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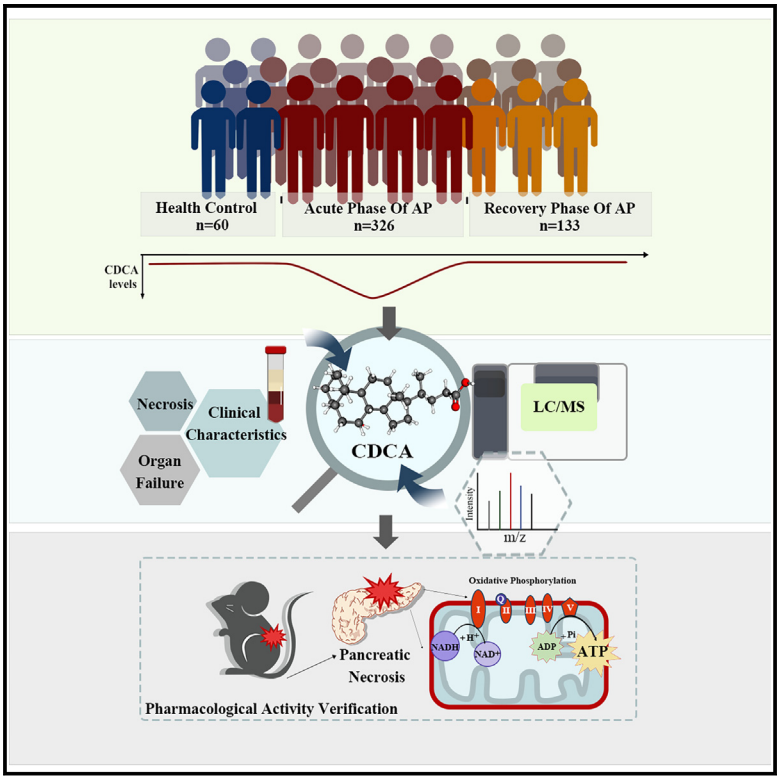
Bile acid metabolomics identifies chenodeoxycholic acid as a therapeutic agent for pancreatic necrosis

Graphical abstract



Highlights

- Bile acids metabolites profiles of individuals with AP differ from healthy controls
- CDCA decreases in the acute and increases in recovery phases of acute pancreatitis
- CDCA is strongly associated with pancreatic necrosis in patients with AP
- CDCA and its receptor, FXR, may be a novel target for acinar cell necrosis treatment

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In brief

Pancreatic necrosis is a significant complication arising from AP. Zhu et al. conduct targeted serum bile acids metabolomics in AP patients and experiments *in vitro* and *in vivo* to demonstrate that the therapeutic targeting of CDCA and its receptor, FXR, holds potential as a strategy for addressing pancreatic necrosis.

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Bile acid metabolomics identifies chenodeoxycholic acid as a therapeutic agent for pancreatic necrosis

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SUMMARY

Bile acids are altered and associated with prognosis in patients with acute pancreatitis (AP). Here, we conduct targeted metabolomic analyses to detect bile acids changes in patients during the acute (n = 326) and the recovery (n = 133) phases of AP, as well as in healthy controls (n = 60). Chenodeoxycholic acid (CDCA) decreases in the acute phase, increases in the recovery phase, and is associated with pancreatic necrosis. CDCA and its derivative obeticholic acid exhibit a protective effect against acinar cell injury *in vitro* and pancreatic necrosis in murine models, and RNA sequencing reveals that the oxidative phosphorylation pathway is mainly involved. Moreover, we find that overexpression of farnesoid X receptor (FXR, CDCA receptor) inhibits pancreatic necrosis, and interfering expression of FXR exhibits an opposite phenotype in mice. Our results possibly suggest that targeting CDCA is a potential strategy for the treatment of acinar cell necrosis in AP, but further verification is needed.

INTRODUCTION

Acute pancreatitis (AP) is an acute inflammation of pancreatic tissue characterized by auto-digestion, edema, and necrosis.[1] Pancreatic necrosis is correlated with the prognosis of patients with AP, the incidence of organ failure, systemic inflammatory response syndrome, and other diseases.[2,3] Mortality can reach 15%–30% in patients who have concomitant AP and pancreatic necrosis.[4] Findings of randomized clinical trials conducted on AP pharmacotherapy have been limited, and effective preventive and therapeutic measures for pancreatic necrosis are still lacking.[5]

Bile acids, the endogenous products of cholesterol metabolism in the body, have various biological functions in addition to their role in the digestion and absorption of fats.[6,7] Bile acids have been reported to function as signaling molecules by targeting receptors that regulate a variety of downstream metabolic, inflammatory, immune, and other pathophysiological processes.[8–10] Experiments have confirmed that supplementation with taurodeoxycholic acid (TUDCA) and ursodeoxycholic acid (UDCA) in various animal models has protective effects against neurodegeneration.[11,12] The farnesoid X receptor (FXR) and Takeda G protein-coupled receptor clone (TGR5) are the two most studied bile acid-specific receptors. FXR, which is highly expressed in the liver and gastrointestinal tract, can be conjugated by bile acids, the highest affinity of which is chenodeoxycholic acid (CDCA).[13] Modulation of inflammatory responses in myeloid immune cells by FXR agonists attenuates multiple sclerosis and experimental autoimmune encephalomyelitis in mice.[14,15]

The traditional view holds that biliary diseases cause bile reflux, and that bile acids, the main constituents of bile, play an important role in the pathogenesis of AP.[16] However, growing research on the properties of bile acids as signaling molecules in recent years has led to a new understanding of the pathophysiological mechanisms underlying AP.[17–19] Clinical study has demonstrated that a transient increase in serum bile acids occurs within 24 h in the acute stage of AP.[20] Furthermore, the level of serum total bile acids was significantly higher in patients with AP of biliary etiology compared with those of alcohol and other

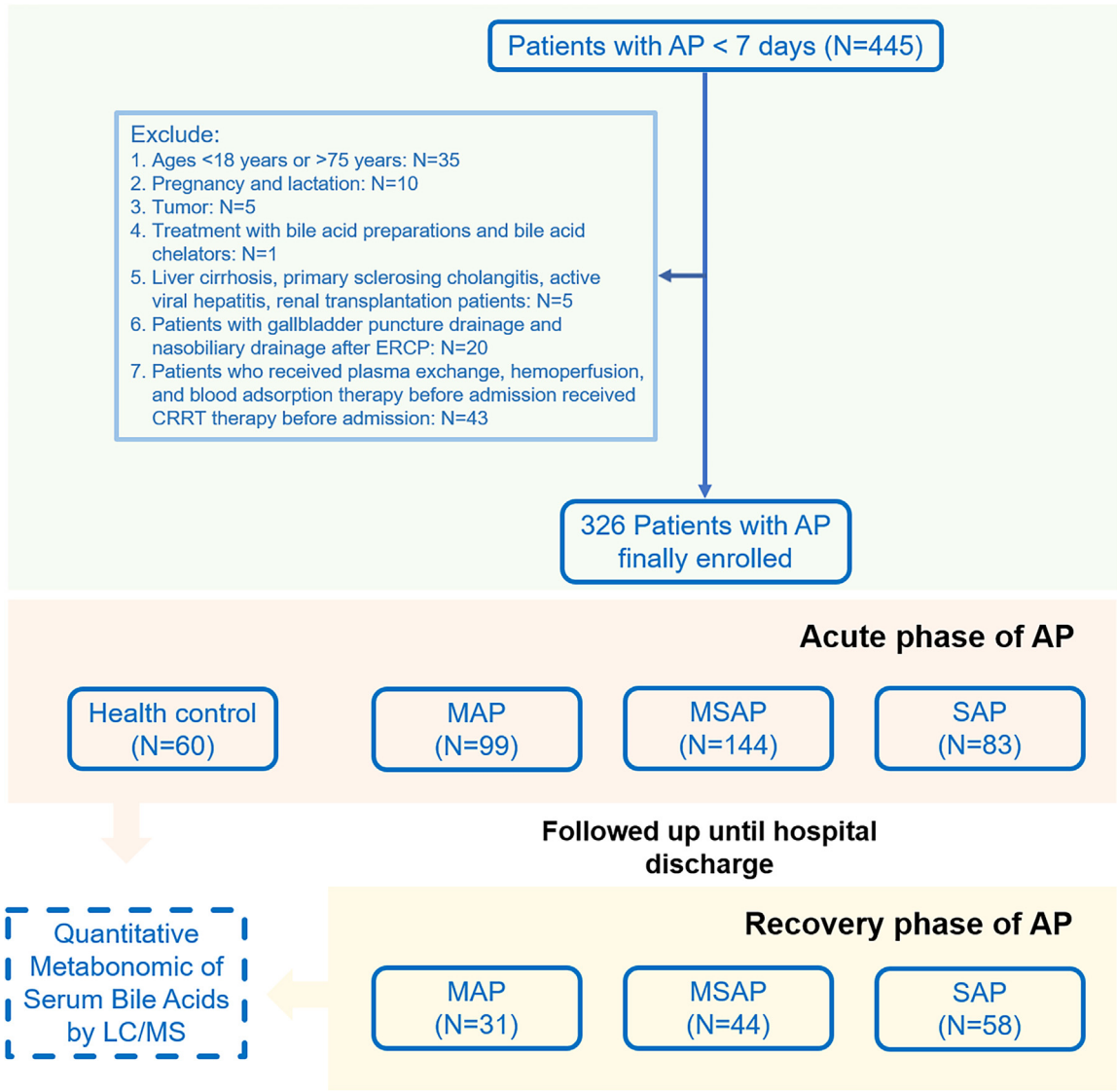

Figure 1. Flow chart of the study design for the cohort

etiologies, These findings suggest that bile acids can help in the early diagnosis of AP. Our group's previous clinical retrospective study showed that patients with high bile acid levels had higher rates of pancreatic necrosis, organ failure, and case fatality than those with normal bile acid levels.[21] Nevertheless, the dynamic changes in bile acid metabolic profiles and their components during AP and their correlation with AP disease severity and pancreatic necrosis remain unknown.

In this study, we aimed to identify the altered bile acid metabolism in AP using targeted metabolomic mass spectrometry analysis in a human population. Furthermore, *in vivo* and *in vitro* investigations using AP murine models and acinar cells were performed to identify the role of the main bile acid CDCA and its receptor FXR in AP. We also focused on observing the pharmacological effects of obeticholic acid (OCA), a CDCA derivative commonly used in clinical practice. Our findings suggest that targeted intervention on the dysregulated bile acid metabolism in AP, particularly CDCA, may provide new therapeutic strategies for this condition.

RESULTS

Changes in the metabolic components of serum bile acids in patients during the acute phase of AP

A total of 326 patients with AP onset of fewer than 7 days and 60 healthy controls were included in the study, and the flow chart of study design is shown in Figure 1. The patients were classified according to the Atlanta classification diagnostic criteria[22] as having mild acute pancreatitis (MAP, n = 99), moderately severe acute pancreatitis (MSAP, n = 144), and severe acute pancreatitis (SAP, n = 83). Clinical data including the general clinical

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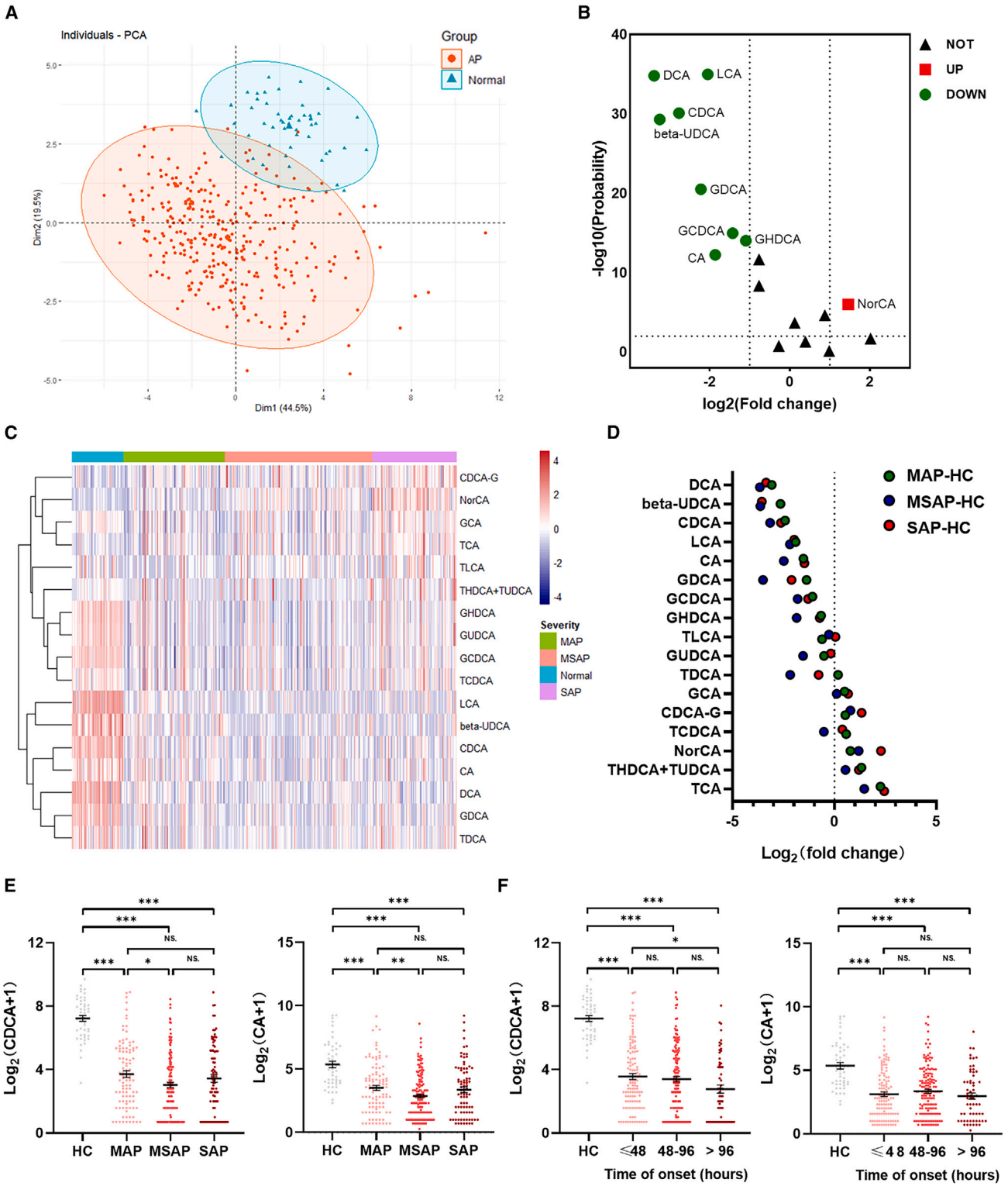

Figure 2. Changes in the metabolic components of serum bile acids in patients during the acute phase of AP

(A) Principal component analysis (PCA) of targeted bile acids metabonomic.

(B) Volcano plot representing the levels of 17 significantly changed bile acids up- or downregulated in the acute phase of AP patients (n = 326) with respect to healthy controls (HCs, n = 60). Components of serum bile acids with significant changes are presented in red (significant increase) or in green (significant decrease).

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and demographic data of healthy controls and patients with AP are shown in Table S1.

To investigate altered bile acid metabolites in patients with AP, we performed targeted bile acid metabolomic mass spectrometry analysis of population serum, which included a total of 39 bile acid metabolism-related metabolites (Table S2). Principal component analysis (PCA) revealed significant differences in the cluster analysis of bile acid metabolites between patients with AP during the acute phase and healthy controls (Figure 2A). A total of 17 bile acids met the quality control criteria and were quantified using volcano plots (Figure 2B) to map the fold changes in serum bile acid metabolite levels. Subgroup analysis was conducted based on the severity of AP patients; the mean normalized concentrations of 17 bile acid metabolites are presented in the heatmap (Figure 2C), and the fold change in different bile acid metabolite concentrations observed was visualized using Cleveland plots (Figure 2D). Overall, the differential metabolites are mainly primary bile acid (CDCA and cholic acid [CA]) and their downstream metabolites or binding forms. Figures 2E and S1 provide a more intuitive display of the significant correlation between the severity of AP and the changes in serum standardized concentration levels of primary bile acid (CDCA and CA), its downstream metabolically derived secondary bile acids, or conjugated modified form. In addition, we divided patients into three groups based on the onset time of AP in acute phase, and the results showed that CDCA or CA showed a significant downward trend in the early onset of AP (within 48 h); moreover, the secondary bile acids or conjugated modification forms derived from CDCA or CA also exhibit a related trend of change (Figure S2). Finally, subgroup analysis was conducted, and we found that the above changes are independent of the etiology of AP (Figure S3). In addition, to validate the reliability and generalizability of our results, data from an external source, the Affiliated Hospital of Yangzhou University, consisted of 38 healthy control subjects, 125 MAP, 35 MASP, and 18 SAP. Consistent with the results from the Nanjing cohort, AP patients also exhibited significant reductions in CDCA and CA (Figure S4), and the extent of reduction demonstrated a correlation with the severity of the disease.

Changes in the metabolic components of serum bile acids in patients during the recovery phase of AP

To assess the correlation between changes in serum bile acid metabolite levels and the course of AP, we dynamically collected convalescent serum samples from MAP (n = 31), MSAP (n = 44), and SAP (n = 58) patients, for a total of 133 AP patients, and we performed targeted metabolomics mass spectrometry analysis. Multiple metabolites were significantly different in the convalescent phase between patients with AP and healthy controls as evidenced by the volcano plots, heatmaps, and Cleveland plots. Only primary bile acids CDCA and CA were significantly elevated in the convalescent phase compared to the acute phase, a finding that matched the PCA results (cluster analysis of the two groups was not significantly different) (Figures 3A–3D). Furthermore, the recovery of CDCA and CA metabolite levels was statistically more significant in patients with MSAP and SAP ($p < 0.05$) (Figure 3E).

Combining the quantitative bile acid metabolomics results of the two different populations, we found that the levels of CDCA and CA significantly changed, whereby they decreased significantly during the acute phase and recovered significantly during the recovery phase of AP; moreover, the decrease in levels of CDCA and CA is significantly correlated with the severity of AP.

Correlation between the normalized concentrations of serum CDCA or CA and clinical features of AP

To evaluate the potential translational value of CDCA and CA, we evaluated the association between the serum level of CDCA or CA and clinical features of AP. Concurrent pancreatic necrosis and organ failure are major contributors to poor outcomes in patients with AP. The results showed that the levels of normalized serum CDCA or CA were significantly lower in the acute necrotic collection (ANC) group compared to the non-ANC group; however, this difference was not observed in patients with pancreatic necrosis infection and organ failure (Figures 4A and 4B). Univariate logistic regression analysis showed that decreased normalized CDCA levels (odds ratio [OR] = 0.862, 95% confidence interval [CI] = 0.775–0.958) and CA levels (OR = 0.867, 95% CI = 0.773–0.974) are risk factors for pancreatic necrosis in patients with AP. Furthermore, after correction for age, gender, BMI, etiology, and comorbid disease parameters, multivariate logistic regression analysis revealed that decreased normalized CDCA levels (OR = 0.865, 95% CI = 0.763–0.98) and CA levels (OR = 0.854, 95% CI = 0.748–0.975) were independent risk factors for pancreatic necrosis in patients with in AP (Figure 4C).

CDCA and its derivative obeticholic acid, but not CA, have shown pharmacological protection activity in acinar cell injury and pancreatic necrosis *in vitro* and *in vivo*

We found a close association between primary bile acid (CDCA and CA) and pancreatic necrosis in our clinical cohort, but the biological effect of CDCA or CA on acinar cell necrosis has not been reported. Here we used caerulein to establish a classical murine AP model and then observed the changes in serum bile acids levels in these mice. Using targeted mass spectrometry, we found

(C) Heatmap of mean normalized bile acids metabolite concentrations derived from targeted bile acids metabonomic profiling in the acute phase of AP patients (n = 326) and healthy controls (HCs, n = 60).

(D) Cleveland dot plot showing a ranked log2 transformation of fold changes of serum bile acids. Green, blue, and red dots represent the fold changes between patients with MAP (n = 99), MASP (n = 144), and SAP (n = 83), with respect to HCs (n = 60), respectively.

(E) Scatterplots showing log2 transformation of the normalized concentrations of serum CDCA and CA in patients with MAP (n = 99), MASP (n = 144), SAP (n = 83), and healthy controls (n = 60).

(F) Scatterplots showing the changes in log2 transformation of the normalized concentrations of serum CDCA and CA in the acute phase of AP patients grouped according to the different intervals between onset and hospital admission (≤48 h, n = 118; 48–96 h, n = 146; >96 n = 62) and healthy controls (HCs, n = 60). Numbers indicate the mean and standard error of the mean (SEM). ***$p < 0.001$, **$p < 0.01$, and *$p < 0.05$. NS, no significance.

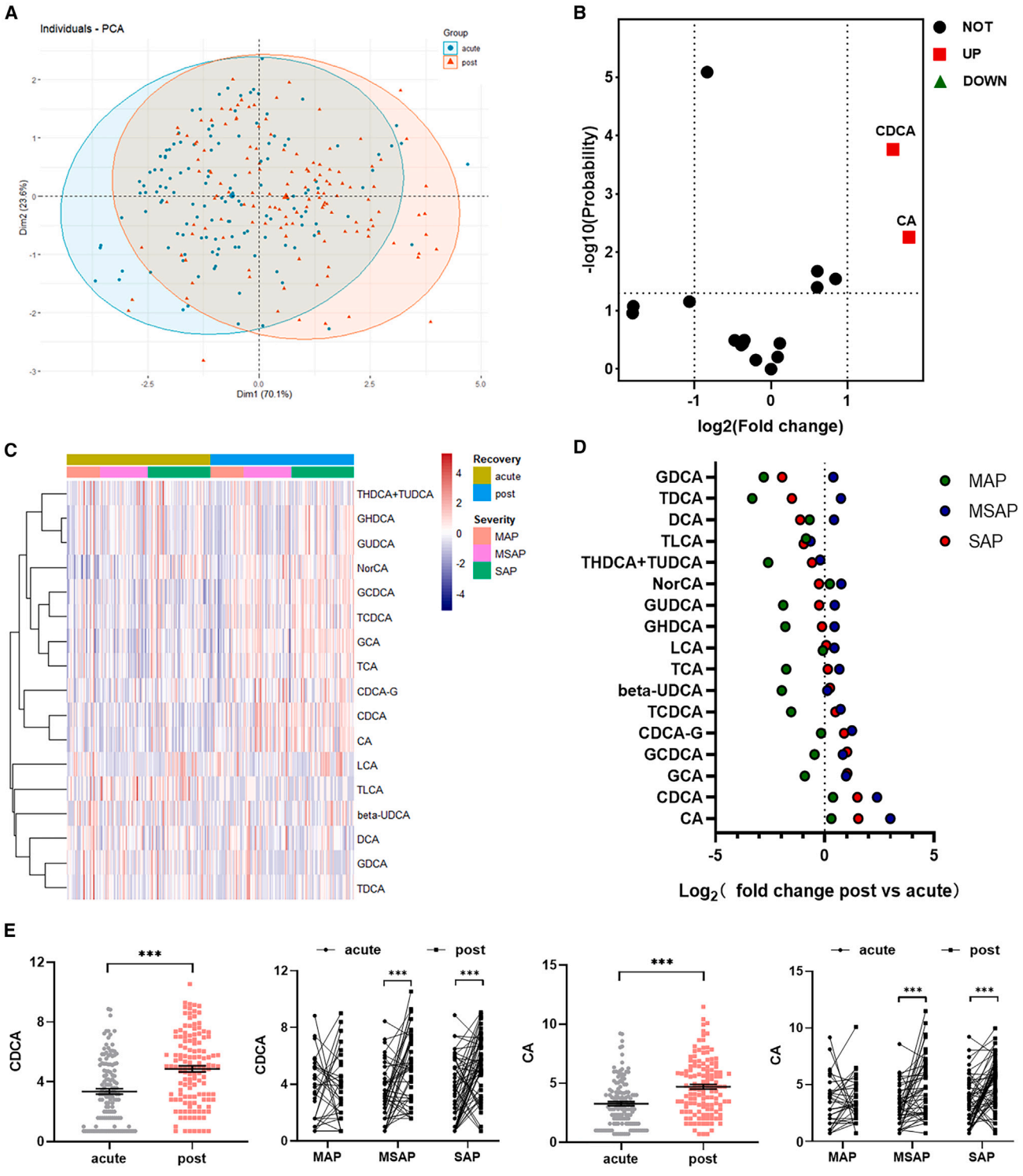

Figure 3. Changes in the metabolic components of serum bile acids in patients during the recovery phase of AP

(A) Principal component analysis (PCA) of targeted bile acids metabonomic.

(B) Volcano plot showing the levels of changed bile acids up- or downregulated in patients during the recovery phase of AP concerning the acute phase of AP. The levels of CDCA and CA with significant increases are presented in red.

(C) Heatmap of mean normalized bile acids metabolite concentrations derived from targeted bile acids metabonomic profiling in patients during the acute phase of AP (acute, n = 133) and the recovery phase of AP (post, n = 133) with MAP (n = 31), MASP (n = 44), and SAP (n = 58).

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that circulating CDCA and CA levels were significantly decreased in mice with AP, consistent with the trend in the human population (Figure 5A). The classical pathway of bile acids synthesis is mediated by the CYP7A1 enzyme, while the alternative pathway is mediated by the CYP27A1 enzyme. These are the two most abundant bile acid synthetic enzymes in humans, and the ratio between them is determined by sterol Cyp8B1.[7] We measured the mRNA levels of four key enzymes of hepatic bile acid synthesis and found that the mRNA levels of CYP7A1 and CYP27A1 were significantly decreased in AP mice compared with normal control mice. This indicates that the decreased level of CDCA and CA during AP may be due to decreased synthesis. No significant differences were observed between the mRNA levels of Cyp7B1 and Cyp8B1, indicating that the metabolic pathway for bile acid synthesis was not significantly altered (Figure 5B).

Subsequently, we evaluated the pharmacological effects of CDCA and CA on acinar cell necrosis *in vitro*. Using two primary acinar cell damage models induced by cholecystokinin octapeptide (CCK) and taurolithocholic acid 3-sulfate disodium salt (TLCS), we found that CDCA significantly reduced lactate dehydrogenase (LDH) release and decreased the number of necrotic acinar cells marked by phosphatidylinositol staining (Figures 5C–5E and S8A), whereas CA did not show similar effects (Figure S5).

OCA is a derivative of CDCA with a favorable biosafety profile, which has a significantly higher binding capacity to downstream receptors than CDCA, and it is already an FDA-approved marketed drug for the treatment of primary biliary cirrhosis.[23–25] Considering the potential for future clinical translation of this drug, we similarly performed *in vitro* cell experiments with OCA. As expected, OCA significantly reduced cell necrosis in two primary acinar cell injury models (Figures 5C–5E and S8A).

Based on the above cellular experimental results, we only conducted subsequent animal experiments using CDCA and OCA. After administering different concentration gradients of OCA or CDCA intraperitoneally to prevent caerulein-induced AP in mice, we identified 20 mg/kg of OCA and 40 mg/kg of CDCA as the optimal protective doses that can exert a pharmacologically meaningful effect *in vivo* (Figures S6 and S7). Furthermore, we observed in another TLCS-induced experimental AP model that therapeutic administration of CDCA or OCA significantly ameliorated pancreatic histopathological damage in mice with AP (Figure S8). To emulate the actual clinical drug administration route, we employed intragastric administration of CDCA or OCA and obtained similar therapeutic effects (Figures S10). Finally, it was noted that the pharmacological protective activity of CDCA and OCA was independent of mouse gender (Figures S8 and S9).

OCA regulates the mitochondrial oxidative phosphorylation pathway to promote cellular ATP production

Obviously, the pharmacological protective activity of OCA is better than that of CDCA in AP mouse model. Hence, we employed OCA for subsequent mechanistic studies, and RNA sequencing was performed by extracting mRNA from CCK-induced damaged pancreatic acinar cells with or without the addition of OCA. Gene enrichment analysis by Kyoto Encyclopedia of Genes and Genomes (KEGG) identified oxidative phosphorylation in the top ten significantly changed pathways (Figure 5I). Similarly, gene set enrichment analysis (GSEA) revealed clear alterations in "mitochondrial ATP synthesis coupled electron transport," "mitochondrial respiratory chain complex assembly," "NADH dehydrogenase complex assembly," and "oxidative phosphorylation" (Figure 5J). Based on these results, we examined ATP production, mitochondrial respiratory chain complex I and V activities and NADP-malate dehydrogenase activity in injured acinar cells before and after OCA intervention. The results validated the RNA sequencing findings that OCA significantly increased ATP production in acinar cells, enhanced mitochondrial function, and improved mitochondrial oxidative phosphorylation (Figure 5K). Mitochondrial ROS (MitROS) and mitochondrial membrane potential JC-1 staining similarly confirmed that CDCA and its derivative OCA improved primary acinar cell mitochondrial function *in vitro* (Figure S11). Taken together, the above results confirm that OCA supplementation *in vitro* and *in vivo* regulates the metabolic pathway of mitochondrial oxidative phosphorylation, reduces the inflammatory response, alleviates acinar cell necrosis, and protects against AP.

Bile acids receptor FXR regulates pancreatic necrosis in AP mice

CDCA and OCA can regulate signaling molecules through their dependent receptors, with FXR being the receptor with the highest affinity. Previous studies have confirmed that FXR is mainly expressed in the liver and gastrointestinal tract and that both CDCA and FXR have inflammatory regulatory effects. However, the role of FXR in AP and acinar cell injury has not been reported. To identify whether FXR changes significantly in pancreatic tissue during AP, we first examined the expression level of FXR in AP acinar cells. Immunohistochemistry and western blot (WB) results showed elevated expression of FXR in pancreatic acinar cells 6 h after caerulein treatment, which was correlated with the expression of its nuclear receptors (Figures 6A and 6B). Moreover, pancreatic-specific FXR overexpression mice were generated by orthotopic injection of AAV-FXR overexpression vector into the pancreas. The protein expression level of FXR was significantly increased by WB of pancreatic tissue indicating successful AAV transfection. In the caerulein-induced murine AP model, we found that pancreas-specific overexpression of FXR significantly ameliorated pancreatic histopathological injury and reduced the extent of pancreatic acinar cell death and serum levels of amylase and lipase in mice with AP (Figures 6C–6E). Similarly, using pancreatic orthotopic injections of AAV-FXR interference vectors to construct pancreas-specific FXR knockdown mice, we found that inhibiting FXR

(D) Cleveland dot plot showing a ranked log2 transformation of fold changes of serum bile acids. Green, blue, and red dots represent the fold changes between the recovery phase of AP with respect to the acute phase of AP with MAP (n = 31), MASP (n = 44), and SAP (n = 58), respectively.
(E) Scatterplots displaying the changes of the normalized concentrations of serum CDCA and CA in patients during the acute phase of AP (acute, n = 133) and the recovery phase of AP (post, n = 133) with MAP (n = 31), MASP (n = 44), and SAP (n = 58). Numbers indicate the mean and standard error of the mean (SEM). ***p < 0.001, **p < 0.01, and *p < 0.05 vs. acute group.

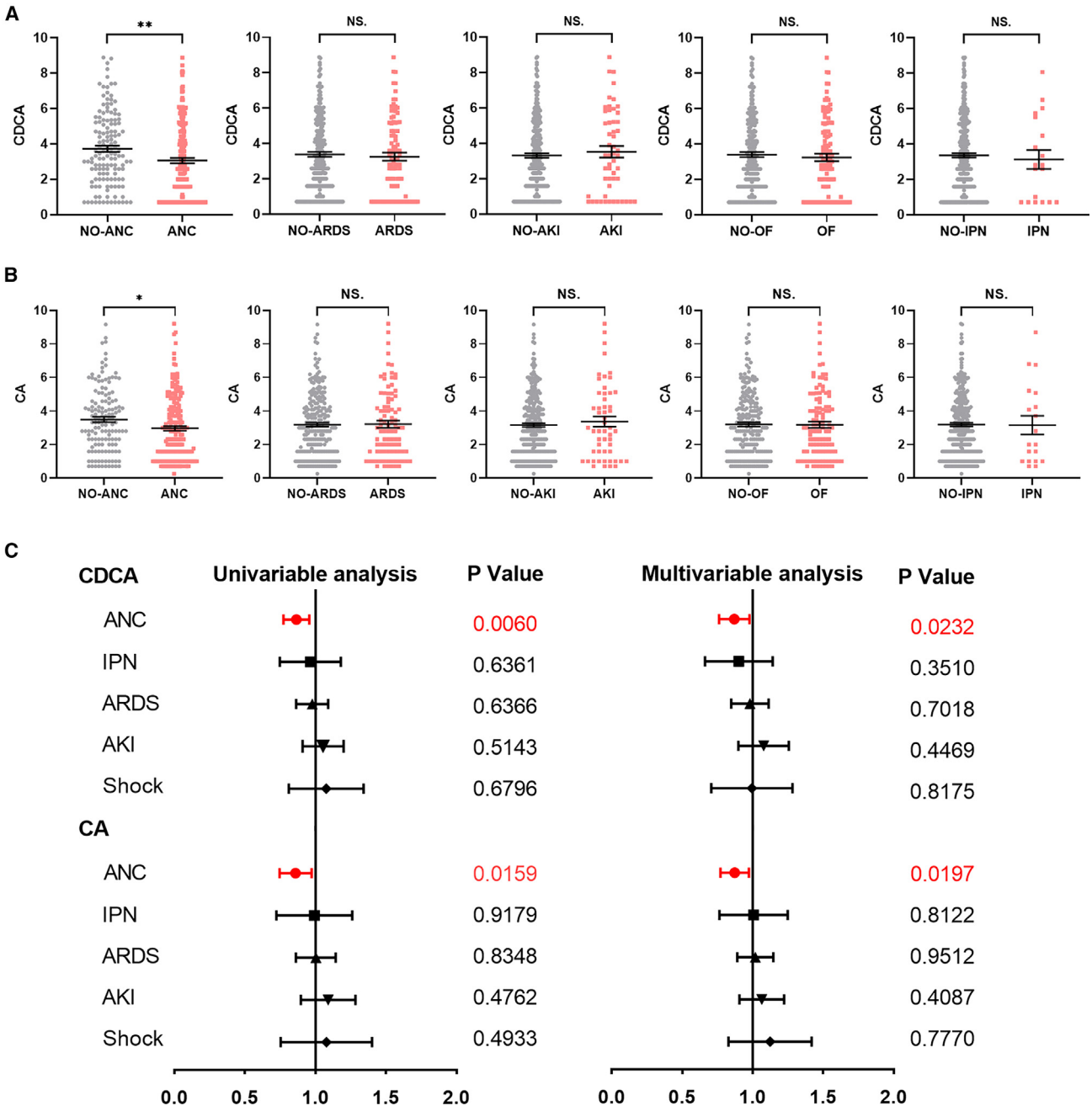


Figure 4. Serum CDCA and CA were significantly associated with pancreatic necrosis

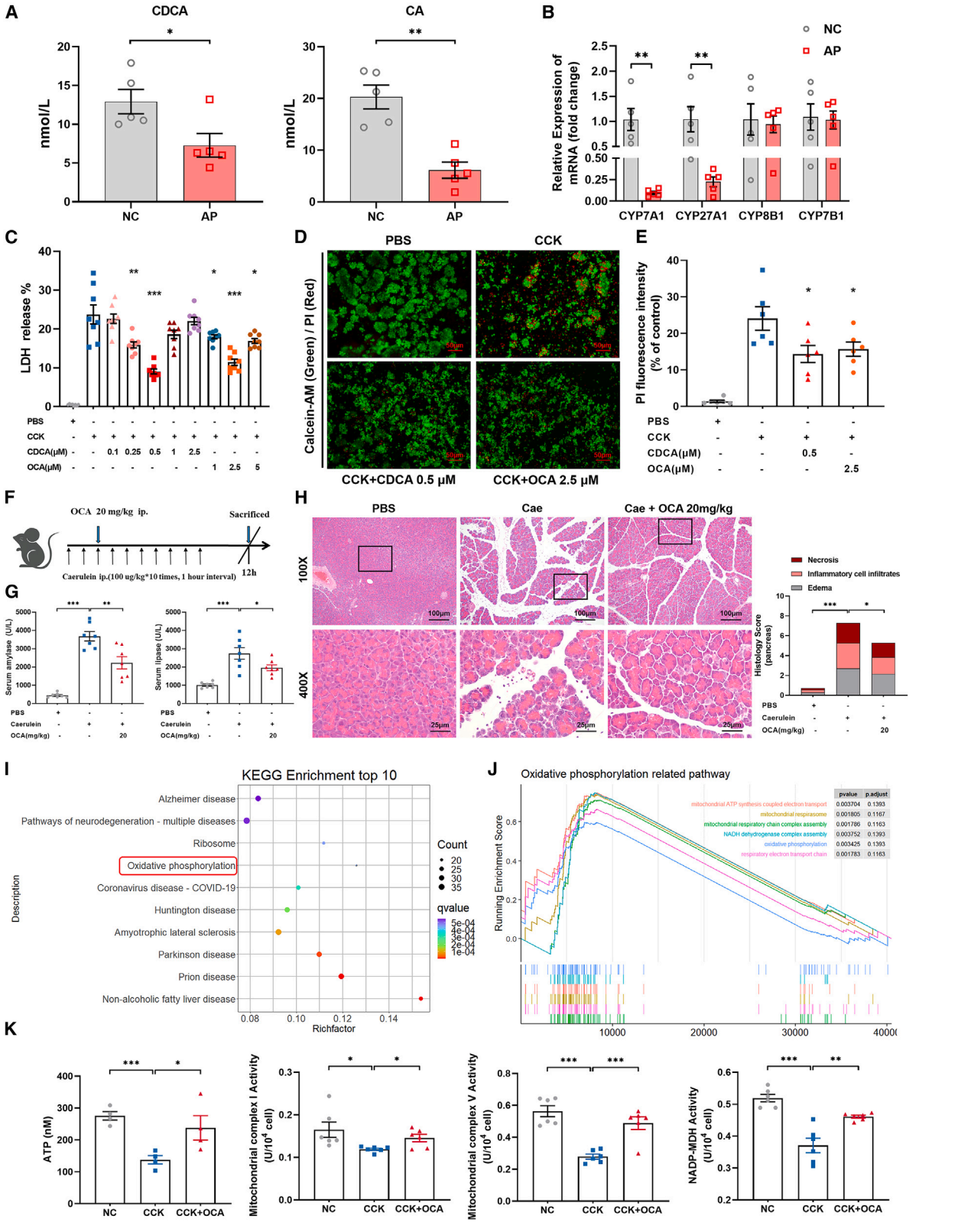
(A and B) Association analysis between the normalized concentrations of serum CDCA and CA and acute necrotic collection (ANC [n = 186] < NO-ANC [n = 140]), organ failure (OF [n = 108], NO-OF [n = 218]), acute respiratory distress syndrome (ARDS [n = 92], NO-ARDS [n = 234]), acute kidney injury (AKI [n = 53], NO-AKI [n = 273]), and pancreatic necrosis infection (IPN [n = 19], NO-IPN [n = 307]). Numbers indicate the mean and standard error of the mean (SEM). **p < 0.01 and *p < 0.05. NS, no significance.

(C) Forest plot of risk factors for pancreatic necrosis, IPN, ARDS, AKI, and shock in AP patients that were analyzed using univariate logistic regression and multivariate logistic regression of corrected age, gender, body mass index, etiology, and disease parameter.

significantly exacerbated pancreatic injury in caerulein-induced AP in mice (Figures 6F–6H). The above results indicate that regulating the CDCA receptor FXR can alleviate acinar cell necrosis and protect against AP in a mouse AP model.

DISCUSSION

To the best of our knowledge, this is the most extensive and detailed bile acid metabolomics study on AP. Our study provides



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a novel view that bile acid metabolism is significantly altered in AP and that CDCA and its receptor, FXR, may be potential therapeutic targets in AP. Our study presents several significant findings. First, significant changes in the metabolism of multiple bile acids have been reported in patients with AP compared with healthy controls. Second, CDCA decreased significantly during the acute phase and increased during the recovery phase of AP, and it was strongly associated with pancreatic necrosis in patients with AP. Third, supplementation with CDCA and its derivative OCA and modulation of its receptor FXR effectively induced acinar cell necrosis in mice with AP. Collectively, the targeted regulation of CDCA could be a novel strategy for the treatment of acinar cell necrosis in AP.

Recent studies in patients with Alzheimer's disease,[11,12] a neurodegenerative disease, and multiple sclerosis,[14] an inflammatory demyelinating disease of the CNS, found that lower levels of circulating bile acid metabolites are strongly associated with the development of cognitive impairment and can affect astrocyte neurotoxicity and pro-inflammatory polarization of microglia. Furthermore, both obesity and diabetes were associated with lower serum concentrations of hyocholic acid (HCA) in a cohort of people with metabolic disorders, and serum HCA levels were strong predictors of the occurrence of metabolic disorders within 5 and 10 years.[26] In this study, 39 bile acid metabolism-related metabolites in humans were quantified by tandem mass spectrometry in the serum of patients with AP and healthy controls, and a total of 17 bile acids, which mainly two primary bile acids (CDCA and CA) and their downstream metabolites or binding forms, met the quality control criteria and were included in the quantification analysis, a finding consistent with previous prospective studies of bile acid levels in colon cancer patient cohorts.[27] Combined with the changes in mRNA levels of key enzymes in liver bile acid synthesis in AP mouse models, CYP7A1 and CYP27A1 mRNA showed significant changes, while CYP8B1 and CYP7B1 showed no significant changes. We suggest that the decrease of CA and CDCA is mainly due to the decrease of hepatic synthesis, which may be associated with liver injury mediated by systemic inflammatory response associated with AP. In addition, further analysis of changes in bile acid levels of different types indicates that CDCA and CA significantly increase the conversion or binding forms of downstream secondary bile acids. In summary, the decrease in bile acid metabolism synthesis and the increase in CDCA metabolism conversion jointly led to a decrease in circulating primary bile acid CDCA and CA in AP patients.

There are differences in the effects bile acids exert on pancreatic cells that may be attributed to the polarity of different bile acids. Quang et al.[19] showed that different types of bile acids exhibited different effects in different animal models of AP. For example, hydrophilic bile acids, represented by TLCS, significantly enhanced pancreatic acinar cell trypsin activity and aggravated AP severity in caerulein-induced, L-arginine-induced, pancreatic duct ligation, or combined bile and pancreatic duct ligation-induced murine AP models. On the other hand, hydrophobic bile acids, represented by TUDCA and LCA, showed significant protective effects in murine AP models induced by caerulein and L-arginine but not in models induced by pancreatic duct ligation or combined bile and pancreatic duct ligation.[28] In the present study, we observed that CDCA had a definite effect on inhibiting acinar cell necrosis, whereas CA had no effect. This could be due to the hydrophilic and hydrophobic properties of the two bile acids. CA is a hydrophilic bile acid with a hydrophobic parameter of 3.6, while CDCA has hydrophobic properties.[29]

Mitochondria produce ATP through oxidative phosphorylation to provide energy for normal physiological activities of cells. Loss of mitochondrial membrane potential due to the opening of the mitochondrial permeability transition pore and the disruption of mitochondrial membrane function results in the release of mitochondrial content, mitochondrial dysfunction, and ATP depletion, all of which are key events in early acinar cell death.[30] Chen et al. found that a pathological increase in deoxysphingolipids in diabetic mice inhibited acinar cell oxidative phosphorylation pathways, reduced ATP production, and exacerbated acinar cell necrosis.[31] Pancreatic necrosis resulting from early acinar cell death is a major contributor to severe illness and poor prognosis in patients with AP. Acinar cell necrosis causes irreversible damage to the pancreas and a series of inflammatory reactions that cause concurrent organ failure, systemic inflammatory response syndrome, and significantly higher mortality in patients with AP.[2,3] Studies have reported that treatment with CDCA increases intracellular ATP levels, which promotes intestinal epithelial cell proliferation by regulating the cell cycle and mitochondrial function.[32] In this

Figure 5. CDCA and its derivative OCA regulate the mitochondrial oxidative phosphorylation pathway to promote cellular ATP production attenuated acinar cell necrosis *in vitro* and *in vivo*

(A) Scatterplots showing changes in serum CDCA and CA levels in mice with AP (n = 5).

(B) qPCR detected the mRNA levels of key enzymes of hepatic bile acids synthesis (CYP7A1, CYP27A1,CYP8B1,CYP7B1) (n = 5).

(C) CCK-induced primary acinar cell damage was detected by LDH release after concentration gradients CDCA (0.1, 0.25, 0.5, 1, 25 μM) or OCA (1, 2.5, 5 μM) were treated.

(D and E) Immunofluorescent imaging (200x, scale bar represents 50 μM) of calcein-AM/PI stained acinar cells and phosphatidylinositol fluorescence intensity of injured acinar cells quantified (n = 6–8).

(F) Diagram of caerulein-induced experimental AP model and OCA intervention in mice.

(G) Change in serum activity of amylase and lipase.

(H) H & E staining images (100x and 400x, scale bars represent 100 μM and 25 μM) and histological scores (edema, inflammation, necrosis) of pancreatic tissues in AP mice (n = 7).

(I) Differential genes enrichment analysis by KEGG in RNA-seq of pancreatic acinar cells with or without the addition of OCA.

(J) GSEA enrichment analysis of differential genes.

(K) The levels of ATP production, mitochondrial respiratory chain complex I and V activities, and NADP enzyme activity in the pancreatic acinar cells (n = 4–6). Numbers indicate the mean and standard error of the mean (SEM). ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$. NS, no significance.

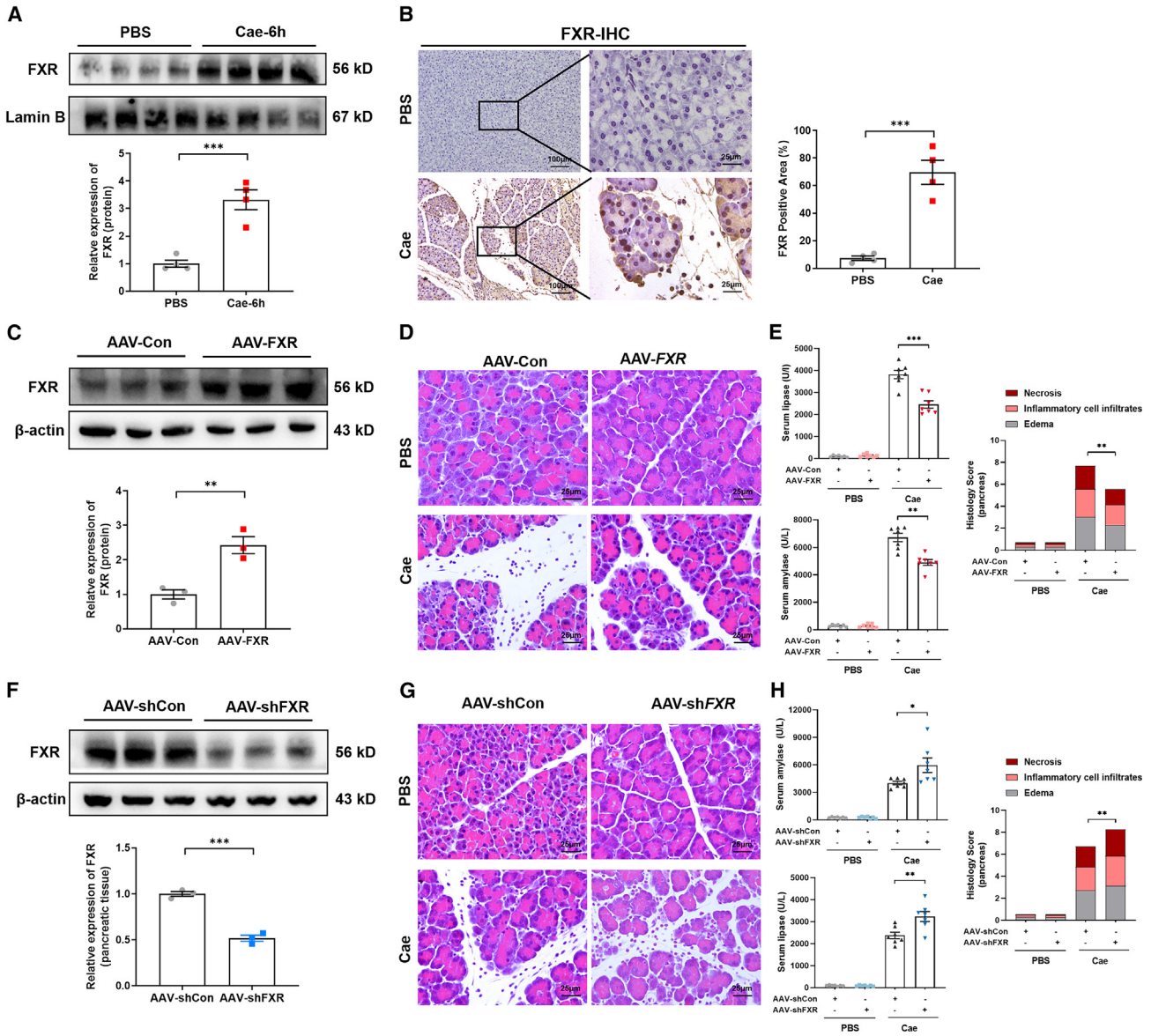

Figure 6. Expression of FXR by acinar cells regulates pancreatic necrosis in mice with AP

(A) Expression and relative quantification of FXR in pancreatic tissue of caerulein-induced AP mice by western blotting.

(B) IHC staining of FXR in tissue homogenates obtained from caerulein-induced AP mice confirmed that FXR expression was significantly elevated (n = 4).

(C) Western blots showing FXR expression in pancreatic tissue of mice was significantly increased after 3 weeks injection of AAV-FXR in mouse pancreatic tissue (n = 3).

(D and E) H&E staining images (400x, scale bar represents 25 μM), histological scores (edema, inflammation, and necrosis) of pancreatic tissues, and changes in serum activity of amylase and lipase after AP induced by caerulein in AAV-FXR overexpressing mice (n = 7).

(F) Western blots showing FXR expression in pancreatic tissue of mice was significantly decreased after 3 weeks injection of AAV-shFXR in mouse pancreatic tissue (n = 3).

(G and H) H&E staining images (400x, scale bar represents 25 μM), histological scores (edema, inflammation, and necrosis) of pancreatic tissues and changes in serum activity of amylase and lipase after AP induced by caerulein in AAV-shFXR interfering mice (n = 7). Numbers indicate the mean and standard error of the mean (SEM). ***p < 0.001, **p < 0.01, and *p < 0.05.

study, we confirmed the significant upregulation of genes involved in mitochondrial respiratory chain complexes I and V associated with oxidative phosphorylation, ATP production, and mitochondrial function in acinar cells after OCA administration, resulting in a significant improvement of acinar cell necrosis, as confirmed by transcriptomic, enzyme activity analysis, mitochondrial ROS, and mitochondrial membrane potential JC-1 staining.

Studies have shown that mutations at the FXR site in humans do not appear to affect pancreatitis susceptibility and

that loss of FXR function does not affect AP severity in a caerulein-induced map model in FXR-KO mice.[33] In this study, we performed exogenous AAV pancreatic orthotopic injection to specifically regulate FXR expression in acinar cells and found that regulating the CDCA receptor FXR alleviated acinar cell necrosis and protected against AP in a murine AP model. The variable conclusions of our study suggest that FXR may play a regulatory role in other cells and organs in a mouse AP model with global endogenous FXR deletion, a phenomenon that needs further study.

In conclusion, this study is the first to apply targeted metabolomic mass spectrometry to analyze the dynamics of serum bile acid metabolism in 326 patients diagnosed with AP. Both *in vivo* and *in vitro*, supplementation with CDCA or its derivative OCA and modulation of its receptor, FXR, attenuate acinar cell necrosis and protect against AP. Targeted regulation of CDCA may be a novel strategy for the treatment of acinar cell necrosis in AP, but further verification is required.

Limitations of the study

Nevertheless, this study has some limitations. First, although CDCA is one of the most significantly changed bile acids, other bile acid species still need to be considered, such as NorCA, whose effects on acinar cells need to be further explored in other studies. Second, although the present study is the first international report of serum bile acid-targeted metabolomic mass spectrometry in patients with clinical AP, the sample size is small, and only single-center patients with AP were included in this study. The source of patients in this cohort was a tertiary referral center of AP, resulting in a relatively high MSAP and SAP ratio. To address this, we collected additional serum samples from AP patients at another hospital to validate the consistency of CDCA and CA changes and to minimize the potential impact of severity distribution bias. However, this still requires validation from clinical cohorts in different countries and regions. The distribution of AP etiologies presents different characteristics due to the difference between East and West ethnicities. There may be some study bias in this study, and the clinical cohort is not representative of the general population with AP. In addition, we did not collect continuous blood samples from the same patient during the early days of onset, resulting in a lack of dynamic presentation of bile acid metabolism changes. Therefore, the findings of this study need to be replicated in large-sample multicenter cohort studies.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xcrm.2023.101304.

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AUTHOR CONTRIBUTIONS

L.H., W.L., and G.L. formulated the paper's idea; Q.Z., C.Y., X.D., and Y.W. performed the research and wrote the manuscript; W.G., Y.D., W.X., and B.L. provided comments and technical advice; Q.Z., C.Y., W.C., and X.X. participated in preparing the figures, tables, and data analysis; B.T., W.G., B.L., and W.C. provided comments on the manuscript, as well as supervised the research and revised the manuscript; all authors reviewed the manuscript. Q.Z., C.Y., X.D., and Y.W. contributed equally to this work.

DECLARATION OF INTERESTS

The authors declare no competing interests.

INCLUSION AND DIVERSITY

We support inclusive, diverse, and equitable conduct of research.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-FXR	Santa Cruz Biotechnology	Cat# sc-25309
Anti-β-actin	Santa Cruz Biotechnology	Cat# sc-8432
Anti-Lamin B	Santa Cruz Biotechnology	Cat# sc-56144
Chemicals, peptides, and recombinant proteins		
Cholecystokinin Octapeptide (CCK)	MedChemExpress	Cat# HY-P0093
Chenodeoxycholic Acid (CDCA)	MedChemExpress	Cat# HY-76847
Obeticholic acid (OCA)	MedChemExpress	Cat# HY-12222
Cholic acid (CA)	MedChemExpress	Cat# HY-N0324
Caerulein	Nanjing Peptide	Cat# Pep03263
Taurolithocholic acid 3-sulfate disodium salt (TLCS)	MedChemExpress	Cat# HY- 137255
MitoSOX Red superoxide indicators	Thermo Fisher Technology Co., LTD	Cat# M36009
JC-1	MedChemExpress	Cat# HY-15534
Amylase assays kit	BioSino Biotechnology & Science Inc.	N/A
Lipase assays kit	Nanjing Jiancheng Corp.	Cat# A054
TNFα ELISA kits	Affymetrix eBioscience	Cat# 88-7013-22
IL6 ELISA kits	Affymetrix eBioscience	Cat# 88-7064-88
MCP-1 ELISA kits	Affymetrix eBioscience	Cat# 88-7503-22
Cytotoxicity LDH test kit	Dojindo Molecular Technologies, Inc.	Cat# C0017
Calcein-AM/PI Double Stain Kit	Yeasen Biotechnology	Cat# 40747ES80
RevertAid First Strand cDNA Synthesis Kit	Thermo Fisher Technology Co., LTD	Cat# K1622
Hieff UNICON® qPCR SYBR Green Master Mix	Yeasen Biotechnology	Cat# 11198ES08
Enhanced ATP Assay Kit	Beyotime Biotechnology	Cat# S0027
Mitochondrial complex I Activity Assay Kit	Abbkine Scientific Co., Ltd	Cat# KTB1850
Mitochondrial complex V Activity Assay Kit	Abbkine Scientific Co., Ltd	Cat# KTB1890
NADP-Malate Dehydrogenase (NADP-MDH) Activity Assay Kit	Abbkine Scientific Co., Ltd	Cat# KTB3020
Critical commercial assays		
LC-MS analysis	Suzhou PANOMIX Biomedical Tech Co. Ltd	N/A
RNA-sequencing	Novogene Co, Ltd	N/A
Deposited data		
Raw and analyzed data	This paper	Science DataBank repository https://doi.org/10.57760/sciencedb.12335
RNA sequencing datasets	This paper	GEO accession GEO: GSE245795
Targeted bile acid metabolomics datasets	This paper	MetaboLights: https://www.ebi.ac.uk/metabolights/MTBLS8786
Software and algorithms		
R software	R-project	N/A
GraphPad Prism v.8	Graphpad	N/A
Other		
panAAV-CMV-EGFP-P2A-FXR-3xFLAG-WPRE	OBiO Technology Corp., Ltd	N/A
panAAV-U6-shRNA (FXR)-CMV-EGFP-WPRE	OBiO Technology Corp., Ltd	N/A
panAAV-CMV-EGFP-P2A-3xFLAG-WPRE	OBiO Technology Corp., Ltd	N/A

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be direct to and will be fulfilled by the lead contact, Guotao Lu, Ph.D. (gtlu@yzu.edu.cn).

Materials availability

This study did not generate new reagents.

Data and code availability

The RNA sequencing data can be downloaded from the NCBI GEO accession code GEO: GSE245795. The targeted bile acid metabolomics datasets generated in this study is available at the MetaboLights website. The data can be accessed directly at MetaboLights: https://www.ebi.ac.uk/metabolights/MTBLS8786. All original data has been deposited at Science DataBank and is publicly available as of the date of publication. DOIs are listed in the key resources table. The code used in this study are all open access R packages in R language software, which have been described in the statistical analysis section, and there are no restrictions to availability. Any additional information required to reanalyze the data reported in this work paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Human subjects

Patients with AP admitted to the Critical Medicine Department of Jinling Hospital, School of Medicine, Nanjing University within 7 days of onset were recruited for this study. The exclusion criteria were as follows: age <18 or >75 years, pregnancy, cancer, cirrhosis, primary sclerosing cholangitis, active viral hepatitis, and renal transplantation. The patients were administered bile acid preparations and treated with bile acid sequestrants before the onset of AP. Patients who underwent cholecystectomy, gallbladder puncture and drainage, endoscopic retrograde cholangiopancreatography, nasobiliary drainage, plasma exchange, blood perfusion, or CRRT before admission were also excluded. Based on these criteria, 326 patients with AP were included in this study. A healthy control cohort of 60 subjects matched for age, gender, and BMI were recruited from the physical examination center excluding prior history of AP, chronic pancreatitis, and other chronic diseases. We collected clinical and demographic data on the relevant subjects from participants as well as fasting intravenous blood samples. We also collected fasting serum samples before discharge from 133 patients during the recovery phase of AP with different severity (31 MAP, 44 MSAP, and 58 SAP). All human samples were collected, stored, and measured in accordance with the standard operating protocols of the Jinling Hospital, School of Medicine, Nanjing University. Blood serum samples were centrifugated and stored at -80°C until analysis. Written informed consent was obtained from all the participants prior to recruitment. The study protocols involved in this study were reviewed and approved by the Ethics Committee of Jinling Hospital, School of Medicine, Nanjing University, and were in accordance with the World Medical Association Declaration of Helsinki. The external validation serum samples were obtained from the clinical biobank of acute pancreatitis of the Affiliated Hospital of Yangzhou University, using the same inclusion and exclusion criteria as mentioned above, including 38 healthy controls, 125 MAP, 35 MSAP, and 18 SAP. The clinical biospecimen repository has received approval from the Clinical Ethics Committee of the Affiliated Hospital of Yangzhou University. Informed consent has been obtained for collecting all the samples.

We used the revised Atlanta classification (RAC) to determine the AP severity classification as follows: MAP was defined as clinical manifestations and biochemical changes of AP without organ dysfunction and local or systemic complications. MSAP was defined as the clinical manifestations and biochemical changes of AP accompanied by transient ($\leq$48 h) organ dysfunction and/or local complications. SAP was defined by clinical manifestations and biochemical changes in AP that are with persistent (>48 h) organ dysfunction. The diagnostic criteria for organ dysfunction were based on the improved Marshall score system, and the presence of organ dysfunction was defined as an organ score $\geq$2 points.

Animals

GemPharmatech supplied C57BL/6J wild-type mice (6–8 weeks, 20–22 g, male and female). We maintained all mice in a pathogen-free environment, with a 12 h light-dark cycle, a temperature of $23 \pm 2^{\circ}$C, a humidity of 45.5%, and normal rodent food and water. This study was based on the Principles of Laboratory Animal Care (NIH Publication No. 85Y23, revised 1996) and approved by the Laboratory Animal Ethics Committee of Yangzhou University.

Experimental AP models in mice and drugs intervention

Caerulein-induced AP: C57BL/6J wild-type mice received continuous intraperitoneal injections of caerulein (100 μg/kg × 7 times, 1 h apart).

TLCS-induced SAP: C57BL/6J wild-type mice were anesthetized and then a 1–2 cm longitudinal incision was made in the mouse abdomen to expose the abdominal cavity, followed by retrograde injection of 2.5% TLCS to the pancreatic bile duct. The sham group that did not receive a retrograde injection was set as a control group.

Drugs intervention: OCA (10, 20, and 40 mg/kg) or CDCA (2.5, 5, 10, 20, and 40 mg/kg) was injected intraperitoneally at 3 h after administration. OCA or CDCA was dissolved in 10% DMSO. The same volume of DMSO was used for the control and AP model groups. Mouse pancreatic-specific panAAV-CBV-mCherry-3xFLAG-WPRE-H1-FXR, panAAV-CBV-mCherry-3xFLAG-WPRE-H1-ShFXR and AAV-PAN-DIO-mCherry were purchased from OBiO Biotechnology (Shanghai) Crop to be used as a control group for AP experiments. Mice were intraperitoneally injected from a right abdominal approach near the pancreas at a dose of 3×10^{10} vg/animal and sacrificed 3 weeks later for either microscopic examination or to confirm the expression and function of FXR overexpression or interference. Mice were sacrificed at 12 h, pancreatic tissue was preserved for histological examination, and the residual tissues were quickly frozen in liquid nitrogen at −80°C for subsequent testing.

Cultured primary pancreatic acinar cells (PACs)

Fresh PACs were quickly isolated from mice using IV collagenase as previously described.[34] PACs injury was induced by adding 1 μM CCK or 1 mM TLCS for 6 h. Cells were incubated with increasing concentrations of CDCA, OCA or CA.

METHOD DETAILS

Bile acids metabolomics

Targeted bile acid metabolomics measured 38 major human primary and secondary bile acids and their respective glycine and taurine conjugates (Table S2). All bile acid standards were weighed, and standards at different concentrations were prepared using the corresponding solvents. The quantitative ranges and standard curves for the various bile acid analytes (based on the quantification of 100 μL analysis of serum) are listed in Table S2. The proteins to be tested were precipitated from serum samples, evaporated to dryness in a centrifugal vacuum concentrator, and analyzed by mass spectrometry using an AB 4000 triple quadrupole mass spectrometer (waters acquity UPLC) from Suzhou PANOMIX Biomedical Tech Co., LTD and the 6460 triple quadrupole liquid chromatography-mass spectrometry system from the Analysis and Testing Center of Yangzhou University. Multiple reaction monitoring (MRM) was used for scanning. Data were collected and processed using ab SCIEX Analyst software version 1.6.3.

RNA-sequencing and quantitative PCR (q-PCR) analysis

Total RNA was extracted from the PACs and a 2 μg sample was reverse-transcribed using the SuperScript VILO cDNA Synthesis kit (Thermo Fisher Scientific, #11754-050). Sequencing Libraries were prepared using the Agilent Bioanalyzer 2100 system. The mRNA levels of four key enzymes involved in hepatic bile acid synthesis were analyzed using q-PCR. Primers used are listed in Table S3. The comparative CT ($2^{-\Delta\Delta CT}$) approach was used to measure the fold change for each mRNA.

Histopathology and immunohistochemistry analysis

Paraffin-embedded sections or frozen tissue sections were used for staining as described in our previous study.[35]

Evaluation of pancreatic acinar cell death

Necrotic cell death was determined using PI (4 μM for dying/dead cells) and calcein-AM (2 μM; for living cells). Cell death was also assessed by measuring the release of LDH in the cell culture supernatant using LDH Cytotoxicity Assay Kit.

MitROS, JC-1 staining and biochemical assays

Serum amylase, inflammatory factors, ATP, mitochondrial complex I Activity, mitochondrial complex V Activity, NADP-Malate Dehydrogenase (NADP-MDH) activity, Mitochondrial ROS (mitROS) and mitochondrial membrane potential JC-1 staining were detected according to the manufacturer's instructions.

Statistical analysis

For human and mouse bile acid-targeted metabolomics data, we excluded metabolites with more than 50% missing values, and finally included 17 bile acid metabolites in humans and 38 bile acid metabolites in mice. The remaining missing data were filled using the lower limit of the corresponding detection range of 0.5*. The concentration of metabolites was then normalized using the logarithm (log2). The "factoextra", "ggplot2", and "pheatmap" packages in R language were used to conduct multivariate statistical analyses on the data after pre-processing and to draw the principal component analysis diagram, volcano diagram, and heatmap. Statistical analyses of RNA-sequencing data were also performed using R software. The R statistical package software DESeq2 was used for differentially expressed genes (DEGs) analysis. Gene Set Enrichment Analysis (GSEA) was used to assess enrichment of the Kyoto Encyclopedia of Genes and Genomes (KEGG) gene sets using the R package software clusterProfiler. Categorical variables are described using frequencies and percentages. Mean ± standard deviation (SD) or median ± quartile distance (IQR) was used to describe continuous variables based on their distribution. The t-test was used to analyze the differences between two groups, and one-way analysis of variance (ANOVA) was used to evaluate the statistical significance among three or more groups. The results are expressed as mean ± standard deviation (SEM) in a scatterplot. When the t-test was used, the data points were displayed in a chart assuming the data distribution was normal, but no formal test was performed. Risk factors for pancreatic necrosis, ARDS, AKI, and shock in AP patients were analyzed using univariate logistic regression and multivariate logistic regression of corrected age, gender, body mass index, etiology, and disease parameter.