


Adjunct Fenofibrate Up-regulates Bile Acid Glucuronidation and Improves Treatment Response For Patients With Cholestasis

Gina M. Gallucci,¹ Jocelyn Trottier,² Christopher Hemme,^{1,3} David N. Assis,⁴ James L. Boyer,⁴ Olivier Barbier,^{2,5} and Nisanne S. Ghonem¹ ¹

Accumulation of cytotoxic bile acids (BAs) during cholestasis can result in liver failure. Glucuronidation, a phase II metabolism pathway responsible for BA detoxification, is regulated by peroxisome proliferator-activated receptor alpha (PPAR α). This study investigates the efficacy of adjunct fenofibrate therapy to up-regulate BA-glucuronidation and reduce serum BA toxicity during cholestasis. Adult patients with primary biliary cholangitis (PBC, n = 32) and primary sclerosing cholangitis (PSC, n = 23), who experienced an incomplete response while receiving ursodiol monotherapy (13–15 mg/kg/day), defined as serum alkaline phosphatase (ALP) \geq 1.5 times the upper limit of normal, received additional fenofibrate (145–160 mg/day) as standard of care. Serum BA and BA-glucuronide concentrations were measured by liquid chromatography–mass spectrometry. Combination therapy with fenofibrate significantly decreased elevated serum ALP (–76%, $P < 0.001$), aspartate transaminase, alanine aminotransferase, bilirubin, total serum BAs (–54%), and increased serum BA-glucuronides (+2.1-fold, $P < 0.01$) versus ursodiol monotherapy. The major serum BA-glucuronides that were favorably altered following adjunct fenofibrate include hyodeoxycholic acid–6G (+3.7-fold, $P < 0.01$), hyocholic acid–6G (+2.6-fold, $P < 0.05$), chenodeoxycholic acid (CDCA)–3G (–36%), and lithocholic acid (LCA)–3G (–42%) versus ursodiol monotherapy. Fenofibrate also up-regulated the expression of uridine 5'-diphosphoglucuronosyltransferases and multidrug resistance-associated protein 3 messenger RNA in primary human hepatocytes. Pearson's correlation coefficients identified strong associations between serum ALP and metabolic ratios of CDCA-3G ($r^2 = 0.62$, $P < 0.0001$), deoxycholic acid (DCA)-3G ($r^2 = 0.48$, $P < 0.0001$), and LCA-3G ($r^2 = 0.40$, $P < 0.001$), in ursodiol monotherapy versus control. Receiver operating characteristic analysis identified serum BA-glucuronides as measures of response to therapy. **Conclusion:** Fenofibrate favorably alters major serum BA-glucuronides, which correlate with reduced serum ALP levels and improved outcomes. A PPAR α -mediated anti-cholestatic mechanism is involved in detoxifying serum BAs in patients with PBC and PSC who have an incomplete response on ursodiol monotherapy and receive adjunct fenofibrate. Serum BA-glucuronides may serve as a noninvasive measure of treatment response in PBC and PSC. (*Hepatology Communications* 2021;5:2035–2051).

Cholestatic liver diseases, such as primary biliary cholangitis (PBC) and primary sclerosing cholangitis (PSC), result in the intracellular accumulation of toxic bile constituents, notably bile acids (BAs). Reducing BA toxicity is a primary goal in treating PBC and PSC. BA-glucuronidation is a key step in BA detoxification and a major product of the phase II pathway of drug metabolism.⁽¹⁾

Abbreviations: ALP, alkaline phosphatase; ALT, alanine aminotransferase; ANOVA, analysis of variance; AST, aspartate aminotransferase; AUC, area under the curve; BA, bile acid; CA, cholic acid; CDCA, chenodeoxycholic acid; Combo, combination; CYP, cytochrome P450; DCA, deoxycholic acid; HCA, hyocholic acid; HDCA, hyodeoxycholic acid; LCA, lithocholic acid; Mono, monotherapy; mRNA, messenger RNA; MRP, multidrug resistance-associated protein; PBC, primary biliary cholangitis; PC, principal component; PCA, principal component analysis; PCR, polymerase chain reaction; PPAR α , peroxisome proliferator-activated receptor; PSC, primary sclerosing cholangitis; ROC, receiver operating characteristic; UGT, uridine 5'-diphosphoglucuronosyltransferase; ULN, upper limit of normal.

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BA-glucuronidation conjugates glucuronic acid to BAs at the 3/6-hydroxyl or 24-carboxyl BA steroid nucleus, to produce corresponding ether/ester or acyl glucuronides, and increase their hydrophilicity for renal excretion.⁽²⁾ The human UDP-glucuronosyltransferase (UGT) family of enzymes catalyze glucuronidation reactions,⁽³⁾ predominantly through genes belonging to the UGT1A and 2B families.⁽⁴⁻⁶⁾ Remarkably, only four UGT enzymes are responsible for hepatic BA-glucuronidation, specifically UGT1A3, UGT1A4, UGT2B4 and UGT2B7, of which the first three are regulated by the nuclear peroxisome proliferator-activated receptor alpha (PPAR α).^(4,5,7) In addition, BA-glucuronides are substrates for the multidrug resistance-associated proteins (MRPs), including MRP3, an efflux transporter located on the sinusoidal membrane of hepatocytes and involved in the hepatic excretion of BA-glucuronides into systemic circulation for subsequent urinary excretion.⁽⁸⁾

Fenofibrate, a Food and Drug Administration (FDA)-approved cholesterol-lowering fibrate and PPAR α agonist, offers potential therapeutic benefit for patients with PBC^(9,10) and PSC⁽¹¹⁾ who are incomplete responders to ursodiol monotherapy. We previously demonstrated that PPAR α activation by fenofibrate transcriptionally activates the hepatic transporter multidrug resistance protein (MDR)-3.⁽¹²⁾ MDR3 is critically involved in biliary phosphatidylcholine secretion, which is needed to inactivate

the toxic detergent action of BAs and prevent damage to epithelial cells lining the bile duct epithelia. Furthermore, the therapeutic efficacy of combination treatment with fenofibrate and ursodiol in patients with PBC and PSC was shown by normalized biochemical markers of cholestasis and reduced toxicity of serum BA and composition, particularly 7 α -hydroxy-4-cholesten-3-one (C4), the BA precursor, as well as reduced primary and secondary BAs, toward levels observed in healthy controls.⁽¹³⁾ Also, fenofibrate has low reported adverse events in patients with PBC.⁽¹⁴⁾

Although glucuronidation plays less of a major role in BA metabolism under homeostatic conditions,⁽¹⁵⁾ this phase II conjugation pathway becomes an important therapeutic pathway during cholestatic conditions to reduce BA toxicity.^(16,17) Individual and total BAs have been examined for their potential as prognostic markers in acute liver injury⁽¹⁸⁻²²⁾; however, BA-glucuronides have yet to be evaluated in chronic cholestatic liver injury. This study investigates the efficacy of fenofibrate to activate PPAR α -mediated BA glucuronidation, as a therapeutic mechanism to reduce the toxicity of serum BAs during adult chronic cholestatic liver diseases. In addition, this study examines serum BA and BA-glucuronides as secondary measures of treatment response for patients experiencing an incomplete response to ursodiol monotherapy and for those receiving combination therapy with fenofibrate.

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ARTICLE INFORMATION:

From the ¹College of Pharmacy, Biomedical and Pharmaceutical Sciences, University of Rhode Island, Kingston, RI, USA; ²Laboratory of Molecular Pharmacology, Endocrinology and Nephrology Axis, CHU de Québec Research Center, Laval, Québec, Canada; ³RI-INBRE Bioinformatics Core, Kingston, RI, USA; ⁴Yale School of Medicine, Liver Center, New Haven, CT, USA; ⁵Faculty of Pharmacy, Laval University, Laval, Québec, Canada.

ADDRESS CORRESPONDENCE AND REPRINT REQUESTS TO:

Nisanne S. Ghonem, Pharm.D., Ph.D.
College of Pharmacy, Biomedical and Pharmaceutical Sciences
University of Rhode Island
Avedisian Hall #395, 7 Greenhouse Road

Kingston, RI 02881, USA
E-mail: nghonem@uri.edu
Tel.: +1-401-874-4805

Materials and Methods

Chemicals are listed in the Supporting Materials and Methods.

STUDY POPULATION

This retrospective study examines the biochemical responses and serum BA and BA-glucuronide concentrations of patients with PBC and PSC who were treated with either (1) ursodiol monotherapy (13-15 mg/kg/day) for at least 6 months and continued to experience an incomplete biochemical response or (2) combination treatment with ursodiol and off-label fenofibrate (145-160 mg/day) after having an incomplete response while receiving ursodiol monotherapy for at least 6 months before the addition of fenofibrate. An incomplete biochemical response is defined as serum alkaline phosphatase (ALP) levels ≥ 1.5 times the upper limit of normal (ULN). Treatments were part of the patients' routine clinical care at the Yale Autoimmune and Cholestatic Liver Diseases Program (New Haven, CT), as previously described.⁽¹³⁾ Patients provided written consent to have their blood samples de-identified and stored for research purposes at the Yale Liver Center Clinical Registry (New Haven, CT). Ongoing registry sample collection has provided new samples included in the biochemical analyses ($n = 5$ PBC and $n = 4$ PSC; combination fenofibrate cohort). All samples were drawn during morning routine clinic visits, which is the only period when patients are seen, and sampling times did not vary between the patient groups. These de-identified samples were determined to be exempt from the institutional review boards at Yale University (New Haven, CT) and the University of Rhode Island (Kingston, RI). The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in *a priori* approval by the institutional review committee. Serum samples from healthy de-identified adult donors (BioIVT, Westbury, NY) did not have any clinically diagnosed liver disease and serve as healthy controls.

SERUM LIVER ENZYMES

Serum ALP, alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin, and lipid panels were measured by standard clinical

laboratories (Yale New Haven Hospital [New Haven, CT] or Quest Diagnostics [Secaucus, NJ]).

BA AND BA-GLUCURONIDE EXTRACTION AND LIQUID CHROMATOGRAPHY COUPLED WITH TANDEM MASS SPECTROMETRY

Serum BA, BA-glucuronide extraction, and liquid chromatography–tandem mass spectrometry methodology are provided in the Supporting Materials and Methods. The total serum BAs include the sum of the following 17 compounds: cholic acid (CA), chenodeoxycholic acid (CDCA), deoxycholic acid (DCA), lithocholic acid (LCA), hyodeoxycholic acid (HDCA), hyocholic acid (HCA), ursodeoxycholic acid (UDCA), glycochenodeoxycholic acid (GCDCA), glycodeoxycholic acid (GDCA), glyoursodeoxycholic acid (GUDCA), glycolithocholic acid (GLCA), glycocholic acid (GCA), tauroolithocholic acid (TLCA), taurocholic acid (TCA), taurochenodeoxycholic acid (TCDCA), taurodeoxycholic acid (TDCA), and taurooursodeoxycholic acid (TUDCA). The following BA-glucuronides were measured: CDCA-3G and CDCA-24G, DCA-3G and DCA-24G, LCA-3G and LCA-24G, HDCA-6G and HCDA-24G, HCA-6G and HCA-24G, and CA-24G. All metabolites are also listed in Supporting Table S1.

CELL CULTURE AND QUANTITATIVE REAL-TIME POLYMERASE CHAIN REACTION ANALYSIS

Cryopreserved human hepatocytes (Sekisui Xenotech, LLC, Kansas City, KS) were maintained on collagen-coated plates and cultured according to the manufacturer's protocol in a 37°C, 5% CO₂ incubator. Hepatocytes were treated with DMSO (vehicle) or fenofibrate (50 μ M) for 24 hours. Total RNA was extracted from hepatocytes using TRIzol (Invitrogen, Carlsbad, CA). Hepatic messenger RNA (mRNA) levels of *PPAR α* , *UGT1A1*, *1A3*, *1A4*, *2B4*, *2B7*, *CYP3A4*, *MRP2*, *MRP3*, *CYP4A11* (positive control for *PPAR α*), and *PPIA* were analyzed using Taqman probes (Applied Biosystems, Foster City, CA). Quantitative real-time polymerase chain reaction (PCR) analysis was performed using a Roche LightCycler Detection System (Basel, Switzerland).

DATA ANALYSIS

Results are reported as mean \pm SEM. Serum liver enzymes and lipids were analyzed using two-tailed unpaired *t*-tests with Welch's correction, and quantitative real-time PCR data were analyzed using two-tailed unpaired Student *t* test (GraphPad Prism 9). Unless otherwise stated, serum BA and BA-glucuronide concentrations were \log_2 -transformed to obtain normal distribution before statistical analysis,⁽²³⁾ then analyzed using one-way analysis of variance (ANOVA) (R Studio, v.4.0.4). Further analyses of data within cohorts were performed using a linear mixed model with subject ID as the blocking factor (limma package v.3.38.3⁽²⁴⁾). Details are provided in Supporting Table S1. Strip charts were made for each metabolite in R using the ggplot function of the ggplot2 package (v.3.3.2). Indicated *P* values between the groups were derived from the contrasts for the linear mixed model. Means of enzyme concentrations were compared in R using ANOVA with treatment (control, monotherapy, and combination) as the fixed effect, and multiple comparisons were conducted using the Tukey method (multcomp package in R). Metabolic ratios were analyzed by the Kruskal-Wallis test with Dunn's multiple comparisons. Within treatment groups, values below the limit of detection were set to the limit of detection. Pearson's correlation coefficient (r^2) was used to measure the association of serum BAs with serum ALP values. Extreme outliers defined as values ≥ 3.0 times the interquartile range either above Q_3 or below Q_1 were excluded from analysis. The ability of serum BAs and BA-glucuronides to indicate the response to therapy for each patient cohort (e.g., ursodiol monotherapy nonresponders or combination therapy with fenofibrate) was measured using receiver operating characteristic (ROC) analysis curves, as implemented in the plotROC package, Rstudio.⁽²⁵⁾ The binary variable for ROC curves was defined as 0 for the healthy control state and 1 for either the mono or combination treatment state; *P* values were calculated for the area under the curve (AUC) for each ROC curve using the Mann-Whitney test in Rstudio. Principal component analysis (PCA) was conducted using R prcomp function and plotted with the ggbiplot package (v.0.55). Heatmaps were constructed using the heatmap.2 function of the gplots package (3.0.4), and values were scaled by row (metabolite).

Results

CLINICAL CHARACTERISTICS AND SERUM MARKERS OF LIVER INJURY

Serum was obtained from patients with PBC ($n = 32$; monotherapy: $n = 16$ and combination: $n = 16$) and PSC ($n = 23$; monotherapy: $n = 11$ and combination: $n = 12$) before and for a median of 16 months (range: 1–64 months) after the addition of fenofibrate to ursodiol (duration of fenofibrate therapy is based on the time from initiating therapy to when sample analyses occurred). One patient with PSC had cirrhosis; no other patients had advanced fibrosis. Patient demographics are presented in Table 1A. Supporting Table S2 provides the clinical characteristics and the associated serum total BAs, BA-glucuronides, and ALP based on fibrosis scores obtained at the time of diagnosis; these scores provide the range in severity of disease for the patients with an incomplete response and receiving ursodiol monotherapy. In the combination therapy cohorts, characteristics obtained before the addition of fenofibrate were available for a subset of patients with fibrosis scores of 1 ($n = 1$, PBC), 2 ($n = 1$, PSC), and 3 ($n = 4$, 2 PBC and 2 PSC), which represent a similar range in the severity of underlying disease compared with the ursodiol monotherapy cohort.

Combination therapy with fenofibrate significantly reduced serum ALP levels by 76% versus ursodiol monotherapy, and normalized levels for 82% of patients. Serum ALT and AST levels were also normalized after combination treatment with fenofibrate, decreasing values by 55% and 46% versus ursodiol monotherapy, respectively. In addition, total serum bilirubin levels decreased by 76% by fenofibrate treatment (Table 1B). These results demonstrate the efficacy of combination treatment with fenofibrate and ursodiol in reducing elevated liver biochemistries in patients with cholestasis who have a subtherapeutic response to ursodiol monotherapy.

Paired analysis was performed in a subset of patients ($n = 7$: PBC = 5 and PSC = 2) who received ursodiol monotherapy ≥ 6 months and experienced an incomplete biochemical response (e.g., ALP ≥ 1.5 times the ULN [baseline]), followed by blood sampling after receiving combination therapy with fenofibrate. As given in Table 2, reductions in all serum biochemistries

TABLE 1. DEMOGRAPHICS OF ALL PATIENTS INCLUDED IN THE STUDY (A) AND SERUM LIVER BIOCHEMISTRIES OF PATIENTS WITH PBC AND PSC (B)

	Controls (n = 33)	PBC		PSC	
		Monotherapy (n = 16)	Combination (n = 16)	Monotherapy (n = 11)	Combination (n = 12)
A. Demographics					
Age, median (range, years)	41 (24-61)	54 (44-70)	60 (48-72)	42 (24-66)	45 (33-76)
Male	18 (55%)	0	0	10 (91%)	5 (42%)
Female	15 (45%)	16 (100%)	16 (100%)	1 (9%)	7 (58%)
White	10 (30%)	11 (69%)	10 (63%)	8 (73%)	10 (84%)
Black	17 (52%)	0	0	3 (27%)	1 (8%)
Hispanic	6 (18%)	3 (19%)	4 (25%)	0	0
Unknown	0	2 (12%)	2 (12%)	0	1 (8%)
B. Serum biochemistries					
ALP (U/L)	52 ± 2.1	391 ± 95	97 ± 10**	621 ± 135	147 ± 32**
ALT (U/L)	6-34 [#]	74 ± 14	31 ± 3**	110 ± 14	51 ± 11**
AST (U/L)	11-33 [#]	62 ± 10	37 ± 4*	104 ± 17	51 ± 11*
Total bilirubin (mg/dL)	<1.2 [#]	0.9 ± 0.3	0.6 ± 0.1	4 ± 1.4	0.4 ± 0*
Creatinine (mg/dL)	0.7 ± 0	0.6 ± 0.1	0.7 ± 0.1	0.6 ± 0.1	0.7 ± 0

Note: Serum samples from healthy de-identified adult donors (BioIVT) serve as healthy controls. [#]Reference ranges obtained from Yale New Haven Hospital Clinical laboratory. Data represent mean ± SEM, analyzed by pairwise t-tests with Welch's correction.

**P* < 0.05.

***P* < 0.01.

TABLE 2. PAIRED ANALYSIS OF SERUM BIOCHEMICAL MARKERS OF LIVER INJURY, TOTAL SERUM BA, AND BA-GLUCURONIDES IN A SUBSET OF PATIENTS (N = 7, PBC = 5, AND PSC = 2)

No. of Patients (Treatment)	ALT (U/L)	AST (U/L)	Total Bili (mg/dL)	ALP (U/L) (% change)	Total Serum BA (μM)	Serum BA Gluc (nM) (fold change)	Feno Duration (months)
<i>PBC</i>							
1 mono	21	32	0.8	271	185	763	—
1 combo	27	31	0.6	96 [−65%]	70	1420 [+1.9]	29
2 mono	25	26	0.26	214	9.2	280	—
2 combo	16	25	0.16	91 [−57%]	3.3	670 [+2.4]	14
2 combo	19	24	0.3	88 [−59%]	30.3	1,457 [+5.2]	49
3 mono	129	155	3.2	1,679	23.6	1,684	—
3 combo	47	78	0.8	77 [−95%]	39.1	1,606	40
3 combo	44	68	1.2	94 [−94%]	51	4,742 [+2.8]	53
4 mono	51	48	0.5	163	45.4	597	—
4 combo	24	27	0.5	73 [−55%]	6.4	1,250 [+2.1]	44
5 mono	18	25	0.5	156	74.3	309	—
5 combo	28	36	0.6	124 [−21%]	19.1	1,456 [+4.7]	7
<i>PSC</i>							
6 mono	40	24	0.48	155	8.1	79	—
6 combo	26	17	0.51	92 [−41%]	21.5	635 [+8.1]	1
6 combo	33	25	0.5	114 [−26%]	15.8	229 [+2.9]	15
7 mono	100	58	0.61	307	11	404	—
7 combo	35	31	0.52	147 [−52%]	8	403 —	7
7 combo	30	31	0.6	117 [−62%]	7.7	732 [+1.8]	19

Abbreviations: Bili, bilirubin; combo, combination with fenofibrate; Feno, fenofibrate; Gluc, glucuronide; mono, ursodiol monotherapy.

occurred following the addition of fenofibrate, most notably in serum ALP. Indeed, the addition of fenofibrate reduces total serum BA levels, which correspond with increased serum BA-glucuronide concentrations.

The FDA-approved indication of fenofibrate is to reduce elevated cholesterol. Although this was not the indication for the addition of fenofibrate in these patients, treatment with fenofibrate reduced serum lipids in a subset of patients with available lipid levels for analysis, including total cholesterol (-20%), low-density lipoprotein (-38% , $P = 0.07$), triglycerides (-34% , $P = 0.06$), and increased high-density lipoprotein ($+23\%$) versus ursodiol monotherapy, respectively (Supporting Table S3), confirming the efficacy

of fenofibrate in reducing serum lipids and providing further evidence of a drug effect.

mRNA EXPRESSION IN HUMAN HEPATOCYTES

To determine the activation of PPAR α and its regulated genes involved in BA-glucuronidation, human hepatocytes were treated with fenofibrate ($50 \mu\text{M}$), and gene expression was measured by quantitative real-time PCR. Treatment with fenofibrate significantly up-regulated the mRNA expression of *PPAR α* , *UGT1A3*, *UGT1A4*, *CYP3A4*, *MRP2*, *MRP3*, and *CYP4A11* versus DMSO (vehicle) (Fig. 1A). No

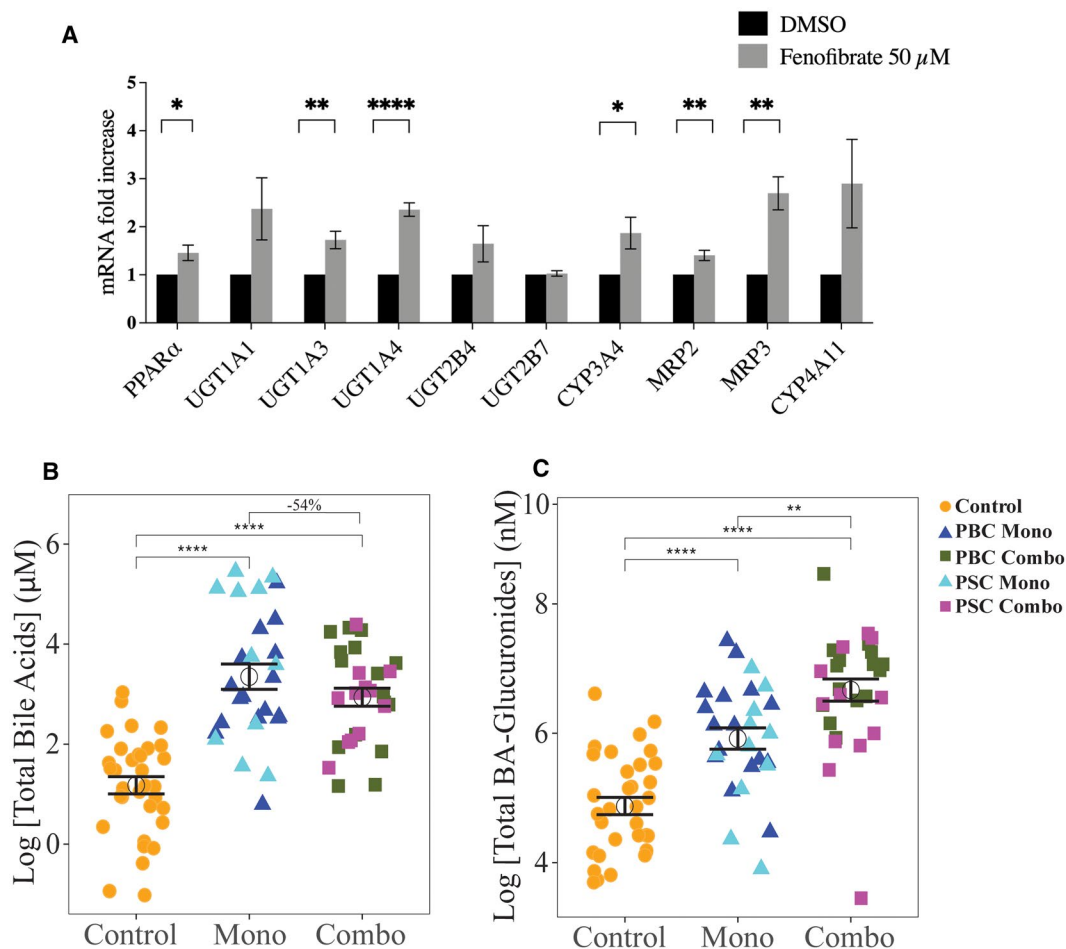


FIG. 1. (A) Quantitative real-time PCR analysis shows that fenofibrate ($50 \mu\text{M}$) treatment for 24 hours up-regulates *PPAR α* , *UGT1A1*, *UGT1A3*, *UGT1A4*, *UGT2B4*, *CYP3A4*, *MRP2*, *MRP3*, and *CYP4A11* mRNA expression in cryopreserved hepatocytes compared with DMSO (vehicle control). Relative mRNA expression was calculated using the $\Delta\Delta\text{Ct}$ method, normalized to *PPIA* and expressed as a fold-change over DMSO. Data are expressed as mean \pm SEM ($n = 3$ -4 individual cases of hepatocytes), analyzed by two-tailed unpaired Student t test. * $P < 0.05$, ** $P < 0.01$, and **** $P < 0.0001$ versus DMSO. Combination treatment with fenofibrate decreases total serum BAs (B) and increases total serum BA-glucuronides (C). Log $_2$ -transformed data were analyzed by one-way ANOVA. ** $P < 0.01$ and **** $P < 0.0001$. Total serum BAs and BA-glucuronides are defined in the Supporting Materials and Methods.

effect on *UGT2B7* expression was expected. These *in vitro* data confirm that fenofibrate/PPAR α -mediated activation of UGTs and MRPs can be involved in BA-glucuronidation and efflux out of the liver for subsequent excretion and detoxification.

TOTAL SERUM BA AND BA-GLUCURONIDE CONCENTRATIONS

The total serum BAs levels observed in patients on ursodiol monotherapy in this study are comparable to serum BA concentrations reported in untreated patients with PBC and PSC.⁽²⁶⁾ Combination therapy with fenofibrate reduced total serum BA concentrations by 54% (28 ± 4 vs. 61 ± 14 μ M) compared with ursodiol monotherapy and trended toward levels found in healthy controls (5 ± 0.8 μ M) (Fig. 1B).

To investigate the role of the UDP-glucuronyl transferase metabolic pathway in reducing the toxicity of serum BAs, we next quantified patient serum BA-glucuronide concentrations. Combination therapy with fenofibrate increased total serum BA-glucuronides in PBC and PSC by about 6.0-fold ($1,064 \pm 166$ vs. 176 ± 26 nM, $P < 0.0001$) and 2.1-fold ($1,064 \pm 166$ vs. 505 ± 77 nM, $P < 0.01$) versus healthy controls and ursodiol monotherapy, respectively (Fig. 1C). Serum BA-glucuronide concentrations were also elevated by 2.8-fold (505 ± 77 vs. 176 ± 26 nM, $P < 0.0001$) in patients on ursodiol monotherapy versus healthy controls, albeit to a much lower degree than by the addition of fenofibrate. Despite this increase in BA-glucuronide levels in patients on ursodiol monotherapy, their total serum BA concentrations remained significantly elevated compared with controls (61 ± 14 μ M vs. 5 ± 0.8 μ M, $P < 0.0001$), suggesting that the ursodiol-mediated increases in BA-glucuronides were not sufficient to reduce the toxicity of the total serum BA pool. In contrast, the reduction of total serum BAs along with PPAR α activation demonstrate that the addition of fenofibrate reduces the toxicity of serum BAs through PPAR α -mediated BA-glucuronidation.

THERAPEUTIC IMPACT OF FENOFIBRATE ON SERUM BA-GLUCURONIDE CONCENTRATIONS AND COMPOSITION

We next assessed the impact of fenofibrate on the individual BA-glucuronide profiles. Of the

11 metabolites studied, we found that 99% of the serum BA-glucuronides in the healthy controls consisted of five BA-glucuronides: HCA-6G (47%), CDCA-3G (36%), DCA-3G (6%), HDCA-6G (10%), and LCA-3G (1%). In PBC and PSC, the addition of fenofibrate increased serum HCA-6G and HDCA-6G concentrations by 2.6-fold and 3.7-fold versus ursodiol alone ($P < 0.01$ and $P < 0.05$), respectively (Fig. 2A,B). Fenofibrate therapy also increased the contribution of HCA-6G in the serum glucuronide pool from 47% in healthy controls to 55% and 66% in patients with PBC and PSC, respectively (Fig. 3). Similarly, fenofibrate treatment increased the contribution of HDCA-6G from 10% of the serum BA-glucuronides in controls to 35% in patients with PBC and 20% in patients with PSC (Fig. 3). Finally, a corresponding decrease in serum HCA-24G (-76%) was observed, whereas HDCA-24G remained unchanged (Supporting Fig. S1A,B). These findings confirm that glucuronidation is preferred at the 6-hydroxyl⁽¹⁷⁾ over the 24-hydroxyl position.

When HepG2 cells are treated with CDCA-3G, DCA-3G, and LCA-3G, they exhibit apoptosis and necrosis,⁽²⁷⁾ confirming the therapeutic importance of glucuronidation of these particular compounds. In this study, ursodiol monotherapy significantly increased serum concentrations of CDCA-3G (+2.2-fold, $P < 0.05$) and LCA-3G (+6.2-fold, $P < 0.001$) levels versus healthy controls, which were markedly reduced by the addition of fenofibrate (-36% and -42%, respectively) versus ursodiol monotherapy (Fig. 2C,D). Notable reductions in the contribution of CDCA-3G and LCA-3G to the serum BA-glucuronide pool were also found, particularly in PSC, decreasing from 52% to 13% and 5% to 0.3%, respectively, in the fenofibrate-treated cohort relative to ursodiol monotherapy (Fig. 3). Fenofibrate did not significantly change DCA-3G serum concentrations, relative to that of ursodiol monotherapy (Fig. 2E). CDCA, DCA, and LCA are glucuronidated at the C3 position by *UGT2B7* during homeostatic conditions, and CDCA undergoes glucuronidation through PPAR α -induced *UGT1A4* when exposed to fenofibrate.⁽⁴⁾ Collectively, the shift away from these three C3 glucuronides and toward C24 glucuronidation (Supporting Fig. S1) likely reduces the toxic impact of these metabolites. Moreover, this latter finding indicates that reducing elevated serum C3 glucuronides in PBC and PSC by fenofibrate is hepatoprotective.

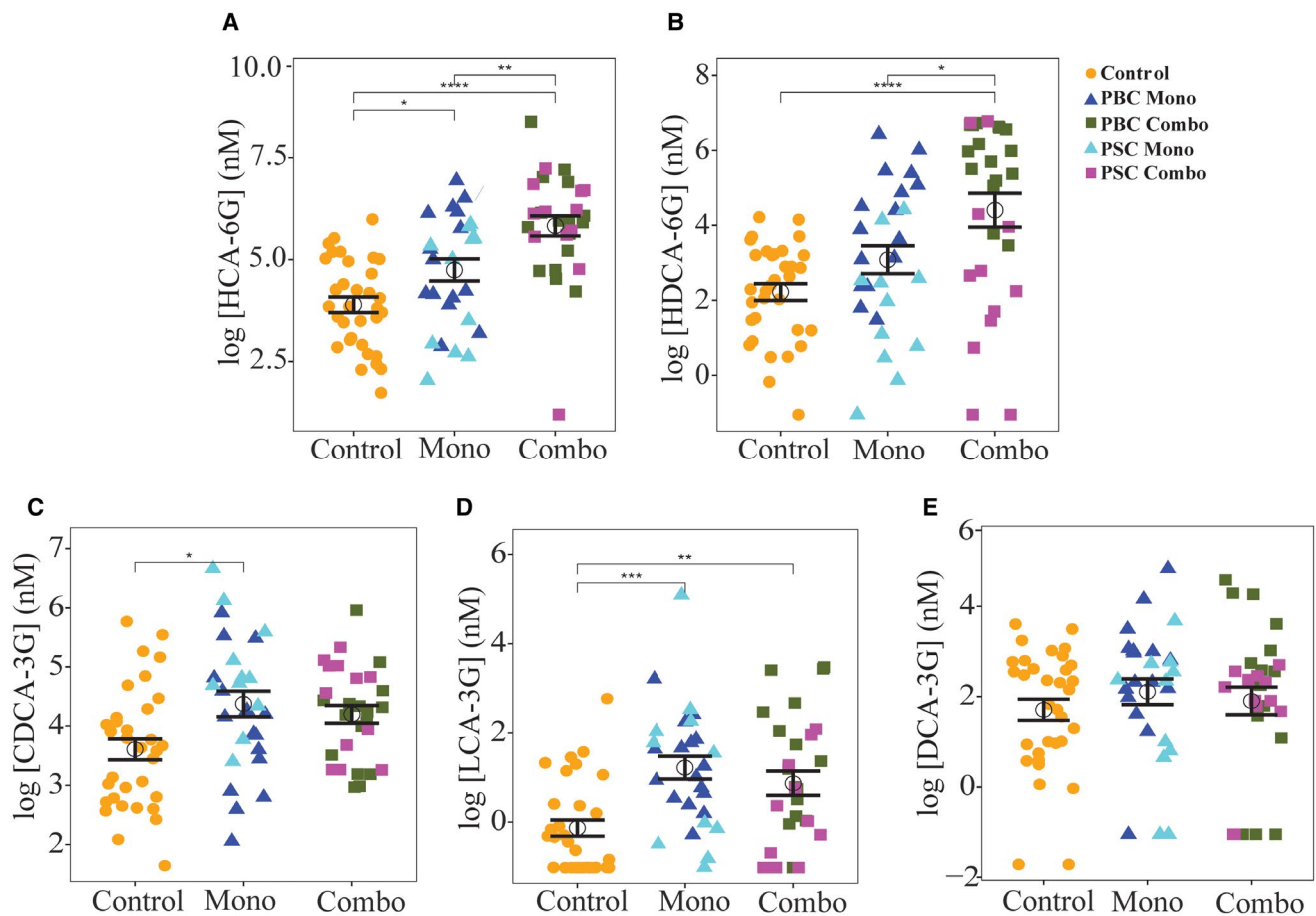


FIG. 2. Combination treatment with fenofibrate alters serum BA-glucuronides in patients with PBC and PSC. Scatter plots show individual patient serum concentrations of HCA-6G (A), HDCA-6G (B), CDCA-3G (C), LCA-3G (D), and DCA-3G (E). Log₂-transformed data were analyzed by one-way ANOVA. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, and *****P* < 0.0001. Abbreviations: Combo, combination; and Mono, monotherapy.

METABOLIC RATIOS OF SERUM BA-GLUCURONIDES AND CORRELATIONS WITH SERUM ALP

To further investigate whether changes in the serum BA-glucuronides reflect the amount of BA substrate available for glucuronidation (e.g., parent BA) or a change in metabolism and transport, we calculated the metabolic ratio for each serum BA-glucuronide compared with their unconjugated parent BA (BA-glucuronide/unconjugated parent BA). The most profound changes occurred among the 3-carboxyl BA-glucuronides. Significant reductions in serum CDCA-3G decreased the metabolic ratio of CDCA-3G/CDCA by 87% in fenofibrate-treated patients with PBC and PSC versus those

receiving ursodiol alone (Fig. 4A). Also, fenofibrate combination treatment reduced serum levels of DCA, resulting in a 77% reduction in the metabolic ratio of DCA-3G/DCA and decreased LCA-3G serum concentrations, which reduced the metabolic ratio of LCA-3G/LCA by 81%, relative to ursodiol monotherapy, and nearly restoring levels for all BA-3Gs to those found in healthy controls (Fig. 3B,C). Additionally, increased metabolic ratios of serum HCA-6G and HDCA-6G support the notion of enhanced BA-glucuronide efflux out of the liver and into circulation through up-regulated MRP3 by fenofibrate (Fig. 4D,E). The increases in these metabolic ratios were due to increases in both the parent BA compound and BA-glucuronide compound after the addition of fenofibrate treatment. Corresponding

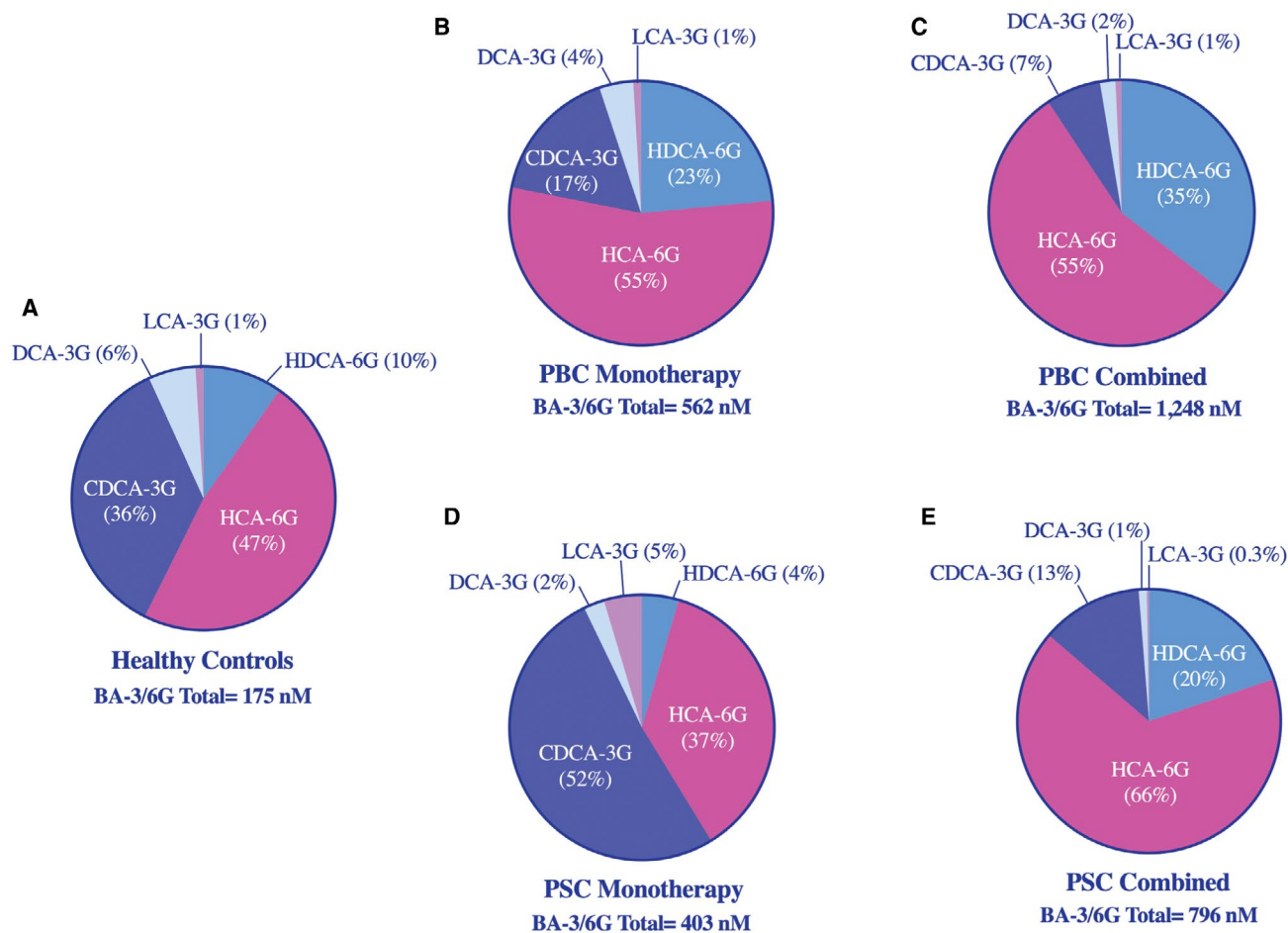


FIG. 3. Combination treatment with fenofibrate shifts the percentage and composition of total 3G and 6G-glucuronides (CDCA-3G, DCA-3G, LCA-3G, HCA-6G, and HDCA-6G) in patients with PBC and PSC, closer to values observed in control subjects: healthy controls (A), PBC monotherapy (B), PBC combined (C), PSC monotherapy (D), and PSC combined (E).

shifts in the metabolic ratios of BA-24Gs were also detected (Supporting Fig. S2).

CORRELATION OF SERUM ALP AND BA-GLUCURONIDE METABOLIC RATIOS

Pearson's correlation coefficients (r^2) between serum ALP and the concentrations of individual BAs, BA-glucuronides, and their respective metabolic ratios were analyzed. The strongest correlations associated with elevated serum ALP levels were found in the patients receiving ursodiol monotherapy, particularly for serum CDCA-3G/CDCA ($r^2 = 0.62$, $P < 0.0001$), DCA-3G/DCA ($r^2 = 0.48$, $P < 0.0001$), and LCA-3G/LCA ($r^2 = 0.40$, $P < 0.001$) (Fig. 5A). These results highlight

the strong association of CDCA-3G, DCA-3G, and LCA-3G metabolic ratios with an incomplete response to ursodiol monotherapy (i.e., serum ALP values > 1.5 times the ULN). Conversely, correlation coefficients obtained from the analysis of combined fenofibrate treatment revealed therapeutic and significant reductions in both ALP and BA-glucuronide metabolic ratios by fenofibrate, similar to levels found in healthy control patients (Fig. 5A).

SERUM BA-GLUCURONIDES INDICATE TREATMENT RESPONSE

We next analyzed the relationship between serum BA-glucuronides and treatment response for the

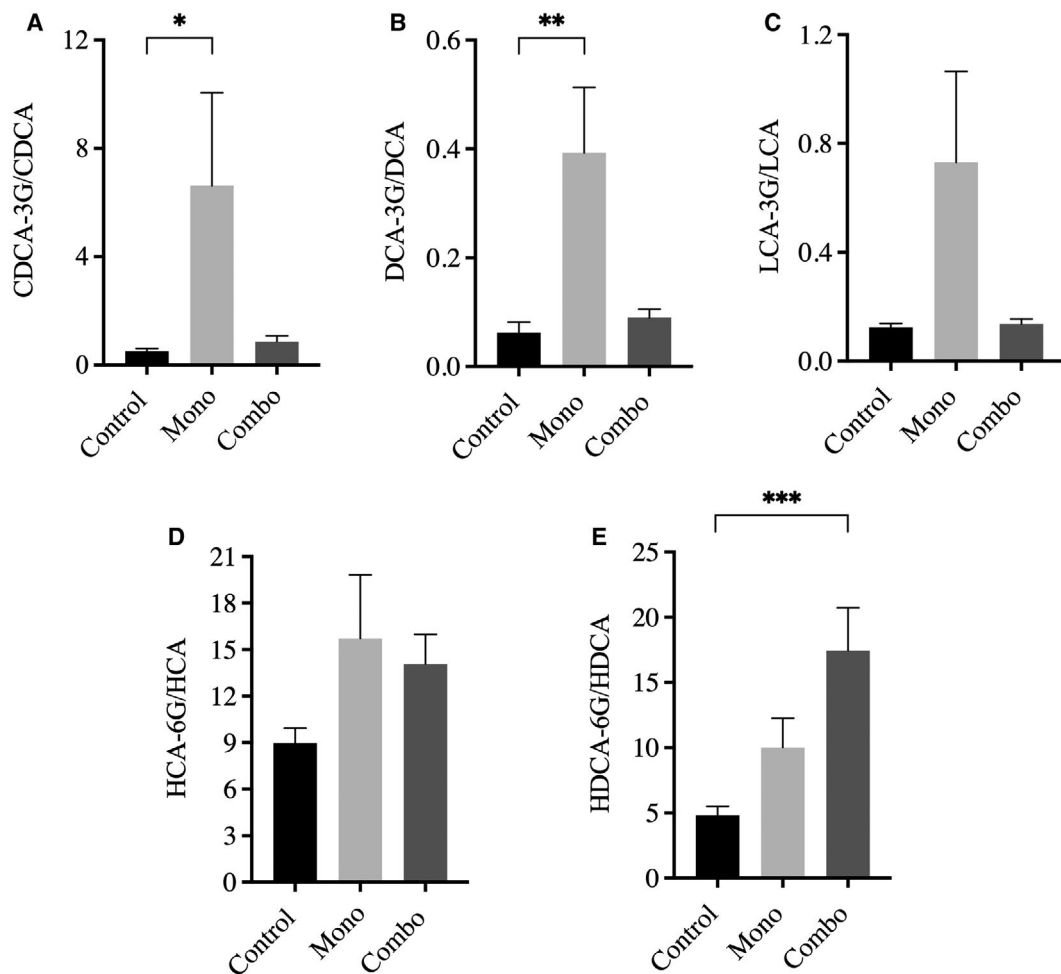


FIG. 4. Combination treatment with fenofibrate alters metabolic ratios (BA-glucuronide/parent BA) in patients with PBC and PSC versus ursodiol monotherapy: CDCA-3G/CDCA (A), DCA-3G/DCA (B), LCA-3G/LCA (C), HCA-6G/HCA (D), and HDCA-6G/HDCA (E). Data were analyzed by the Kruskal-Wallis test with Dunn's multiple comparisons. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

ursodiol monotherapy (incomplete responders) versus healthy controls by ROC curves. The AUC reveals that CDCA-3G (AUC = 0.69, $P = 0.01$), LCA-3G (AUC = 0.8, $P < 0.0001$), as well as the metabolic ratios of CDCA-3G/CDCA (AUC = 0.69, $P = 0.01$) and DCA-3G/DCA (AUC = 0.76, $P < 0.001$), respectively, are strong indicators of the incomplete response to ursodiol monotherapy (Fig. 5B,C). Further ROC curve analyses were performed on the ursodiol monotherapy cohort based on their fibrosis score (Supporting Fig. S3). Those with a fibrosis score of 1, 2, and 3 resulted with AUC values and significance proportional to the degree of their liver injury, including CDCA-3G (AUC = 0.6, $P = 0.63$; AUC = 0.63, $P = 0.35$; and AUC = 0.72, $P = 0.06$; respectively) and

LCA-3G (AUC = 0.8, $P = 0.9$; AUC = 0.93, $P < 0.001$; and AUC = 0.82, $P = 0.01$; respectively). Similarly, the AUCs for the metabolic ratios of CDCA-3G/CDCA (AUC = 0.47, $P = 0.89$; AUC = 0.7, $P = 0.12$; and AUC = 0.78, $P = 0.02$; respectively) and DCA-3G/DCA (AUC = 0.39, $P = 0.55$; AUC = 0.85, $P = 0.01$; and AUC = 0.8, $P = 0.01$; respectively) demonstrate that these metabolites are strong indicators of the incomplete response to ursodiol monotherapy.

To further support the hypothesis that fenofibrate treatment improves BA-glucuronidation and reduces the toxicity of serum BAs, ROC analysis was performed with the combined fenofibrate cohort versus healthy controls. HDCA-6G (AUC = 0.79, $P = 0.0001$) and HCA-6G (AUC = 0.93, $P < 0.0001$)

and their metabolic ratios ($AUC = 0.77$, $P < 0.001$ and $AUC = 0.67$, $P < 0.05$, respectively) were identified as an indicator of the therapeutic response to combined fenofibrate treatment (Fig. 5D,E). Overall, these results support the use of serum BA-6Gs as measures of the therapeutic efficacy of combined treatment with fenofibrate. Additional analyses of serum BA-glucuronides in measuring the treatment

response were assessed by ROC curves (Supporting Table S4).

BA-GLUCURONIDE PATTERNS AND VARIABILITY

Finally, to identify which metabolites are responsible for the variation in BA-glucuronide concentrations

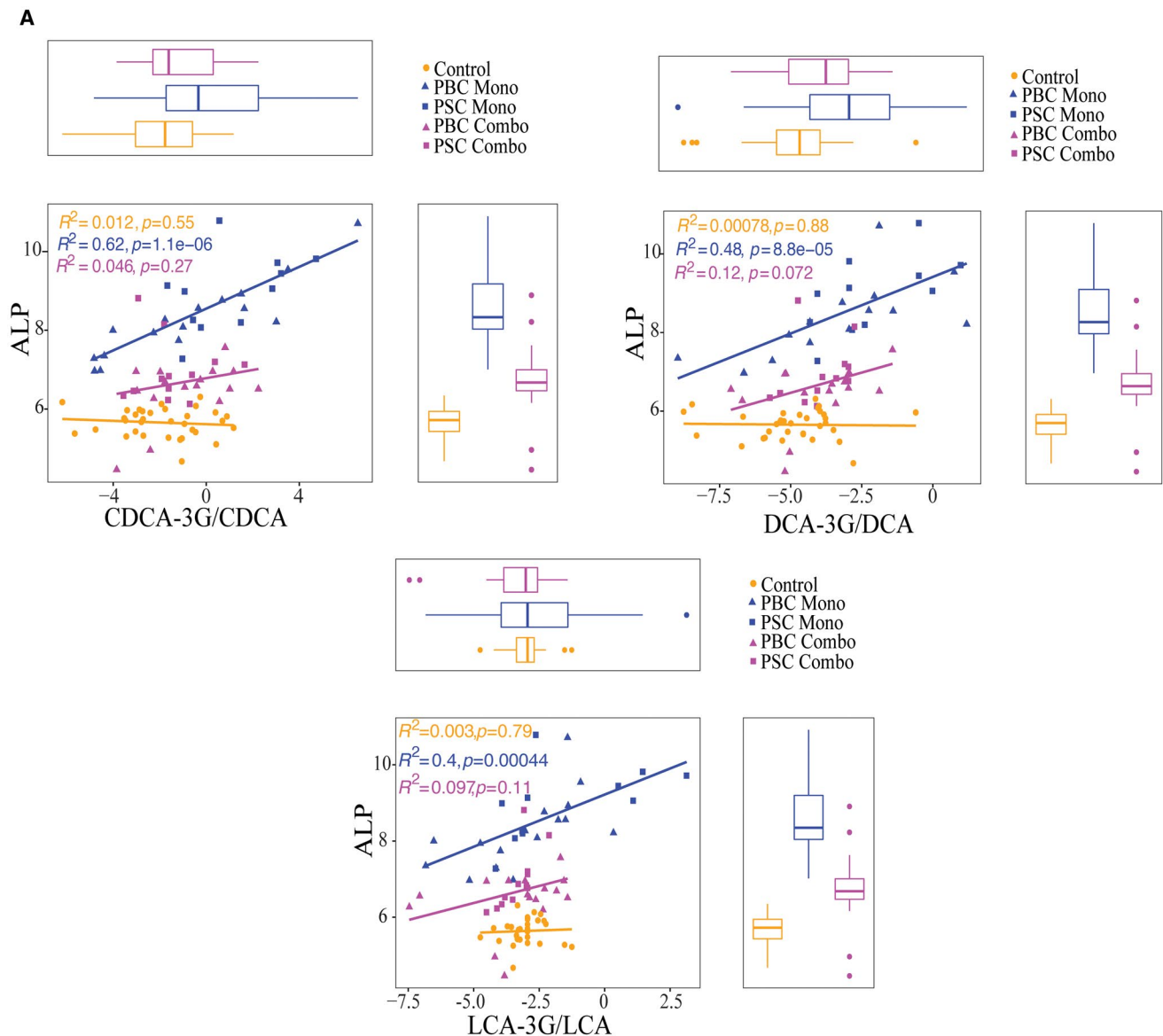


FIG. 5. Pearson's correlation coefficient of BA-glucuronide metabolic ratios with ALP levels: CDCA-3G/CDCA, DCA-3G/DCA, and LCA-3G/LCA (A). ROC analysis in ursodiol monotherapy versus control: CDCA-3G and LCA-3G (B), CDCA-3G/CDCA and DCA-3G/DCA ratios (C). ROC curve analyses in combined fenofibrate treatment versus control: HDCA-6G and HCA-6G (D) and HDCA-6G/HDCA and HCA-6G/HCA ratios (E). Data was \log_2 -transformed before analysis.

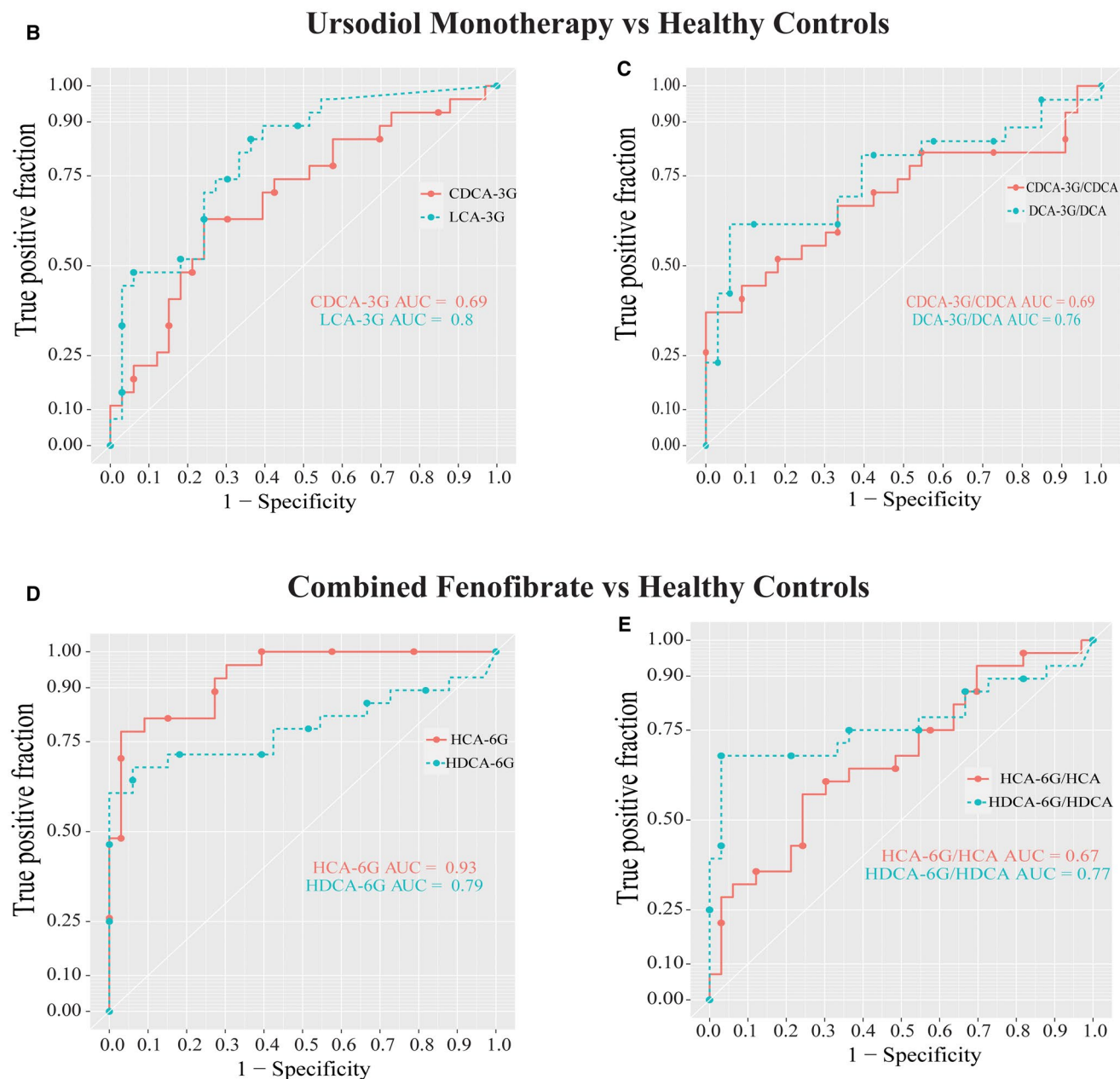


FIG. 5. Continued.

between patient cohorts, we performed principal component (PC) and heatmap analyses. Approximately 68% of the variability in BA-glucuronide levels between patient cohorts are accounted for by three PCs. The first PC accounts for about 36% of the variability among all patient cohorts, which is driven by the healthy control groups having significantly lower concentrations of all BA-glucuronides (Fig. 6A). The second PC accounts for 20% of the

variability, in which the major decreases in LCA-3G and CDCA-3G separated the combination treatment cohorts away from the ursodiol monotherapy cohorts, creating a clear distinction between the treatment groups (Fig. 6A). In the third PC, 12% of the variability is accounted for by the result of DCA-3G, which did not show significant differences between monotherapy and combination cohorts, (+1.8-fold vs. +1.7-fold, respectively), compared with healthy

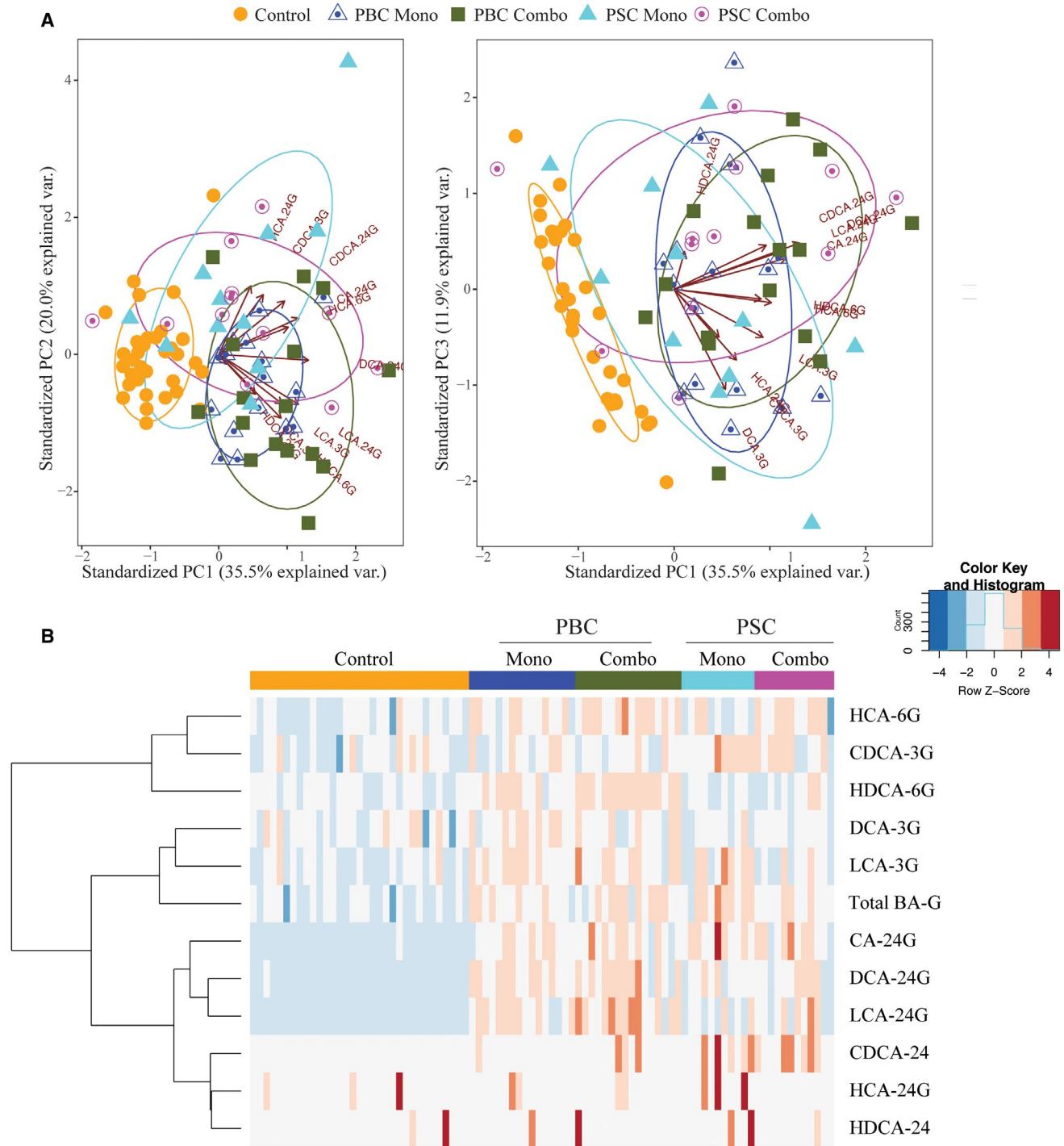


FIG. 6. (A) Two PCA score plots of individual BA-glucuronides from the serum of healthy controls, ursodiol monotherapy, and combination fenofibrate-treated patients to account for approximately 68% of variability. Data was \log_2 -transformed before PCA analysis and grouped as Control, PBC Mono, PBC Combo, PSC Mono, and PSC Combo. (B) Heatmap analysis of individual glucuronides from the serum of healthy subjects, ursodiol monotherapy, and combination fenofibrate-treated patients. Data were \log_2 -transformed before constructing the heatmap. Rows represent BA-glucuronides. Columns represent individual samples and are clustered based on cohort (Control, PBC Mono, PBC Combo, PSC Mono, and PSC Combo).

controls (Fig. 6A). When plotted on the heatmap, the variability of the metabolites results partially from the higher concentrations of the BA-glucuronides in both the PBC and PSC combination fenofibrate therapy groups in relation to ursodiol mono, thereby creating separation between those receiving ursodiol monotherapy and patients receiving combination treatment with fenofibrate (Fig. 6B).

Discussion

In this study, we hypothesized that fenofibrate exerts its therapeutic role in cholestasis in part through PPAR α -mediated UGT up-regulation to promote BA glucuronidation in patients with PBC and PSC who have remained inadequately responsive to ursodiol monotherapy. We also examined whether BA-glucuronides have the potential to serve as measures of efficacy to mono or combination therapy. During chronic cholestatic liver diseases, BAs accumulate within the liver and result in inflammation, fibrosis, and disease progression.⁽²⁸⁾ This pathophysiologic process emphasizes the importance of reducing serum BAs as a therapeutic target for patients with PBC and PSC, especially those with an incomplete response to ursodiol monotherapy. In the past decade, clinical studies have demonstrated the efficacy of fibrates, particularly fenofibrate^(9,11) and bezafibrate,⁽²⁹⁾ as therapeutic alternatives for patients with PBC or PSC who have an incomplete response to ursodiol. We⁽¹³⁾ and others⁽¹⁴⁾ have shown fenofibrate to be safe and well-tolerated in PBC, although much of these data demonstrate that fibrates lower elevated serum liver enzymes during cholestasis, very little data exist on their effect on BA metabolism, specifically BA-glucuronidation. Here, our data show that combination therapy with fenofibrate significantly increased the serum concentrations of BA-glucuronides in patients with PBC and PSC compared with ursodiol monotherapy and healthy controls, in addition to normalizing serum ALP in 94% of patients with PBC and 67% in patients with PSC versus ursodiol monotherapy. We also found that BA-glucuronides comprise 4% of the serum BAs in healthy controls, whereas after monotherapy with ursodiol, serum BA-glucuronides comprise less than 1% of the total serum BA pool, likely due to the significant increases in total BA concentrations and progressive liver disease in patients with an incomplete

response to ursodiol monotherapy. In stark contrast, combination treatment with fenofibrate enhanced BA glucuronidation and restored serum BA-glucuronide levels to normal levels, comprising approximately 4% of the total BA pool in PBC and PSC. The PCA and heatmap plots show a clear separation between the healthy and treated patients along PC1, which is driven by the general increase in BA-glucuronide concentrations in the treated patients. Additionally, the major serum BA-glucuronides were favorably altered by combination treatment with fenofibrate, including elevated serum HDCA-6G ($P < 0.05$) and HCA-6G ($P < 0.01$) levels, which represent most of the BA-glucuronide pool, compared with ursodiol monotherapy. Both of these BA-glucuronides undergo glucuronidation by UGT2B4, which is transcriptionally regulated by PPAR α .⁽⁵⁾ The data presented herein reveal a mechanism in which fenofibrate increases serum BA-glucuronidation and reduces BA toxicity during cholestatic liver diseases in patients who have an incomplete response to ursodiol monotherapy.

Among secondary BAs, DCA and LCA are the most toxic. Elevated concentrations of LCA induce cholestatic liver damage⁽³⁰⁾ and hepatotoxicity,⁽³¹⁾ highlighting the importance of reducing LCA serum concentrations. However, LCA-3G exhibits cholestatic⁽³²⁾ and apoptotic properties.⁽²⁷⁾ Indeed, decreasing LCA-3G levels and promoting LCA detoxification through additional pathways, such as sulfation, is associated with improved markers of disease for patients with PBC and PSC who are incomplete responders to ursodiol monotherapy.⁽¹³⁾ Hydroxylation is also an alternative route for LCA detoxification,⁽³³⁾ where it serves as a substrate for cytochrome P450 (CYP) 3A4-mediated metabolism to HDCA.⁽³⁴⁾ Bezafibrate, a pan-PPAR agonist, transcriptionally activates PXR and subsequently increases *CYP3A4* mRNA expression in HepaRG cells.⁽³⁵⁾ However, whether bezafibrate also increases LCA hydroxylation remains to be determined. In agreement with our current data showing that fenofibrate up-regulates the *CYP3A4* mRNA expression in human hepatocytes (Fig. 1A), *CYP3A4* mRNA expression increased in primary human hepatocytes treated with the PPAR α agonist Wy-14,643,⁽³⁶⁾ which was significantly decreased in response to silencing PPAR α expression.⁽³⁷⁾ Herein, the increase in serum HDCA parent concentrations in the combination fenofibrate cohorts allow for subsequent HDCA 6-glucuronidation by UGT2B4 and

UGT2B7,^(4,5) thereby enhancing its urinary excretion. These findings shed light on an additional mechanism of LCA detoxification regulated, in part, by PPAR α .

All of the BA-24Gs that we studied are formed by UGT1A3,⁽⁴⁾ in which the most significant changes following fenofibrate therapy occurred with LCA-24G and DCA-24G. Our findings agree with reports that PPAR α activation increased UGT1A3 expression and activity, including the production of LCA-24G.^(1,38) In fact, LCA-24G is more actively produced compared with LCA-3G in healthy human liver microsomes.⁽¹⁷⁾ In support of this action, a PPAR α response element has been identified in human *UGT1A3*,⁽⁷⁾ which is supported by our *UGT1A3* mRNA data. Up-regulation of *UGT1A3* is also important for LCA 24-glucuronidation to reduce the hydrophobic and cytotoxic properties of LCA. However, many acyl glucuronides, including serum CA-24G (Supporting Fig. S2F), were found at low concentrations, likely because the carboxyl group at C24 remains intact until glycine or taurine conjugation occurs.⁽¹⁵⁾

Activation of PPAR α also increases the mRNA expression of *MRP3*,^(39,40) an efflux transporter responsible for secreting BA-glucuronides into the systemic circulation for urinary excretion, which is likely to be involved in the BA detoxification process in the current study. In support of this notion, urinary levels of BA-glucuronides were increased in patients with cholestasis during biliary stenting, suggesting that this detoxification pathway may serve as a protective measure to reduce toxic BAs during cholestasis.⁽¹⁷⁾ Although urinary BA-glucuronides were not available for study, the up-regulated *MRP3* mRNA expression following fenofibrate treatment in human hepatocytes supports our hypothesis that PPAR α therapy enhances the urinary excretion of BA-glucuronides, ultimately to reduce BA toxicity during cholestasis.

In addition, combination treatment with fenofibrate decreased total bilirubin concentrations in serum by 35% and 89% in PBC and PSC, respectively. Bilirubin is conjugated to bilirubin diglucuronide by UGT1A1 and 1A4 to facilitate its biliary excretion through MRP2.^(34,41-43) In support of this pathway, our data show that fenofibrate also up-regulates human *UGT1A1*, *1A4*, and *MRP2* mRNA expression, suggesting that activation of PPAR α by fenofibrate also promotes bilirubin excretion through conjugation to glucuronic acid and excretion into bile through

MRP2, thereby reducing elevated serum levels during cholestasis.

Serum and urinary total BAs and individual BAs have been evaluated as potential mechanistic biomarkers of liver injury.^(20,21) Recently, He et al. showed that CA and CDCA were predictive diagnostic markers in severe alcohol-associated hepatitis.⁽²²⁾ However, the role of serum BA-glucuronides as secondary measures of treatment response in cholestatic liver injury remains unknown. In this study, Pearson correlation coefficients demonstrate that metabolic ratios of serum CDCA-3G and LCA-3G concentrations are strongly associated with elevated serum ALP levels in patients experiencing an incomplete response to ursodiol monotherapy. Similarly, PCA plots identified a strong separation between treatment groups as a result of the decreases found in LCA-3G and CDCA-3G after combined fenofibrate treatment in comparison to ursodiol monotherapy (Fig. 3A). Together with the ROC curve results, these findings support the possible role of serum BA and serum BA-glucuronides as mechanistically relevant secondary endpoints in clinical trials for PBC and PSC with anti-cholestatic agents.

Furthermore, the ROC curves generated from serum HCA-6G and HDCA-6G concentrations in combination fenofibrate group versus healthy controls (AUCs > 0.79, $P < 0.0001$) are consistent with the heatmap in which increases for HDCA-6G and HCA-6G are found in both PBC and PSC fenofibrate combination groups in comparison to ursodiol monotherapy and healthy controls. Importantly, HDCA-6G and HCA-6G are not cytotoxic *in vitro*.⁽²⁷⁾ Together, these data demonstrate the importance of serum BA-glucuronides, in addition to reduced serum ALP levels, as measures of response to therapy (e.g., incomplete response to ursodiol monotherapy versus therapeutic efficacy of adjunct fenofibrate treatment).

Toxic BA accumulation is a major concern for both patients with PBC and PSC; thus, both cholestatic patient cohorts have been included in our study. Data for each cohort are presented to demonstrate how combination fenofibrate therapy affects BA glucuronidation in each disease. Human BA-glucuronides have been shown to have higher concentrations in urine than in serum.^(4,27) Nevertheless, favorable differences in reduced serum liver enzymes and increased serum BA-glucuronide concentrations following the addition of fenofibrate remain evident, supporting the

role of serum BA-glucuronides as secondary markers of response to therapy for cholestatic liver injury. Our study provides compelling evidence that fenofibrate-mediated BA-glucuronidation is a mechanism to detoxify the toxic primary (e.g., CDCA, CA) and secondary BAs (e.g., DCA, LCA) during cholestasis. In particular, the serum concentrations of CDCA-3G and LCA-3G greatly decreased after combination treatment with fenofibrate (Fig. 2C,D) compared with ursodiol monotherapy and approached healthy control levels, thereby reducing their toxicity. These trends in concentrations changes are also supported by the correlation data and ROC curves.

In summary, this is a study of BA-glucuronidation in patients with PBC and PSC receiving combination therapy with fenofibrate and ursodiol. The findings shed light on therapeutic mechanisms for future treatment options for patients with chronic cholestatic liver diseases who remain subtherapeutic while taking ursodiol monotherapy.

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request. Some data may not be made available because of privacy or ethical restrictions.

REFERENCES

- Trottier J, Milkiewicz P, Kaeding J, Verreault M, Barbier O. Coordinate regulation of hepatic bile acid oxidation and conjugation by nuclear receptors. *Mol Pharm* 2006;3:212-222.
- Barbier O, Trottier J, Kaeding J, Caron P, Verreault M. Lipid-activated transcription factors control bile acid glucuronidation. *Mol Cell Biochem* 2009;326:3-8.
- Kiang TK, Ensom MH, Chang TK. UDP-glucuronosyltransferases and clinical drug-drug interactions. *Pharmacol Ther* 2005;106:97-132.
- Trottier J, Perreault M, Rudkowska I, Levy C, Dallaire-Theroux A, Verreault M, et al. Profiling serum bile acid glucuronides in humans: gender divergences, genetic determinants, and response to fenofibrate. *Clin Pharmacol Ther* 2013;94:533-543.
- Barbier O, Duran-Sandoval D, Pineda-Torra I, Kosykh V, Fruchart JC, Staels B. Peroxisome proliferator-activated receptor alpha induces hepatic expression of the human bile acid glucuronidating UDP-glucuronosyltransferase 2B4 enzyme. *J Biol Chem* 2003;278:32852-32860.
- Gall WE, Zawada G, Mojarrabi B, Tephly TR, Green MD, Coffman BL, et al. Differential glucuronidation of bile acids, androgens and estrogens by human UGT1A3 and 2B7. *J Steroid Biochem Mol Biol* 1999;70:101-108.
- Senkeo-Effenberger K, Chen S, Brace-Sinnokrak E, Bonzo JA, Yuch MF, Argikar U, et al. Expression of the human UGT1 locus in transgenic mice by 4-chloro-6-(2,3-xylylidino)-2-pyrimidinylthioacetic acid (WY-14643) and implications on drug metabolism through peroxisome proliferator-activated receptor alpha activation. *Drug Metab Dispos* 2007;35:419-427.
- Mostarda S, Passeri D, Carotti A, Cerra B, Colliva C, Benicchi T, et al. Synthesis, physicochemical properties, and biological activity of bile acids 3-glucuronides: novel insights into bile acid signalling and detoxification. *Eur J Med Chem* 2018;144:349-358.
- Levy C, Peter JA, Nelson DR, Keach J, Petz J, Cabrera R, et al. Pilot study: fenofibrate for patients with primary biliary cirrhosis and an incomplete response to ursodeoxycholic acid. *Aliment Pharmacol Ther* 2011;33:235-242.
- Lindor KD, Bowlus CL, Boyer J, Levy C, Mayo M. Primary biliary cholangitis: 2018 practice guidance from the American Association for the Study of Liver Diseases. *Hepatology* 2019;69:394-419.
- Lemoine S, Pares A, Reig A, Ben Belkacem K, Kemgang Fankem AD, Gaouar F, et al. Primary sclerosing cholangitis response to the combination of fibrates with ursodeoxycholic acid: French-Spanish experience. *Clin Res Hepatol Gastroenterol* 2018;42:521-528.
- Ghonem NS, Ananthanarayanan M, Soroka CJ, Boyer JL. Peroxisome proliferator-activated receptor α activates human multidrug resistance transporter 3/ATP-binding cassette protein subfamily B4 transcription and increases rat biliary phosphatidylcholine secretion. *Hepatology* 2014;59:1030-1042.
- Ghonem NS, Auclair AM, Hemme CL, Gallucci GM, de la Rosa RR, Boyer JL, et al. Fenofibrate improves liver function and reduces the toxicity of the bile acid pool in patients with primary biliary cholangitis and primary sclerosing cholangitis who are partial responders to ursodiol. *Clin Pharmacol Ther* 2020;108:1213-1223.
- Carrion AF, Lindor KD, Levy C. Safety of fibrates in cholestatic liver diseases. *Liver Int* 2021;41:1335-1343.
- Hofmann AF. Why bile acid glucuronidation is a minor pathway for conjugation of endogenous bile acids in man. *Hepatology* 2007;45:1083-1084; author reply 1084-1085.
- Dawson PA, Karpen SJ. Intestinal transport and metabolism of bile acids. *J Lipid Res* 2015;56:1085-1099.
- Perreault M, Wunsch E, Bialek A, Trottier J, Verreault M, Caron P, et al. Urinary elimination of bile acid glucuronides under severe cholestatic situations: contribution of hepatic and renal glucuronidation reactions. *Can J Gastroenterol Hepatol* 2018;2018:8096314.
- Horvatits T, Drolz A, Roedl K, Rutter K, Ferlitsch A, Fauler G, et al. Serum bile acids as marker for acute decompensation and acute-on-chronic liver failure in patients with non-cholestatic cirrhosis. *Liver Int* 2017;37:224-231.
- Horvatits T, Drolz A, Rutter K, Roedl K, Langouche L, Van den Berghe G, et al. Circulating bile acids predict outcome in critically ill patients. *Ann Intensive Care* 2017;7:48.
- Woolbright BL, McGill MR, Staggs VS, Winefield RD, Gholami P, Olyae M, et al. Glycodeoxycholic acid levels as prognostic biomarker in acetaminophen-induced acute liver failure patients. *Toxicol Sci* 2014;142:436-444.
- Luo L, Aubrecht J, Li D, Warner RL, Johnson KJ, Kenny J, et al. Assessment of serum bile acid profiles as biomarkers of liver injury and liver disease in humans. *PLoS One* 2018;13:e0193824.
- He L, Vatsalya V, Ma X, Zhang J, Yin X, Kim S, et al. Metabolic profiling of bile acids in the urine of patients with alcohol-associated liver disease. *Hepatol Commun* 2021;5:798-811.
- Manna LB, Ovadia C, Lövgren-Sandblom A, Chambers J, Begum S, Seed P, et al. Enzymatic quantification of total serum bile acids as a monitoring strategy for women with intrahepatic cholestasis

- of pregnancy receiving ursodeoxycholic acid treatment: a cohort study. *BJOG* 2019;126:1633-1640.
- 24) Smyth GK. *limma: Linear Models for Microarray Data. Bioinformatics and Computational Biology Solutions Using R and Bioconductor*. New York, NY: Springer; 2005:397-420.
 - 25) Sachs MC. *plotROC: a tool for plotting ROC curves*. *J Stat Softw* 2017;79:2.
 - 26) Trottier J, Bialek A, Caron P, Straka RJ, Heathcote J, Milkiewicz P, et al. Metabolomic profiling of 17 bile acids in serum from patients with primary biliary cirrhosis and primary sclerosing cholangitis: a pilot study. *Dig Liver Dis* 2012;44:303-310.
 - 27) Perreault M, Bialek A, Trottier J, Verreault M, Caron P, Milkiewicz P, et al. Role of glucuronidation for hepatic detoxification and urinary elimination of toxic bile acids during biliary obstruction. *PLoS One* 2013;8:e80994.
 - 28) Cai S-Y, Ouyang X, Chen Y, Soroka CJ, Wang J, Mennone A, et al. Bile acids initiate cholestatic liver injury by triggering a hepatocyte-specific inflammatory response. *JCI Insight* 2017;2:e90780.
 - 29) Corpechot C, Chazouillères O, Rousseau A, Le Gruyer A, Habersetzer F, Mathurin P, et al. A placebo-controlled trial of bezafibrate in primary biliary cholangitis. *N Engl J Med* 2018;378:2171-2181.
 - 30) Woolbright BL, Li F, Xie Y, Farhood A, Fickert P, Trauner M, et al. Lithocholic acid feeding results in direct hepato-toxicity independent of neutrophil function in mice. *Toxicol Lett* 2014;228:56-66.
 - 31) Song P, Zhang Y, Klaassen CD. Dose-response of five bile acids on serum and liver bile acid concentrations and hepatotoxicity in mice. *Toxicol Sci* 2011;123:359-367.
 - 32) Takikawa H, Minagawa K, Sano N, Yamanaka M. Lithocholate-3-O-glucuronide-induced cholestasis. A study with congenital hyperbilirubinemic rats and effects of ursodeoxycholate conjugates. *Dig Dis Sci* 1993;38:1543-1548.
 - 33) Kitada H, Miyata M, Nakamura T, Tozawa A, Honma W, Shimada M, et al. Protective role of hydroxysteroid sulfotransferase in lithocholic acid-induced liver toxicity. *J Biol Chem* 2003;278:17838-17844.
 - 34) Bock KW. Human UDP-glucuronosyltransferases: feedback loops between substrates and ligands of their transcription factors. *Biochem Pharmacol* 2012;84:1000-1006.
 - 35) Honda A, Ikegami T, Nakamuta M, Miyazaki T, Iwamoto J, Hirayama T, et al. Anticholestatic effects of bezafibrate in patients with primary biliary cirrhosis treated with ursodeoxycholic acid. *Hepatology* 2013;57:1931-1941.
 - 36) Rakhshandehroo M, Hooiveld G, Muller M, Kersten S. Comparative analysis of gene regulation by the transcription factor PPARalpha between mouse and human. *PLoS One* 2009;4:e6796.
 - 37) Klein K, Thomas M, Winter S, Nussler AK, Niemi M, Schwab M, et al. PPARA: a novel genetic determinant of CYP3A4 in vitro and in vivo. *Clin Pharmacol Ther* 2012;91:1044-1052.
 - 38) Verreault M, Senekeo-Effenberger K, Trottier J, Bonzo JA, Bélanger J, Kaeding J, et al. The liver X-receptor alpha controls hepatic expression of the human bile acid-glucuronidating UGT1A3 enzyme in human cells and transgenic mice. *Hepatology* 2006;44:368-378.
 - 39) Dai M, Yang J, Xie M, Lin J, Luo M, Hua H, et al. Inhibition of JNK signalling mediates PPARalpha-dependent protection against intrahepatic cholestasis by fenofibrate. *Br J Pharmacol* 2017;174:3000-3017.
 - 40) Zhao Q, Yang R, Liu F, Wang J, Hu D-D, Yang X-W, et al. Metabolomics reveals that PPARα activation protects against lithocholic acid-induced liver injury. *RSC Adv* 2017;7:49849-49857.
 - 41) Bosma PJ. Inherited disorders of bilirubin metabolism. *J Hepatol* 2003;38:107-117.
 - 42) Ritter JK, Crawford JM, Owens IS. Cloning of two human liver bilirubin UDP-glucuronosyltransferase cDNAs with expression in COS-1 cells. *J Biol Chem* 1991;266:1043-1047.
 - 43) Huang W, Zhang J, Chua SS, Qatanani M, Han Y, Granata R, et al. Induction of bilirubin clearance by the constitutive androstane receptor (CAR). *Proc Natl Acad Sci U S A* 2003;100:4156-4161.

Supporting Information

Additional Supporting Information may be found at onlinelibrary.wiley.com/doi/10.1002/hep4.1787/supinfo.