

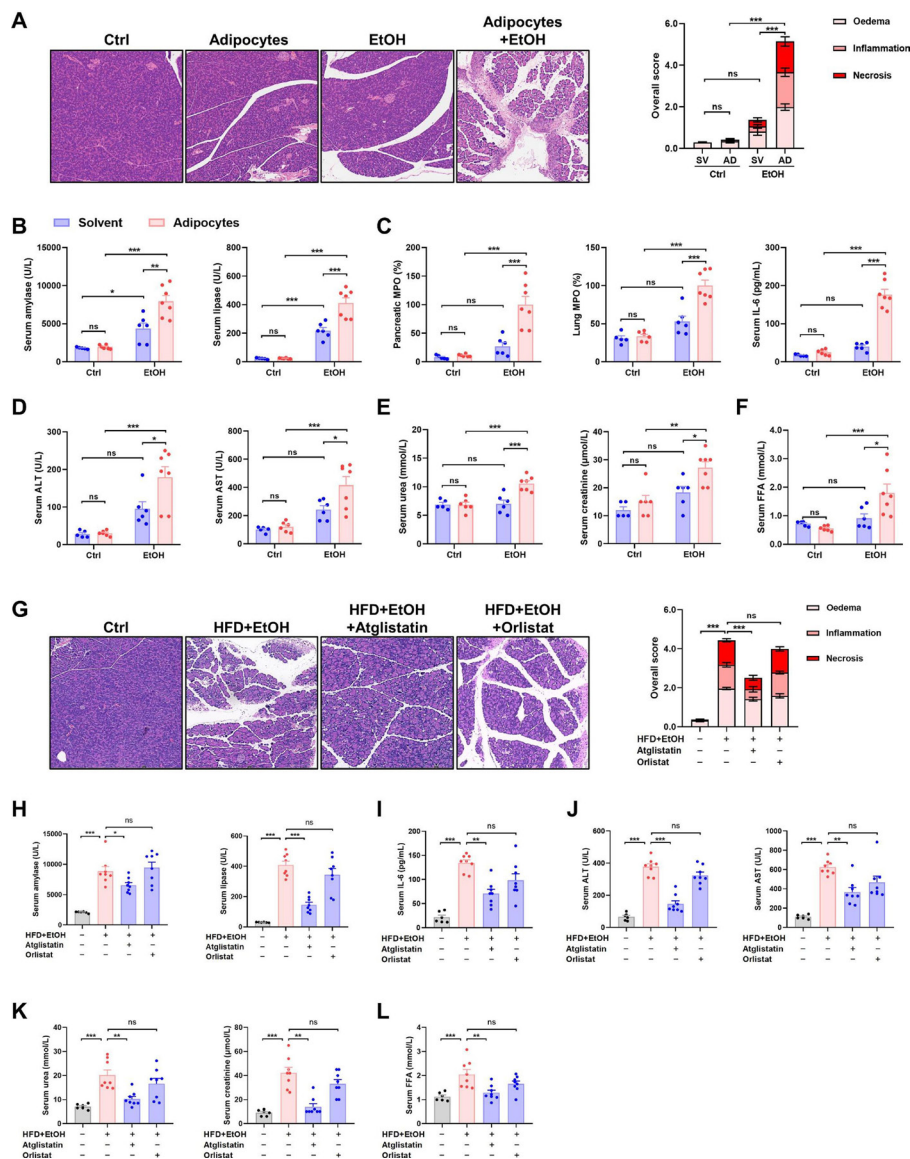
**Figure 1** Alcohol predisposes obese mice to AP with systemic organ injury. (A) Experimental protocol of establishing OA-AP. C57BL/6J mice were fed a CD (lean) or HFD (obese) for 12 weeks, then were injected intraperitoneally with 2 g/kg EtOH two times at 1 hour apart: (B) Body weight. (C) Representative images of pancreatic histopathology and histopathological scores (oedema, inflammation and necrosis; magnification  $\times 200$ ), (D) Serum amylase and lipase and (E) pancreatic MPO, lung MPO, and serum IL-6 levels of the OA-AP mice. (F) Serum FFA levels and (G) FFA and glycerol release in collected adipose tissues. (H) Amylase and lipase levels in epididymal adipose tissue. (I) Immunoblot analysis of ATGL proteins in epididymal adipose tissue. In all experiments, mice were sacrificed at 12 hours after the first injection of EtOH and assessed for disease severity and/or lipolytic parameters. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ . AP, acute pancreatitis; ATGL, adipose triglyceride lipase; CD, control diet; Ctrl, control; EtOH, ethanol; FFA, free fatty acid; HFD, high-fat diet; IL, interleukin; MPO, myeloperoxidase; ns, not significant; OA-AP, obese alcoholic acute pancreatitis.

## Alcohol predisposes obese mice to acute pancreatitis via adipose triglyceride lipase-dependent visceral adipocyte lipolysis

We read with great interest articles by Wang *et al*<sup>1</sup> and Hegyi *et al*,<sup>2</sup> in which the authors reported that alcohol increased the risk of hereditary susceptibility to chronic pancreatitis. These results indicated an

interaction effect between environmental and genetic risk factors on the development of pancreatitis. Epidemiological data suggested that alcohol abuse increased the risk of acute pancreatitis (AP) in people with type 2 diabetes mellitus (adjusted HR

86.3 (65.3–111.0)).<sup>3</sup> However, no study investigated the synergistic effect between obesity and alcohol excess on AP development. Here we report the combination of acute alcohol intake and obesity causes AP with multiorgan injury (MOI) in mice,



**Figure 2** Obesity and acute alcohol intake cause AP through visceral adipocyte lipolysis mediated by ATGL. Lean mice received intraperitoneal injection adipocytes 1 hour prior to two injection of 2 g/kg EtOH at a 1-hour interval: (A) Representative images of pancreatic histopathology (magnification  $\times 200$ ) and histopathological scores. (B) Serum amylase and lipase, and (C) pancreatic MPO, lung MPO and serum IL-6 levels. (D) Serum ALT and AST levels, (E) serum urea and creatinine and urea levels, and (F) serum FFA levels. In another experiment, the effect of an ATGL inhibitor, atglitatin, and a pancreatic lipase inhibitor, orlistat, in the OA-AP mice was tested. (G) Representative images of pancreatic histopathology (magnification  $\times 200$ ) and histopathological scores. (H) Serum amylase and lipase activity, (I) serum IL-6 levels, (J) serum ALT and AST levels, (K) serum urea and creatinine levels, and (L) serum FFA levels. In all experiments, mice were sacrificed at 12 hours after the first injection of EtOH and were assessed for disease severity and/or lipolytic parameters. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ . ALT, alanine aminotransferase; AP, acute pancreatitis; AST, aspartate aminotransferase; ATGL, adipose triglyceride lipase; Ctrl, control; EtOH, ethanol; FFA, free fatty acid; HFD, high-fat diet; IL, interleukin; MPO, myeloperoxidase; ns, not significant; OA-AP, obese alcoholic acute pancreatitis.

mediated by visceral adipocyte tissue (VAT) lipolysis.

The schedule of high-fat feeding and ethanol administration is shown in figure 1A. Body weight was significantly higher in the high-fat (obese) than the chow (lean) group after 12 weeks

(figure 1B). Acute ethanol administration in obese mice induced significant increases in pancreatic histopathology scores (oedema, inflammation and necrosis; figure 1C), elevated circulating pancreatic enzymes (figure 1D), pancreatic and lung myeloperoxidase, and serum

interleukin-6 levels (figure 1E). Time-course changes in this obese alcoholic acute pancreatitis (OA-AP) model showed pancreatic injury parameters were significantly elevated from 3 to 6 hours after the first ethanol injection, with rises in MOI indices (online supplemental figure 1A–F); almost all parameters peaked at 12 hours. In contrast, acute ethanol administration in lean mice caused only mild pancreatic oedema without discernable pancreatic necrosis or elevations of MOI indices.

We speculated that lipolysis from excess abdominal fat is critical to OA-AP, releasing free fatty acids (FFAs) from ethanol-induced VAT lipolysis. Indeed, fat saponification was seen in the peritoneal cavity and around the pancreas of ethanol-treated obese mice (online supplemental figure 2A,B). Circulating baseline FFA levels were higher in obese mice than in lean mice, which were further increased after acute ethanol administration (figure 1F). FFA and glycerol release over 3 hours from freshly isolated epididymal VAT of ethanol-treated obese mice was higher than that of lean mice (figure 1G). While pancreatic amylase or pancreatic triglyceride lipase (PNLIP) were comparable (figure 1H), adipose triglyceride lipase (ATGL) of ethanol-treated epididymal VAT taken from obese mice was, however, significantly higher than from lean mice (figure 1I).

To confirm our hypothesis, we injected ethanol and adipocytes simultaneously into the abdominal cavity of lean mice, which recapitulated all features of OA-AP (figure 2A–F). Inhibition of lipolysis using specific ATGL inhibitor atglitatin significantly reduced pancreas histopathology scores, serum pancreatic enzymes, serum MOI indices and serum FFA levels, while PNLIP inhibitor orlistat had a minimal effect (figure 2G–L). These findings indicate ethanol-induced VAT lipolysis via ATGL activation is central to the pathogenesis of OA-AP. This mechanism parallels the protective systemic effects of ATGL inhibition in burn injury,<sup>4</sup> which is distinct from systemic lipotoxicity consequent on leakage of PNLIP from the injured pancreas.<sup>5</sup> Interestingly, we found both atglitatin and orlistat were protective against caerulein-induced AP in obese mice (online supplemental figure 2C–G), mirroring patients with COVID-19 where PNLIP-mediated and ATGL-mediated lipotoxicity may both take place after disease onset.<sup>6</sup>

In summary, our study reports that obesity and alcohol act synergistically in the pathogenesis of onset and

development of MOI in OA-AP through induction of ATGL-mediated VAT lipolysis. High amounts of ethanol alone may be insufficient to induce clinical AP and is not sufficient to induce murine experimental AP.<sup>7</sup> A genetic predisposition or a susceptible precondition may be required,<sup>1,2,8,9</sup> if not the presence of a cofactor, as in murine fatty acid ethyl ester AP induced by ethanol with palmitoleic or palmitic acid.<sup>7,10</sup> Obesity is an alternative, which our model suggests may be targeted by ATGL inhibition.

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**Contributors** XY and LY are joint first authors. QX and WHu are joint senior authors. WHu, QX and XY designed the study. QX and WHu obtained funding and supervised students. XY and WHu drafted the manuscript. XY, LY, MY, LD and TL planned and conducted the experiments and analysed the data.

WHu, XF and RS had important intellectual input. JX and RS critically revised the manuscript. All authors read and approved the final version of the article.

**Funding** National Nature Science Foundation of China (number 81973632, to WHu; number 81860122, to WHu; number 81774120, to QX; number 81800575 to TL; numbers 92157205 and 81 970561 to XF); Programme of Science and Technology Department of Sichuan Province (number 22ZDYF1206, to XY); UK NIHR Senior Investigator Award (to RS).

**Competing interests** None declared.

**Patient consent for publication** Not applicable.

**Ethics approval** Not applicable.

**Provenance and peer review** Not commissioned; externally peer reviewed.

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► Additional supplemental material is published online only. To view, please visit the journal online (<http://dx.doi.org/10.1136/gutjnl-2022-326958>).

XY and LY are joint first authors.

QX and WH are joint senior authors.



**To cite** Yang X, Yao L, Dai L, *et al.* *Gut* 2023;**72**:212–214.

Received 12 January 2022

Accepted 12 March 2022

Published Online First 22 April 2022

*Gut* 2023;**72**:212–214. doi:10.1136/gutjnl-2022-326958

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# **Alcohol predisposes obese mice to acute pancreatitis via adipose triglyceride lipase dependent visceral adipocyte lipolysis**

## **Supplementary methods**

### **Animals and ethics**

C57BL/6J male mice were purchased from Beijing Huafukang Bioscience Corporation (Beijing, China). Mice were housed at 22°C with a 12-h light-dark cycle and had free access to food and water throughout experiments. All animal procedures were assessed and authorised by the Ethics Committee of West China Hospital of Sichuan University (20211086A).

### **Obesity and alcohol-induced acute pancreatitis**

Mice of 4-5 weeks old mice were randomly divided into chow diet (CD) group and high-fat diet (HFD) group, and respectively fed a CD (10 kcal % fat, H10010) and HFD (60 kcal % fat, H10060 Huafukang Bioscience Corporation; Beijing, China) for 12 weeks. Then, alcohol stimulation was conducted by two hourly intraperitoneal injections of ethanol at a dose of 2 g/kg. Ethanol (459836, Sigma, Shanghai China) was dissolved in sterile normal saline at 37.5% concentration (v/v). Control mice were given equal-volume saline injections. Animals were sacrificed at 3, 6, 12, 24, and 48 h after the first injection.



### **Adipocytes and alcohol-induced acute pancreatitis**

Adipocytes were freshly isolated from HFD obese mouse as previously described with minor modifications.<sup>1</sup> Briefly, mouse white adipose tissue fat pads were dissected, weighted, and minced thoroughly until no obvious tissue pieces were visible. The minced tissue was incubated at 37°C with shaking at ~220 rpm for 1 h with collagenase solution (Krebs Ringer Bicarbonate HEPES [KRBH] buffer containing 2% bovine serum albumin [BSA], 500 nM adenosine, and 3 mg/ml Collagenase I; 3 ml per gram of tissue). After digestion, the mixture was filtered through a 300 µm nylon mesh strainer, where after adipocytes were washed 3 times by free floating using a total of 40 ml wash buffer (2% BSA, 500 nM adenosine in KRBH) at room temperature. For each round of washing cells were allowed to float for > 5 min and the infranatant removed with a long needle and syringe. After the final wash, about  $1.5-2 \times 10^7$  in 0.3 ml volume was intraperitoneally injected into 8-10 weeks-old mice using an 18-gauge needle. After 1 h, alcohol stimulation was conducted as above described. Animals were sacrificed at 12 h after the first alcohol injection.

### **Caerulein-induced acute pancreatitis**

CD or HFD mice were given seven intraperitoneal injections of caerulein (50 µg/kg, Tocris, Shanghai) at hourly intervals as described previously.<sup>2, 3</sup> Control mice were given equal-volume saline injections. Animals were sacrificed at 12 h after the first injection.

### **Atglistatin and orlistat treatment**

Atglistatin (HY-15859, MedChemExpress, Shanghai) was dissolved in dimethyl sulfoxide at a stock (0.3 M), and then further dissolved in olive oil. Atglistatin treatment was administered orally by gavage (200 µmol/kg) before 1 h of first ethanol or caerulein injection.<sup>4</sup> Orlistat (Cayman Chemical, Ann Arbor, MI) was dissolved in ethanol at a stock (20 mg/ml). Orlistat treatment (50 mg/kg) was administered intraperitoneally 2 h after the first ethanol or caerulein injection. Animals were sacrificed at 12 h after induction of acute pancreatitis.

### **Histology**

The tissues were collected and fixed in 10% formalin for 24 hours, embedded with paraffin, and cut into slices before haematoxylin and eosin (H&E) staining. The severity of pancreatic injury was evaluated by two independent, blinded investigators grading oedema, inflammatory cell infiltration, and acinar necrosis (scale, 0-4) on × 10 high-power fields/slide/mouse.<sup>5</sup>

### **Serum biochemical marker measurement**

Biochemical parameters (amylase, lipase, alanine aminotransferase, aspartate aminotransferase, urea, and creatinine) were measured by an automatic biochemical analyser (Roche Cobas 8000, Shanghai, China). Interleukin-6 (IL-6) was determined by enzyme-linked immunosorbent assay (R&D Systems, Shanghai, China), free fatty acids (FFA) were determined by a commercial kit

(Abcam, Shanghai, China).

### **Myeloperoxidase activity**

Myeloperoxidase (MPO) activity was measured as described.<sup>6</sup> Pancreatic or lung tissue was homogenised, resuspended in 100 mM potassium phosphate buffer (pH 5.4) containing 0.5% hexadecyltrimethyl ammonium bromide, 10 mM ethylene diamine tetraacetic acid and protease inhibitors, freeze-thawed three times, sonicated for 30 sec and centrifuged for 15 min at 16,000 × g. MPO activity was measured in supernatants mixed with 3,3',5,5'-tetramethylbenzidine as substrate (1.6 mM, final) with freshly added H<sub>2</sub>O<sub>2</sub> (3 mM, final). Absorbance was measured at 655 nm and MPO was calculated as the difference between absorbance at 0 and 3 minutes. The activity was calculated by standard curve and expressed as units/mg protein or normalised to control.

### **Ex vivo lipolysis measurement**

Lipolysis of adipose tissue was measured as the release of glycerol and FFA into the culture medium *ex vivo*.<sup>7</sup> Briefly, the fresh epididymal adipose tissue explants were isolated from mice. Approximately 30 mg of adipose tissue was transferred in 1 ml KRBH medium containing 2% FFA-free BSA, minced into small pieces, and then incubated at 37°C for 3 h. The contents of glycerol and FFA (Abcam) in incubation media were quantified according to the manufacturer's protocols.

### Western blot analysis

After collection, the adipose tissues were centrifuged at  $250 \times g$  for 5 min, followed by removal of supernatant and addition of RIPA lysis buffer with protein extraction cocktail (100  $\mu$ l). Protein was extracted by ultrasonication and centrifuged at  $12000 \times g$  for 15 min  $4^{\circ}\text{C}$ . The protein concentration was quantified by a bicinchoninic acid assay. Proteins (20  $\mu$ g) were subjected to 8% or 15% SDS-PAGE and transferred to polyvinylidene difluoride membranes, which were blocked with 5% non-fat milk for 1 h at room temperature. Then the membranes were further incubated overnight at  $4^{\circ}\text{C}$  with respective primary antibodies: adipose triglyceride lipase (ATGL; Santa Cruz Biotechnology, Shanghai, China), and  $\beta$ -actin (Proteintech, Wuhan, China). After primary antibody incubation, membranes were washed with phosphate-buffered saline containing 0.1% Tween 20 and followed by the incubation of secondary goat anti-mouse IgG-HRP antibody (Proteintech) for 1 h at room temperature. Protein bands were visualised using a Chemiluminescent Detection System (Bio-Rad, Hercules, CA).  $\beta$ -actin was used as the internal reference control.

### Statistical analysis

Data were presented as mean  $\pm$  SEM and analysed using GraphPad Prism 8.4.1 (San Diego, CA). For two-group comparisons, mean differences were analyzed by 2-tailed Student's *t* test. For multi-group comparisons, mean differences were analysed by one-way ANOVA analysis of variance with the



Tukey's multiple comparison post-test.  $P$  value < 0.05 was considered significant.

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