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Non-invasive liquid biopsy based on metabolomic profiling improves diagnosis and early warning of severe acute pancreatitis

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To identify novel diagnostic biomarkers for acute pancreatitis (AP) and facilitate the early prediction of severe AP (SAP), this investigation characterized the serum metabolomic profiles of patients across distinct disease phases and integrated metabolomics with artificial intelligence to construct bile acid-based predictive models. The observational protocol was registered with the Chinese Clinical Trial Registry (ChiCTR2000034117) on June 24, 2020. Comparative metabolomic analysis revealed significant alterations in 303 metabolites and 461 lipid species in AP. Subsequent weighted gene coexpression network analysis demonstrated robust correlations between clinical parameters and specific metabolic clusters, particularly bile acids (BAs) and lipid species. Targeted quantification of 63 BAs was subsequently performed within a multicentre validation cohort ($n = 948$). Machine learning algorithms applied to these data facilitated the derivation of two distinct BA panels. The first panel, comprising nine BAs, demonstrated high diagnostic accuracy for AP, including among individuals with negative conventional enzymatic biomarkers, and effectively discriminated AP from acute cholangitis, as reflected by elevated area under the curve (AUC) values. A second panel, consisting of 13 BAs, reliably identified patients at elevated risk for SAP progression. Collectively, these results validate the translational potential of machine learning-driven metabolic biomarkers for the precision management of acute abdominal conditions, underscore the clinical utility of BAs as promising diagnostic and prognostic biomarkers in acute pancreatitis, and provide a new paradigm for the development of dynamic risk early-warning systems (Clinical Trial Registration Our study is an observational study registered in ChiCTR (ChiCTR2000034117) on 2020/06/24, not a prospective interventional clinical trial, and therefore does not fall under the ICMJE definition of a clinical trial requiring CONSORT compliance).

Acute pancreatitis (AP) represents a significant and growing gastrointestinal disease burden, characterized by its acute onset and rapid progression^{1,2}. Current guidelines for AP primarily emphasize both early fluid resuscitation and comprehensive supportive care^{3–5}. While AP diagnosis currently relies

on detecting elevated levels of amylase or lipase in bodily fluids, the clinical utility of these enzymes is constrained by suboptimal sensitivity and specificity, often necessitating supplementary diagnostic information^{6–8}. Moreover, these markers do not provide insights into potential molecular

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targets for treatment. Notably, approximately 20% of patients progress to severe acute pancreatitis (SAP), with a mortality rate of up to 30%, and a high burden of immediate and long-term complications among survivors^{9,10}. This may be attributed to some extent to the lack of biomarkers that contribute to the early detection of patients at risk of SAP who require intensive therapy^{11,12}. The early identification of patients at high risk of SAP has long been a major challenge. A potential pathway to overcome this challenge may involve the precise characterization of patient-specific molecular phenotypes and the integration of data from multiple molecular species to construct a more robust predictive model for SAP risk assessment.

The metabolome serves as an integrated representation of systemic physiological status and a functional indicator of gut microbiota activity¹³. Metabolomic profiling offers a novel framework for elucidating pathological processes by characterizing global alterations in the complement of metabolites associated with disease states^{14–16}. The pancreas is an essential metabolic organ with endocrine and exocrine functions, regulating the intake of exogenous nutrients and glucose and lipid metabolism homeostasis^{17,18}. AP is characterized by local pancreatic inflammation accompanied by a systemic inflammatory response caused by various etiologies^{19,20}. Studies have shown that an abnormal metabolic network increases the pancreas's susceptibility to risk factors and ultimately leads to the initiation and progression of AP²¹. Global metabolic alterations indicate the potential translational value of AP-associated metabolites as tools for diagnostic and predictive applications²².

Circulating biomarkers can facilitate the screening of various diseases and the prediction of outcomes^{23–25}. Previous studies have shown that serum metabolites in AP reflect pathophysiological alterations, and serum metabolites can differentiate etiologies²⁶. These studies revealed the specific metabolic characteristics of AP. However, due to the small sample sizes in these studies and the lack of validation, these candidate applications are limited. Consequently, large-scale, multicentre studies are required to confirm the reproducibility of these candidate biomarkers.

This study sought to address this gap by analyzing a large clinical cohort of AP patients and integrating multiple bile acid (BA) biomarkers to improve AP diagnosis and enable the early prediction of SAP. A total of 948 subjects were enrolled from five medical centers to explore the abnormal metabolic characteristics and pathways of AP. A three-step analysis strategy, including discovery, internal validation, and external validation, was used to identify and verify a new panel. A cohort of patients with acute cholangitis (AC) was utilized to evaluate the differential value of the diagnostic panel. In addition, we aimed to identify a panel of biomarkers for the accurate early risk stratification of SAP upon patient admission.

Results

Study design and workflow

For the discovery phase, untargeted metabolomic analysis was performed on serum samples from 126 participants (MAP, $n = 32$; MSAP + SAP, $n = 30$; controls, $n = 64$) to characterize the global metabolic profile of AP. Weighted gene co-expression network analysis (WGCNA) was applied to identify BA species associated with disease onset and progression. To validate these findings, a targeted BA metabolomics study was conducted using a multicentre validation cohort (total $n = 948$; MAP, $n = 188$; MSAP, $n = 86$; SAP, $n = 43$; AC, $n = 53$; controls, $n = 578$), enabling the quantification of 50 distinct BAs in human serum. Utilizing this BA profile, a machine learning algorithm was employed to construct diagnostic and predictive biomarker panels. The study workflow is summarized in Fig. 1.

The clinical characteristics of all samples in this study, including age, sex, and white blood cell count, are shown in Table 1. A global representation of the untargeted metabolomic data is provided by the total ion chromatograms (Supplementary Fig. 1a–h). The quality control (QC) samples demonstrated analytical robustness, as evidenced by the distribution of coefficients of variation, as shown in Supplementary Fig. 2a–b. In the principal component analysis (PCA) score plot of polar metabolites and lipids, QC samples were closely clustered together (Supplementary Fig. 2c–d).

Distinct metabolic profiles between AP and con groups

In the discovery study, the orthogonal projections to latent structures discriminant analysis (OPLS-DA) score plot showed a significant global metabolic difference in polar metabolites (Fig. 2a, Supplementary Fig. 3a and Supplementary Data 1) and lipids (Fig. 2b, Supplementary Fig. 3b and Supplementary Data 2) between the AP and Con groups. A distinct pattern was observed in the heatmap, showing differential concentrations of polar metabolites (e.g., amino acids, bile acids (BAs), and dipeptides) between groups. Correspondingly, most lipids were significantly increased in the AP group, while lysophosphocholines (LPCs) were decreased (Fig. 2c).

To explore metabolic molecules associated with AP progression, we performed a stratified analysis based on AP severity. All detected polar metabolites and lipids were compared as follows: AP group vs. Con group, MAP group vs. Con group, and MSAP + SAP group vs. Con group. The Venn diagram analysis demonstrated a high degree of overlap among the significantly altered polar metabolites and lipids identified across all three comparative analyses. A total of 245 polar metabolites (Fig. 2d) and 399 lipids (Fig. 2e) may be associated with the development of AP. The changes in differential metabolites in each group are shown in Fig. 2f–g. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses²⁷ of the differentially expressed polar metabolites and lipids were performed, which revealed that AP-induced metabolic reprogramming was mainly related to the metabolism of amino acids, BAs, sphingolipids, and glycerophospholipids (Supplementary Fig. 3c–d).

To explore the metabolic remodeling closely associated with AP characteristics, WGCNA, an innovative analysis method considering weighted factors, was utilized to identify metabolites that were closely related to AP onset and establish a weighted metabolite interaction network. A total of four modules were screened, which mainly included BAs and lipids and were related to the onset, severity and etiology of AP (Supplementary Fig. 4a–f, Supplementary Data 3). Furthermore, the above differentially expressed metabolites were classified into subgroups. Triacylglycerols (TGs), glycerophosphoethanolamines (PEs), diacylglycerols (DGs), and glycerophosphocholines (PCs) were higher in the AP group than in the Con group, whereas LPCs were higher in the Con group. The concentrations of free fatty acids (FFAs) and BAs varied differently between the two groups (Fig. 2h, Supplementary Fig. 4g–h, Supplementary Data 4). Interestingly, lipids such as TGs, PEs, DGs, and PCs were highly heterogeneous within AP. In contrast, the profiles of BAs, FFAs, and LPCs exhibited greater consistency within the AP cohort. To exclude potential interference from etiology, we compared the relative concentrations of BAs, FFAs, and LPCs in patients with different etiologies. The results showed that BAs, FFAs and LPCs exhibited significant changes in different clinical etiological groups. These three types of molecules, BAs, FFAs, and LPCs, may represent a common metabolic remodeling feature in the occurrence and development of AP (Supplementary Fig. 5a–c).

Further classification of BAs suggested that conjugated BAs and primary BAs were higher in the AP group, while secondary BAs were decreased in the AP group. Moreover, the total BA content remained comparable between the two groups (Supplementary Fig. 6a).

We used BioPAN to explore FFA metabolism and the mutual transformation between subclasses of lipids (Supplementary Data 5). We found that saturated fatty acids (SFAs) and monounsaturated fatty acids (MUFAs) (specifically C16:0, C16:1 n-7 and C18:1 n-9) were elevated in patients with AP. These results revealed that lipolysis of peripheral fat was increased, which elevated blood FFAs and TG levels. Under the action of activated lipase, TGs were hydrolyzed, and the conversion of TGs, DGs, and PEs to PCs was enhanced, resulting in an increase in PCs in the blood. We found an increase in long-chain unsaturated FFAs, especially omega-6 FFAs, in AP patients. Interestingly, as important precursors of prostaglandin, leukotriene and other inflammatory mediators, FFA 20:3 and FFA 20:4 were decreased in AP, while their upstream FFA 20:2 and downstream molecule FFA 20:5 were significantly increased. We speculated that the synthesis pathway of 20-carbon FFAs was increased in AP. FFA 20:4 was consumed more as a substrate for inflammatory molecules, such as leukotrienes,

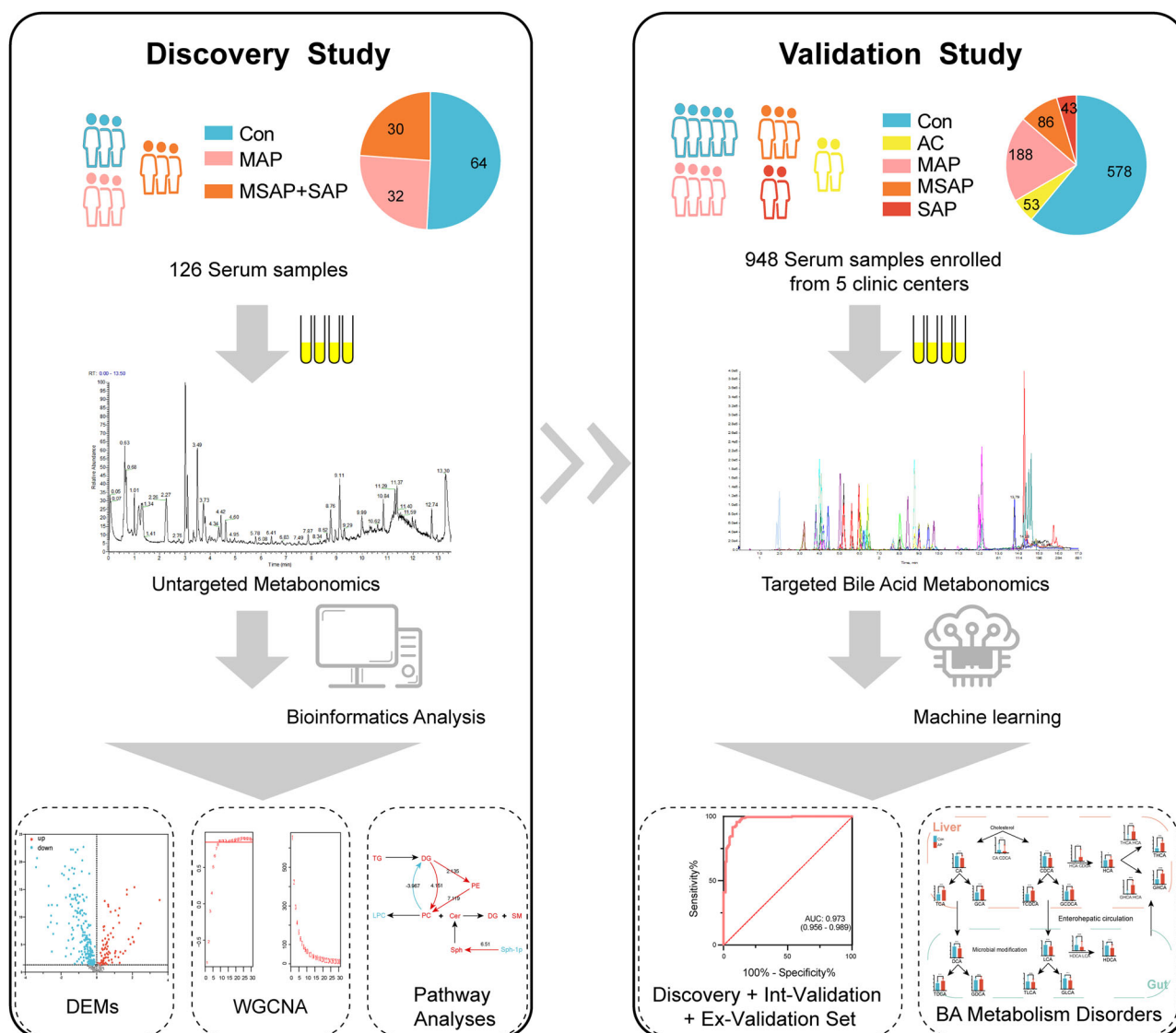


Fig. 1 | Overall study design and workflow. The study consisted of two major stages: a discovery phase (left) and a validation phase (right). In the discovery study, untargeted metabolomic profiling of 126 serum samples (including Con, MAP, and MSAP + SAP) was performed to identify key metabolic alterations. Bioinformatics analyses, including differential expression metabolite screening, weighted gene co-expression network analysis, and pathway enrichment, revealed bile acid metabolism as a major dysregulated pathway in acute pancreatitis. In the

validation study, targeted bile acid metabolomics was conducted in 948 serum samples collected from five clinical centers (Con, AC, MAP, MSAP, and SAP). Machine learning-based modeling was applied to construct and validate a bile acid-derived diagnostic biomarker panel to assist in diagnosing amylase-negative acute pancreatitis and to enable early prediction of SAP. Con: control, MAP mild acute pancreatitis, MSAP moderately severe acute pancreatitis, SAP severe acute pancreatitis, AC acute cholecystitis.

resulting in lower blood levels (Supplementary Fig. 6b, Supplementary Data 5).

BA_s are associated with the clinicopathological features of AP

To further analyze the relationship among BA_s, lipids, and clinical features, DSPC was performed (Fig. 3a and Supplementary Data 6). Specifically, positive correlations were found between D-glucuronic acid and total bilirubin (TBIL), glycochenodeoxycholic acid 3-glucuronide (GCDCA-3G) and alanine aminotransferase (ALT), and LPC (16:1p) and total TG_s. In addition, negative correlations were found between LPC (15:0/0:0) and procalcitonin (PCT) and between lysophosphatidylethanolamine P-18:0/0:0 (LPE P-18:0/0:0) and creatinine (Cr) (Fig. 3b–f). Network analysis revealed that nearly all statistically significant BA_s and a substantial proportion of lipids, predominantly LPC_s, demonstrated strong correlations with key clinical indicators. Since the expression of BA_s increased in AP, while that of LPC_s decreased, BA_s may be key metabolic molecules in the occurrence and development of AP and have the potential to serve as biomarkers.

To identify the role of BA_s in AP, 18 BA_s detected by untargeted metabolomics were used to construct an OPLS-DA classification model. The participants were randomly divided into a discovery set and a validation set in a 7:3 ratio. A total of five models were established: AP vs. Con, AP with negative enzyme vs. Con, and etiology, severity, and recurrence (Supplementary Figs. 7–8). AP and its etiology can always be well distinguished. However, the predictive performance of the models for disease recurrence and severity progression was limited. BA_s represent a large and diverse group of compounds whose analytical quantification can pose significant challenges. Given the relatively few BA_s detected in untargeted metabolomics, it was necessary to perform in-depth targeted quantitative detection of BA_s.

Metabolic characteristics of BA_s in AP

To validate the results of the discovery study, further BA-targeted metabolomics analysis was conducted. Given that we analyzed several dozen structurally specific individual BA_s, 16 BA ratios were added to provide a more comprehensive illustration of the biological significance of BA

Table 1 | Baseline information

Characteristics	Untargeted Metabolomics				Targeted Metabolomics							
	Con	MAP	MSAP	P	Con	MAP	MSAP	SAP	AC	P		P
N	64	32	30	----	578	188	86	43	53	----		----
Age (years)	53.47 ± 18.6	54.81 ± 15.29	53.13 ± 20.43	0.92	55.96 ± 14.89	54.67 ± 16.52	51.78 ± 19.02	50.49 ± 17.25	67.06 ± 15.61	1.57*10 ⁻⁷		
Sex (male/female)	29/35	13/19	16/14	0.60	379/199	119/69	56/30	28/15	33/20	0.97		
White blood cell (*10 ⁹ /L)	5.62 ± 1.24	10.49 ± 3.54	15.49 ± 4.55	7.71*10 ⁻¹⁹	5.98 ± 1.47	10.25 ± 3.72	13.86 ± 4.1	13.1 ± 5.8	9.5 ± 4.39	4.74*10 ⁻¹¹⁷		
Neutrophils (%)	57.04 ± 6.6	79.57 ± 10.52	85.58 ± 5.95	1.01*10 ⁻²⁵	56.23 ± 8.33	77.73 ± 11.77	83.37 ± 10.29	84.49 ± 10.07	74.73 ± 18.19	2.38*10 ⁻¹⁴⁷		
Hematocrit (%)	38.89 ± 5.36	35.88 ± 14.38	41.85 ± 9.57	0.09	42.88 ± 5.13	32.57 ± 17.42	34.58 ± 20.09	32.8 ± 20.29	40.65 ± 5.31	3.52*10 ⁻²⁴		
Creatinine (μmol/L)	61.26 ± 15.54	57.65 ± 21.01	57.87 ± 20.27	0.71	69.34 ± 25.98	63.33 ± 17.78	67.18 ± 26.69	96.68 ± 103.44	74.04 ± 32.09	8.62*10 ⁻⁷		
Blood urea nitrogen (μmol/L)	5.9 ± 1.79	4.66 ± 1.69	5.61 ± 1.79	0.02	5.4 ± 1.67	5.25 ± 3.56	5.55 ± 1.85	6.92 ± 4.62	6.03 ± 2.13	1.19*10 ⁻³		
Glucose (mmol/L)	5.45 ± 0.86	9.18 ± 3.84	12.83 ± 5.99	2.35*10 ⁻⁹	6.71 ± 23.03	8.25 ± 3.44	11.14 ± 5.17	10.82 ± 5.38	8.58 ± 5.38	0.20		
Alanine transaminase (U/L)	21.88 ± 17.33	125.8 ± 150.96	74.02 ± 81.67	3.11*10 ⁻⁴	22.83 ± 17	117.73 ± 161.07	71.81 ± 93.53	130.71 ± 160.13	209.28 ± 258.76	2.25*10 ⁻³⁷		
Aspartate transaminase (U/L)	22.82 ± 10.29	94 ± 108.14	84.47 ± 129.26	0.01	21.64 ± 8.34	98.24 ± 144.04	80.31 ± 130.79	327.79 ± 1293.76	193.62 ± 283.91	2.30*10 ⁻⁹		
Total bilirubin (μmol/L)	12.48 ± 3.81	29.86 ± 42.05	33.89 ± 36.47	0.02	15.23 ± 20.67	29.69 ± 36.97	28.93 ± 29.06	35.12 ± 28.94	66.25 ± 65.93	7.06*10 ⁻²⁷		
Direct Bilirubin (μmol/L)	3.58 ± 1.41	14.4 ± 13.94	12.2 ± 8.72	3.54*10 ⁻⁵	4.22 ± 1.77	15.85 ± 22.49	11.56 ± 11.58	17.97 ± 28.8	25.76 ± 37.55	1.65*10 ⁻²⁴		
Total cholesterol (mmol/L)	4.82 ± 0.8	7.47 ± 3.97	8.31 ± 4.54	2.34*10 ⁻³	5.05 ± 1.1	5.49 ± 2.6	7.12 ± 4.67	5.8 ± 2.82	----	1.25*10 ⁻¹⁰		
Triglyceride (mmol/L)	1.59 ± 0.75	8.8 ± 8.11	10.89 ± 10.53	1.58*10 ⁻⁴	1.56 ± 1.8	4.55 ± 6.28	8.7 ± 13.52	5.87 ± 5.89	----	3.73*10 ⁻²²		
High-density lipoprotein (mmol/L)	1.18 ± 0.27	1.53 ± 1.43	1.86 ± 1.58	0.18	1.88 ± 8.67	1.2 ± 0.71	1.53 ± 1.46	1.05 ± 0.59	----	0.74		
Low-density lipoprotein (mmol/L)	2.6 ± 0.55	2.65 ± 1.04	3.45 ± 1.7	0.04	2.87 ± 1.68	3.08 ± 6.45	2.75 ± 1.29	2.27 ± 1.1	----	0.59		
Biliary AP (%)	----	50%	40%	----	----	39.89%	31.40%	30.23%	----	----		
Hyperlipidemia AP (%)	----	37.5%	36.67%	----	----	19.68%	38.37%	37.21%	----	----		
Other AP (%)	----	12.5%	23.33%	----	----	40.43%	30.23%	32.56%	----	----		

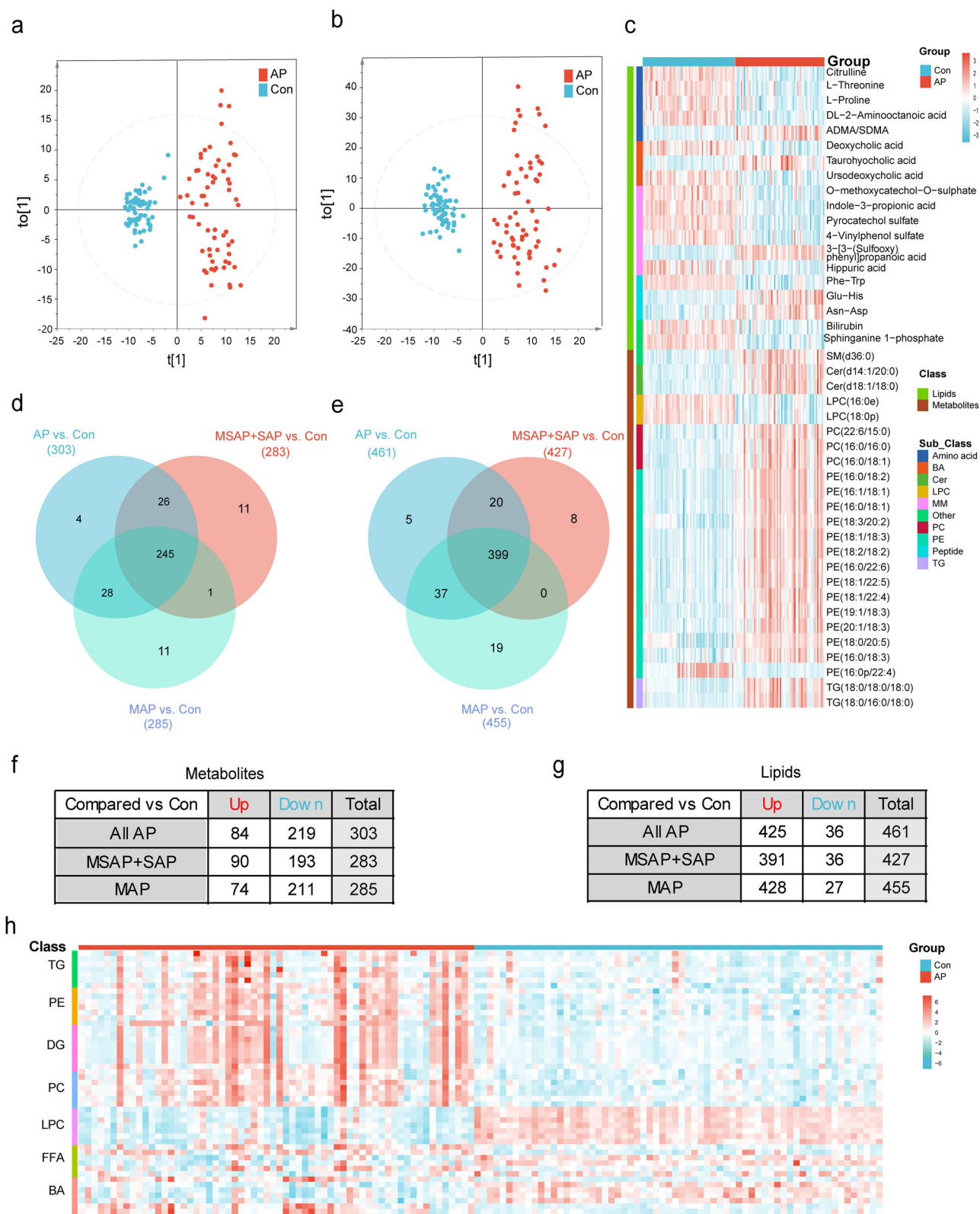


Fig. 2 | Distinct serum metabolomic and lipidomic profiles between AP and Con groups. Orthogonal partial least squares-discriminant analysis score plots of untargeted polar metabolites (**a**) and lipids (**b**) differentiating AP from Con groups. **c** Heatmap showing the relative intensities of differential metabolites and lipids. Blue and red indicate lower and higher relative abundances, respectively. Color bars on the left represent the chemical class and subclass of each compound. Venn diagrams illustrating the overlap of differential polar metabolites (**d**) and lipids (**e**) among three pairwise comparisons (AP vs. Con, MAP vs. Con, and MSAP + SAP vs. Con). (**f–g**) Summary of upregulated and downregulated metabolites (**f**) and lipids (**g**) in each comparison. **h** Heatmap showing the relative concentrations of triglycerides, phosphatidylethanolamines, diglycerides, phosphatidylcholines, lysophosphatidylcholines, free fatty acids, and bile acids between AP and Con groups. AP acute pancreatitis, Con control, MAP mild acute pancreatitis, MSAP moderately severe acute pancreatitis, SAP severe acute pancreatitis.

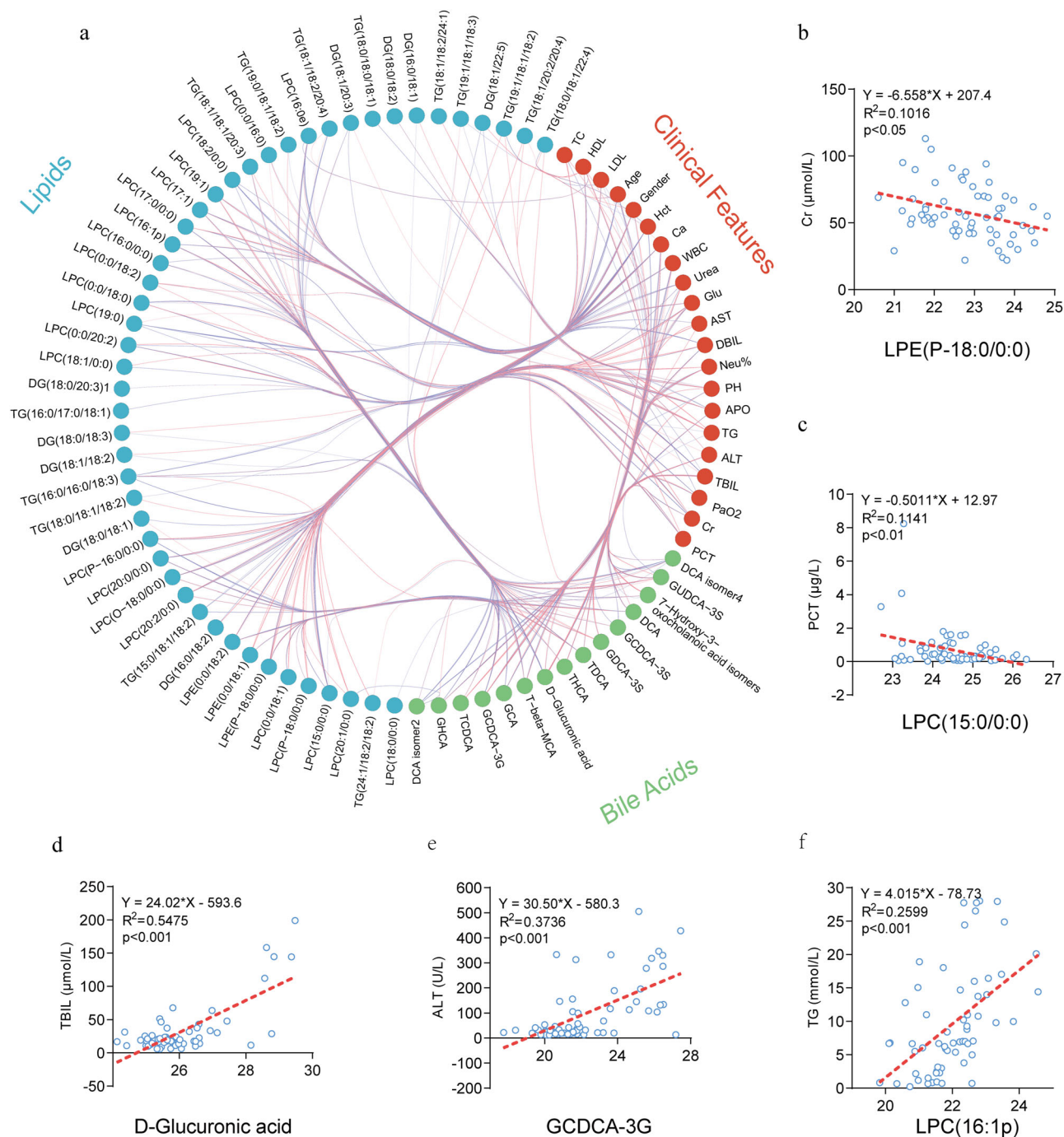


Fig. 3 | Correlations among bile acids, lipids, and clinical indicators in AP.

a Network visualization of pairwise correlations among bile acids, lipid metabolites, and clinical parameters. Red and blue lines indicate positive and negative correlations, respectively; line width corresponds to the absolute value of the correlation coefficient. Representative scatter plots with linear regression analyses showing the

relationships between **(b)** LPE (P-18:0/0:0) and serum creatinine, **(c)** LPC (15:0/0:0) and procalcitonin, **(d)** D-glucuronic acid and total bilirubin, **(e)** GCDCA-3G and alanine aminotransferase, and **(f)** LPC (16:1p) and total triglycerides. AP acute pancreatitis.

changes. Structurally defined serum BAs and various subclasses of BAs in AP were significantly altered compared to Con, and the concentrations of BAs were mainly increased in AP (Supplementary Data 7–8, Supplementary Fig. 9). AP was associated with a larger circulating BA pool size.

To gain further insights into the relationship between BAs and AP, we evaluated BA patterns in relation to AP severity. We found that BA metabolism in MSAP and SAP differed significantly from that in MAP. MSAP and SAP exhibited many similarities in BA metabolism. A significant proportion of the elevated serum levels of total BAs was due

to higher levels of conjugated BAs. There was no difference in the levels of total BAs and conjugated BA between individuals with MSAP and those with definite SAP. We observed a strong trend for higher sulfate BA levels with higher indices of severity (Fig. 4a). Besides, two specific secondary BAs, deoxycholic acid (DCA) and deoxycholic acid 3-sulfate (DCA-3S), derived from cholic acid (CA), were associated with increasing severity of AP (Fig. 4b).

A validation study confirmed serum BA alterations throughout the entire spectrum of AP. Abnormal BA metabolism also occurred in AP

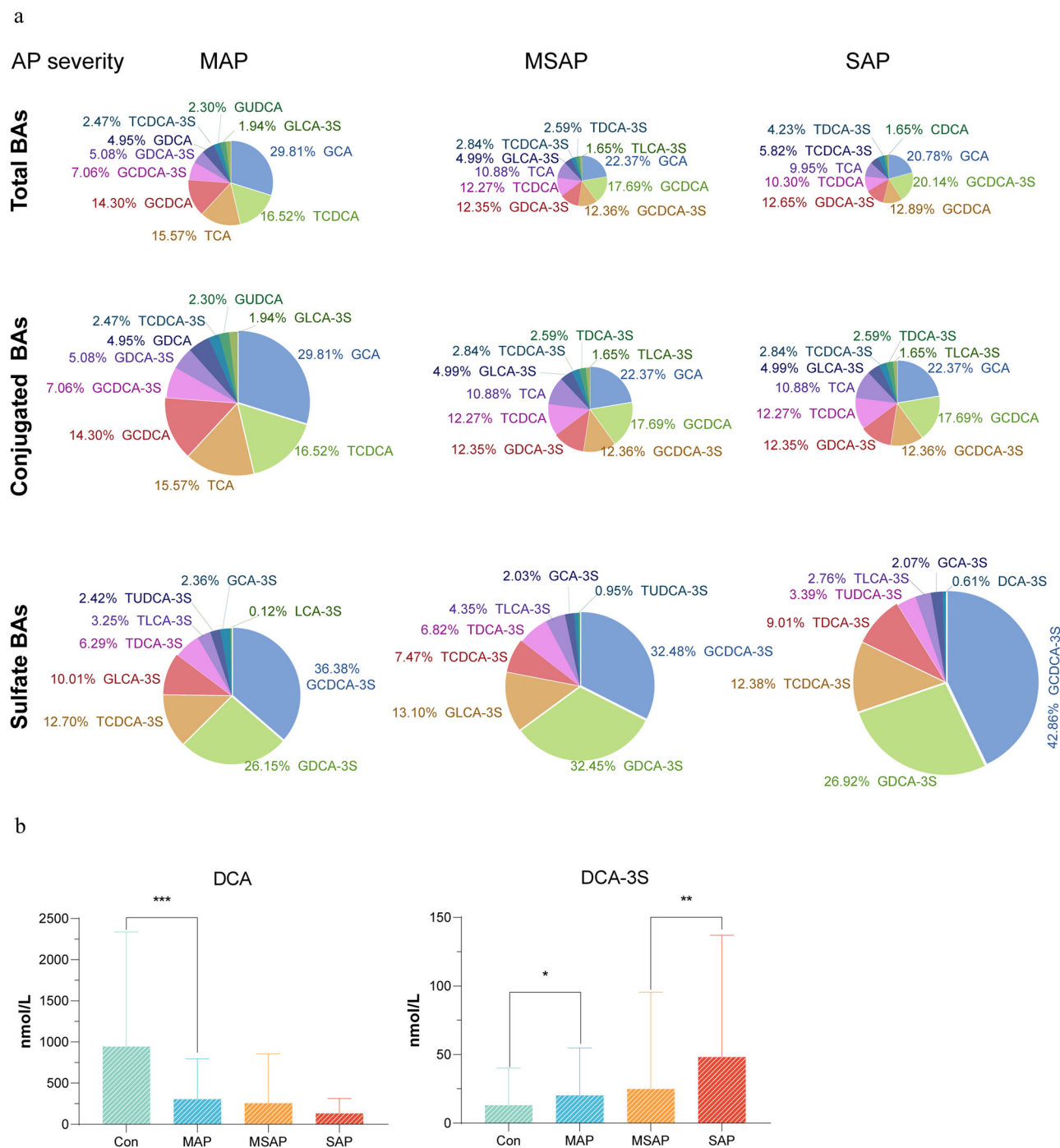


Fig. 4 | Alterations in serum BA composition associated with AP severity. **a** Pie charts showing the proportional composition of individual BAs within the total, conjugated, and sulfate BA pools across MAP, MSAP, and SAP groups. Each pie size reflects the total concentration of the corresponding BA pool, and percentage values indicate the most abundant or significantly altered species. **b** Relative concentrations

of DCA and DCA-3S in Con, MAP, MSAP, and SAP groups. For all comparisons, adjusted *p*-values are shown: *p* < 0.05; **p* < 0.01; ***p* < 0.001. BA bile acid, AP acute pancreatitis, MAP mild acute pancreatitis, MSAP moderately severe acute pancreatitis, SAP severe acute pancreatitis, DCA deoxycholic acid, DCA-3S deoxycholic acid-3-sulfate, Con control.

patients with different etiologies (Supplementary Fig. 10). BA metabolism disorder is a molecular characteristic of AP.

Establishment of BA panels for AP and AC

To screen BAs with diagnostic efficacy, we selected the top 20 differentially expressed BAs from the classification results of the AP and Con groups based on mean decrease in accuracy, and then conducted least absolute shrinkage and selection operator (LASSO) regression. Nine BAs were obtained under optimal conditions: chenodeoxycholic acid (CDCA), beta-

muricholic acid (β -MCA), ursodeoxycholic acid (UCA), norcholeic acid (NorCA), isolithocholic acid (Iso-LCA), tauroursodeoxycholic acid 3-sulfate (TUDCA-3S), 7-ketolithocholic acid (7-ketoLCA), hyocholic acid/CDCA (HCA/CDCA), and unconjugated BAs (Table 2, Supplementary Data 9 and Supplementary Fig. 11).

Next, a random forest (RF) model, trained on all samples, revealed that the Con group showed a lower mean predicted probability than the AC group and AP group (0.198 vs. 0.624 vs. 0.762) (Fig. 5a). There were no associations between predicted probability and age or sex (Supplementary

Table 2 | Performance of BA panels in the diagnosis of AP

	Discovery Set			Internal Validation Set			External Validation Set					
	N	AUC (95% CI)	Sensitivity	Specificity	N	AUC (95% CI)	Sensitivity	Specificity	N	AUC (95% CI)	Sensitivity	Specificity
9-BA panel												
AP vs. Con	353	0.973 (0.956–0.989)	89.7 (83.1–93.9)	95.6 (92.1–97.6)	148	0.988 (0.972–1.00)	96.2 (87.0–99.3)	97.9 (92.7–99.6)	394	0.897 (0.862–0.932)	71.9 (64.0–78.7)	95.7 (92.4–97.6)
AP vs. AC	143	0.940 (0.893–0.987)	83.3 (75.9–88.8)	94.1 (73.0–99.7)	69	0.938 (0.879–0.996)	88.5 (77.0–94.6)	88.2 (65.7–97.9)	158	0.849 (0.768–0.930)	72.7 (64.7–79.4)	89.5 (86.6–98.1)
AP Neg vs. AC	79	0.912 (0.850–0.975)	92.0 (81.2–96.8)	75.9 (57.9–87.8)					70	0.889 (0.814–0.964)	89.1 (77.0–95.3)	75.0 (55.1–88.0)
Enzyme												
AP vs. AC	143	0.869 (0.810–0.928)	73.8 (65.5–80.7)	100 (81.6–100)	69	0.837 (0.746–0.928)	67.3 (53.8–78.5)	100 (81.6–100)	158	0.835 (0.770–0.899)	66.9 (58.7–74.2)	100 (83.2–100)
9-BA panel+Enzyme												
AP vs. AC	143	0.981 (0.960–1.00)	93.7 (88.0–96.7)	94.1 (73.0–99.7)	69	0.966 (0.911–1.00)	94.2 (84.4–98.4)	94.1 (73.0–99.7)	158	0.958 (0.928–0.988)	85.6 (78.8–90.5)	100 (83.2–100)
13-BA panel												
SAP vs. MSAP	66	0.936 (0.879–0.993)	85.0 (64.0–94.8)	89.1 (77.0–95.3)					63	0.918 (0.845–0.991)	91.3 (73.2–98.5)	82.5 (68.1–91.3)

Fig. 12a–b). The participants from the First Affiliated Hospital of Dalian Medical University (FAHDMU) were randomly divided into a discovery set and an internal validation set at a 7:3 ratio. The participants from the subcentres were used as the external validation set.

We found that the 9-BA panel showed promising diagnostic efficacy in distinguishing between the AP and Con groups. The discovery, internal validation, and external validation sets were combined, and a model with an AUC of 0.956 and a 95% confidence interval (95% CI) of 0.942 to 0.969 was obtained (Fig. 5b). The diagnostic model demonstrated high performance across all cohorts. In the discovery set, the AUC, sensitivity, and specificity were 0.973, 89.7%, and 95.6%, respectively. Corresponding values for the internal validation set were 0.988, 96.2%, and 97.9%, while the external validation set yielded values of 0.897, 71.9%, and 95.7% (Table 2).

Next, we assessed the diagnostic performance of the 9-BA panel in distinguishing AP from AC. The model of the combined sets exhibited an AUC of 0.890 and a 95% CI of 0.847 to 0.932 (Fig. 5c). Performance metrics are summarized in Table 2. The discovery, internal validation, and external validation sets yielded AUCs of 0.940, 0.938, and 0.849, with corresponding sensitivities of 83.3%, 88.5%, and 72.7% and specificities of 94.1%, 88.2%, and 89.5%, respectively. Conventional diagnostic biomarkers, such as amylase and lipase, were not elevated in all patients presenting with AP. The 9-BA panel also demonstrated significant efficacy among patients who presented with negative enzymatic results for AP. However, due to the relatively small sample size, all participants from the FAHDMU were used as the discovery set, and those from the subcentre as the external validation set. In the discovery set, the AUC, sensitivity, and specificity were 0.912, 92.0%, and 75.9%, respectively. Corresponding values in the external validation set were 0.889, 89.1% and 75.0%, respectively (Table 2).

In the present study, enzymatic indicators exhibited superior specificity in distinguishing AP from AC, but poor sensitivity (Table 2). To compensate for the enzymatic indicator's limited sensitivity, we built a model that combined the 9-BA panel. The model and cut-off value established in the discovery set were applied to the external validation set, and the results are presented by a box plot. Based on the optimal cut-off probability of 0.568, individuals were stratified into AP (probability >0.568) or AC (probability ≤0.568) diagnostic groups (Fig. 5d). In the combined sets, the AUC was 0.969 (95% CI: 0.953 to 0.985) (Fig. 5e). The AUC, sensitivity, and specificity for the discovery, internal validation, and external validation sets were (0.981, 93.7%, 94.1%), (0.966, 94.2%, 94.1%), and (0.958, 85.6%, 100%), respectively (Table 2, Fig. 5f).

A comparative assessment was conducted between the predictive performance of nomograms utilizing enzymatic biomarkers and those incorporating the 9-BA biomarkers. To assess the accuracy of the nomograms, we constructed a calibration plot that showed good agreement between the predicted probabilities and actual observations (Supplementary Fig. 13). Furthermore, the confusion matrices for these biomarker combinations showed high accuracy in classifying and clustering different groups (Supplementary Fig. 14).

The BA panel enables early identification of SAP

Based on clinical guidelines, it is standard practice to stratify AP into mild (MAP), moderately severe (MSAP), and severe (SAP) categories. However, the initial 9-BA panel demonstrated limited efficacy for this specific stratification. Consequently, we sought to identify a more suitable BA panel for severity classification. To this end, all BAs quantified in the targeted metabolomics analysis were subjected to LASSO regression. This analysis yielded an optimal model comprising thirteen candidate BAs: HCA, NorCA, Iso-LCA, omega-muricholic acid (ω-MCA), taurohyocholic acid (THCA), 7-ketodeoxycholic acid (7-ketoDCA), taurochenodeoxycholic acid-glucuronide (TCDCA-G), TUDCA-3S, nordesoxycholic acid (NorDCA), norursodeoxycholic acid (NorUDCA), 7-ketoLCA, glycodeoxycholic acid (GDCA)/CA, and secondary BAs (Supplementary Data 9). Normalized expression of each biomarker across the different groups is shown in Supplementary Fig. 15.

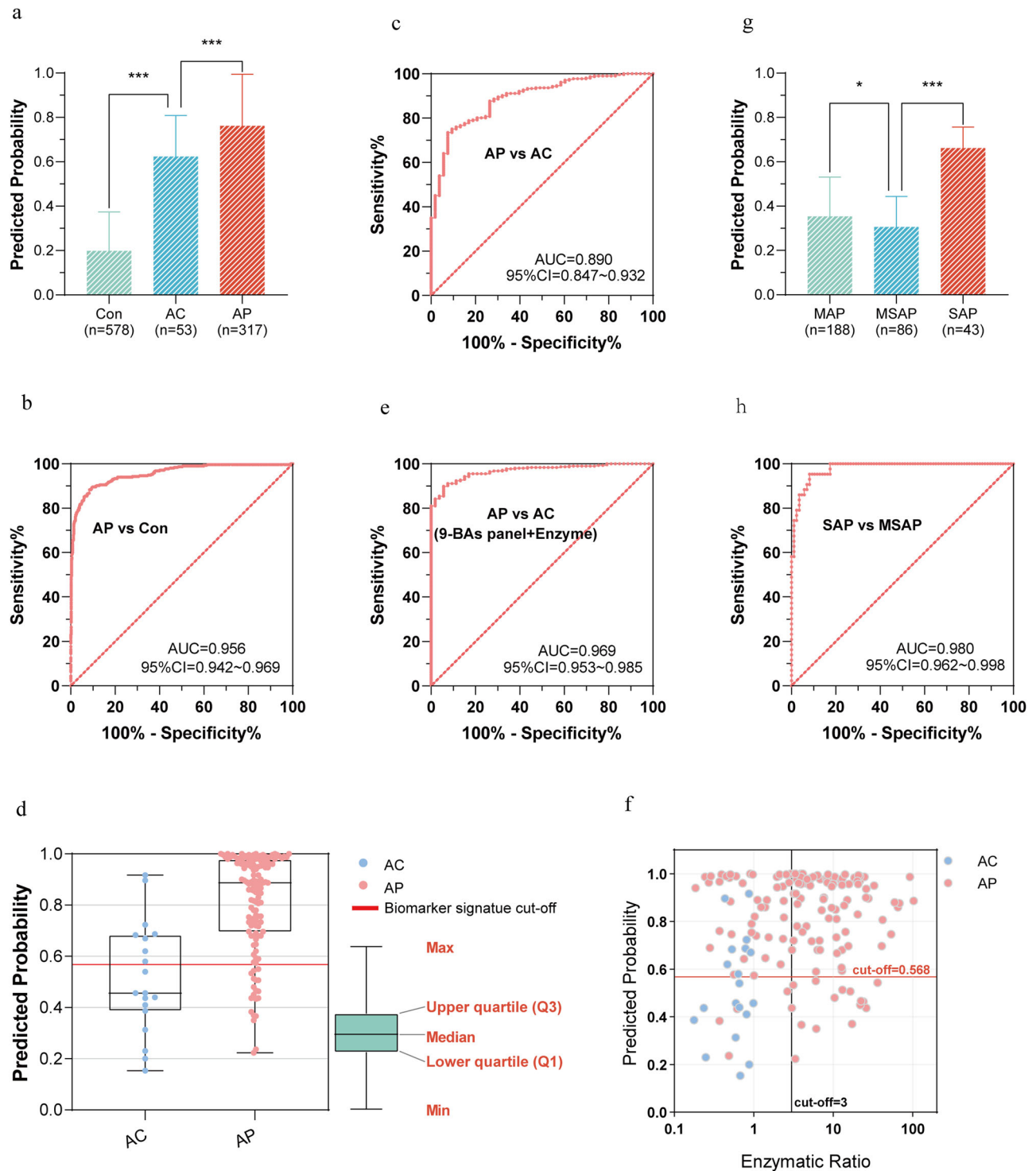


Fig. 5 | BA panel profiling in the diagnosis of AP and AC. The 9-BA panel's predicted probability in healthy controls ($n = 578$), AC patients ($n = 53$) and AP patients ($n = 317$) (a). ROC curve of the 9-BA panel between AP patients and healthy controls in the combined set (b). ROC curve of the 9-BA panel between AP patients and AC patients in the combined set (c). ROC curve of the 9-BA panel + enzymatic indicators between AP patients and AC patients in the combined set (d). The predicted probability of the 13-BA panel in MAP patients ($n = 188$), MSAP patients ($n = 86$) and SAP patients ($n = 43$) (e). ROC curve of the 13-BA panel between MSAP patients and SAP patients in the combined set (f). Predicted probability of the

9-BA panel identified in the discovery set and applied to the external validation set (g). Scatter plot comparing the 9-BA panel and enzymatic indicator efficiencies (h). The serum concentrations of amylase and lipase were divided by the upper limit of the normal value, and a larger value was selected as the enzymatic ratio. The enzymatic ratio of AC was randomly selected from the upper and lower limits of the normal value. For all figures, FDR-adjusted p value: * $p < 0.05$; *** $p < 0.001$. BA bile acid, AP acute pancreatitis, AC acute cholecystitis, ROC receiver operating characteristic, MAP mild acute pancreatitis, MSAP moderately severe acute pancreatitis, SAP severe acute pancreatitis.

We used all AP samples to establish an RF model and found that the MAP and MSAP groups had lower mean predicted probabilities than the SAP group (0.353 vs. 0.306 vs. 0.662), and there were no associations between the predicted probability and age or sex (Supplementary Fig. 12c–d). However, the mean predicted probability of the MAP and MSAP groups did not increase with severity (0.353 vs. 0.306, Fig. 5g). Accordingly, MAP and MSAP can be distinguished by clinical features at the early stage of admission.

Due to the low incidence of SAP, the sample size for SAP was limited. Hence, this model used all participants from the FAHDMU as the discovery set and those from the subcentres as the external validation set. In the combined sets, the AUC was 0.980 (95% CI: 0.962 to 0.998) (Fig. 5h). The model achieved strong discriminative performance in both cohorts, with an AUC of 0.936 (sensitivity 85.0%, specificity 89.1%) in the discovery set and 0.918 (sensitivity 91.3%, specificity 82.5%) in the external validation set (Table 2). Nomograms and a calibration plot were constructed (Supplementary Fig. 16). Furthermore, the confusion matrices for these biomarker combinations showed high accuracy in classifying and clustering different groups (Supplementary Fig. 17).

Discussion

The timely recognition of AP, especially enzyme-negative AP and SAP, remains a significant challenge in clinical practice. Indeed, a panel of disease-specific molecular signals could be an excellent candidate biomarker²⁸. To our knowledge, this is the first integrated metabolomics study to describe the metabolic characteristics of patients with AP in a large, multicentre cohort. First, in the untargeted metabolomics study, we found that lipid metabolism and BA metabolism were closely linked to the clinicopathological features of AP, and BAs showed huge potential as diagnostic biomarkers. Primary BAs are synthesized from cholesterol in the liver and transformed into secondary BAs in the gut by microbiota. Therefore, these compounds may be involved in the development of AP. Next, we studied 948 subjects from five medical centers and used a targeted metabolomics platform to validate the dysregulation of BA metabolism in AP. A diagnostic panel comprising nine BAs or ratios (CDCA, β -MCA, UCA, NorCA, Iso-LCA, TUDCA-3S, 7-ketoLCA, HCA/CDCA, and unconjugated BAs) was established using a machine learning algorithm and validated in the discovery, internal validation, and external validation sets. Besides, the model demonstrated high selectivity for AP patients compared with AC patients. Notably, the 9-BA panel complemented serum enzymatic biomarkers. The early identification of patients at risk of SAP is of great significance. Thus, we constructed a model comprising 13 BAs (HCA, NorCA, Iso-LCA, ω -MCA, THCA, 7-ketoDCA, TCDCA-G, TUDCA-3S, NorDCA, NorUDCA, 7-ketoLCA, GDCA/CA, and secondary BAs) that accurately stratified patients at risk of SAP at admission. These results highlighted that impaired BA metabolism may be a characteristic molecular phenotype of AP and that BAs have potential as an early indicator of SAP.

The molecular phenotype accurately reflects the pathological features of the disease^{29,30}. In exploring the molecular phenotype of AP, we found that lipids and BAs play a crucial role in the occurrence and development of AP. Correlation analysis showed that BAs were closely related to LPCs, glycerides and clinical characteristics. Linear regression analysis revealed a close relationship between metabolic molecules and clinicopathological features. Circulating metabolites can be used as indicators of AP characteristics. Compared with the decrease in LPCs in AP, the expression of BAs was increased, which may be a key proinflammatory factor and an ideal candidate marker of AP. To validate the hypothesis, a discriminant model incorporating all significant BAs was developed. The model successfully discriminated AP state and etiology but failed to accurately stratify disease severity. This result suggests that the BA profile necessary for severity discrimination may not be fully captured by untargeted metabolomic analysis. Therefore, in-depth targeted BA metabolomics is needed to confirm our hypothesis. However, there was no significant change in the BA profile between initial and recurrent AP. We hypothesized that BAs might be the driving molecules in AP, warranting further research.

BAs are increasingly recognized as critical signaling molecules. BAs regulate glucose and lipid homeostasis and energy expenditure by regulating farnesoid X receptor (FXR) and the G protein-coupled BA receptor TGR5 in peripheral tissues such as the liver, intestine, fat, and pancreas^{31–33}. The expression and activity of FXR are closely related to TG homeostasis³¹. Mitochondrial dysfunction may be one of the essential pathways by which BAs cause cellular dysfunction in inflammation³⁴. BAs can also serve as damage-associated molecular pattern (DAMP) molecules to participate in the activation of the NOD-like receptor thermal protein domain-associated protein 3 (NLRP3) inflammasome in immune cells in coordination with ATP and to participate in inflammatory reactions³⁵. On the other hand, BAs are also closely related to the gut microbiota. Secondary BAs are manufactured by microorganisms, and different kinds of secondary BAs may reflect the state of the species of the flora³⁶. Our validation study confirmed significant alterations in AP patients' total circulating BA pool and BA composition profile. Increased total BAs in patients with AP indicated elevated hepatic BA anabolism. The liver and gut flora affect the pathophysiological process of AP by regulating the metabolic network of BAs. We found an interesting phenomenon: in patients with AP, primary BAs increased, whereas total secondary BAs did not change significantly. Glycocholic acid (GCA) and lithocholic acid (LCA), the main components of secondary BAs, significantly decreased, indicating that gut flora was less catalytic for BAs. Regardless of etiology, intestinal dysfunction is a central pathophysiological event in AP that disrupts the gut microbiome and secondary BA metabolism. Therefore, BA metabolism disorder may be an AP molecular phenotype independent of etiology. Conjugated BAs, glycolaldehyde-acidified BAs, and sulphated BAs were significantly increased in AP, but their pathophysiological effects in AP need to be further explored. The ratio of upstream and downstream metabolic molecules can indirectly reflect the catalytic enzymatic activity³⁷. In the present study, we compared the ratios of 5 types of BAs to elucidate the mechanism of BA changes. The results showed that BA metabolism was altered from the classical pathway to the alternative pathway (CA/CDCA). The BAs metabolized by nonclassical pathways were primarily transformed into HCA species (HCA/CDCA). HCAs are a group of 6A-hydroxylated BAs that play an essential role in regulating glucose homeostasis and are considered key regulatory molecules in resisting the development of type 2 diabetes in pigs fed a high-energy diet³⁸. The ratios of THCA/HCA and glycohyocholic acid (GHCA)/HCA reflect the activities of taurine-binding enzymes and glycine-binding enzymes in the liver on HCA. As the function of the gut microbiota was affected, the activity of the HCA-related catalytic enzymes, hydoxychocholic acid (HDCA)/LCA, decreased. These results suggested that BA enterohepatic circulation played an important role in the pathological process of AP.

The early and rapid diagnosis of an acute disease can significantly benefit patients. However, enzyme-negative patients with AP are often missed. Enzymatic markers have good specificity for diagnosing AP, but their sensitivity is low. To reduce missed diagnoses in enzyme-negative patients with AP, further reference to imaging features or repeated detection of enzymatic markers is needed, leading to delayed treatment. The 9-BA panel we constructed exhibited a complementary effect with enzymatic biomarkers, and the combination contributed to early and accurate differential diagnosis of AP from AC, with an AUC of 0.969 across the three combined cohorts. MSAP and SAP share similar clinical manifestations within 48 hours of the onset of organ dysfunction, but their clinical outcomes differ significantly. Identifying patients at high risk of organ dysfunction is an urgent problem. The accurate identification of SAP at an early stage can optimize medical resources and improve outcomes. Commonly used clinical AP classifications include RAC and determination-based classification (DBC). RAC is mainly applicable to patients in the early stage of AP. The purpose of this study was to screen AP patients at risk of developing SAP at admission, so the severity was assessed primarily by RAC. Our prediction model based on 13 BAs was highly effective for stratifying SAP. Given this model's predictive power, we did not compare it with existing scoring systems, such as the bedside index for severity in AP

(BISAP) and systemic inflammatory response syndrome (SIRS). The panels we constructed were composed of multiple molecules. Compared with a single molecule, multiple molecules can effectively reduce molecular noise and improve diagnostic accuracy. Our results suggested that BAs not only serve as diagnostic markers but also have the potential to be therapeutic targets. It is well-established that LCA is transformed into iso-LCA through the iso-BAs pathway of the gut microbiota, which has antibacterial and anti-inflammatory effects³⁹. A study revealed that the secondary BA ω -MCA increased forkhead box P3+ (FOXP3+) T cells and Treg cells, regulating intestinal immune homeostasis⁴⁰. NorUDCA, a derivative of ursodeoxycholic acid (UDCA), improved liver steatosis, reduced blood cholesterol and played an anti-inflammatory role⁴¹. However, the role of these BAs in AP needs further study.

This study has the following strengths: (1) We used an integrated metabolomics strategy to describe the overall metabolic characteristics of AP and conducted targeted validation in a large cohort. These results contributed to the design of therapeutic targets and the identification of markers. (2) Previous studies have considered that disorders of BA metabolism are only associated with biliary pancreatitis. Our results showed that BA metabolism disorders might be a typical molecular phenotype of AP under natural etiology, expanding the current understanding of AP. (3) This study was the largest AP metabolomics study with a total of 948 subjects, and a three-step analysis strategy was adopted to identify novel biomarkers. More importantly, we established an external validation cohort comprising patients from four medical centers to evaluate the diagnostic panel's scalability. (4) The identified biomarkers exhibited a complementary effect on serum amylase or lipase. These biomarkers were based on urgent demands in clinical practice and had good clinical transformation significance. A diagnosis of AP is frequently considered for patients exhibiting upper abdominal pain with negative serum amylase but positive CT findings, such as pancreatic swelling or peripancreatic fluid. In such cases, the BA biomarkers identified in this study may serve as useful adjuncts to further support the clinical suspicion. Moreover, in newly admitted patients with AP, quantification of BA abundance may help to predict those at higher risk of progressing to SAP. For these high-risk individuals, clinicians should provide closer monitoring and consider early, proactive interventions.

Although our study provided original insights into the pathogenesis of AP, it also had limitations. First, as an observational study, the causal relationship between lipid and BAs metabolism disorders and AP needs further confirmation. Second, although serum BAs or their ratios can accurately identify patients at risk of SAP, the number of SAP cases in this cohort was relatively small. Indeed, the model comparing SAP and MSAP lacked an internal validation cohort. Therefore, the results require further validation in larger populations. Third, we did not compare the BA biomarkers derived from this study with the BISAP score, as specific components of the BISAP score, such as impaired consciousness and SIRS, are delayed and retrospective. In contrast, the BA biomarkers identified in our study are intended to provide clinicians with an early warning at the time of admission, thereby facilitating timely risk stratification. Such early predictive markers are urgently needed in current clinical practice.

In summary, this study characterized the serum metabolic profile of AP, highlighting dysregulated lipid and BA pathways (Fig. 6). The diagnostic panels constructed from BAs can be used as a complementary index for enzyme-negative AP patients and could identify patients at risk of SAP early. The clinical efficacy of these panels also needs further study.

Methods

Patients and clinical features

This study was registered in the Chinese Clinical Trial Registry (No. ChiCTR2000034117) on June 24, 2020. A total of 948 participants were enrolled in this study, including patients with AP ($n = 317$), patients with AC ($n = 53$), who presented similar symptoms and biochemical changes to the AP group, and healthy controls (Con, $n = 578$). Con patients were recruited to match AP patients in terms of age and sex. A consecutive cohort of patients with AP was recruited from the FAHDMU between June 2017

and May 2021. In addition, 139 AP patients were recruited from four subcentres between August 2020 and April 2021: The Second Affiliated Hospital of Dalian Medical University, Baotou Central Hospital, Yingkou Central Hospital, and Inner Mongolia Autonomous Region People's Hospital. All participants signed an informed consent form, and their blood samples were collected at 6:00 am within 24 hours after admission. The collection of clinical specimens in this study was conducted in accordance with the Declaration of Helsinki. This multicenter study received ethical approval from the following institutional review boards: the Ethics Committee of the Second Affiliated Hospital of Dalian Medical University, the Ethics Committee of Baotou Central Hospital (approval no: KYLL2021006), the Ethics Committee of Yingkou Central Hospital, the Ethics Committee of Inner Mongolia Autonomous Region People's Hospital, and the Ethics Committee of the First Affiliated Hospital of Dalian Medical University (approval no: PJ-KS-KY-2019-99).

The inclusion criteria were based on the 2012 revised Atlanta classification (RAC), which stratified AP into MAP, MSAP, and SAP. Participants were excluded based on the following criteria: (1) age under 18 or above 85 years; (2) presence of advanced pulmonary, cardiac, or renal diseases, including chronic kidney disease stages 4 to 5; (3) liver cirrhosis classified as modified Child-Pugh grade 2 or 3; (4) cases where malignancy, pregnancy, or trauma were identified as the underlying cause; (5) a history of chronic pancreatitis during an acute exacerbation; (6) long-term use of medications known to significantly alter metabolic function.

Metabolomics preparation and detection

The procedures for metabolomic sample preparation and analytical detection were described in our previous study⁴².

Sample pretreatment

All samples were promptly stored at -80°C and thawed at 4°C prior to pretreatment. First, 600 μL of methanol (Fisher Scientific, USA) was added to 150 μL of serum. The resulting solution was vortexed and centrifuged. Two replicates of 200 μL of supernatant were transferred to a 450 μL 96-well plate (Thermo Scientific, USA), and dried by a centrifugal vacuum concentrator (Labconco Corporation, USA). The remaining supernatant was mixed and similarly distributed in 200 μL of each replicate as a QC sample. The polar extractions were redissolved for electrospray ionization (ESI+ / ESI-) untargeted metabolomics analysis.

To extract lipids from serum, we added 120 μL of methanol to the 20 μL serum. Then, 360 μL of methyl tert-butyl ether (MTBE, Sigma-Aldrich, USA) and 100 μL of ultrapure water were added to the solution. The mixture was vortexed for 10 minutes, kept at room temperature for another 10 minutes, and finally centrifuged for 15 minutes. The 200 μL supernatant lipid extraction was transferred to a 1.5 mL EP tube for drying. Then, the lipid extracts were redissolved, and lipidomics analysis was performed. Meanwhile, QC samples of lipids were also prepared.

For targeted BA pretreatment, 80 μL of serum was transferred to a 1 mL 96-deepwell plate. Then, 320 μL of methanol: acetonitrile (1:1, with a 50 ng/mL BA isotope internal standard) was added to the plate. The mixture was vortexed for 5 minutes and then centrifuged for 20 minutes. Next, 260 μL of supernatant was transferred to a 450 μL 96-well plate and dried as mentioned above. The mixture of QC samples was also prepared. The extractions were redissolved for BA-targeted metabolomics analysis.

Untargeted metabolite detection

An Ultimate 3000 ultrahigh-performance liquid chromatography system and a Q Exactive Quadrupole-Orbitrap high-resolution mass spectrometer (Thermo Scientific, USA) were used for untargeted metabolomics analysis. The polar metabolites were separated by an Excel C18-PFP column (ACE Co., UK, 3.0 μm , 2.1×100 mm) for ESI+ detection and an Acquity HSS C18 column (Waters Co., USA, 1.8 μm , 2.1×100 mm) for ESI- detection. Lipids were separated by an Accucore C30 core-shell column (Thermo Scientific, USA, 2.6 μm , 2.1×100 mm) for ESI+ detection. The flow rate, injection volume, and column temperature of polar metabolites in ESI+ and ESI-

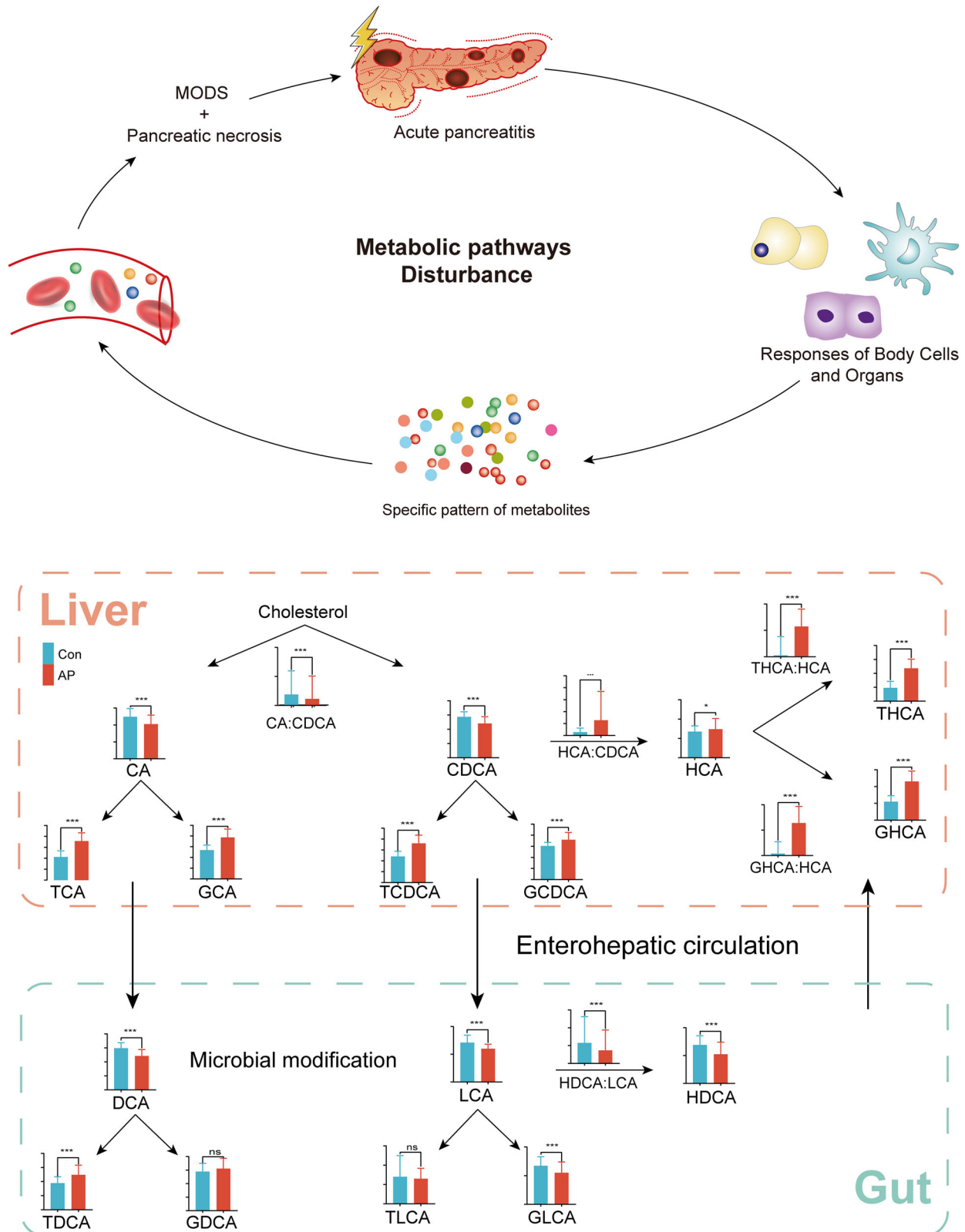


Fig. 6 | Dysregulation of BA metabolism in AP. The upper panel illustrates that AP triggers extensive inflammatory responses in multiple organs, leading to systemic metabolic disturbances. These abnormal metabolic profiles in blood and tissues further aggravate inflammation and pancreatic necrosis, forming a positive feedback loop that contributes to multiple organ dysfunction syndrome. The lower panel depicts the BA metabolic network between the liver and intestine, highlighting the

altered hepatic synthesis, microbial modification, and enterohepatic circulation of BAs during AP. Relative concentrations of key BAs and their ratios are presented as bar plots comparing Con and AP groups. For all comparisons, adjusted *p*-values are shown: ns, not significant (*p* > 0.05); **p* < 0.05; ***p* < 0.001. BA bile acid, AP acute pancreatitis, Con Control.

modes were set at the same conditions of 0.4 mL min⁻¹, 5 µL and 50 °C, while in the lipid separation, the conditions were set at 0.35 mL min⁻¹, 2 µL and 50 °C.

Targeted metabolomics analysis

In targeted metabolomics, we established the following concentration gradient using the mixed standards solution: 465 µg/L, 232.5 µg/L, 116.25 µg/L, 58.125 µg/L, 29.0625 µg/L, 14.531 µg/L, 7.266 µg/L, 3.633 µg/L, 1.816 µg/L, 0.908 µg/L, 0.454 µg/L, 0.227 µg/L, and 0.114 µg/L.

The targeted metabolomics system was composed of a Waters Acquity UPLC (Waters Co., USA) and a Sciex 5500+ triple quadrupole (QQQ) mass spectrometer (AB Sciex, Singapore). BAs were chromatographically separated on a C18-PFP column (ACE Co., UK, 3 µm, 2.1 × 50 mm). Then, 2.5 µL of the BA extract was injected at a flow rate of 0.4 mL min⁻¹. The BAs were ionized by the ESI- mode and detected by multiple reaction monitoring modes.

Data processing

The untargeted metabolomics annotation was based on the recommendation of the Metabolomics Standardization Initiative (MSI). The primary mass spectrometry (MS) and secondary mass spectrometry (MS/MS) information were compared with local databases, KEGG, the Human Metabolome Database (HMDB), the mzCloud library, and LIPID MAPS. Finally, the AUC values of the metabolites were extracted as relative quantification data using TraceFinder software (Thermo Scientific, USA). Internal calibration was performed using Analyst and OS-MQ software (AB SCIEX, Singapore) for quantitative analysis of BAs.

Details of WGCNA

The correlation between the metabolic co-expression module and the clinical phenotype of AP was analyzed using the WGCNA software package, based on the relative intensity data of all untargeted metabolites. First, hierarchical clustering was performed to identify potential outliers, with a height threshold set to 80. No outliers were detected. WGCNA calculates the appropriate soft-thresholding power for network construction by evaluating the scale-free topology fit index across a range of powers. If the scale-free topology fit index exceeds 0.85 for lower powers, it indicates a scale-free network topology and suggests the absence of batch effects. The optimal soft-thresholding power was found to be 9 (scale-free topology R² = 0.876, slope = -0.622, mean connectivity = 73.6). The merged cut height was then set to 0.1, with a minimum module size of 10, resulting in 8 modules. Among these, the gray module represented metabolites that could not be incorporated into the co-expression network and should be minimized to enhance model robustness. Pearson correlation analysis revealed that the black and blue modules were significantly negatively correlated with the occurrence and progression of AP, while the green and turquoise modules were positively correlated (Supplementary Data 3). Next, the DSPC algorithm was applied to describe the correlation of metabolites in each module (Supplementary Data 4). The black and green modules were mainly composed of BAs, and there was a close correlation between the two kinds of BAs with opposite trends. In addition, TGs and DGs were mainly classified into the turquoise module, and LPCs were classified into the blue module. The lipids in the two modules were also closely related. To elucidate the relationships among key metabolites within each module, an MMI network was constructed based on the metabolites' internal connectivity.

Model establishment

Optimal variables from the targeted BA panel were screened using LASSO regression analysis of candidate BAs and their ratios, implemented with the glmnet package in R (v4.0.3). The resulting candidate biomarkers were uploaded to the Biomarker Analysis tool on the MetaboAnalyst website for multivariate modeling, using the random forest method. This process established a random forest-based diagnostic model, and its prediction probabilities were exported for further ROC analysis.

Statistical analysis

Metabolites with more than 50% missing values were excluded, and the K-nearest neighbor (KNN) algorithm (sample-wise) was applied to estimate the missing values. To ensure the uniqueness of polar metabolites and lipids, molecules detected by multiple modes were retained only once. Before the final statistical analysis, log2 transformation was conducted to obtain a normal distribution^{43–45}. The mass concentration of BAs was converted into the molar concentration of the original sample. Multivariate analyses were processed by SIMCA-P software (Umetrics, Sweden). Univariate analysis, including independent samples Student's t test and Benjamini-Hochberg (B-H) false discovery rate (FDR) adjustment, was performed with SPSS Statistics 26.0 software (IBM, USA). We applied the WGCNA⁴⁶, RF⁴⁷, and glmnet packages in the R software version 4.0.3 (R Core Team, 2020). Venn plots were generated by the InteractiVenn website⁴⁸. BioPAN, an innovative tool that can reveal the trends and relationships between lipids and FFAs, was also used. Moreover, receiver operating characteristic (ROC) curves and bar plots were generated with GraphPad Prism 8.0 (GraphPad Software Inc., USA). The R package rms was used to construct a nomogram including BAs and enzymatic biomarkers. The debiased sparse partial correlation (DSPC) algorithm and heatmap construction were performed on the MetaboAnalyst website. Cytoscape 3.8.0 was used for biological network construction and visualization⁴⁹.

Data availability

The metabolic sequencing data are provided in the Supplementary Data. For any questions regarding the data, please feel free to contact the corresponding author.

Code availability

The code can be acquired from the corresponding author upon reasonable request.

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Competing interests

Zeming Wu is co-founder of iPhenome Biotechnology Inc. Dalian (Yun Pu Kang). Other authors have declared that no competing interest exists.

Additional information

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