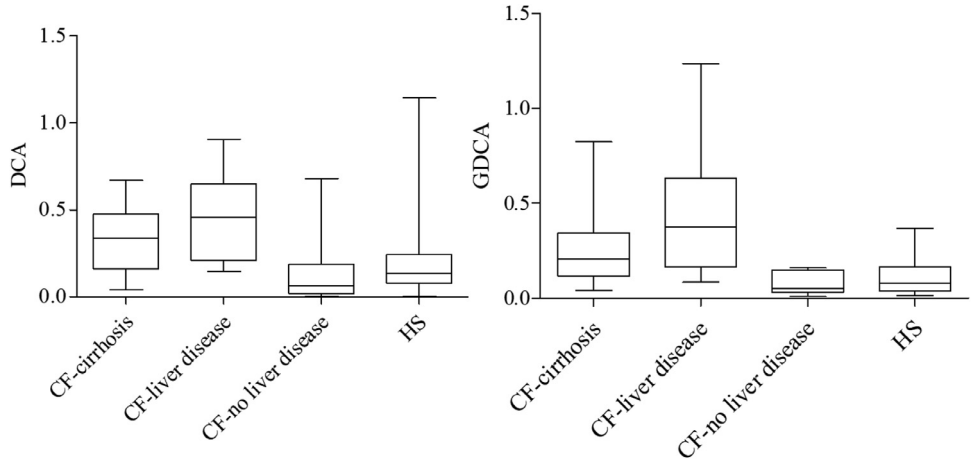

Fig. 2. Deoxycholic acid and glycodeoxycholic acid levels in subgroups of cystic fibrosis (CF) patients and healthy subjects (HS)
DCA-Deoxycholic acid, GDCA-Glycodeoxycholic acid.

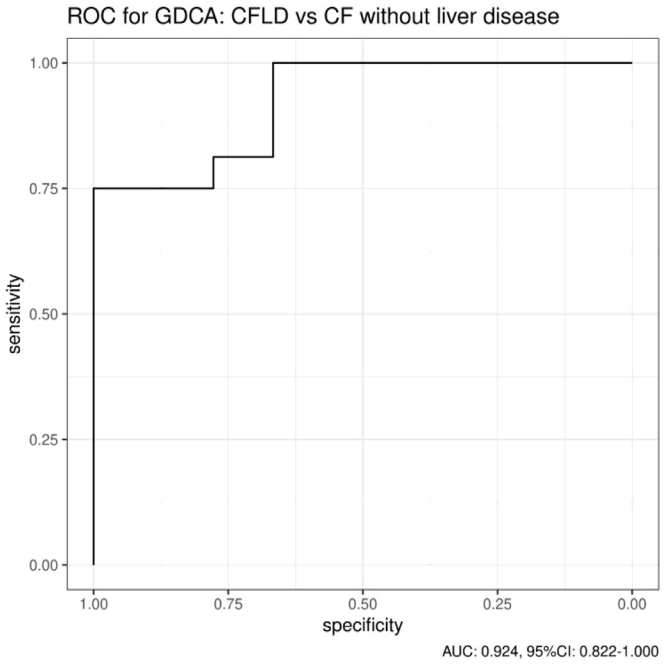

Fig. 3. Receiver operating characteristic curve for distinguishing cystic fibrosis (CF) patients with other liver diseases from patients with CF without liver disease.

transplant, and chronic liver injury [50]. However, our study suggested that GDCA is the most promising biomarker for delineation of non-cirrhotic liver involvement in CF patients.

3.4. Limitations and strengths

The main limitation of this study is the lack of information regarding the composition of the intestinal microbiota. Small intestinal bacterial overgrowth occurs frequently in CF patients [51,52] and can affect the function of the digestive system [53,54]. Faecal analysis is appropriate for the assessment of the bacterial profile in the lower part of the gastrointestinal tract but is not sufficient to observe changes occurring due to bacterial overgrowth of the small intestine. Hence, sampling duodenal juice is necessary, but this is an invasive procedure. Similarly, the study of bile acid secretion would be a valuable supplement to our research, but due to the invasiveness, it was also abandoned. Unfortunately, due to technical limitations, we did not evaluate UDCA concentrations. Therefore, we could neither assess the differential effect of liver cirrhosis on DCA and UDCA levels nor a relationship between UDCA intake and serum bile acid concentrations. The direct comparison to the results obtained by Colombo et al. [21] is not possible since the authors did not present concentrations of particular free, glyco- and tauro-conjugated bile acids. It is essential since the ratios of particular free and conjugated bile acids in CF are differentiated. Nonetheless, the main strengths of this research include a large sample size (at various stages of CFLD), a comprehensive characterisation of CF-related clinical factors, the investigation of exogenous and endogenous determinants of bile acids profile and determining a considerable number of major bile acids.

4. Conclusions

Serum bile acid concentrations were higher in CF patients (except GDCA) than in HS, with GCA and GCDCA levels higher in CF patients without liver disease. GDCA and DCA were proved to significantly differentiate patients with non-cirrhotic liver involvement from those with no detectable liver disease, thus are potential markers for the assessment of liver disease progression in CF.

Declaration of Competing Interest

None.

Acknowledgments

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Author contributions

SDC and JW conceived the study, SDC, AN, JN and JW recruited patients; KD, AS, and PKJ performed laboratory and statistical analyses; SDC and JW drafted the manuscript; RA and SD collected the data; all authors acquired data and interpreted them, revised the manuscript and approved its final version; SDC, and JW obtained funding; SDC, NK and JW supervised the study.

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