

Lipotoxic inflammation drives pancreatic injury in hypertriglyceridemic acute pancreatitis: a proteomic study in APOE^{-/-} mice

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Abstract

Hypertriglyceridemia-induced acute pancreatitis (HTG-AP) is a severe disease with rising global incidence and limited therapies, but its pathogenesis remains unclear, largely owing to the lack of suitable animal models. In this study, we established an HTG-AP model in *APOE*^{-/-} mice by feeding them a high-fat diet for four months, followed by caerulein injection. Pancreatic injury was assessed using histopathology, serum biochemistry, and inflammatory markers. We then performed proteomic profiling, qPCR, and immunohistochemistry to identify differentially expressed proteins (DEPs). We found that HTG-AP mice showed more severe pancreatic injury, elevated serum amylase, lipase, lactate dehydrogenase, and C-reactive protein levels, reduced calcium and glucose levels, and aggravated liver and lung damage compared to AP mice. Proteomic analysis identified 884 DEPs, with KEGG enrichment revealing PPAR signaling, cholesterol metabolism, and fatty acid metabolism as the most significantly altered pathways. Key proteins, including zinc finger and BTB domain-containing protein 20 (Zbtb20), fatty acid synthase (Fasn), stearoyl-CoA desaturase 1 (Scd1), and apolipoprotein B (ApoB), were upregulated in HTG-AP mice. In conclusion, we established a reliable HTG-AP model in *APOE*^{-/-} mice and demonstrated that HTG exacerbates AP through lipotoxic inflammation, possibly via the Zbtb20/Fasn/Scd1 axis, suggesting a therapeutic target for HTG-AP.

Introduction

Acute pancreatitis (AP) is a devastating inflammatory disease of the exocrine pancreas that can cause pancreatic necrosis and dysfunction of remote organs and systems^[1]. The global incidence of AP is 34 cases per 100,000 population per year, with a mortality rate of 1.16 per 100,000 population per year^[2]. The increasing incidence and severe sequelae of AP, including chronic pancreatitis, post-pancreatitis diabetes mellitus, and pancreatic exocrine and/or endocrine insufficiency, seriously affect the patients' quality of life and impose a growing socioeconomic burden^[3].

The aetiologies of AP include cholelithiasis, ethanol, hypertriglyceridemia (HTG), drugs, and endoscopic retrograde cholangiopancreatography^[3]. With the improvement of living standards, the global incidence of hypertriglyceridemia-induced acute pancreatitis (HTG-AP) is increasing^[4]. In China, HTG has surpassed alcohol consumption as the second leading cause of AP^[5]. One study reported that the incidence of HTG-AP increased from 14.0% in 2001 to 34.0% in 2016 at a tertiary hospital in China^[6]. Compared with AP of other aetiologies, HTG-AP is more likely to result in local and systemic complications, multiple organ dysfunction, and higher mortality^[4]. However, the underlying pathogenesis of HTG-AP remains incompletely understood, largely owing to the scarcity of robust and reproducible animal models^[7].

Apolipoprotein E (ApoE) is a fat-binding protein primarily responsible for lipid transport and cholesterol homeostasis. ApoE gene knockout (*APOE*^{-/-}) mice exhibit significant hyperlipidemia and are internationally recognized as models of dyslipidemia^[8]. Therefore, we used *APOE*^{-/-} mice in

combination with caerulein to generate an HTG-AP model and employed proteomic profiling to explore the underlying mechanisms.

Results

Establishment of HTG model

First, we measured the serum levels of total cholesterol (T-CHO), triglyceride (TG), low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C) in each group. As shown in Fig. 1A, after four months of high-fat diet feeding, APOE mice exhibited significantly increased levels of T-CHO, TG, and LDL-C compared with the normal control (NC) group, confirming that the HTG model was successfully established in *APOE*^{-/-} mice. After caerulein injection, the AP group showed no significant changes in blood lipid levels compared with the NC group, the low-dose caerulein-induced (HTG-APL) group exhibited significantly reduced TG and LDL-C levels compared with the APOE group. The HTG-APL and high-dose caerulein-induced (HTG-APH) groups showed significantly increased T-CHO and LDL-C levels compared with the AP group. The HDL-C levels did not differ significantly among the groups.

We also measured liver function indices and pathological changes. The results showed that the alanine aminotransferase (ALT) levels in the HTG-APL groups were higher than those in the APOE group, and the aspartate aminotransferase (AST) levels in the HTG-APL and HTG-APH groups were higher than those in the APOE group. Moreover, AST levels in HTG-APL and HTG-APH groups were higher than in AP group (Fig. 1A). In the liver, hepatocytes in NC and AP mice were neatly arranged, with no obvious deformation or necrosis. In contrast, APOE, HTG-APL, and HTG-APH mice exhibited marked hepatocyte-induced steatosis. Hepatocellular oedema and twisted hepatic cords were also observed in the HTG-APL and HTG-APH mice (Fig. 1B).

HTG aggravated pancreatic injury in AP

No obvious pathological changes were observed in either C57BL/6 or *APOE*^{-/-} mice injected with saline. In contrast, C57BL/6 mice administered caerulein developed pancreatic injury, including edema, neutrophil infiltration, hemorrhage, and necrosis. Notably, *APOE*^{-/-} mice injected with either a low or high dose of caerulein showed more severe pancreatic injury than AP mice (Fig. 2A).

As serum amylase (AMS), lipase (LPS), and lactate dehydrogenase (LDH) levels are diagnostic and prognostic indicators of AP, we measured their concentrations in all groups. As shown in Fig. 2B, the AP group showed no significant changes in AMS, LPS, or LDH levels compared to the NC group, whereas the HTG-APL and HTG-APH groups exhibited significantly elevated AMS and LPS levels relative to the APOE group. Moreover, the AMS and LPS levels in the HTG-APL group were significantly higher than those in the AP group, and the LDH levels in both the HTG-APL and HTG-APH groups were significantly higher than those in the AP group. Serum calcium (Ca²⁺) levels can predict the severity of AP. In our study, the

Ca²⁺ levels were significantly increased in the APOE group compared to the NC group and significantly reduced in the HTG-APL group compared to the APOE group.

AP is a devastating inflammatory disease; therefore, we also measured serum levels of inflammatory cytokines. As shown in Fig. 2C, the C-reactive protein (CRP) levels were significantly elevated in HTG-APL group compared to the APOE group. In addition, although the CRP and interleukin-6 (IL-6) levels in the HTG-APL and HTG-APH groups showed an increasing trend relative to those in the AP group, the differences were not statistically significant. Serum glucose (GLU) levels were also measured. The APOE group had significantly higher GLU levels than the NC group. After caerulein-induced pancreatic injury, GLU levels in the HTG-APL and HTG-APH groups were significantly reduced compared to those in the APOE group.

Acute lung injury (ALI) is a common and life-threatening complication of severe acute pancreatitis (SAP). Clinical evidence has demonstrated that patients with HTG-AP exhibit a significantly higher incidence of ALI than those with other etiological subtypes of AP [9]. In this study, varying degrees of inflammatory cell infiltration, haemorrhage, and capillary congestion were observed in the alveolar septa of the lungs of APOE, AP, HTG-APL, and HTG-APH mice. Notably, the pathological lesions in the HTG-APL and HTG-APH groups were more severe than those in the APOE and AP groups. These findings indicate that both HTG and caerulein can induce lung damage, and their combination exerts a more pronounced effect on lung pathology than either treatment alone.

Identification of pancreatic tissue proteins in NC, AP, and HTG-APH mice

The PCA score plot (Fig. 3A) revealed clear separation among the NC, AP, and HTG-APH groups based on principal components 1 (PC1) and 2 (PC2), respectively. The small intra-group variability indicated good sample reproducibility. Label-free quantitative analysis identified 76,642 peptides corresponding to 5,003 proteins in the pancreatic tissues of NC, AP, and HTG-APH mice.

The Gene Ontology (GO) database is the world's largest source of gene function information and serves as the foundation for large-scale computational analyses in molecular biology and genetics. The GO database describes genes and gene products from three aspects: biological process (BP), cellular component (CC), and molecular function (MF) [10]. GO functional annotation of the 5,003 identified proteins revealed that the top ten enriched biological processes were as follows: primary metabolic process, organic substance metabolic process, metabolic process, cellular process, cellular metabolic process, organonitrogen compound metabolic process, nitrogen compound metabolic process, cellular component organisation or biogenesis, cellular component organisation, and localisation. According to the CC classification, the ten most significantly enriched cellular components were organelles, membrane-bound organelles, intracellular organelles, intracellular membrane-bound organelles, intracellular anatomical structures, cytosol, cytoplasm, cellular anatomical entities, protein-containing

complexes, and mitochondria. Under the MF classification, the top ten enriched molecular functions were protein binding, catalytic activity, binding, ion binding, small molecule binding, identical protein binding, anion binding, nucleotide binding, nucleoside phosphate binding, and protein-containing complex binding (Fig. 3B).

Further analysis of the subcellular localisation of the 5,003 proteins showed that they were distributed as follows: cytoplasm, membrane, mitochondrion, endoplasmic reticulum, extracellular region, Golgi apparatus, nucleolus, lysosome, centrosome, nuclear membrane, ribosome, and peroxisome (Fig. 3C).

The Kyoto Encyclopedia of Genes and Genomes (KEGG) is a knowledge base for systematic gene function analysis that links genomic information with higher-order functional information^[11]. KEGG pathway analysis revealed that the 5,003 identified proteins were primarily involved in the following pathways: metabolic pathways, diabetic cardiomyopathy, chemical carcinogenesis-reactive oxygen species, amyotrophic lateral sclerosis, carbon metabolism, prion disease, Parkinson disease, oxidative phosphorylation, protein processing in the endoplasmic reticulum, and cytoskeleton in muscle cells (Fig. 3D).

Analysis of differentially expressed proteins (DEPs) between HTG-APH and AP groups

To explore the mechanism of HTG-AP, we further analyzed the DEPs between the HTG-APH and AP groups. A total of 884 DEPs were identified, of which 603 were upregulated and 281 were downregulated. Volcano plots illustrate all upregulated and downregulated proteins, heatmaps display the top 60 upregulated and top 30 downregulated proteins, and the proteins of interest are highlighted in Fig. 4A,B.

KEGG enrichment analysis of the DEPs showed that the top ten most significantly enriched pathways were as follows: complement and coagulation cascades, PPAR signaling pathway, peroxisome, cholesterol metabolism, pyruvate metabolism, ECM-receptor interaction, *Staphylococcus aureus* infection, fatty acid metabolism, fatty acid degradation, and histidine metabolism. Among these, the PPAR signaling pathway, cholesterol metabolism, fatty acid metabolism, and fatty acid degradation are closely related to lipid metabolism (Fig. 4C). The proteins involved in these pathways are shown in Fig. 4D.

Among these, the PPAR signaling pathway was enriched in proteins including *Acs11*, *Cd36*, *Plin4*, *Plin1*, *Me1*, *Scp2*, *Fabp4*, *Acadm*, *Acaa1a*, *Acadl*, *Fabp1*, *Scd1*, *Dbi*, *Adipoq*, *Sorbs1*, *Apoa2*, *Pck1* and *Acsbg1*. Cholesterol metabolism was enriched in proteins including *Apoa4*, *Cd36*, *Apoc1*, *Apoe*, *Apob*, *Lcat*, *Apoh*, *Sort1*, *Tspo*, *Lrpap1* and *Apoa2*. Fatty acid metabolism was enriched in proteins including *Acaca*, *Fasn*, *Acs11*, *Scp2*, *Hsd17b4*, *Acadm*, *Acaa1a*, *Acadl*, *Acadvl*, *Scd1* and *Acsbg1*. Fatty acid degradation was enriched in proteins including *Aldh2*, *Acs11*, *Acadm*, *Acaa1a*, *Acadl*, *Acadvl*, *Eci2*, *Acsbg1* and *Aldh1b1*.

Experimental verification of key DEPs in HTG-APH and AP groups

To further validate these results, we selected the most significantly expressed proteins from four lipid-related pathways: the PPAR signaling pathway, cholesterol metabolism, fatty acid metabolism, and fatty acid degradation. These proteins are labeled in Fig. 4A, in which Scd1, Plin1, Fasn, and Apob were upregulated, Acsbg1 was downregulated in HTG-AP group compared to AP group. Furthermore, Zbtb20, which participates in the regulation of lipid metabolism, showed significantly higher expression in the HTG-AP group than in the AP group. In addition, the inflammation-related proteins Map3k20 and Nfkb2 showed significantly upregulated in HTG-AP group compared to AP group, suggesting that HTG may exacerbate the inflammatory response in AP (Fig. 5A). mRNA expression analysis revealed that the mRNA levels of Scd1, Plin1, Fasn, Apob, Acsbg1, Zbtb20, Map3k20, and Nfkb2 were significantly reduced in the HTG-APH group compared to the AP group (Fig. 5B). Consistently, protein expression analysis showed that the positive staining ratios of Scd1, Fasn, Apob, and Zbtb20 were significantly increased in the HTG-APH group compared to the AP group (Fig. 5C).

Analysis of DEPs between AP and NC and multi-group comparison

A total of 2,145 DEPs were identified between the AP and NC groups, of which 865 were upregulated and 1,280 were downregulated. Volcano plots illustrate all DEPs, and heatmaps display the top 25 upregulated and top 25 downregulated proteins (Fig. 6A,B).

KEGG enrichment analysis of these DEPs revealed the following top 20 most significantly enriched pathways: metabolic pathways, carbon metabolism, diabetic cardiomyopathy, cytoskeleton in muscle cells, pyruvate metabolism, citrate cycle (TCA cycle), glycolysis/gluconeogenesis, chemical carcinogenesis-reactive oxygen species, Parkinson disease, oxidative phosphorylation, biosynthesis of amino acids, fatty acid metabolism, prion disease, fatty acid degradation, thermogenesis, propanoate metabolism, Huntington disease, platelet activation, pancreatic secretion, and valine, leucine, and isoleucine degradation (Fig. 6C).

We also performed a multi-group comparison among the HTG-APH, AP, and NC groups. The trends in protein expression changes across the three groups and the main AP-related pathways enriched by the identified proteins are presented in Fig. 6D.

Proteins of interest identified in the previous analysis were further examined. Proteomic data showed that the AP group exhibited significantly downregulated expression of Scd1, Plin1, Fasn, Zbtb20, and Map3k20 and significantly upregulated expression of Acsbg1 compared to the NC group. In contrast, the HTG-APH group showed significantly upregulated expression of Scd1, Plin1, Fasn, Zbtb20, Map3k20, and ApoB and significantly downregulated expression of Acsbg1 compared to the AP group (Fig. 7A).

Next, we examined mRNA levels to confirm these changes. Nfkb2 mRNA was higher in the AP group than in the NC group, whereas Nfkb2 and Plin1 mRNA levels were lower in the HTG-APL and HTG-APH groups than in the AP group. Scd1 mRNA was also reduced in the HTG-APL group compared to that in the AP group (Fig. 7B). Consistent with these results, IHC staining further supported the observed changes. The positive staining ratios of Scd1, Fasn, ApoB, and Zbtb20 were negligible in the NC and

APOE groups. In the AP group, weak positive staining was observed in pancreatic interstitial cells and inflammatory cells. In contrast, markedly increased positive staining ratios of these proteins were observed in the HTG-APL and HTG-APH groups (Fig. 7C).

Discussion

Over the past 20 years, the incidence of HTG-AP has increasing^[4]. In order to explore the pathogenesis of HTG-AP, researchers have developed various animal models, but existing models have limitations to varying degrees^[7]. Therefore, we used *APOE*^{-/-} mice, a well-recognized model of dyslipidemia, to establish HTG. After four months on a high-fat diet, *APOE*^{-/-} mice had significantly increased serum T-CHO, TG, and LDL-C levels compared with C57BL/6 mice fed a normal diet, whereas serum ALT and AST levels did not increase significantly. This indicates that the four-month high-fat diet successfully established an HTG model in *APOE*^{-/-} mice.

After caerulein injection, *APOE*^{-/-} mice injected with either 25 µg/kg or 50 µg/kg caerulein developed more severe pancreatic injury (e.g., edema and neutrophil infiltration) than AP mice. Notably, caerulein injection lowered serum TG levels in *APOE*^{-/-} mice, which was most pronounced in the HTG-APL group. The free fatty acid (FFA) hypothesis is widely accepted in the pathogenesis of HTG-AP, suggesting that TG itself is not inherently toxic to the pancreas; pancreatic LPS hydrolyzes TG into free fatty acids (FFAs), which then cause lipotoxicity^[13]. In our study, LPS levels were significantly elevated in *APOE*^{-/-} mice injected with 25 µg/kg or 50 µg/kg caerulein. This indicates that excessive LPS in HTG-APL and HTG-APH mice breaks down TG into FFAs, thereby leading to reduced TG levels and severe pancreatic injury. Furthermore, HTG-APL and HTG-APH mice showed higher serum AST levels and more severe hepatic pathological changes than AP mice, suggesting that HTG-AP is more prone to causing liver damage.

AP is usually diagnosed based on abdominal pain together with elevated serum AMS and/or LPS, and LPS is considered more sensitive than AMS^[14]. In our study, serum AMS and LPS levels did not increase significantly in AP mice but were markedly elevated in HTG-APL mice. LDH is widely used to assess AP severity, and elevated LDH levels are associated with a higher incidence of AP complications, including systemic inflammatory response syndrome, pleural effusion, ascites, myocardial infarction, and acute kidney injury^[15]. In our study, LDH levels were significantly higher in HTG-APL and HTG-APH mice than in AP mice. Low serum Ca²⁺ and high CRP levels are risk factors for severe AP in patients with HTG-AP^[16]. In line with this, HTG-APL mice in our study showed lower serum Ca²⁺ and higher CRP levels after caerulein injury. FFAs can directly promote the release of inflammatory cytokines such as TNF-α, IL-6, and IL-1β^[4]. Although serum IL-6 tended to be higher in the HTG-APL and HTG-APH groups, the difference was not statistically significant, possibly because of large intergroup variability and the small sample size. Furthermore, *APOE*^{-/-} mice displayed higher serum GLU levels after four months of high-fat diet feeding, whereas caerulein-induced pancreatic injury led to a significant decrease in GLU levels, indicating islet cell damage. More severe lung injury was also observed in the HTG-APL and HTG-APH groups compared to the AP group. In conclusion, HTG-AP showed higher levels of AMS, LPS, LDH, CRP,

and lower levels of Ca^{2+} and GLU, and more severe lung injury than AP. These results suggest that HTG exacerbates pancreatic injury. Moreover, the damage induced by 25 $\mu\text{g}/\text{kg}$ caerulein in *APOE*^{-/-} mice was more severe than that induced by 50 $\mu\text{g}/\text{kg}$ caerulein in C57BL/6 mice, demonstrating that HTG increases susceptibility to AP.

Together, these results show that the HTG-AP model was successfully established in *APOE*^{-/-} mice by combining a four-month high-fat diet with caerulein injection. Given the well-established nature of *APOE*^{-/-} mice, this approach is relatively simple and practical. We confirmed the model using serum biochemistry, inflammatory markers, and pancreatic histopathology. In the future, stratifying HTG-AP into mild, moderate, and severe grades based on serum lipid levels may help address the major challenge facing animal models of HTG-AP: the lack of a unified diagnostic standard for this disease.

Interestingly, in this study, the HTG-APL group showed milder pancreatic pathological injury but higher serum AMS, LPS, CRP, and IL-6 levels, as well as lower serum Ca^{2+} and GLU levels than the HTG-APH group, although none of these differences reached statistical significance. This trend may support the “enzyme-injury dissociation phenomenon”: low-dose caerulein induced stronger release of enzymes and inflammatory factors, whereas in the high-dose group, rapid acinar necrosis and accumulation of lipotoxic products inhibited their entry into the blood [17]. The lack of significance may be attributable to the small sample size, large interindividual variability, and the sampling time point not capturing the peak levels, warranting future validation with larger samples and dynamic time points.

Proteomics has been applied to AP research for several years [18]; however, proteomic studies on HTG-AP remain limited. To investigate the biological functions altered in the pancreas of AP and HTG-AP mice, we used LC-MS to construct a pancreatic proteomic database from NC, AP, and HTG-APH mice. GO and KEGG analysis showed that 5,003 proteins were mainly distributed in the cytoplasm or membrane and were primarily involved in metabolic pathways. Metabolic pathways regulate the catabolism and anabolism of small molecules in cells or tissues through enzyme-catalyzed reactions. Studies have shown that metabolic pathways contribute substantially to the development of SAP [19], which supports the possibility that HTG exacerbates AP in our study.

We next compared protein expression profiles between the HTG-APH and AP groups. This led to the identification of 884 DEPs, of which 603 were upregulated and 281 downregulated. KEGG analysis of these DEPs showed that the most enriched pathways were largely related to lipid metabolism, including the peroxisome proliferator-activated receptor (PPAR) signaling pathway, cholesterol metabolism, fatty acid metabolism, and fatty acid degradation. Therefore, we selected these pathways and their most differentially expressed proteins for further analysis.

The PPAR signaling pathway is central to lipid metabolism and inflammation and is therefore an important factor in AP pathogenesis. PPARs interact with other transcription factors such as nuclear factor κB (NF- κB), which plays a key role in AP pathogenesis [20]. Stearoyl-CoA desaturase-1 (Scd1) regulates fatty acid profiles and TG synthesis in adipocytes [21], and its expression is upregulated in rat

models of HTG-AP [22]. Perilipin-1 (Plin1) controls lipolysis by recruiting lipases to lipid droplets and regulating their interaction with mitochondria [23]. In INS-1 beta cells, perilipin overexpression increases lipid storage and suppresses lipolysis [24]. Plin1 knockout in 3T3-L1 adipocytes enhances lipolysis by exposing lipids within lipid droplets and increasing lipase activity [25]. Acyl-CoA synthetase bubble gum 1 (Acsbg1) catalyzes the thioesterification of fatty acids to CoA, which is the initial step in fatty acid β -oxidation, synthesis, and other lipid metabolic pathways [26]. In our study, Scd1 and Plin1 were upregulated in HTG-AP, Acsbg1 was downregulated, suggesting a role for these proteins in lipid metabolism during HTG-AP.

Cholesterol metabolism and inflammation are closely related. For example, elevated circulating cholesterol promotes lipid reprogramming in macrophages and triggers inflammatory responses in atherosclerotic plaques [27]. High plasma cholesterol affects pancreatic β -cell function and survival [28]. Although triglyceride metabolism has received the most attention, growing evidence suggests that cholesterol metabolism also contributes to HTG-AP pathogenesis. For instance, one study showed that total cholesterol levels showed a negative correlation with the risk of severe HTG-AP when total cholesterol was < 6.09 mmol/L, but a positive correlation when total cholesterol was > 6.09 mmol/L in HTG-AP patients [29]. Apolipoproteins regulate lipoprotein metabolism by facilitating the transport and redistribution of lipids, acting as cofactors for lipid-metabolizing enzymes, and maintaining the structural integrity of lipoprotein particles [30]. Apob is the major structural component of low-density lipoprotein, intermediate-density lipoprotein, and very low-density lipoprotein and is believed to promote inflammation in the vessel wall [31]. Our study found that Apob expression was upregulated in HTG-AP. However, another study reported a negative correlation between Apob and progression to severe HTG-AP [32]. Therefore, the role of cholesterol metabolism in HTG-AP requires further investigation.

Fatty acid metabolism encompasses synthesis, degradation, and utilization of fatty acids. Studies have indicated that LPS breaks down TG into FFAs, leading to lipotoxicity [4]. FFAs are categorized into two types: saturated fatty acids (SFAs) and unsaturated fatty acids (UFAs). Cellular accumulation of long-chain SFAs is closely linked to lipotoxicity [33]. Similarly, the superior aqueous stability of UFAs allows them to exist as monomers at higher concentrations than SFAs, contributing to lipotoxic inflammation and organ failure [34]. When FFAs exceed the binding capacity of albumin, they directly cause dysfunction, leakage, and coagulation of the vascular endothelium. At high concentrations, FFAs form detergent-like micelles, which induce ischemia, acidosis, and trypsin activation, leading to further tissue damage [35]. FFAs also interact with extracellular Ca^{2+} , leading to hypocalcemia in SAP [36], and can activate Toll-like receptors, thereby triggering NF- κ B-mediated cytokine production [37]. Fatty acid synthase (Fasn) is a key enzyme in fatty acid synthesis [38]. Studies have shown that Fasn expression is upregulated in human pancreatic cancer tissues [39]. In our study, Fasn was significantly upregulated in HTG-AP mice, combined with the elevated serum LDH and decreased TG and Ca^{2+} levels, suggesting that fatty acid metabolism contributes substantially to HTG-AP.

zinc finger and BTB domain-containing protein 20 (Zbtb20) is a transcription factor involved in neurogenesis, β -cell function, glucose homeostasis, and postnatal growth ^[40, 41]. Liraglutide can downregulate Zbtb20 to inhibit adipogenesis, and Zbtb20 overexpression suppresses LPS ^[42]. Zbtb20 inactivation increases LPS activity and enhances postprandial TG clearance, thereby lowering plasma TG in hepatocytes ^[43]. Zbtb20 upregulated was also observed in HTG-AP mice in our study, indicating that its high expression is correlated with the increase in TG levels in HTG-AP.

Chemokines and cytokines drive inflammatory cascades in HTG-AP ^[44]. The Mitogen-Activated Protein Kinase signaling pathway (MAPK) and NF- κ B pathways are widely recognized inflammatory signaling pathways ^[45]. MAPKs and NF- κ B can regulate cell proliferation, differentiation, survival, apoptosis, inflammation, and immune responses ^[45, 46]. In our study, Map3k20 and Nfkb2 were higher in HTG-AP mice than in AP mice, implying that elevated TG may exacerbate inflammation through FFA-triggered inflammatory cascades. Multi-group proteomic analysis showed that Scd1, Plin1, Fasn, Zbtb20, and Map3k20 were downregulated, whereas Acsbg1 was upregulated in AP mice compared to NC mice. In contrast, HTG-AP mice exhibited higher expression of Scd1, Plin1, Fasn, Apob, Zbtb20, and Map3k20 and lower expression of Acsbg1 than AP mice. However, when we verified these findings experimentally, the positive staining ratios for Scd1, Fasn, Apob, and Zbtb20 were only slightly higher in AP mice than in NC or APOE mice, and these differences were not statistically significant. But in HTG-AP mice, the positive staining ratios of Scd1, Fasn, Apob, and Zbtb20 were increased relative to AP mice, suggesting that high expression of these proteins is closely associated with the severe pancreatic injury observed in HTG-AP mice.

Studies have shown that Zbtb20 acts as a key transcriptional regulator in hepatic lipogenesis by modulating the expression of carbohydrate response element-binding proteins (ChREBPs), which in turn control downstream lipogenic genes, such as Scd1 and Fasn. Although Apob expression was assessed in this study, no direct regulatory effect of Zbtb20 on Apob was observed ^[47]. Fasn and Scd1 are central to fatty acid metabolism. Fasn mediates the de novo synthesis of long-chain SFAs ^[48], and Scd1 converts SFAs into monounsaturated fatty acids (MUFAs) ^[49]. So we speculate that upregulated Zbtb20 led to increased Fasn and Scd1 expression in HTG-AP mice. Then promoted the synthesis of SFAs and MUFAs, which were subsequently esterified into TG and stored in pancreatic acinar cells. When AP developed, acinar cell necrosis released TG into the extracellular space, where elevated pancreatic LPS hydrolyzed it into FFAs, resulting in a lipotoxic inflammatory cascade aggravated pancreatic injury.

To date, no study has demonstrated that the Zbtb20/Fasn/Scd1 axis is involved in HTG-AP. However, we have shown that lipid metabolic disorders are closely linked to HTG-AP, and that each of these proteins has well-known roles in lipid metabolism. This indirect evidence supports the axis and points to directions for future research.

In conclusion, we successfully established a reliable HTG-AP model in *APOE*^{-/-} mice using a four-month high-fat diet with caerulein injections. Our results showed that pre-existing HTG worsens pancreatic

injury and increases AP susceptibility by promoting lipotoxic inflammation. Proteomic analysis revealed PPAR signaling, cholesterol metabolism, fatty acid metabolism, and fatty acid degradation in HTG-AP. Among the proteins in these pathways, Zbtb20, Scd1, Plin1, Fasn, Apob, and Acsbg1, may contribute to disease progression. In particular, the Zbtb20/Fasn/Scd1 axis seems to mediate lipotoxic inflammation, which is worth further study as a potential therapeutic target for HTG-AP.

Materials and Methods

Experimental Design

Nine-week-old female C57BL/6J and *APOE*^{-/-} mice were obtained from the Animal Experiment Center of the Shaanxi University of Chinese Medicine. All animals were housed in a specific pathogen-free (SPF) facility under a 12 h light-dark cycle at 24 ± 2°C, with standard laboratory chow and water ad libitum. The experimental protocols were approved by the Experimental Animal Ethics Committee of Shaanxi University of Chinese Medicine (Approval No. SUCMDL20220304015). All experimental procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and abided by the ARRIVE guidelines.

Mice were randomly divided into five groups (n = 10 per group): (1) NC group: C57BL/6J mice fed a normal diet for four months and then intraperitoneally injected with saline (seven injections at 1 h intervals); (2) APOE group: *APOE*^{-/-} mice fed a high-fat diet (40% fat, 40% carbohydrates, 20% proteins; Jiangsu Sihuan Bioengineering Pharmaceutical Co., Ltd., China) for four months and then intraperitoneally injected with saline (seven injections at 1 h intervals); (3) AP group: C57BL/6J mice fed a normal diet for four months and then intraperitoneally injected with caerulein at 50 µg/kg (seven injections at 1 h intervals); (4) HTG-APL group: *APOE*^{-/-} mice fed a high-fat diet for four months and then intraperitoneally injected with caerulein at 25 µg/kg (seven injections at 1 h intervals); (5) HTG-APH group: *APOE*^{-/-} mice fed a high-fat diet for four months and then intraperitoneally injected with caerulein at 50 µg/kg (seven injections at 1 h intervals). At 24 h after the last caerulein injection, mice were anesthetized with sodium pentobarbital (60 mg/kg, i.p.). Blood samples were collected via cardiac puncture, and euthanasia was then performed by an overdose of sodium pentobarbital (≥ 200 mg/kg, i.p.). Pancreatic tissues were also collected for further analysis.

Plasma Biochemical Assay

Blood samples were centrifuged at 35,000 rpm for 10 min at 4°C, and the serum was collected and stored at - 80°C until analysis. Serum levels of T-CHO, TG, LDL-C, HDL-C, ALT, AST, GLU, LDH, AMS, LPS, and Ca²⁺ were measured using an automatic biochemical analyser (Chemray 800, Shenzhen Leidu Life Technology Company, China). The serum samples were diluted three-fold and assayed using commercial kits (Shenzhen Leidu Life Technology, Nanjing Jiancheng, or Changchun Huili, China) according to the manufacturer's instructions.

Plasma Inflammatory indicators measurement

Serum IL-6 and CRP levels were measured using commercial ELISA kits (GEM0001 for IL-6 and GEM0007 for CRP; Servicebio, China) according to the manufacturer's instructions. Before measurement, the serum samples were diluted three-fold for IL-6 detection and 20,000-fold for CRP detection.

Histological Examination

Pancreatic tissue samples were fixed in 4% formaldehyde for 24 h, dehydrated, embedded in paraffin, and sectioned using a Leica RM2016 ultramicrotome. The sections were stained with haematoxylin and eosin (H&E; Servicebio, China).

Immunohistochemistry(IHC)

Pancreatic sections (3 µm thick) were de-paraffinised, and antigen retrieval was performed by boiling in EDTA solution (BL617A; Biosharp, China). Endogenous peroxidase activity was blocked by incubating the sections with 3% H₂O₂ (SP kit-A3, Maxim, China) for 10 min, followed by blocking with normal goat serum (ZLI-9056, Zsbio, China) for 20 min. The sections were then incubated with primary antibodies at 37°C for 60 min at the following concentrations: anti-Scd1 (ab236868, Abcam, 1:2000), anti-Fasn (pb9865, Boster, 1:100), anti-Apob (84047-1-RR, Proteintech, 1:250), and anti-Zbtb20 (A04274-1, Boster, 1:100). Thereafter, the sections were incubated with the appropriate secondary antibody at 37°C for 30 min. Immunoreactivity was visualised using DAB substrate (Kit-5230, Maxim, China), followed by counterstaining with haematoxylin (BA4041, Beso, China), dehydration, and cell mounting.

RNA isolation and real-time PCR

Total RNA was extracted from pancreatic tissue using the RNAeasy Animal RNA Isolation Kit with Spin Column (R0026, Beyotime, China) and reverse-transcribed into cDNA using the BeyoRT III First Strand cDNA Synthesis Kit (D7178M, Beyotime, China). Real-time PCR was performed using the BeyoFast SYBR Green reaction mix (D-7265, Beyotime, China) on an Applied Biosystems instrument (Thermo Fisher Scientific, Shanghai, China). The PCR conditions were as follows: initial denaturation at 95°C for 2 min, followed by 40 cycles of denaturation at 95°C for 15 s and annealing at 60–64°C for 1 min.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control to calculate the relative expression levels of the target genes. The primer sequences are presented in Table 1.

Table 1
Primer sequences used for RT-PCR analysis

Gene	Forward sequence (5'-3')	Reverse sequence (5'-3')
Scd1	AGTTCCTACACGACCACCACTA	GGACGGATGTCTTCTTCCAGAT
Plin1	GAGAGGAGACAGACGACGAGGAG	GGTCACTGCGGAGATGGTGTTC
Fasn	CATGACCTCGTGATGAACGTGT	CGGGTGAGGACGTTTACAAAG
Apob	CGTGGGCTCCAGCATTCTA	TCACCAGTCATTTCTGCCTTTG
Acsbg1	GCAGACTGGCGGATTACCTA	TGTAAAGCCGATAGTTGTAGGG
Zbtb20	CAGCCAAACAGACTACGTCA	GCATCACCATGTGCTTGATA
Map3k20	AATAGCGACACTTTCTGCGTG	GGCTGTACTGTGTTGGGGTTA
Nfkb2	GTCCTGGAGTTAACTTTCAACC	TTCCGACTTCTTTCCGAGC

Proteomics

Protein preparation and digestion

Proteins were extracted from pancreatic tissue samples (n = 3 per group) using SDT lysis buffer (4% sodium dodecyl sulfate, 100 mM dithiothreitol, 100 mM Tris-HCl, pH 8.0). The samples were boiled for 5 min, ultrasonicated, and boiled again for 5 min. Undissolved cellular debris was removed by centrifugation at 16,000 × g for 15 min. The supernatant was collected, and the protein concentration was quantified using a BCA Protein Assay Kit (Bio-Rad, USA). For each sample, 200 µg of protein was digested using the FASP method described by Wisniewski, Zougman et al ^[50]. Briefly, detergent, dithiothreitol, and iodoacetamide in UA buffer were added to block reduced cysteine residues. Subsequently, the protein suspension was digested with trypsin (Promega) at an enzyme-to-substrate ratio of 1:50 (w/w) overnight at 37°C. The resulting peptides were collected by centrifugation at 16,000 × g for 15 min, desalted using C18 StageTips, and then subjected to LC-MS/MS analysis (performed by Shanghai Bioprofile Technology Co., Ltd.). Peptide concentrations were determined by measuring the absorbance at 280 nm using a Nanodrop One device.

Liquid chromatography-Tandem mass spectrometry (LC-MS/MS) Analysis

LC-MS/MS analysis was performed using a Q Exactive Plus mass spectrometer coupled with an Easy 1200 nLC system (Thermo Fisher Scientific). Peptides were first loaded onto a trap column (100 µm × 20 mm, 5 µm, C18, Dr. Maisch GmbH, Ammerbuch, Germany) in buffer A (0.1% formic acid in water). Reverse-phase high-performance liquid chromatography (RP-HPLC) separation was performed using an EASY-nLC system (Thermo Fisher Scientific, Bremen, Germany) with a self-packed column (75 µm × 150 mm; 3 µm ReproSil-Pur C18 beads, 120 Å, Dr. Maisch GmbH, Ammerbuch, Germany) at a flow rate of 300

nL/min. The mobile phase consisted of A (0.1% formic acid in water) and B (0.1% formic acid in 95% acetonitrile). Peptides were eluted over 120 min with a linear gradient of buffer B. MS data were acquired in data-dependent top20 mode, selecting the most abundant precursor ions from the survey scan (300–1800 m/z) for higher-energy collisional dissociation (HCD) fragmentation. The instrument was run with peptide recognition enabled. A lock mass of 445.120025 Da served as an internal standard for mass calibration. Full MS scans were acquired at a resolution of 70,000 at m/z 200, and MS/MS scans were acquired at 17,500 at m/z 200. The maximum injection time was set to 50 ms for both MS and MS/MS scan modes. The normalised collision energy was 27, and the isolation window was set to 1.6 Th. The dynamic exclusion duration was 60 s.

Sequence Database Searching and Data Analysis

The MS data were analyzed using MaxQuant software (version 1.6.0.16). The data were searched against the UniProtKB Rattus norvegicus database (36,080 total entries, downloaded on August 14, 2018). The search results were filtered to achieve a false discovery rate (FDR) of < 1% at both the peptide spectrum match and protein levels. Label-free quantification (LFQ) was performed using MaxQuant with intensity determination and normalisation algorithms^[51–53]. The LFQ intensity for each protein across samples was determined as the best estimate from all pairwise peptide comparisons and then scaled to the sum of peptide intensities. MaxQuant was used to weight and normalize protein ratios to the median ratio. Proteins with a fold change ≥ 1.5 and p-value < 0.05 were considered significantly differentially expressed.

Bioinformatics analysis

Bioinformatics analyses were performed using Perseus^[54], Microsoft Excel, and R. Hierarchical clustering was performed using the pheatmap package in R, with Euclidean distance and complete linkage. Sequence annotation used information from UniProtKB/Swiss-Prot^[55], KEGG^[56], and GO^[10]. GO and KEGG enrichment were tested using Fisher's exact test with FDR correction for multiple comparisons. Enriched terms and pathways with $p < 0.05$ were considered nominally significant.

Statistical analysis

All experiments were performed at least three times. Bar graphs and statistical analyses were generated using GraphPad Prism version 8.4.2 (GraphPad Software, San Diego, CA, USA). Data are presented as the mean $\pm$ standard deviation (SD). Multigroup comparisons were performed using one-way ANOVA, followed by Tukey's multiple comparison test. Statistical significance was set at $p < 0.05$ and highly significant at $p < 0.01$.

Data availability statement

The datasets used and/or analysed in the current study are available from the corresponding author upon reasonable request.

Declarations

Acknowledgements

Not applicable.

Author contributions

Dongli Yang: Writing – original draft, Writing – review & editing, Visualization, Project administration, Methodology, Investigation, Formal analysis, Data curation, Funding acquisition, Conceptualization. Wenjiang Zhang: Validation, Project administration, Methodology, Investigation, Conceptualization. Xue Han, Lan Liang, Chenming He, Xiayu Cui: Validation, Methodology. Hong Zhang: Supervision, Resources, Funding acquisition, Conceptualization. All authors read and approved the final manuscript.

Additional Information

Competing interests

The authors declare no conflicts of interest.

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Figures

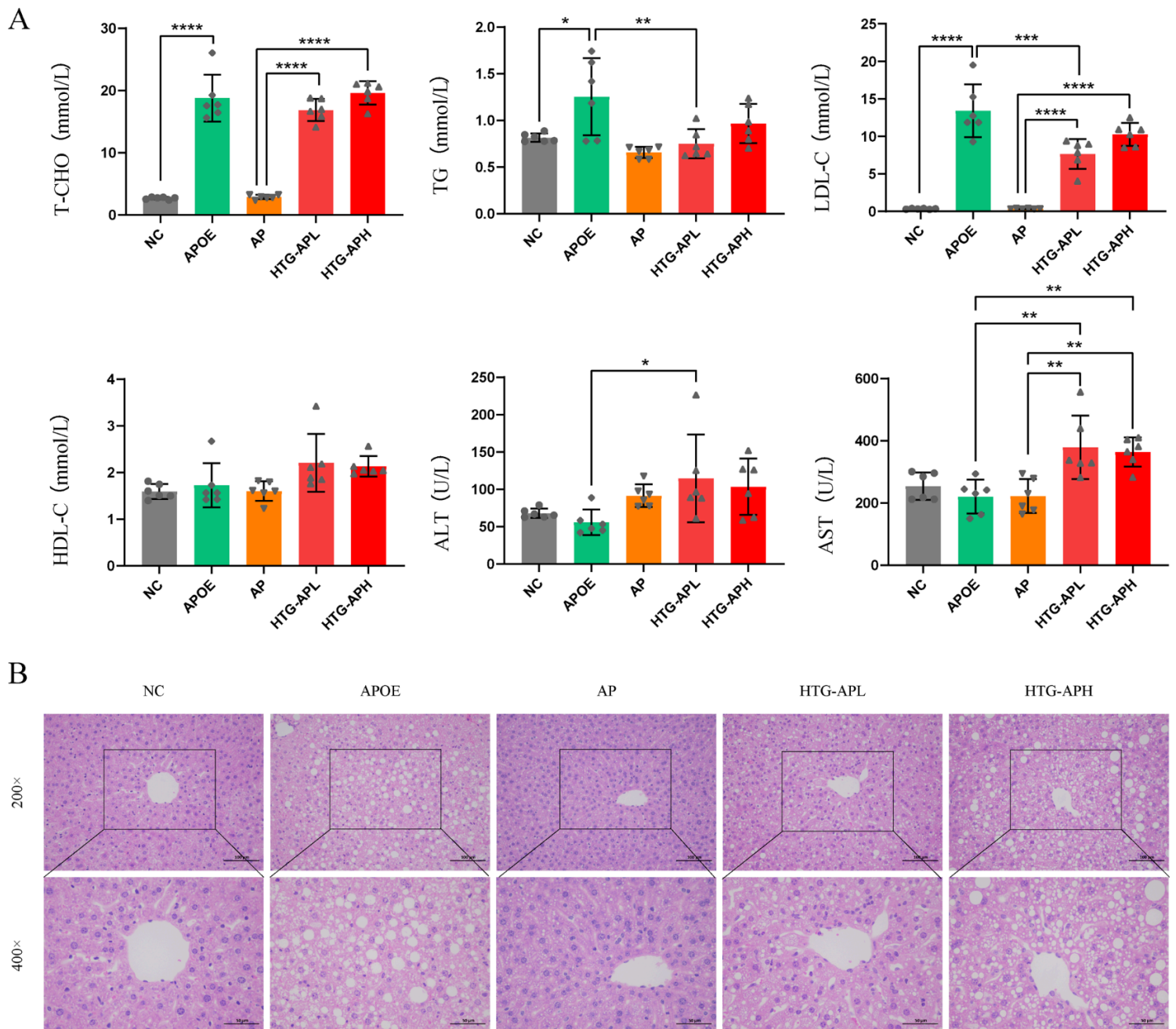


Figure 1

Establishment of the HTG model in *APOE*^{-/-} mice. (A) Serum levels of total cholesterol (T-CHO), triglycerides (TG), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), alanine aminotransferase (ALT), and aspartate aminotransferase (AST) in mice (n = 6 per group). Data are presented as mean ± SD. *, *p* < 0.05; **, *p* < 0.01; ***, *p* < 0.001; ****, *p* < 0.0001. (B) Representative histopathological changes in the liver (magnification: ×200 and ×400).

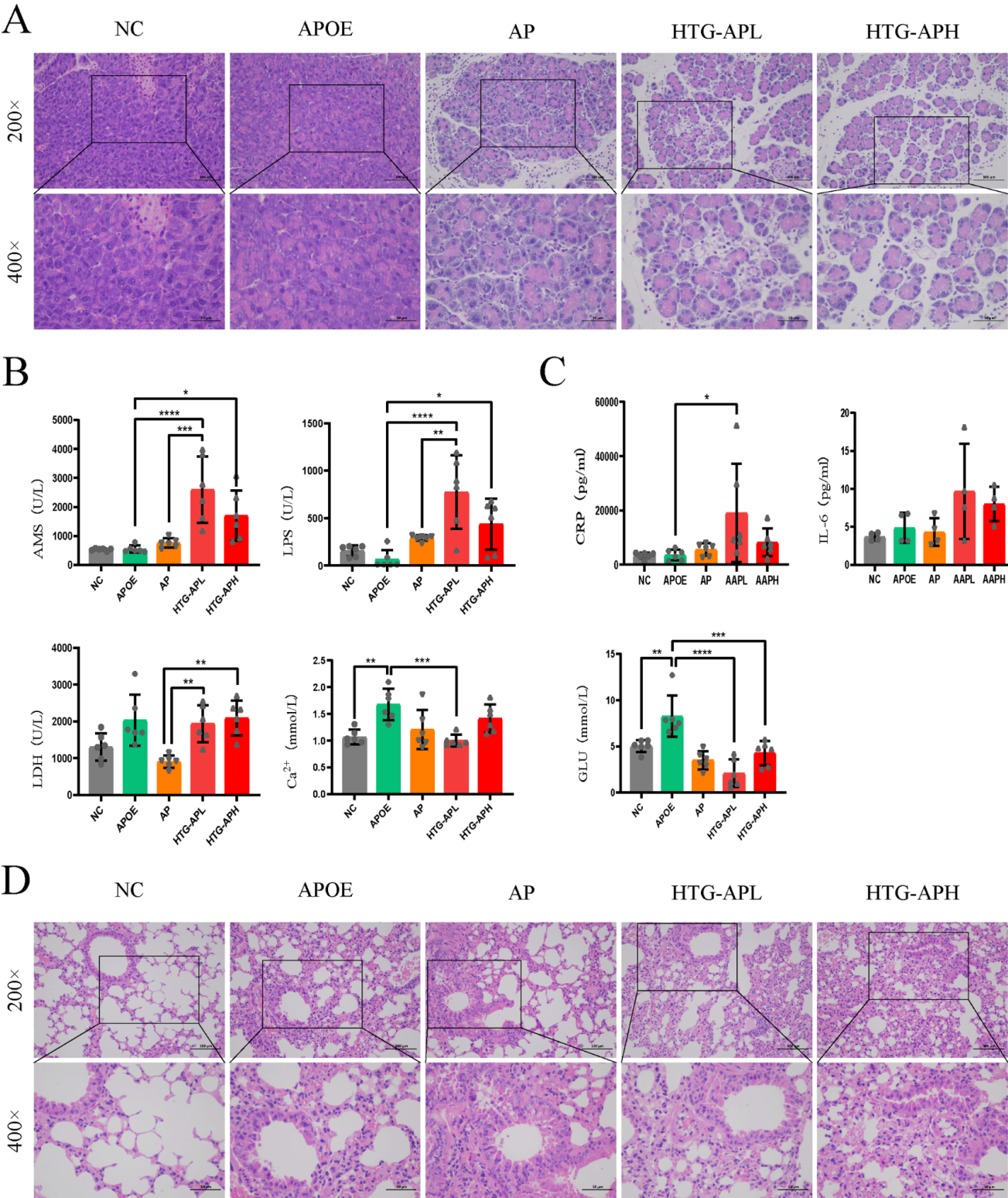


Figure 2

HTG aggravates pancreatic injury in a mouse model of AP. (A) Representative histopathological changes in the pancreas (original magnification $\times 200$ and $\times 400$). (B) Serum levels of amylase (AMS), lipase (LPS), lactate dehydrogenase (LDH), and calcium (Ca^{2+}) in mice ($n = 6$ per group). (C) Serum levels of C-reactive protein (CRP), interleukin-6 (IL-6), and glucose (GLU) in mice ($n = 6$ per group). (D) Representative histopathological changes in the lung (original magnification $\times 200$ and $\times 400$). Data are presented as mean $\pm$ SD. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

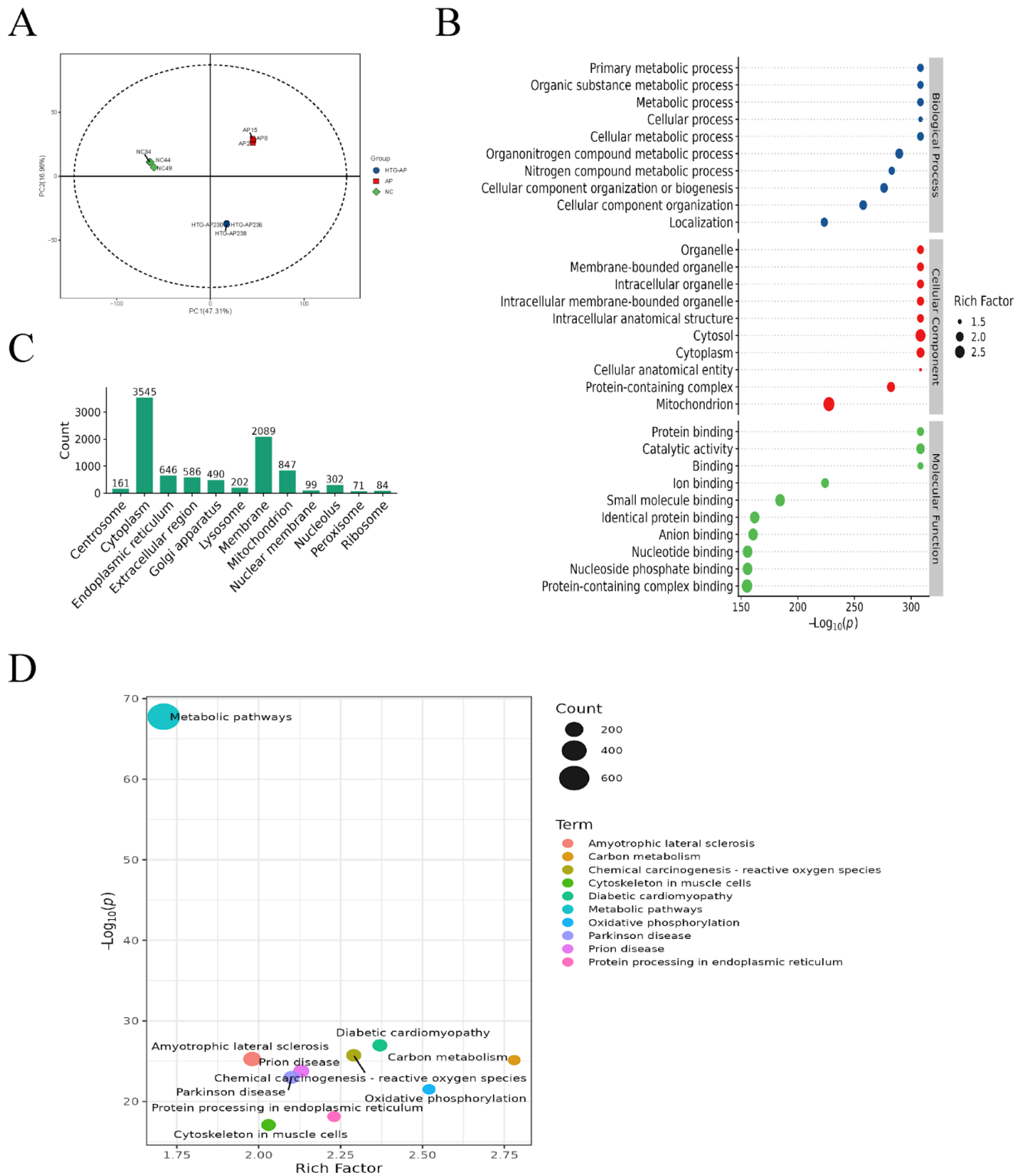


Figure 3

Proteomic profiling of pancreatic tissues from NC, AP, and HTG-APH mice. (A) PCA score plot of the NC, AP, and HTG-APH groups (n = 3 per group). Each point represents a sample; the closer the distance between samples, the higher the similarity, and the farther the distance, the greater the difference between the samples. (B) Top 10 enriched GO terms (bubble chart). The horizontal axis represents the negative logarithm of the enrichment significance p-value, and the vertical axis represents GO terms.

Each circle represents a GO term; the circle size indicates the rich factor, and different colors represent the three main GO branches (biological process, cellular component, and molecular function). (C) Subcellular localization of all identified proteins based on GO cellular component annotation. (D) Top 10 enriched KEGG pathways (bubble chart). The horizontal axis represents the rich factor of pathway enrichment, and the vertical axis represents the negative logarithm of the enrichment significance p-value. Each circle corresponds to a pathway, with the color indicating its category and size reflecting the number of enriched proteins. Data source: KEGG pathway database [12].

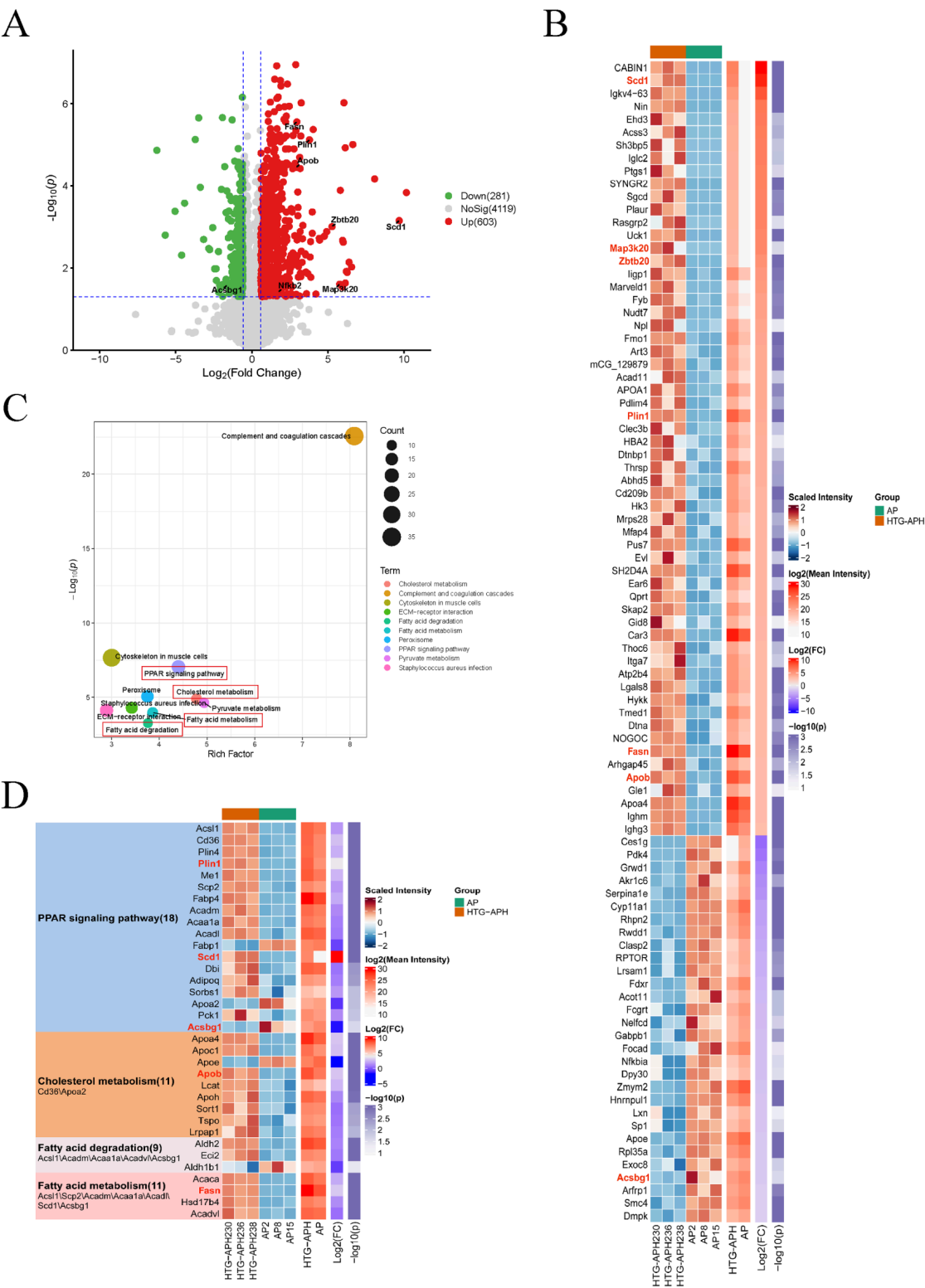


Figure 4

Identification and functional enrichment of DEPs between the HTG-APH and AP groups (n = 3 per group). (A) Volcano plot of DEPs between the HTG-APH and AP groups. DEPs were defined using a threshold of $p < 0.05$ and a fold change of ≥ 1.5 or $\leq 1/1.5$. Red dots represent significantly upregulated proteins, green dots represent significantly downregulated proteins, and gray dots represent non-differentially expressed proteins. (B) Heatmap showing the top 60 upregulated (red) and top 30 downregulated (blue) proteins. (C) Bubble chart of the top 10 enriched KEGG pathways. The horizontal axis represents the rich factor of pathway enrichment, and the vertical axis represents the negative logarithm of the enrichment significance p-value. Each circle corresponds to a pathway, with color indicating its category and size reflecting the number of enriched proteins. Data source: KEGG pathway database^[12]. (D) Heatmap showing the expression levels of proteins involved in the selected pathways of interest.

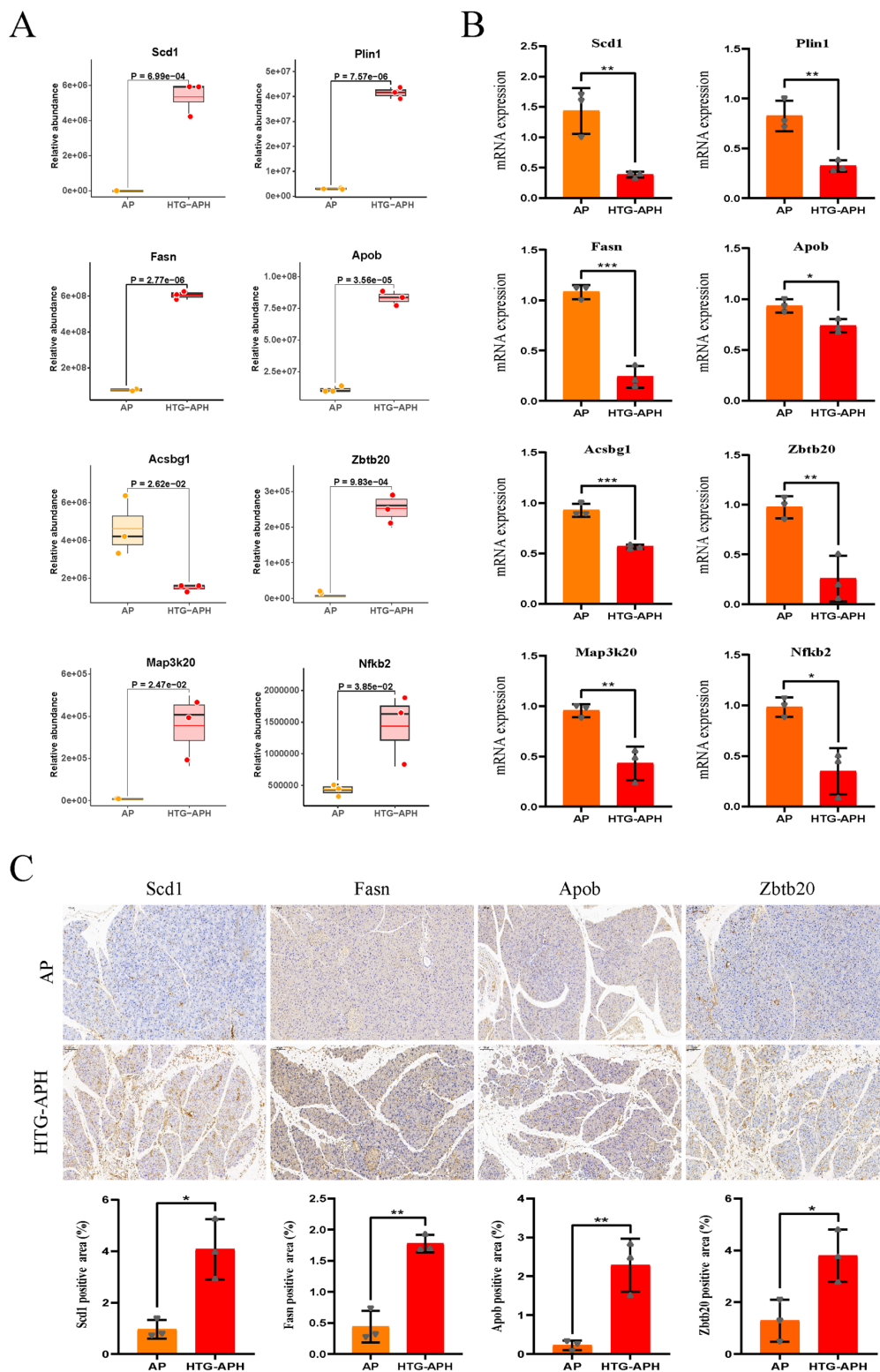


Figure 5

Validation of key proteins in AP and HTG-APH groups. (A) Relative abundance of Scd1, Plin1, Fasn, ApoB, Acsbg1, Zbtb20, Map3k20, and Nfkb2 in pancreatic tissue samples from AP and HTG-APH mice (n = 3 per group). (B) mRNA expression levels of Scd1, Plin1, Fasn, ApoB, Acsbg1, Zbtb20, Map3k20, and Nfkb2 in pancreatic tissue samples from AP and HTG-APH mice, measured by qPCR (n = 3 per group). (C) Representative immunohistochemical staining of Scd1, Plin1, Fasn, ApoB, and Zbtb20 in pancreatic

A B C



Identification and functional enrichment of DEPs between AP and NC and multi-group comparison (n = 3 per group). (A) Volcano plot of the DEPs between the AP and NC groups. DEPs were defined using a

threshold of $p < 0.05$ and a fold change ≥ 1.5 or $\leq 1/1.5$. Red dots represent significantly upregulated proteins, green dots represent significantly downregulated proteins, and gray dots represent non-differentially expressed proteins. (B) Heatmap showing the top 25 upregulated (red) and top 25 downregulated (blue) proteins. (C) Bubble chart of the top 20 enriched KEGG pathways. The horizontal axis represents the negative logarithm of the enrichment significance p-value. The circle color reflects the rich factor, and circle size indicates the number of enriched proteins. Data source: KEGG pathway database ^[12]. (D) Protein expression trends and main enriched pathways in the NC, AP, and HTG-APH groups.

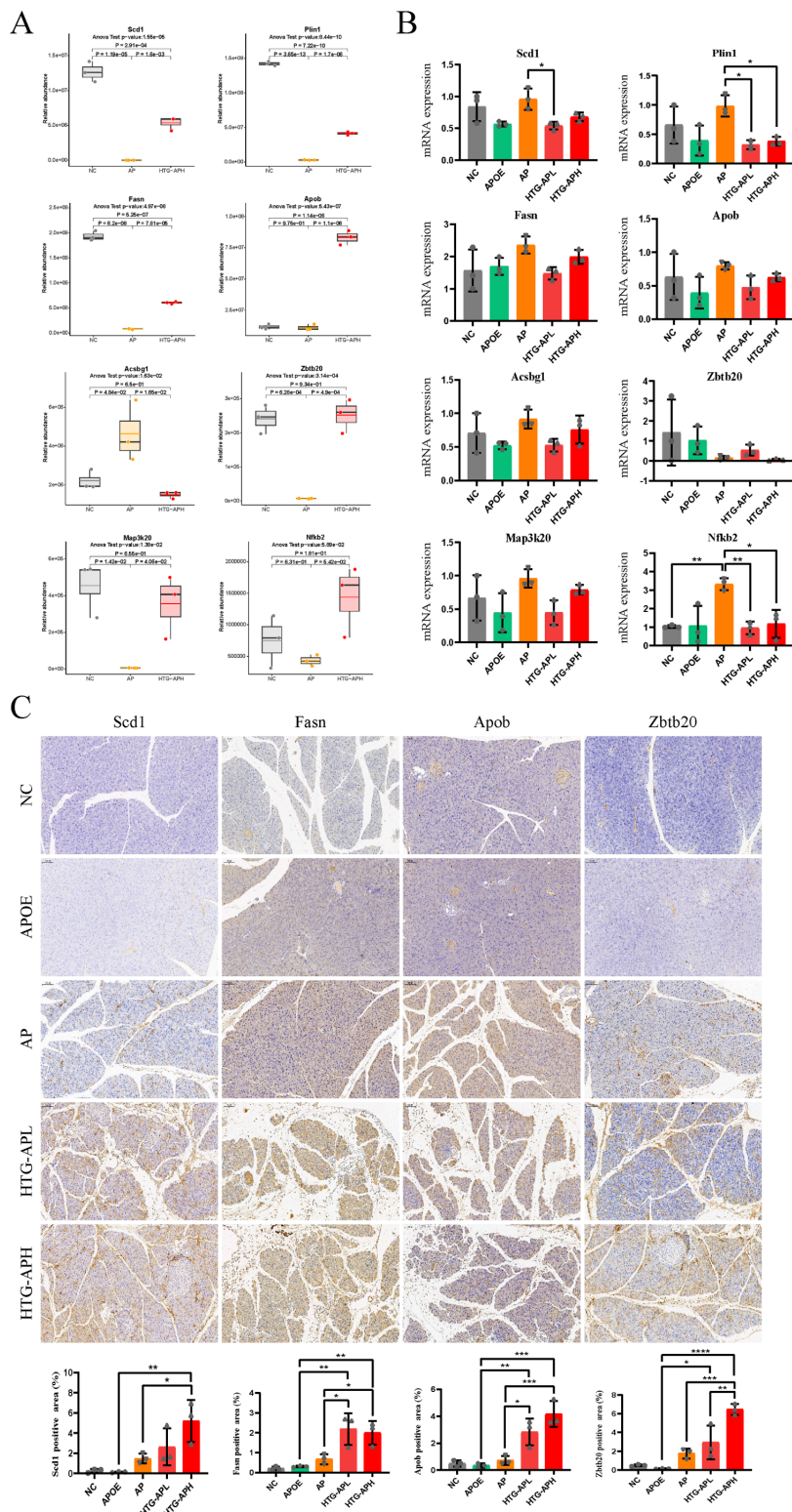


Figure 7

Comparative analysis of protein and mRNA expression levels in the NC, AP, and HTG-APH groups. (A) Relative abundance of Scd1, Plin1, Fasn, ApoB, Acsbg1, Zbtb20, Map3k20, and Nfkb2 in pancreatic tissue samples from NC, AP, and HTG-APH mice (n = 3 per group). (B) mRNA expression levels of Scd1, Plin1, Fasn, ApoB, Acsbg1, Zbtb20, Map3k20, and Nfkb2 in pancreatic tissue samples from NC, AP, and HTG-APH mice, as measured by qPCR (n = 3 per group). (C) Representative immunohistochemical

staining of Scd1, Plin1, Fasn, ApoB, and Zbtb20 in pancreatic tissue samples from NC, AP, and HTG-APH mice (magnification: ×200). Data are presented as mean ± SD. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

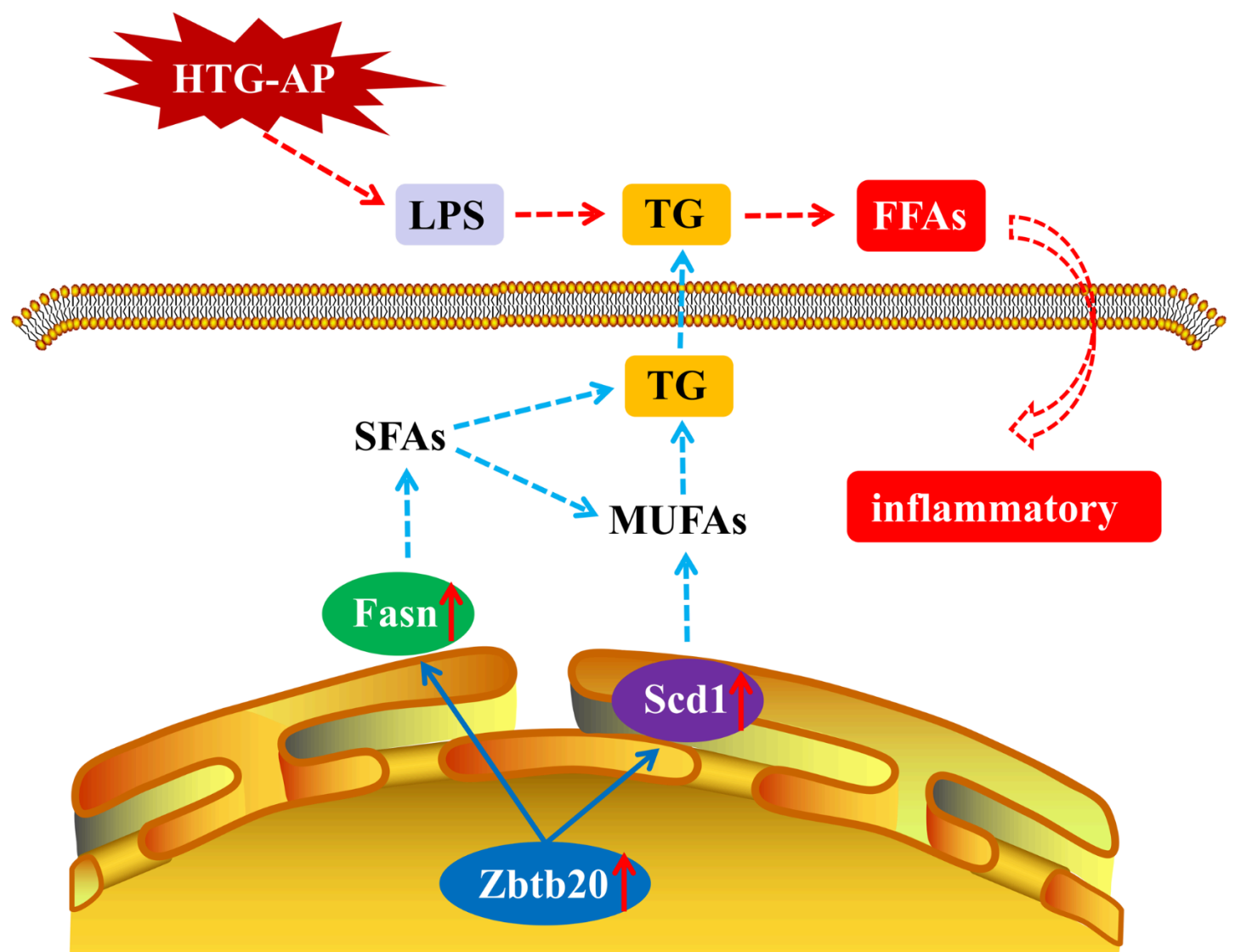


Figure 8

Proposed Zbtb20/Fasn/Scd1 axis in HTG-AP pathogenesis. In the HTG-AP pancreas, upregulated zinc finger and BTB domain-containing protein 20 (Zbtb20) drives the expression of fatty acid synthase (Fasn) and stearoyl-CoA desaturase 1 (Scd1), promoting the synthesis of saturated (SFAs) and monounsaturated (MUFAs) fatty acids, which are esterified into triglycerides (TG) for intracellular storage. During acinar necrosis in AP, the released TG is hydrolyzed by pancreatic lipase (LPS) into free fatty acids (FFAs), triggering lipotoxic inflammation and exacerbating pancreatic injury. Solid arrows indicate transcriptional regulation; dashed arrows indicate conversion or progression; ↑ indicates upregulation.