Frontiers in Bioscience Landmark

*Front. Biosci. (Landmark Ed)* **2025**; 30(6): 39259
https://doi.org/10.31083/FBL39259

*Original Research*

# Norcholic Acid Promotes M1 Macrophage Polarization in Acute Pancreatitis by Activating the Wnt/$\beta$-Catenin Pathway

Xingyu Liu[1,†], Jun Yu[1,†], Junning Liu[1,2], Qing Zhou[1], Linfeng Yang[1], Qian Dai[1], Jianshui Li[1,3], Chuan Lan[1,3,*], Dawei Deng[1,3,*]

[1]Department of Clinical Medicine, North Sichuan Medical College, 637000 Nanchong, Sichuan, China
[2]Department of Cardiovascular Surgery, Beijing Anzhen Nanchong Hospital of Capital Medical University & Nanchong Central Hospital, The Second Clinical Medical College of North Sichuan Medical College, 637000 Nanchong, Sichuan, China
[3]Department of Hepatobiliary and Pancreatic Surgery, Affiliated Hospital of North Sichuan Medical College, 637000 Nanchong, Sichuan, China
*Correspondence: hxlanchuan@163.com (Chuan Lan); ddwtougao@163.com (Dawei Deng)
[†]These authors contributed equally.

Academic Editors: Alfredo Budillon and Natascia Tiso
Submitted: 25 March 2025 Revised: 8 May 2025 Accepted: 27 May 2025 Published: 24 June 2025

**Abstract**

**Background**: Acute pancreatitis (AP) is a common gastrointestinal emergency and critical condition worldwide. Given the absence of specific therapeutic targets, managing the progression of AP to severe phases and the accompanying systemic inflammatory response remains challenging. We detected an abnormally elevated expression of norcholic acid (NorCA) in the serum of patients with various types of AP and found that this bile acid is closely associated with the Wnt/$\beta$-catenin signaling pathway in the context of AP. This study was designed to investigate NorCA's dual role as a novel diagnostic biomarker and molecular therapeutic target in AP, with particular emphasis on elucidating its mechanistic regulation of M1 macrophage polarization in RAW 264.7 murine macrophages during AP pathogenesis. **Methods**: Serum samples from AP patients were collected and screened to identify the levels of NorCA and the extent of metabolic abnormalities using bile acid targeting detection. Transcriptome sequencing and bioinformatics analyses were conducted to investigate the role of the Wnt/$\beta$-catenin pathway. To evaluate NorCA's regulatory effect on M1 macrophage polarization through the Wnt/$\beta$-catenin signaling pathway in AP development, we employed flow cytometry, western blotting, and qRT-PCR analyses. **Results**: NorCA demonstrated a significant elevation in the peripheral blood across different AP subtypes, showing promising diagnostic potential with high sensitivity and specificity. NorCA promotes the polarization of M1 macrophages by activating the Wnt/$\beta$-catenin pathway, leading to further inflammation. Treatment with JW74, a specific Wnt/$\beta$-catenin inhibitor, significantly reduced the degree of NorCA-induced M1 macrophage polarization. **Conclusion**: NorCA demonstrates dual clinical utility as both a novel diagnostic biomarker for AP and a promising molecular target for therapeutic intervention in severe AP and its concomitant systemic inflammatory response syndrome (SIRS).

**Keywords:** pancreatitis; norcholic acid; systemic inflammatory response syndrome; Wnt/$\beta$-catenin; macrophages

## 1. Introduction

Acute pancreatitis (AP) is a common gastrointestinal emergency and critical condition worldwide that is characterized by rapid onset and progression [1]. The prevalence of AP has risen significantly in recent years. Intravenous fluid resuscitation (IVFR) is the primary early treatment for this disease, and aggressive volume resuscitation is a crucial aspect of therapy [2]. However, due to the absence of targeted therapies, approximately 20% of AP cases progress to severe acute pancreatitis (SAP). These patients frequently develop systemic inflammatory response syndrome (SIRS) and subsequent multiple organ dysfunction syndrome (MODS), carrying a significant mortality risk of 20–30% [3]. Consequently, the identification of novel therapeutic targets to mitigate inflammatory cascades in AP represents a critical unmet clinical need.

SIRS exhibits a well-established pathophysiological correlation with both the progression and critical outcomes of AP [4–6]. During the course of acute pancreatitis, peripheral blood macrophages undergo polarization and differentiate into pro-inflammatory M1 and M2 macrophages that exhibit anti-inflammatory and tissue repair functionality [7]. AP induces the activation and phenotypic polarization of pancreatic macrophages toward the pro-inflammatory M1 subtype, resulting in substantial release of inflammatory cytokines and mediators that ultimately trigger localized pancreatic inflammation [7]. Furthermore, macrophage polarization is closely related to AP-associated lung and kidney injuries [8,9]. A previous study has shown that regulating the polarization state of macrophages can significantly improve the systemic inflammatory response in AP [10]. Thus, regulation of macrophage polarization may provide new interventions for the occurrence and development of AP.

It is well known that biliary pancreatitis accounts for 30–50% of the total incidence of AP, and bile acid

metabolism disorders are a typical feature of AP that are closely related to a poor prognosis in patients [11]. Compelling evidence demonstrates that bile acids play a central role in AP pathogenesis by initiating intracellular $Ca^{2+}$ overload, which precipitates mitochondrial membrane potential collapse and ultimately leads to programmed necrosis of acinar cells [12]. However, the relationship between abnormal bile acid metabolism and macrophage polarization in the systemic inflammatory response caused by AP is yet to be elucidated. Therefore, investigating the effect of bile acids on macrophage polarization in AP is of great significance for the intervention of severe AP and its associated systemic inflammatory responses.

In our study, we found that the levels of norcholic acid (NorCA), a nordeoxycholic acid, were significantly elevated in the peripheral blood of patients with AP. In this study, we hypothesized that an appropriate concentration of NorCA could further promote the release of inflammatory factors from M1 macrophages, thereby exacerbating the inflammatory progression of AP. Our findings provide a new perspective on the treatment of AP.

## 2. Materials and Methods

### *2.1 Sample Collection and Inclusion/Exclusion Criteria*

Fasting serum samples were collected from all 100 participants during morning rounds following hospital admission. Based on the 2012 Atlanta classification criteria, AP patients were stratified into three clinical severity groups: (1) mild AP (MAP) characterized by absence of both organ failure and local/systemic complications; (2) moderately severe AP (MSAP) defined by transient organ failure (<48 hours) and/or localized complications; and (3) severe AP (SAP) featuring persistent organ failure (>48 hours) regardless of complication status. Exclusion criteria included patients with rheumatic diseases, neoplastic diseases, gastrointestinal diseases, chronic kidney diseases, or liver diseases, as well as patients whose AP symptoms had persisted for more than 48 h. The healthy control cohort was prospectively matched with AP patients using the following matching criteria: age (within 3-year strata), sex distribution, and BMI categories (WHO classification). Strict exclusion criteria comprised: (a) history of chronic diseases affecting major organ systems; (b) pregnancy/lactation; and (c) use of metabolically active medications within the past 3 months. All collected serum samples were immediately aliquoted into 500 µL cryovials and preserved at –80 °C in ultralow temperature freezers (Thermo Fisher Scientific, Waltham, MA, USA) until subsequent biochemical analyses.

Patients or their families/legal guardians informed consent was obtained for specimen collection, which was approved and supervised by the Ethics Committee of North Sichuan Medical College Affiliated Hospital (Approval Number: 2021ER133-1).

### *2.2 The Targeted Detection of Bile Acids*

Serum samples (80 µL) were aliquoted into 96-well plates and mixed with 320 µL of methanol:acetonitrile (1:1, v/v) spiked with 50 ng/mL bile acid isotope internal standards. Following 5-min vortexing, samples underwent centrifugation (5300 rpm, 20 min, 4 °C), with 260 µL supernatant aliquoted into 450 µL 96-well plates for drying. The resulting extracts were reconstituted for targeted bile acid metabolomic analysis. Scanning and quantification were performed using an Acquity UPLC (Waters, Milford, MA, USA) and a Sciex 5500+ triple quadrupole mass spectrometer (Sciex, Framingham, MA, USA) in tandem. The bile acid extract was injected at a volume of 2.5 µL and separated on a C18-PFP (3 µm, 2.1 × 50 mm) chromatographic column. The mobile phase system comprised: (A) 2 mM ammonium acetate in water and (B) acetonitrile. The gradient elution program progressed as follows: 17% to 30% B over 10 min, then to 55% B in 3 min, rapidly increased to 95% B in 1 min (held for 3 min), followed by a 5-min re-equilibration. Chromatographic separation was performed at 0.4 mL/min with metabolites ionized via TurboV-heated electrospray ionization (ESI) and detected in multiple reaction monitoring (MRM) mode, using the following optimized parameters: negative ion spray voltage (–4.5 kV), curtain gas (35 psi), and ion source temperature (550 °C).

### *2.3 Cell Culture*

The murine macrophage cell line RAW264.7 was obtained from SUNNCELL (Wuhan, China) and maintained in high-glucose DMEM (C3113-0500, VivaCell Biosciences, Shanghai, China) supplemented with 5% (v/v) heat-inactivated fetal bovine serum (FBS, Biological Industries, Kibbutz Beit Haemek, Israel) and 1% (v/v) penicillin-streptomycin solution (Biological Industries, Kibbutz Beit Haemek, Israel). Cell cultures were grown at 37 °C in a 5% $CO_2$ humidified incubator. Cells at 70% confluence were divided into four groups (n = 6/group): (1) Vehicle (DMSO 10 µL/mL, 12 h); (2) Lipopolysaccharide (LPS, Sigma-Aldrich, St. Louis, MO, USA) (1 µg/mL, 12 h); (3) LPS + NorCA (Sigma-Aldrich, St. Louis, MO, USA, 1 + 10 µg/mL, 12 h); (4) LPS/NorCA→JW74 (MedChemExpress, Monmouth Junction, NJ, USA, 1/10 µg/mL→10 µg/mL, 12 h each phase). The cell line utilized in this study was authenticated by short tandem repeat (STR) profiling and confirmed that the mycoplasma test result was negative.

### *2.4 Cell Toxicity Assays*

RAW264.7 cell viability after NorCA treatment was quantified using a commercial CCK-8 kit (K1018, APExBIO Technology LLC, Houston, TX, USA) per the standard protocol. Briefly, RAW264.7 cells were seeded in 96-well plates at a density of 5000 cells/well and treated with six different concentrations of NorCA (0, 2.5, 5, 10, 15, and 20 µg/mL) in culture medium. After 24 h incuba-

**Table 1. Primer sequences used for quantitative PCR (qPCR).**

| Targets | Forward | Reverse |
|---|---|---|
| *CD11c* | CTGGATAGCCTTTCTTCTGCTG | GCACACTGTGTCCGAACTCA |
| *CD86* | TCAATGGGACTGCATATCTGCC | GCCAAAATACTACCAGCTCACT |
| *GAPDH* | CAGTGGCAAAGTGGAGATTGTTG | TCGCTCCTGGAAGATGGTGAT |
| *β-catenin* | ATGGAGCCGGACAGAAAAGC | TGGGAGGTGTCAACATCTTCTT |

tion, cells received CCK-8 reagent (10% in medium) for 4 h at 37 °C. Cell viability was quantified by measuring absorbance at 450 nm using a microplate reader (M1000 PRO, Tecan, Männedorf, Switzerland), and the maximum effective concentrations of the two bile acids on RAW264.7 cells were calculated. This experiment was repeated thrice.

### *2.5 Flow Cytometry Analysis*

RAW264.7 murine macrophages were cultured in DMEM (C3113-0500, VivaCell Biosciences, Shanghai, China) supplemented with 10% heat-inactivated FBS and maintained at 37 °C in a humidified 5% $CO_2$ incubator. At >80% confluence, cells were randomized into four experimental groups: (1) Vehicle control (1% DMSO), (2) LPS stimulation (1 µg/mL), (3) LPS (1 µg/mL) + NorCA (10 µg/mL) co-treatment, and (4) LPS + NorCA (1 + 10 µg/mL) priming followed by JW74 (10 µg/mL) treatment. The cells were maintained under these culture conditions for 24 hours. Subsequently, RAW264.7 macrophages were PBS-washed (3×, 5 min each, pH 7.4) and centrifuged (300 ×g, 5 min, 4 °C) for harvesting. Cell pellets were fixed with freshly prepared 4% paraformaldehyde (PFA) (BL539A, Biosharp, Nanjing, China) in PBS for 15 min at room temperature (22–25 °C) with gentle agitation. Following fixation, residual PFA was completely removed through three additional PBS washes (5 min each) to ensure proper cellular preparation for downstream applications. The washed cells were incubated with fluorescence-labeled anti-CD86 (11-0862-82, Invitrogen, Carlsbad, CA, USA) and anti-CD11c antibodies (14-0114-82, Invitrogen, Carlsbad, CA, USA) at room temperature for 30 minutes. After staining, cells were washed three times with PBS to remove unbound antibodies, followed by analysis using a Novocyte flow cytometer (ACEA Biosciences, San Diego, CA, USA) to quantify surface marker expression.

### *2.6 Quantitative Real-Time Polymerase Chain Reaction (qPCR)*

Total RNA from each group of cells was isolated using the TRIzol reagent (R401-01, Vazyme, Nanjing, China). RNA samples underwent reverse transcription with HiScript III RT SuperMix for qPCR (+gDNA wiper) (Vazyme), followed by quantitative PCR employing ChamQ Universal SYBR qPCR Master Mix (Vazyme) per manufacturer's protocol. Quantitative PCR was carried out on a LightCycler 96 System (Roche Diagnostics, Basel, Switzerland) using gene-specific primers (sequences provided in Table 1).

Finally, the relative expression levels of target genes across experimental groups (DMSO control, LPS, LPS + NorCA, and LPS + NorCA + JW74) were quantified using the $2^{-\Delta\Delta Ct}$ method with GAPDH normalization.

### *2.7 Western Blot Analysis*

RAW264.7 cells were treated for 24 h with DMSO (10 µL/mL), LPS (1 µg/mL), LPS (1 µg/mL) + NorCA (10 µg/mL), or LPS (1 µg/mL) + NorCA (10 µg/mL) + JW74 (10 µg/mL). After treatment, cells were lysed in 1 mL RIPA buffer (R0010, Solarbio, Beijing, China) supplemented with 10 µL PMSF (P0100, Solarbio, Beijing, China) for protein extraction. Cell lysate protein quantification was performed using a commercial BCA assay kit (PC0020, Solarbio, Beijing, China) following standard protocols. Subsequently, 25 µg of protein from each lysate was separated via SDS-PAGE (10% gel) and transferred to PVDF membranes at 240 mA for 1.5 hours for subsequent immunoblotting analysis. Blocked PVDF membranes (protein-free buffer) underwent TBST washing before overnight 4 °C incubation with CD86/CD11c/β-catenin/β-tubulin antibodies (DF6332/DF2542/AF7011/AF6266, Affinity Biosciences, Nanjing, China; 1:1000 in Beyotime buffer). Following triple TBST washes, membranes received HRP-anti-rabbit secondary antibody (S0001, Affinity Biosciences, Nanjing, China; 1:3000) for 1 h at room temperature (RT), using β-tubulin as loading control. The protein signals were visualized using Immobilon® HRP substrate (Millipore Corporation, Burlington, MA, USA) and imaged on an Amersham Imager 600 (GE Healthcare, Chicago, IL, USA).

### *2.8 Transcriptome Sequencing and Differentially Expressed Genes (DEGs) Analysis*

Transcriptome sequencing of the DMSO, LPS, and LPS + NorCA groups of RAW264.7 cells was performed by Shanghai LingEn Biotechnology Co., Ltd. (Shanghai, China) Total RNA was extracted from the three groups of RAW264.7 cells using the TRIzol method. We next measured the concentration and purity of the extracted total RNA using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Subsequent library preparation was performed following successful quality control assessment. The constructed cDNA libraries underwent paired-end sequencing on an Illumina NovaSeq 6000 system (Illumina, San Diego, CA, USA), generating 150 bp average read lengths. Subsequently, the expression differences between samples for each transcript

were analyzed using edgeR (https://bioconductor.org/packages/release/bioc/html/edgeR.html) to identify differentially expressed genes (DEGs). The screening criteria for significant DEGs comprised a minimum 2-fold change (log2 scale) and 0.05 *p*-value cutoff.

### *2.9 Pathway Enrichment Analysis*

Differential gene expression analysis between the LPS + NorCA-treated group and control groups in LPS-stimulated RAW264.7 cells was conducted using the limma package (R software; https://bioconductor.org/packages/limma/). DEGs were defined as those meeting the threshold criteria of $p < 0.05$ with |log2 fold change| $\geq$1. The ggplot2 (version 3.5.2, https://cran.r-project.org/package=ggplot2) and pheatmap R packages (version 1.0.13, https://cran.r-project.org/web/packages/pheatmap/index.html) were employed to visualize the DEGs through volcano plots and hierarchical clustering heatmaps, respectively. Functional enrichment analysis of the DEGs was performed using the clusterProfiler package in R (version 4.16.0, https://bioconductor.org/packages/clusterProfiler) to identify statistically significant pathways. Gene ontology (GO) term categorization (BP/CC/MF) was performed followed by graphical representation. The false discovery rate-adjusted *p*-value (referred to as the Q-value) was determined, and a cutoff of $<$0.01 was applied for GO analysis. GO enrichment analysis was performed using the Goatools software (https://github.com/tanghaibao/GOatools) using Fisher's exact test. DEGs meeting the cutoff criteria (log2-fold change $\geq$2; adjusted $p \leq 0.05$) were considered significant.

### *2.10 Statistical Analysis*

Statistical analyses were performed using GraphPad Prism 9.4 (GraphPad Software, San Diego, CA, USA), with data expressed as mean $\pm$ SD. Intergroup differences were analyzed using one-way ANOVA followed by Tukey's multiple comparisons test (significance threshold: $p < 0.05$).

## 3. Results

### *3.1 NorCA Can Serve as a Potential Biomarker for Diagnosing and Assessing the Severity of AP*

NorCA has a relative molecular weight of 394.5 g/mol, its molecular structure is shown in Fig. 1a; and its structural formula is shown in Fig. 1a. In addition, the mass-to-charge ratio of the molecular ion during the targeted detection was 393.3; this molecular ion exhibited the highest peak at a retention time of 6.127 min (Fig. 1b). NorCA did not have any structural isomers within the adjacent retention time, making it less prone to interference in peak area calculations, and thus highly reliable for absolute concentration quantification. NorCA demonstrated significant differential expression between healthy controls and AP patients, as well as across AP subgroups ($p < 0.05$), suggesting its potential as a diagnostic biomarker. As shown in Fig. 1c, the serum levels of NorCA in patients with AP were significantly higher than those in the healthy control group. When subdividing patients with AP based on the etiology of the disease, it was evident that the concentrations of NorCA in the serum of patients with biliary AP (BAP) (Fig. 1d), hyperlipidemic AP (HLAP) (Fig. 1e), and idiopathic AP (Fig. 1f) were all significantly higher than those in the healthy control group. Furthermore, NorCA serum levels demonstrated significant upregulation in SAP compared to MSAP cases (Fig. 1g). Therefore, NorCA could serve as a potential biomarker for diagnosing and assessing the severity of AP. ROC curve analysis revealed NorCA's strong diagnostic value for AP (AUC 0.905), achieving 82.5% sensitivity and 85% specificity (Fig. 1h). For AP diagnosis in amylase-negative patients, the ROC analysis yielded an AUC of 0.847, showing 82.5% sensitivity and 77.8% specificity (Fig. 1i), strongly supporting the feasibility of NorCA as a complementary biomarker to amylase, a commonly used clinical diagnostic marker for AP. Moreover, Moreover, NorCA demonstrated discriminative capacity between MSAP and SAP (AUC = 0.702), with 93.3% sensitivity and 60% specificity (Fig. 1j).

### *3.2 NorCA Further Promoted the Expression of Surface Markers of M1 Macrophages During the Progression of AP*

To establish a cellular model of AP, we first induced RAW264.7 cells to differentiate from the M0 state to pro-inflammatory M1 macrophages by administering LPS (**Supplementary Fig. 1a,b,d–f**). Subsequently, we evaluated the cytotoxic effects of NorCA on the macrophages. We selected a concentration of 10 µg/mL of NorCA for this experiment because it had a minimal impact on cell viability (**Supplementary Fig. 1c**). At this concentration, flow cytometric analysis revealed markedly elevated CD86 and CD11c expression levels in NorCA-treated cells relative to LPS-only controls Fig. 2a–c). Quantitative RT-PCR analysis revealed significantly elevated mRNA expression of M1 macrophage markers CD86 and CD11c in the LPS + NorCA group compared to LPS-only controls (Fig. 2d,e). Protein imprinting analysis confirmed these results (Fig. 2f–h).

### *3.3 Differential Expression Analysis Demonstrated Significant Modulation of Wnt-Associated Genes by NorCA Intervention*

Next, we analyzed the correlation between DEGs and the Wnt pathway based on sequencing data from the LPS and LPS + NorCA groups. As shown in Fig. 3a, we first validated the differences in gene expression in the sequencing data from RAW264.7 cells between the DMSO, LPS, and LPS + NorCA groups using PCA (Principal Component Analysis). Next, we plotted a volcano plot (Fig. 3b) to demonstrate the expression of DEGs between control and NorCA-treated groups. In addition, we generated a heatmap (Fig. 3c) to visualize the extent of the upregulation or downregulation of certain DEGs. Of these genes, CTNNB1, an important gene encoding the Wnt pathway

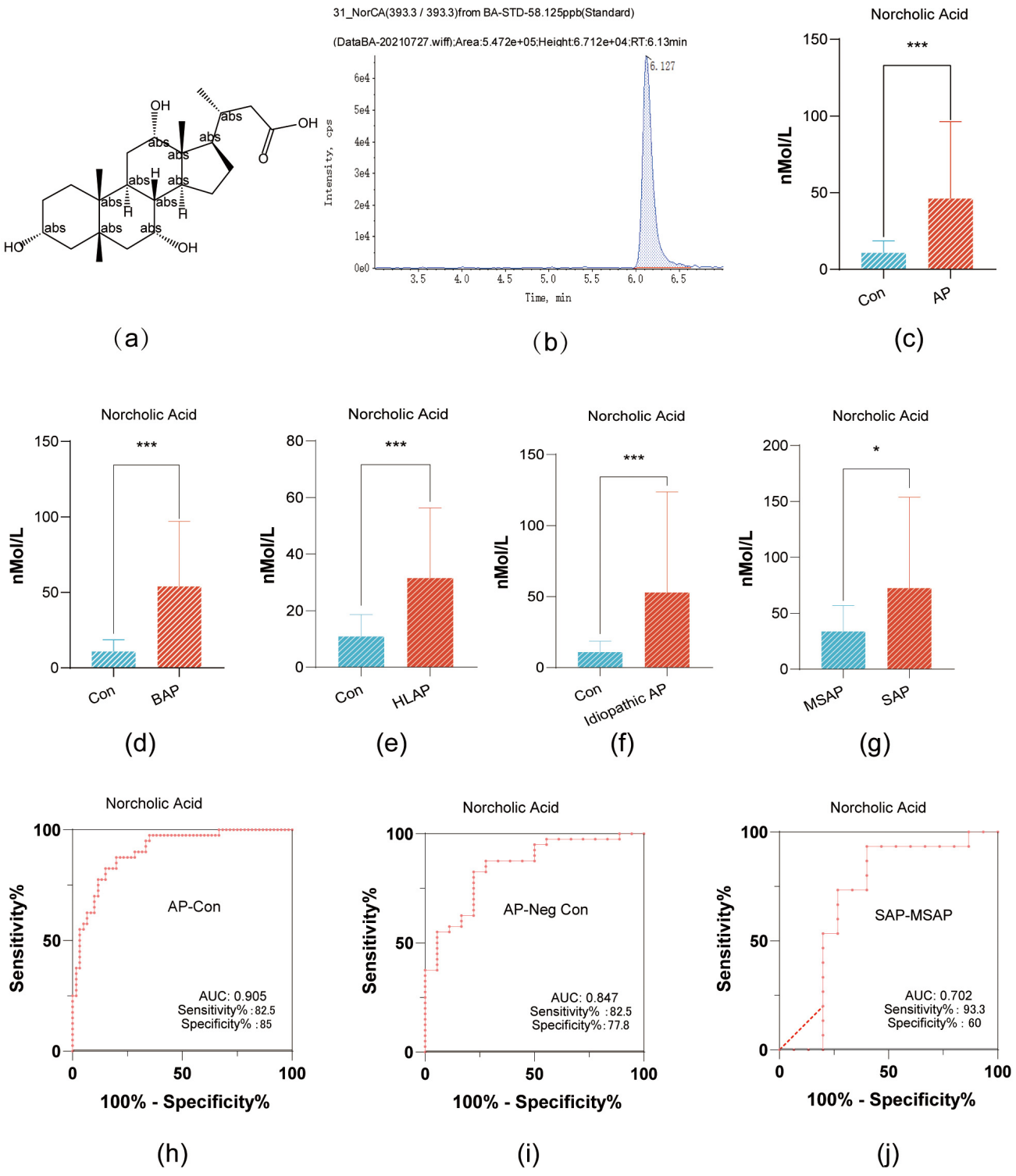


**Fig. 1. NorCA Expression in Pancreatitis Variants and Its Diagnostic Accuracy.** (a) The chemical structure of NorCA. (b) The mass-to-charge ratio and chromatographic separation time of molecular ions in NorCA. (c) Comparative serum NorCA concentrations: AP patients vs healthy controls (bar graph). (d) BAP and control serum NorCA quantification (bar graph). (e) Serum NorCA comparison: HLAP vs healthy controls (bar graph). (f) Bar graph showing the difference in serum NorCA levels between idiopathic pancreatitis and healthy controls. (g) Bar graph showing the difference in serum NorCA levels between severe acute pancreatitis (SAP) and moderately severe acute pancreatitis (MSAP) groups. (h) The area under the ROC curve, sensitivity, and specificity of NorCA as a diagnostic biomarker for AP. (i) The area under the ROC curve, sensitivity, and specificity of NorCA for diagnosing AP patients with negative amylase. (j) The area under the ROC curve, sensitivity, and specificity of NorCA for differentiating SAP and MSAP. *$p < 0.05$, ***$p < 0.001$. NorCA, norcholic acid; AP, acute pancreatitis; BAP, biliary AP; HLAP, hyperlipidemic AP; ROC, receiver operating characteristic.

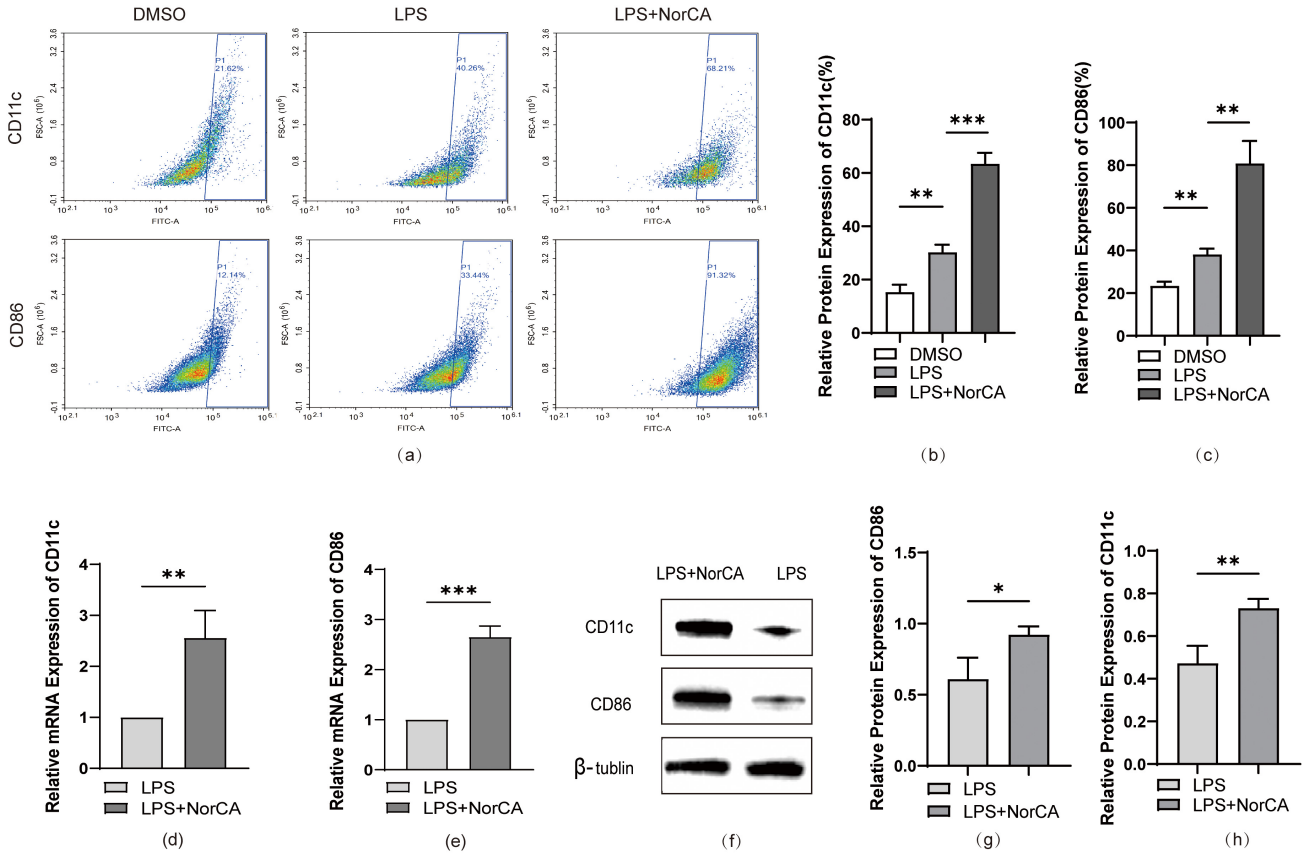


**Fig. 2. NorCA enhances LPS-induced M1 macrophage polarization.** (a) Flow cytometric analysis quantified CD86/CD11c expression in LPS and LPS + NorCA groups. (b,c) Flow cytometry-derived CD86/CD11c expression in LPS vs LPS + NorCA groups (bar graphs). (d,e) qRT-PCR analysis quantified CD86/CD11c expression in LPS vs LPS + NorCA groups. (f–h) The expression levels of CD86 and CD11c in the LPS group and LPS + NorCA group were detected by western blotting. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. LPS, lipopolysaccharide; qRT-PCR, quantitative reverse transcription PCR; FITC-A, fluorescein isothiocyanate - area; FSC-A, forward scatter - area.

protein $\beta$-catenin, was significantly different between the control and NorCA-treated groups (Fig. 3g).

Based on these findings, we conducted gene ontology (GO) term analysis of the sequencing data, including biological processes (BPs) (Fig. 3d), cellular components (CCs) (Fig. 3e), and molecular functions (MFs) (Fig. 3f). We identified a significant enrichment of Wnt-related genes. These findings suggest NorCA likely mediates pro-inflammatory responses in AP through canonical Wnt/$\beta$-catenin signaling. To validate this, we performed RT-qPCR (Fig. 3h) and western blotting (Fig. 3i,j), thus confirming that NorCA activated the $\beta$-catenin pathway protein.

### *3.4 The Wnt/β-Catenin Pathway Blockade Markedly Attenuated NorCA-Induced M1 Macrophage Polarization in AP Progression*

To validate our hypothesis, we added a specific Wnt/$\beta$-catenin pathway inhibitor, JW74, to the LPS + NorCA group. We first analyzed the expression levels of CD86 and CD11c in the LPS, LPS + NorCA, and LPS + NorCA + JW74 groups using flow cytometry (Fig. 4a). The analysis showed a significant reduction in the expression levels of both surface markers after addition of the pathway inhibitor (Fig. 4b,c). Next, qRT-PCR was performed to measure the expression levels of CD86 and CD11c in the LPS, LPS + NorCA, and LPS + NorCA + JW74 groups (Fig. 4d,e). Consistent with this pattern, JW74 co-treatment significantly downregulated both CD86 and CD11c surface expression compared to LPS + NorCA controls. Western blot analysis yielded similar results (Fig. 4f–h). Finally, we present the basic experimental section in a flowchart (Fig. 5).

## 4. Discussion

AP is a common, acute, and severe inflammation of the digestive tract. Owing to the lack of early warning markers and effective specific therapeutic targets, AP often leads to a poor prognosis. Therefore, a thorough understanding of the pathological process of AP is of great research significance and clinical value for accurate diagnosis, prevention, treatment, and prognosis. In this study, we

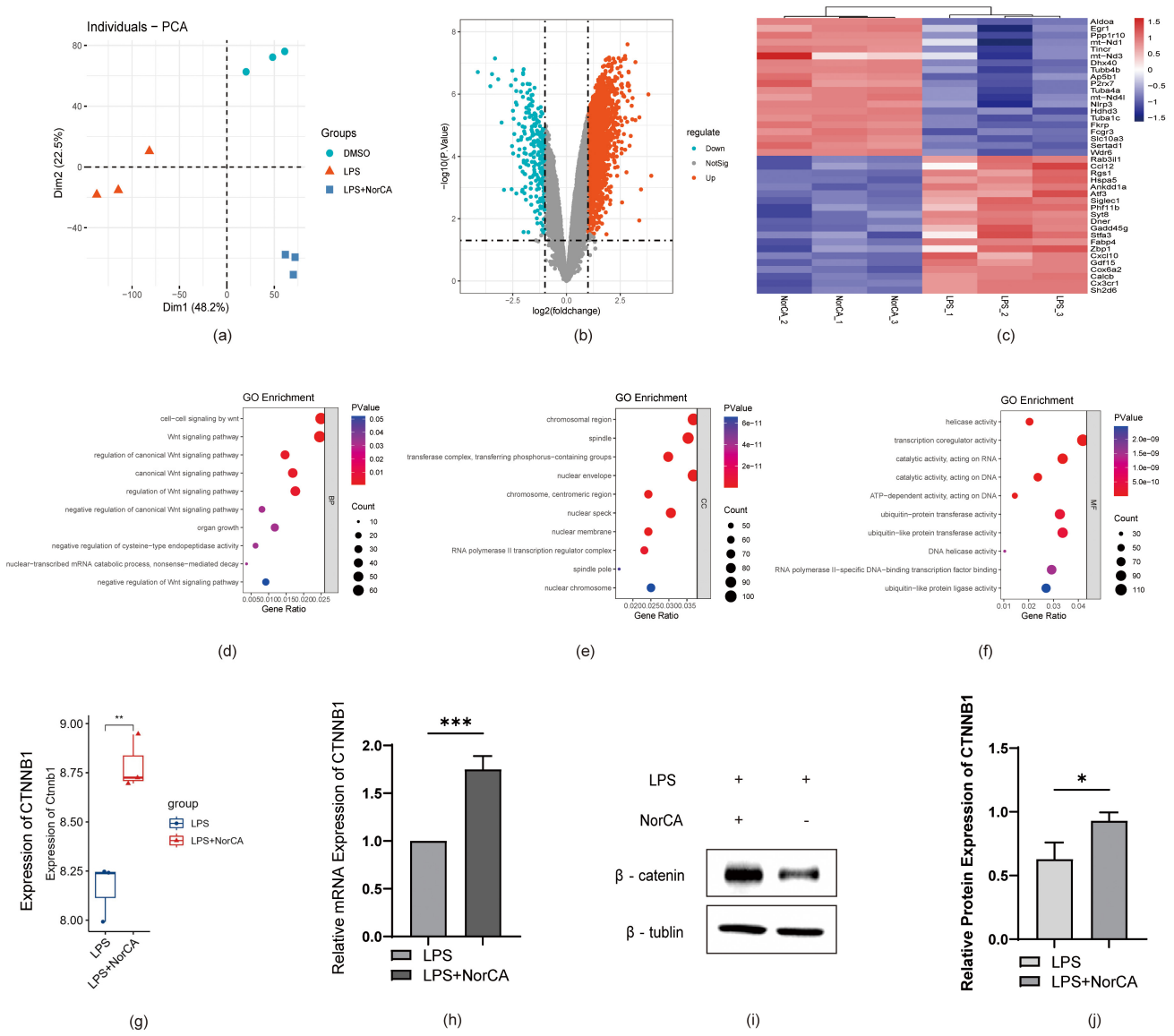


**Fig. 3. RNA-seq reveals Wnt-related gene alterations, with NorCA activating Wnt/$\beta$-catenin via CTNNB1 upregulation.** (a) Principal Component Analysis (PCA) was performed on transcriptomic data to evaluate gene expression variations across DMSO controls, LPS-treated, and LPS + NorCA groups. (b) Volcano plot demonstrating NorCA-modulated gene expression changes in LPS-stimulated conditions. (c) Heatmap showing the expression levels of the top 20 upregulated and downregulated genes in the LPS group and LPS + NorCA group in the sequencing data. Gene Ontology (GO) enrichment analysis of Wnt pathway-associated DEGs in (d) biological processes, (e) cellular components, and (f) molecular functions. (g) Box plot of Ctnnb1 (Wnt/$\beta$-catenin pathway) expression comparing LPS-treated and LPS + NorCA groups. (h) The expression levels of CTNNB1 in the LPS group and the LPS + NorCA group were detected by qPCR-RT. (i,j) Western blot analysis quantified $\beta$-catenin expression in LPS-treated versus LPS + NorCA groups. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. DEGs, differentially expressed genes.

investigated the relationship between bile acid metabolism disorders and the progression and severity of AP for the first time. Our study results showed that NorCA is abnormally elevated in various types of AP progression and exhibits high sensitivity and specificity as a clinical diagnostic and prognostic marker for AP. Moreover, elevated levels of NorCA can promote the polarization of M1 macrophages via the Wnt/$\beta$-catenin pathway during the systemic inflammatory response caused by AP, thereby acting as an inflammatory driver. Our experimental data demonstrate that inhibition of the Wnt/$\beta$-catenin signalling pathway can effectively reduce the systemic inflammatory response associated with NorCA-induced AP.

Bile acids (BAs) are metabolic products synthesized from cholesterol in the liver *via* both classical and non-classical pathways [13]. Increasing evidence suggests that various types of BAs are closely associated with numerous digestive disorders, inflammation, and even tumors. Re-

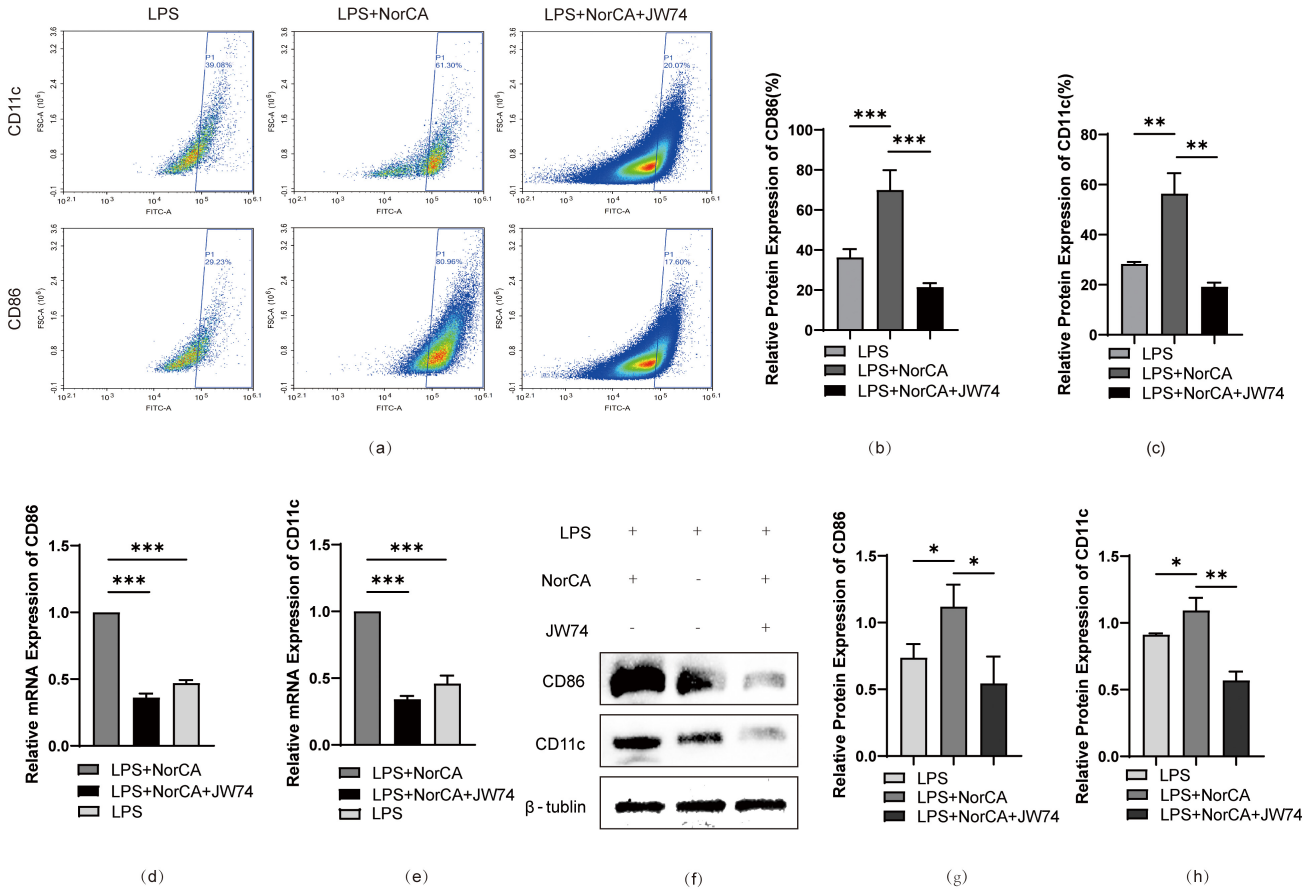


**Fig. 4. Inhibition of Wnt/$\beta$-catenin signaling significantly attenuates NorCA-mediated M1 macrophage polarization.** (a–c) Flow cytometric analysis quantified CD86/CD11c expression across LPS, LPS + NorCA, and LPS + NorCA + JW74 groups. (d,e) qRT-PCR quantified CD86/CD11c expression in LPS, LPS + NorCA, and LPS+NorCA+JW74 groups. (f–h) The expression levels of CD86 and CD11c in the LPS group, LPS + NorCA group, and the LPS + NorCA + JW74 group were detected by western blotting. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

search has shown that patients with inflammatory bowel disease [14] experience varying degrees of BA metabolism disorders, which are also associated with a poor prognosis. The excessive accumulation of BAs in cells has cytotoxic effects and is considered to be one of the most important factors in tumor development. Furthermore, BA metabolic disorders can promote the occurrence of hepatocellular carcinoma associated with obesity and fatty liver [15]. NorCA, also known as nordeoxycholic acid, is a metabolite of BAs and belongs to the category of organic compounds containing trihydroxy bile acids, alcohols, and derivatives. NorCA is a highly selective and potent agonist of farnesoid X receptor (FXR). NorCA is biotransformed by bacteria in the intestine and primarily exists in the free form in the bile [16]. Furthermore, NorCA helps regulate various physiological processes in the human body, including bile acid metabolism, lipid metabolism, glucose metabolism, and inflammatory response. However, to date, researchers still have a limited understanding of the pathogenic effects of NorCA on the human body. In the 1970s, Almé *et al.* [17] detected and quantified NorCA in the urine of healthy controls and patients with congenital intrahepatic cholestasis. Subsequently, NorCA was found to be the major trihydroxy BA present in the urine of patients with liver cirrhosis, suggesting that elevated levels of NorCA may be associated with liver function failure [18]. In 1985, NorCA was discovered in patients with an extremely rare genetic disorder called cerebrotendinous xanthomatosis, which is characterized by the accumulation of cholestanol in various tissues, reduced bile acid synthesis, and excessive accumulation of cholesterol [19]. In recent years, elevated levels of NorCA have been detected in drug-induced liver injury (DILI), suggesting that NorCA may serve as a specific biomarker for predicting the severity of DILI [20,21]. However, researchers are yet to investigate the significance of NorCA-related data and its effects and mechanisms in disease. However, a recent study showed that NorCA can promote the progression and escape of Hepatocellular Carcinoma (HCC) *via* negative feedback regulation of the FXR receptor [22], thus confirming the pathogenic effects and mechanisms of NorCA in the human body for the first time. Consistent with existing literature documenting NorCA up-

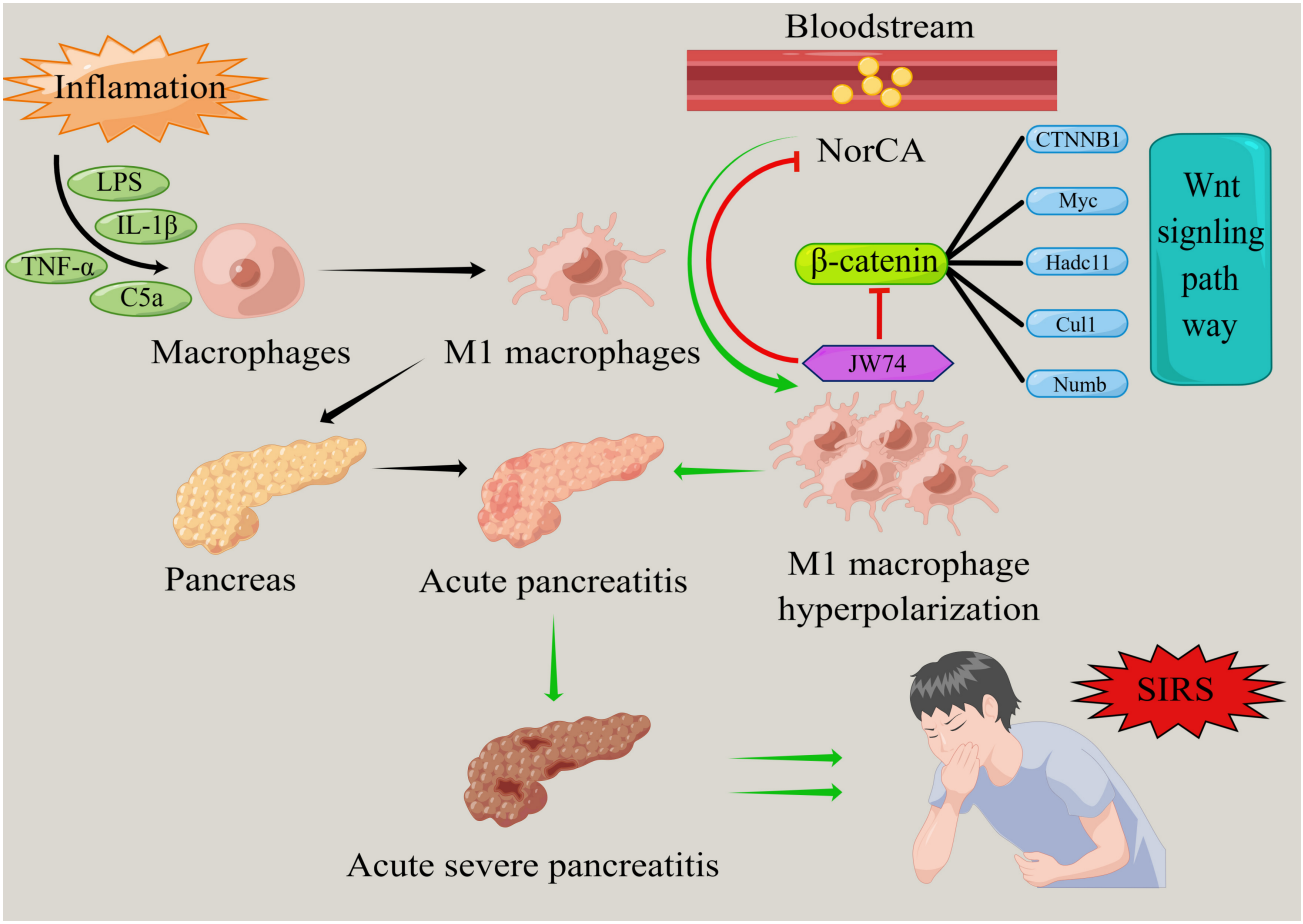


**Fig. 5. Related experimental mechanism.** By Figdraw.

regulation in disease states, we observed statistically significant NorCA expression differences both between healthy controls and AP patients, and across AP clinical subtypes. Subsequent validation experiments established NorCA's sustained upregulation of pro-inflammatory cytokines in M1-polarized macrophages, which exacerbated both AP progression and systemic inflammation. Analytical results identified NorCA as a key pro-inflammatory mediator contributing to AP pathogenesis, leading us to postulate its potential as a specific biomarker for AP severity and prognosis prediction.

The Wnt signalling pathway is a complex and widespread cellular signalling pathway in the human body. The Wnt/$\beta$-catenin pathway has been a focus of research as it has been shown to represent a potential regulatory target in a range of tumor diseases such as breast cancer, lung cancer, and haematological malignancies [23]. Related studies have confirmed that the Wnt/$\beta$-catenin pathway is a tumor suppressor pathway in diseases such as pancreatic ductal adenocarcinoma [24], esophageal cancer [25], colon cancer [26], and glioblastoma [27]. Similarly, the Wnt pathway has been shown to be involved in the occurrence of inflammation and can regulate the activation of macrophages [28]; these previous findings concur with those of our present study. Transcriptomic analysis of RAW264.7 cell variants revealed Wnt pathway-associated differentially expressed genes (DEGs), with CTNNB1 showing significant upregulation in LPS+NorCA-treated cells versus LPS controls. These results were validated by western blotting. Additionally, we observed that the presence of NorCA not only promoted the polarization of M1 macrophages but also continuously activated the Wnt/$\beta$-catenin pathway. Upon addition of the specific Wnt/$\beta$-catenin inhibitor JW74, the expression levels of cell surface markers associated with M1 macrophages (CD86 and CD11c) were significantly reduced. These results demonstrate that NorCA potentiates M1 macrophage polarization through Wnt/$\beta$-catenin pathway activation, thereby exacerbating acute pancreatitis progression and systemic inflammatory cascade. In this study, we successfully demonstrated the role of NorCA as a pro-inflammatory driver in the progression of AP, and that it may serve as a specific marker for determining the severity and prognosis of AP. Furthermore, our validation of the NorCA-Wnt/$\beta$-catenin axis provides novel therapeutic perspectives for managing both AP and its systemic inflammatory complications.

However, several limitations should be acknowledged in this study. First, the experimental validation was exclu-

sively performed *in vitro*, lacking *in vivo* confirmation. Second, although we demonstrated that NorCA modulates AP progression via the Wnt/$\beta$-catenin signaling pathway, the underlying regulatory network and molecular crosstalk remain to be elucidated. Most notably, our flow cytometry and Western blot analyses consistently showed that pharmacological inhibition of the Wnt/$\beta$-catenin pathway effectively reversed LPS-triggered M1 macrophage polarization. These compelling findings strongly suggest the pivotal role of the Wnt/$\beta$-catenin signaling axis in AP pathogenesis, although the precise mechanistic links require systematic exploration in subsequent research.

## 5. Conclusion

Collectively, our findings establish NorCA as a multifunctional mediator in AP pathogenesis with both diagnostic and therapeutic significance. Mechanistically, we provide compelling evidence that NorCA drives AP progression through sustained activation of the Wnt/$\beta$-catenin signaling cascade, resulting in persistent M1 macrophage polarization. Notably, targeted inhibition of this pathway effectively suppressed M1 polarization, subsequently mitigating AP progression, preventing severe complications, and attenuating systemic inflammatory responses. These results not only validate NorCA's dual utility as a novel diagnostic biomarker and severity indicator for AP, but more importantly, reveal its therapeutic potential as a molecular target for intercepting the progression to severe AP and associated systemic inflammatory syndrome.

## Availability of Data and Materials

The data that support the findings of this study are available upon reasonable request from the corresponding author.

## Author Contributions

DD and CL conceived of the experiments. XL, QZ, and JY conducted the experiments; LY, Jianshui L and QD performed the statistical analysis; Junning L performed figure generation. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

## Ethics Approval and Consent to Participate

Informed consent for specimen collection was obtained from all patients or their legal guardians. The study was approved and overseen by the Ethics Committee of the Affiliated Hospital of North Sichuan Medical College (Approval Number: 2021ER133-1). All procedures were conducted in accordance with the principles of the Declaration of Helsinki.


## Acknowledgment

The authors express their gratitude to EditSprings (https://www.editsprings.cn) for providing expert linguistic services.

## Funding

This research was supported by the National Natural Science Foundation of China (Grant No.82300737) and the Office of Science and Technology of Nanchong (No. 22SXQT0110).


## Conflict of Interest

The authors declare no conflict of interest.

## Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/FBL39259.

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