INVESTIGATION OF THE EFFECTS OF AETIOLOGICAL FACTORS AND THERAPEUTIC AGENTS ON THE SEVERITY OF ACUTE PANCREATITIS

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Ph.D. Thesis

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PUBLICATIONS

Articles closely related to the subject of the thesis

I. **Bálint ER**, Fűr G, Kui B, Balla Z, Kormányos ES, Tóth B, Horváth G, Pallagi P, Maléth J, Venglovecz V, Hegyi P, Kiss L, Rakonczay Z Jr. Fentanyl but not morphine or buprenorphine treatment improves the severity of necrotizing acute pancreatitis in rats. Int J Mol Sci. 2022; 23(3):1192. PMID: 35163111 **[IF$_{2020}$: 5.542]**.

II. Tóth E, Maléth J, Závogyán N, Fanczal J, Grassalkovich A, Erdős R, Pallagi P, Horváth G, Tretter L, **Bálint ER**, Rakonczay Z Jr, Venglovecz V, Hegyi P. Novel mitochondrial transition pore inhibitor N-methyl-4-isoleucine cyclosporin is a new therapeutic option in acute pancreatitis. J Physiol. 2019; 597(24):5879-5898. PMID: 31631343 **[IF$_{2019}$: 4.547]**.

III. **Bálint ER**, Fűr G, Kiss L, Németh DI, Soós A, Hegyi P, Szakács Z, Tinusz B, Varjú P, Vincze Á, Erőss B, Czimmer J, Szepes Z, Varga G, Rakonczay Jr.. Assessment of the course of acute pancreatitis in the light of aetiology: a systematic review and meta-analysis. Sci Rep. 2020; 10(1):17936. PMID: 33087766 **[IF$_{2020}$: 3.998]**.

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I. Szentesi A, Tóth E, **Bálint E**, Fanczal J, Madácsy T, Laczkó D, Ignáth I, Balázs A, Pallagi P, Maléth J, Rakonczay Z Jr, Kui B, Illés D, Márta K, Blaskó Á, Demcsák A, Párniczky A, Pár G, Gódi S, Mosztbacher D, Szücs Á, Halász A, Izbéki F, Farkas N, Hegyi P; Hungarian Pancreatic Study Group. Analysis of research activity in gastroenterology: pancreatitis is in real danger. PLoS One. 2016; 24(11)10:e0165244. PMID: 27776171 **[IF$_{2016}$: 3.057].**

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V. Balla Z, Kormányos ES, Kui B, **Bálint ER**, Fűr G, Orján EM, Iványi B, Vécsei L, Fülöp F, Varga G, Harazin A, Tubak V, Deli MA, Papp Cs, Gácser A, Madácsy T, Venglovecz V, Maléth J, Hegyi P, Kiss L, Rakonczay Z Jr. Kynurenic acid and its analogue SZR-72 ameliorate the severity of experimental acute necrotizing pancreatitis. Front Immunol. 2021; 12:702764. PMID: 34745090 **[IF$_{2020}$: 7.561]**.

VI. Fűr G, **Bálint ER**, Orján EM, Balla Z, Kormányos ES, Czira B, Szűcs A, Kovács DP, Pallagi P, Maléth J, Venglovecz V, Hegyi P, Kiss L, Rakonczay Jr Z. Mislocalization of CFTR expression in acute pancreatitis and the beneficial effect of VX-661 + VX-770 treatment on disease severity. J Physiol. 2021; 599(22):4955-4971. PMID: 34587656 **[IF$_{2020}$: 5.182]**.

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LIST OF ABBREVIATIONS

AAP: alcohol-induced/alcoholic acute pancreatitis

AP: acute pancreatitis

BAP: biliary acute pancreatitis

CER: caerulein

CI: 95% confidence interval

CYA: cyclosporin A

Cyp D: cyclophilin D

ERCP: endoscopic retrograde cholangiopancreatography

EtOH: ethanol

FA: fatty acid

FE: fentanyl

HTG: hypertriglyceridaemia

HTG-AP: hypertriglyceridaemia-induced acute pancreatitis

ICU: intensive care unit

i.d.: intraductal

i.p.: intraperitoneal

IL-1β: interleukin-1β

IL-6: interleukin-6

LO: L-ornithine

MO: morphine

MOF: multiple organ failure

MPO: myeloperoxidase

mPTP- mitochondrial transition pore, mitochondrial membrane potential- ψ

NF-kB: nuclear factor kappa B

NIM811: N-methyl-4-isoleucine cyclosporin

OF: organ failure

OR: odds ratio

PAP: post-ERCP-induced acute pancreatitis

POF: persistent organ failure

PS: physiological saline

RAC: Revised Atlanta Classification

TOF: transient organ failure

I. INTRODUCTION

I.1. Physiology of the pancreas

The pancreas functions both as exocrine and endocrine gland. The endocrine part regulates blood glucose concentration by secreting hormones (insulin, glucagon, somatostatin, etc.). The exocrine part of the pancreas secretes 1.5-2 litres of isotonic fluid containing digestive enzymes and high concentrations of bicarbonate (up to 140 mM)[1]. The two most important cell types of the exocrine pancreas are acinar and ductal cells. Acinar cells produce digestive proenzymes and Cl^--rich isotonic fluid. Digestive proenzymes are packed in zymogen granules within the cells. After secretion, enzymes reach the gut lumen, where enterokinase catalyses the conversion of trypsinogen to trypsin. Then trypsin further activates other trypsinogen molecules as well as other proenzymes[2,3]. Ductal cells secrete HCO_3^--rich isotonic fluid. The physiological function of this fluid is to prevent premature activation of trypsinogen inside the ductal lumen, to wash out the digestive enzymes of the ductal tree into the duodenum and to provide optimal pH for the function of the digestive enzymes by neutralising gastric acid[4].

I.2. Acute pancreatitis

Acute pancreatitis (AP) is one of the most common reasons for hospitalization in case of gastrointestinal diseases[5], which has an overall mortality of about 2%[6]. The incidence of the disease is more than 30 per 100 000 population in Europe and this number has shown increasing tendency over time 20%[7].

Gallstones represent the main aetiological background of AP globally (approx. 40%; Figure 1), which are diagnosed by imaging techniques and liver function tests[8,9]. Gallstone-related or biliary AP (BAP) occurs twice as often as alcohol-induced AP (AAP)[8]. AAP is caused by regular, excessive alcohol consumption usually with a clinical history of >5 years and >50-100g/day[10,11]. Severe hypertriglyceridaemia (HTG) with serum triglyceride concentrations >11.3 mM is the third most common (approx. 9%) known aetiological factor of the disease[12,13]. Less frequent causes of AP include endoscopic retrograde cholangiopancreatography (ERCP), hypercalcaemia, pancreas divisum, tumours, genetic polymorphisms and drugs. To date, no standardized diagnostic criteria exist for post-ERCP AP (PAP). The guidelines recommended by Cotton *et al.*[14] are most commonly applied, which suggest PAP to be diagnosed if pancreatitis develops within 24 h after the procedure.

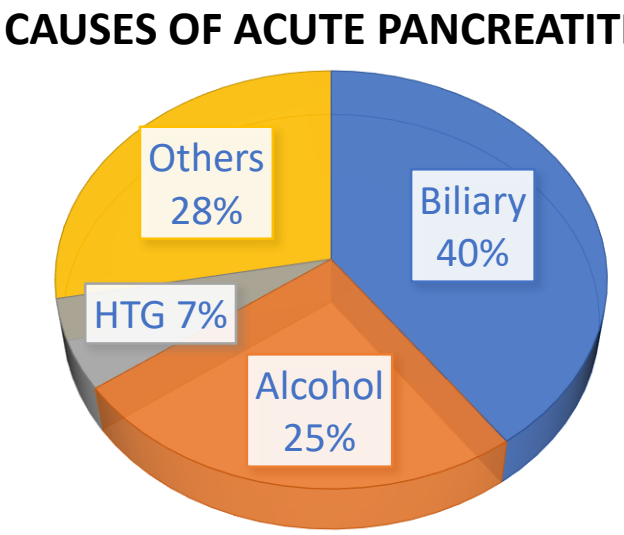


Figure 1. Global incidence of acute pancreatitis based on the publication of Forsmark *et al.*[6] HTG: hypertriglyceridaemia.

I.2.1. Pathomechanism

The factors mentioned above cause pathological Ca^{2+} signaling, which triggers premature trypsinogen activation[15], nuclear factor kappa B (NF-κB) activation, decreased ductal bicarbonate and fluid secretion, inhibition of digestive enzyme secretion, decreased blood flow in the gastrointestinal tract, inhibition of cystic fibrosis transmembrane conductance regulator, decrease of intracellular adenosine triphosphate (ATP), opening of mitochondrial permeability pore, loss of mitochondrial transmembrane potential, mitochondrial damage and endoplasmic reticulum stress[4,16,17]. Mitochondrial damage and decreased ATP levels in the acinar and ductal cells lead to cell death. From the factors mentioned above, the most important ones will be highlighted in terms of the thesis.

I.2.1.1. Premature trypsinogen activation

Normally, digestive enzymes are enfolded in secretory granules isolated from lysosomal hydrolases in order to prevent intracellular zymogen activation[16]. Noxious stimuli induce block of zymogen secretion into the ductal space[18] and colocalization of proenzymes with lysosomal enzymes such as cathepsin B. Cathepsin B activates trypsinogen, which will further activate other trypsinogen and different inactive digestive enzymes (e.g. pro-elastase, pro-collagenase), leading to self-destruction of the acinar cells.

I.2.1.2. Nuclear factor kappa B activation

NF-κB plays a crucial role in regulating the expression of numerous genes involved in inflammation, embryonic development, tissue injury and repair[19]. NF-κB is normally inactive and is bound to inhibitors of κB (IκBs) in the cytoplasm. When cells are stimulated, IκBs get phosphorylated by IκB kinases, polyubiquinated and degraded by 26S proteasomes[20]. When IκB degrades, nuclear translocation signal of NF-κB is revealed and the transcription factor translocates into the nucleus. There, NF-κB binds to DNA sequences and induces the transcription of specific genes which initiate inflammatory cascades; e.g. interleukin (IL)-1β, IL-6, tumour necrosis factor-α (TNF-α), platelet activating factor, intercellular adhesion molecule-1.

I.2.1.3 Mitochondrial damage, cell death

One of the earliest events in AP is mitochondrial dysfunction[2,17,21,22]. It has been shown in acinar cells that agents inducing AP - bile acids or ethanol and fatty acids (EtOH+FA) - open the membrane transition pore (mPTP) channel via cyclophilin D (Cyp D) activation, keeping the channel continuously opened and thus resulting in mitochondrial depolarization, lower ATP synthesis and cell necrosis[2,23,24]. This mechanism was confirmed by Mukherjee *et al*. in four AP models where all relevant local and systemic pathological responses were diminished or eliminated in Cyp D knockout mice and wild type mice injected with mPTP inhibitors[24].

To date, cyclosporin A (CYA) is the only licensed substance applied experimentally to inhibit mPTP (via Cyp D)[25], although its clinical utility is highly controversial for several reasons. In a pilot study, CYA was reported to decrease the rate of damage caused by myocardial infarction, while larger studies did not confirm these results, not to mention its immunosuppressive effect, which could not be diminished[25–27]. In addition, the CYA derivative Debio025 (Alispovirir, Debiopharm, Lausanne, Switzerland) proved effective against the hepatitis C virus, but some of the patients developed pancreatitis. For this reason, a clinical hold was placed on the global Debio025 trial programme[28,29]. Another CYA derivative, TRO40303 (3,5-seco-4-nor-cholestan-5-one oxime-3-o, TROPHOS, Roche, Indianapolis, IN, USA), was not beneficial against acute myocardial infarction in a phase 2 trial[30]. Indeed, it has recently been shown that TRO40303 does not even bind to Cyp D directly[25,31]. With regard to AP, both Debio025 and TRO40303 were reported to be beneficial in experimental studies, but neither of them showed potential for human therapeutic use due to the clinical failures mentioned above.

Recently, a non-immunosuppressive CYA derivative, NIM811 has been shown to have a favourable pharmacokinetic profile and similar oral bioavailability as CYA[32]. NIM811 has been reported to be beneficial in both experimental and clinical settings. No adverse reactions occurred during the studies where NIM811 was applied[32,33].

I.2.1.4. Pancreatic ductal HCO_3^- and fluid secretion

Ductal HCO_3^- and fluid secretion play a crucial role in the physiology of the exocrine pancreas. It washes out toxic agents (such as bile acids) and maintains extracellular pH in order to prevent early zymogen activation. Impaired ductal function modifies the volume and composition of the fluid, which can lead to further aggravate the early enzyme activation and acinar cell damage. Low concentrations of AP triggering factors (EtOH or bile acids) stimulate while high concentrations inhibit HCO_3^- secretion[34,35]. Furthermore, pancreatic duct obstruction can alter acinar cell membrane trafficking, which can facilitate the progression of AP[4].

I.2.2. Diagnosis and treatment

The diagnosis of AP requires the presence of at least two of the following three features: abdominal pain, at least a threefold increase in serum amylase/lipase activity, characteristic findings on contrast-enhanced computed tomography or ultrasonography[36]. To date, there is no specific therapy for AP. The initial treatment is supportive including fluid replacement, nutrition and analgesia. As pain is the most prominent symptom of AP, its relief is priority in clinical settings. Unfortunately, recent guidelines for AP treatment do not have clear recommendations for the types of analgesics to be used[37–39]. Most commonly, the WHO pain management guideline is utilized and treatment ranges from nonsteroidal anti-inflammatory drugs to highly potent opioids. The latter are applied in cases of severe AP and include fentanyl (FE), buprenorphine (BQ), pethidine, pentazocine and morphine (MO)[40]. Although opioids are the most effective pain killers which makes them valuable in clinical settings, there is a scientific debate on their use due to their side effects like constipation or immunosuppression[41,42]. Actually, Meng *et al.*[43] attempted to collect all randomized controlled trials which investigated the side effects of analgesics (opioids and non-opioids) in AP, but the included studies were of low quality, without clear outcome. However, the use of MO is often not preferred in humans due to spasm of sphincter of Oddi, which might worsen the outcome of AP[44]. Even more importantly, Barlass *et al.*[45] have also shown the drawbacks of MO use in AP and the pathological processes of its side effects in a mouse model.

I.2.3. Classification of severity

Based on the Revised Atlanta Classification (RAC), AP severity can be categorized into three groups: mild, moderately severe and severe[36]. Although the majority of cases are mild with a self-limiting course[36], the mortality rate of severe AP can reach 30% which underlies the desperate need of finding proper treatment[46]. Organ failure (OF) is the most important determinant of this classification system[36]. Patients with mild AP have no organ dysfunction and usually recover within a week. Moderately severe AP resolves slower and might require interventions because of the presence of transient OF (TOF, <48 h). Severe AP results in persistent organ failure (POF) which lasts >48 h. Multiple organ failure (MOF) is defined as failure of two or more organ systems, which can be transient or persistent[47]. The three extrapancreatic organs most commonly affected by AP are the lungs, the heart and the kidneys[36]. Approximately 25% of AP patients develop severe complications and have to be admitted to an intensive care unit (ICU)[48]. Local complications can also occur in cases of moderately severe and severe AP, which include acute peripancreatic fluid collections, pancreatic pseudocysts, acute necrotic collections and walled-off necrosis[36]. About 25-30% of patients experience recurrent AP, which refers to a clinical condition defined by repeated episodes of AP[49]. Recurrent AP has a high risk of progression to chronic pancreatitis or pancreatic cancer.

Although there are several risk factors, it is difficult to predict which patient will develop mild, moderately severe or severe AP. To date, numerous clinical studies have investigated the effect of aetiology on AP progression. However, to the best of our knowledge, there have been no efforts to summarize clinical data on how various aetiological backgrounds affect the severity and course of AP.

II. AIMS

The main aim of this work was to assess the effects of analgesia and mitochondrial protection on the course of AP. Furthermore, we wanted to reveal the predisposing effect of aetiology on AP severity. Our detailed aims were the following:

1. to investigate the effects of opioid (FE and MO) administration (pre-or post-treatments) on the course of AP in different rat disease models
2. to test the effects of the novel CYA derivative NIM811 on the severity of AP during *in vivo* experiments in mice
3. to reveal the impact of the aetiological factors for AP on disease severity by performing thorough literature search and meta-analysis on available clinical data

III. MATERIALS AND METHODS

III.1. Animal experiments

III.1.1. Animals and ethical approval

Wistar rats weighing 200-250 g were used for the experiments related to opioid treatment, while mPTP was targeted in C57Bl/6 J mice weighing 25-30 g. The animals were kept at a constant room temperature of 24 °C with a 12-h light–dark cycle and were allowed free access to water and standard laboratory chow (Biofarm, Zagyvaszántó, Hungary). All experiments were performed in compliance with the European Union Directive 2010/63/EU and the Hungarian Government Decree 40/2013 (II.14.). Experiments were approved by both local (University of Szeged) and national ethics committees (X/3354/2017 and XII/4988/2015) for investigations involving animals.

III.1.2. Chemicals

All chemicals were purchased from Sigma-Aldrich (Budapest, Hungary) unless indicated otherwise.

III.1.3. Induction of experimental acute pancreatitis

Necrotizing AP was induced in rats by a single intraperitoneal (i.p.) injection of 3 g/kg L-ornithine-HCl (LO, 30%, pH=7.4). Oedematous AP was induced by hourly i.p. injections of 20 μg/kg caerulein (CER, 50 μg/ml) four times in rats as well. EtOH+FA AP was induced by i.p. injection of 1.75 g/kg ethanol and 750 mg/kg palmitic acid as described previously[50,51] Control groups were given physiological saline (PS: 0.9% NaCl) solution instead of LO/CER/EtOH+FA respectively. Animals were sacrificed at 24 h in the LO- and EtOH+FA-induced experimental pancreatitis models, and at 12 h in case of the CER-model.

At the end of experiments/treatments, deep anaesthesia was induced by pentobarbital injection (85 mg/kg i.p. for the rats and 200 mg/kg for the mice; Bimeda MTC, Cambridge, Canada). Blood was collected through cardiac puncture, then the pancreas was rapidly removed. Pancreata were cleaned from fat and lymph nodes on ice, then cut into pieces. Two parts of the pancreatic tissue were immediately frozen in liquid nitrogen and stored at –80 °C until biochemical assays or dry-wet weight measurements were performed. The third part of the pancreas was fixed in 8% neutral formaldehyde solution for histological analysis. Blood samples were centrifuged at 2500 RCF for 15 min at 4°C, the sera were collected and stored at –20 °C until use.

III.1.4. Opioid treatments

FE was administered at doses of 0.1 and 0.2 mg/kg based on literature data[52]. Different timing arrangements were applied for FE in various AP models; repeated injections were performed when the analgesic effect of FE was decreased (this was determined in preliminary experiments or by literature data). In addition, FE was used as pre- or post-treatment.

In the pre-treatment groups, the first FE injection was given 1 h prior to the induction of AP and it was repeated every 10 h in CER- or 11 h in LO-induced AP, respectively (Figure 2A). In the post-treatment setup, animals received the first FE injection 1 h after AP induction in case of the LO-model or 0.5 h after AP induction in case of the CER-model.

In the post-treatment setup, 5 mg/kg MO was administered i.p. 8 times every 2 h in case of the LO-model (Figure 2B). The dose and timing of MO was chosen based on literature data[53]; repeated injections were performed when the analgesic effect of MO was decreased (this was determined by preliminary experiments or literature data). During pre-treatment, 10 mg/kg MO was injected i.p. 9 times every 2 h (Figure 2B). When AP was induced by CER, 4x5 mg/kg dose of MO was used i.v. every 2 h and analgesia started simultaneously with AP induction (Fig 2B). Animals were sacrificed 24 or 12 h after AP induction with LO or CER, respectively.

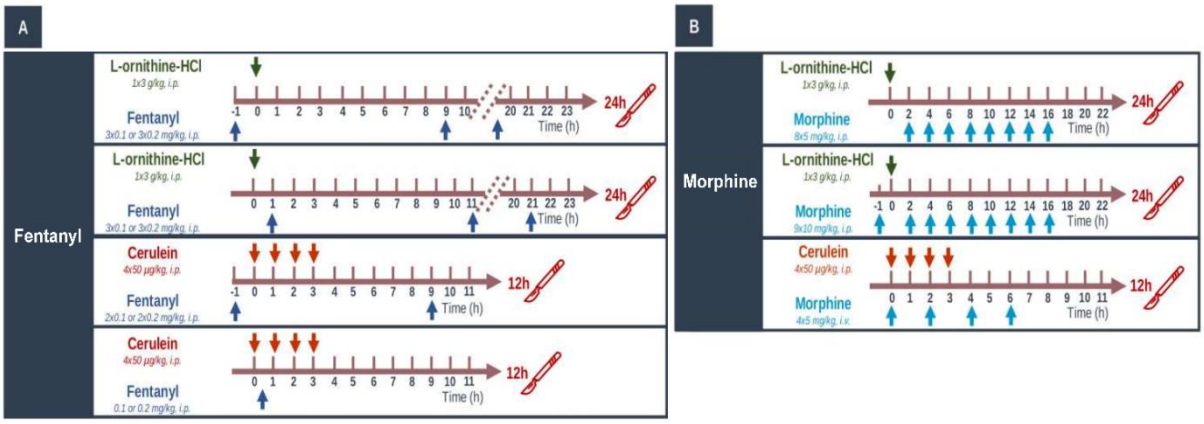


Figure 2. Schematic view of opioid treatments.
Treatment arrangements for acute pancreatitis induction and opioid administration in Wistar rats. Arrows above or below the timeline show the injections. Control animals were injected with physiological saline. I.p: intraperitoneal; i.t.: intrathecal; i.v.: intravenous.

III.1.5. NIM811 administration

NIM811 (MedChem Express Europe, Sollentuna, Sweden) was gavaged orally 1 h prior to EtOH+FA AP induction in the pre-treatment setup and 12 h after AP induction in the post-treatment groups. NIM811 was administered at doses of 5 and 10 mg/kg. Oral gavage treatment were performed by the use of plastic feeding tubes (20 gauge × 38 mm, Instech Laboratories,

Plymouth Meeting, PA, USA). NIM811 were solubilized in a vehicle which contained 8.3% polyoxyl 40 hydrogenated castor oil (Kolliphor RH40) and 8.3% EtOH[54]. Mice were sacrificed 24 h after AP induction.

III.1.6. Laboratory measurements

Serum amylase activity was measured on a Fluorostar Optima plate reader (BMG Labtech, Ortenberg, Germany) with a colorimetric kinetic method using a commercial kit purchased from Diagnosticum ZRt. (Budapest, Hungary).

To evaluate pancreatic water content, the wet weight (WW) of the pancreata was measured, then the tissues were dried for 24 h at 100 °C and the dry weight (DW) was also measured. The wet/dry weight ratio was calculated as: [(WW-DW)/WW]×100.

Pancreatic myeloperoxidase (MPO) activity is a hallmark of leukocytic infiltration and was measured according to Kuebler *et al.*[55]. MPO activities were normalized to total protein content as measured by the Lowry method[56].

To determine the extent of inflammatory response in the pancreata, we measured IL-1β levels by a commercial ELISA kit from R&D Systems (Minneapolis, MN, USA) as described by the manufacturer.

III.1.7. Histological examination

Formalin-fixed pancreatic tissues were sectioned to 3 μm. These sections were prepared and stained with hematoxylin and eosin and were analysed and scored by two independent experts blinded to the experimental protocol. Oedema was scored between 0-3 points (0: none; 1: patchy interlobular; 2: diffuse interlobular; 3: diffuse interlobular and intra-acinar), leukocytic infiltration between 0-4 points (0: none; 1: diffuse/mild; 2: diffuse/moderate; 3: diffuse/severe; 4: diffuse/very severe), vacuolisation between 0-3 points (0: none; 1: diffuse/mild; 2: diffuse/moderate; 3: diffuse/severe), and the percentage of acinar cell damage was also evaluated.

III.1.8. Statistical analysis

Data are presented as means ± SEM. Experiments were evaluated by one- or two-way ANOVA followed by Holm–Sidak post hoc test (SPSS, IBM, Armonk, NY, USA). $P<0.05$ was accepted as statistically significant.

III.2. Meta-analysis

III.2.1. Protocols applied

The systematic review and meta-analysis followed the recommendations of Stroup *et al.*[57] and was conducted in line with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses guidelines[58]. The analysis was based on the Problem, Intervention, Comparison intervention and Outcome (PICO) model as follows: AP patients with alcoholic, biliary, hypertriglyceridaemic and post-ERCP aetiologies were compared in order to examine the effect of aetiology on disease outcomes. Primary outcome was severity, secondary outcomes were POF, MOF, TOF, ICU admission, recurrence rate, mortality, pancreatic necrosis, pulmonary failure (PUF), renal failure, length of hospital stay (LOS), pseudocyst, fluid collection, and systematic inflammatory response syndrome. The protocol for the meta-analysis was registered in the PROSPERO database on 05/15/2018 (https://www.crd.york.ac.uk/PROSPERO/, ID: CRD42018093574).

III.2.2. Search strategy

Literature search was conducted in the electronic databases Embase and Pubmed from publication date 01/01/2012 to 05/31/2018. The reason for the start date is that the RAC was introduced in 2012, which provides the most accepted and widespread criteria for determining AP severity. The following search query was used for Embase: (alcohol* OR ethanol* OR biliary OR gallstone OR cholelithiasis OR 'post-ercp' OR 'post ercp' OR idiopathic OR triglyceride OR hypertriglyceridemia OR hyperlipidemia OR severity OR severe OR mild OR moderate) AND acute AND pancreatitis NOT ('conference abstract'/it OR 'review'/it) AND (2012:py OR 2013:py OR 2014:py OR 2015:py OR 2016:py OR 2017:py OR 2018:py). In Pubmed the following search terms were applied: (alcohol* OR ethanol* OR biliary OR gallstone OR cholelithiasis OR "post-ercp" OR "post ercp" OR idiopathic OR triglyceride OR hypertriglyceridemia OR hyperlipidemia OR severity OR severe OR mild OR moderate) AND acute AND pancreatitis NOT Review[ptyp] NOT Case Reports[ptyp]. Due to the limitations of the length of the thesis, only the results of AAP, BAP and HTG-AP patients are analysed and presented here. For further details please see the Appendix. The search was restricted to studies written in English or in Hungarian.

III.2.3. Eligibility criteria

All randomised trials, retrospective and prospective cohort studies were included that involved adult patients with AP and relevant data are categorized according to the aetiology of the disease. Four major disease backgrounds were included: alcohol abuse, HTG, biliary disease and post-ERCP. Articles that studied only one aetiological group or compared one aetiological group with another group called others or non-… (e.g. alcohol vs. non-alcohol) were excluded. Non-human studies or articles with data from patients younger than 18 years of age were not included. In case of cohort overlap between studies, only the most recent study was included unless a prior study had higher quality.

When assessing AP severity, only studies were included where severity was defined according to the RAC, because in this case it was crucial to present a consistent and clear definition for the analysis. Articles were also excluded if only one or two of the three severity groups were analysed. Both local complications and OFs could lead to serious conditions and death which are characteristic features of moderately severe and severe AP. Therefore, these two groups were combined in our study, and are referred to as "non-mild" disease forms and compared to the mild group. In cases of outcomes other than severity, using only the RAC was not in the criteria.

III.2.4. Study selection and data extraction

Titles and abstracts of publications were screened independently by two review authors to identify studies that potentially meet inclusion criteria. The full texts of these potentially eligible studies were independently assessed for eligibility by the same two review authors. Disagreement between reviewers was resolved by discussion with other two colleagues. Two review authors independently extracted study characteristics (author, title, journal, study location, inclusion period, number of centres involved, type of study, number of participants) and outcome data (severity, POF, MOF, TOF, ICU admission, recurrence rate, mortality, pancreatic necrosis, PUF, renal failure, LOS, pseudocyst, fluid collection, systematic inflammatory response syndrome), which were recorded on a standardized Microsoft Excel spreadsheet. Discrepancies were resolved by discussion. Due to the limitations of the thesis, only the outcomes of severity, POF, MOF, ICU admission, recurrence rate, mortality and pancreatic necrosis are included here. Please find more details in our Scientific Reports publication.

III.2.5. Quality assessment

Methodological quality of the articles was assessed by applying the Quality In Prognosis Studies (QUIPS) tool[59]. This considers the following domains: study participation, study attrition, prognostic factor measurement, outcome measurement, study confounding, and statistical analysis and reporting. All domains were scored by three individual researchers (each article was assessed by at least two of them). The overall risk of bias was considered:

- low if <3 domains were rated a moderate risk of bias and all others were rated a low risk of bias,
- moderate if ≥3 domains were rated a moderate risk of bias and all others were rated a low risk of bias,
- high if ≥1 domain was rated a high risk of bias, irrespective of all other domains.

Consensus was reached after classification by the individual researchers.

III.2.6. Data analyses

Statistical analysis was performed with Stata 11 SE (StataCorp LLC, College Station, TX, USA). Odds ratios (ORs) calculated from patient numbers were used to compare outcomes in different aetiologic groups. ORs were pooled using the random effects model with the DerSimonien–Laird estimation and displayed on forest plots. Summary OR estimation, p value and 95% confidence interval (CI) were calculated. $P<0.1$ was considered as significant difference from summary OR=1. BAP is defined as primary reference group, the other aetiologies are ranked in the following order: AAP, HTG-AP.

Statistical heterogeneity was analysed using the I^2 statistic and the chi-square test to acquire probability values; $p<0.1$ was defined to indicate significant heterogeneity. The small-study effect (in case of comparisons with at least 10 articles) was visually investigated on funnel plots and was also confirmed by Egger's test. Sensitivity analysis was performed to examine the robustness of our results.

IV. RESULTS

IV.1. Animal experiments

IV.1.1. The effect of fentanyl pre-treatment on acute pancreatitis severity

The pancreata of the control group displayed normal morphology, and FE alone did not induce any structural changes in the pancreas (Figures 3, 4A). LO-induced AP resulted in about 60% pancreatic necrosis and intensive leukocyte infiltration (Figure 4A-C). These signs even worsened due to FE pre-treatment (Figure 4A-C). The extent of tissue necrosis significantly increased when the higher dose (3x0.2 mg/kg) of FE was applied, whereas the level of leukocyte infiltration was higher in the 3x0.1 mg/kg FE & AP group compared to the AP group not receiving FE. FE treatment did not cause any significant change in pancreatic water content in the AP groups (Figure 4D). The serum amylase activities markedly increased in the AP groups versus the control group (Figure 4G). Importantly, 3x0.1 mg/kg FE significantly increased serum amylase activity during AP. MPO activity was greatly elevated in the AP groups compared to the control group (Figure 4F), and the dose of 3x0.2 mg/kg FE further increased MPO activity in AP. Interestingly, the concentration of pancreatic IL-1β significantly decreased due to 3x0.1 mg/kg FE treatment in the AP group.

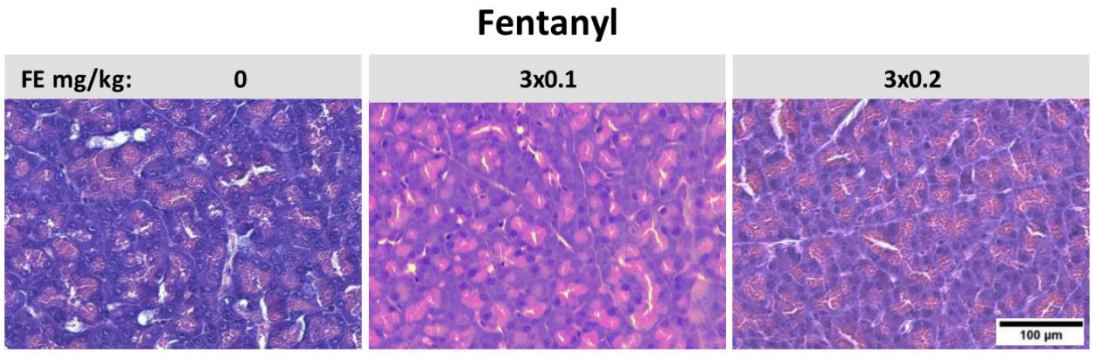


Figure 3. Representative histopathological images of pancreatic tissues of the control groups in cases of fentanyl (FE). Doses of FE used are provided above each image.

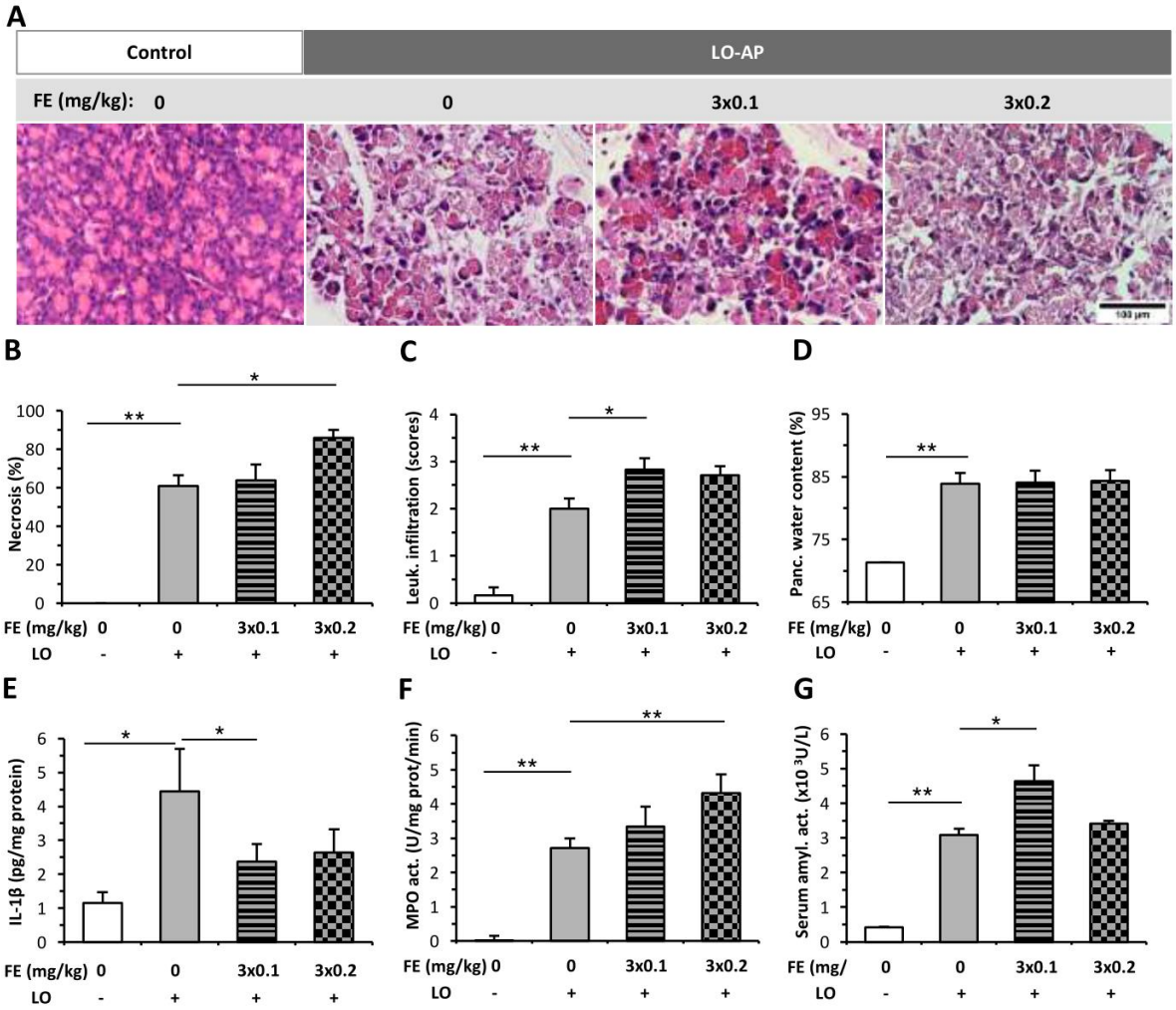


Figure 4. Fentanyl (FE) pre-treatment in L-ornithine (LO)-induced acute pancreatitis.
A) Representative histopathological images of pancreatic tissues of the treatment groups. Bar charts show the extent of pancreatic B) necrosis, C) leukocyte infiltration, D) water content, E) interleukin (IL)-1β concentration, F) myeloperoxidase (MPO) activity, and G) serum amylase activity measurements. Values represent means ± standard error, n=9-11. *: $p<0.05$; ** $p<0.001$.

I.p. injections of CER induced mild AP and increased the extent of pancreatic vacuolisation, leukocyte infiltration and oedema (Figure 5A-D) compared to the control group (histology of control is shown in Figure 3). FE pre-treatment did not cause any change during AP progression in histological parameters or water content (Figure 5A-D). CER-induced AP resulted in elevated serum amylase activity and pancreatic IL-1β content, whereas it did not significantly affect MPO activity (Figure 5 E-G). FE pre-treatment did not alter serum amylase activity or pancreatic IL-1β level in the AP groups.

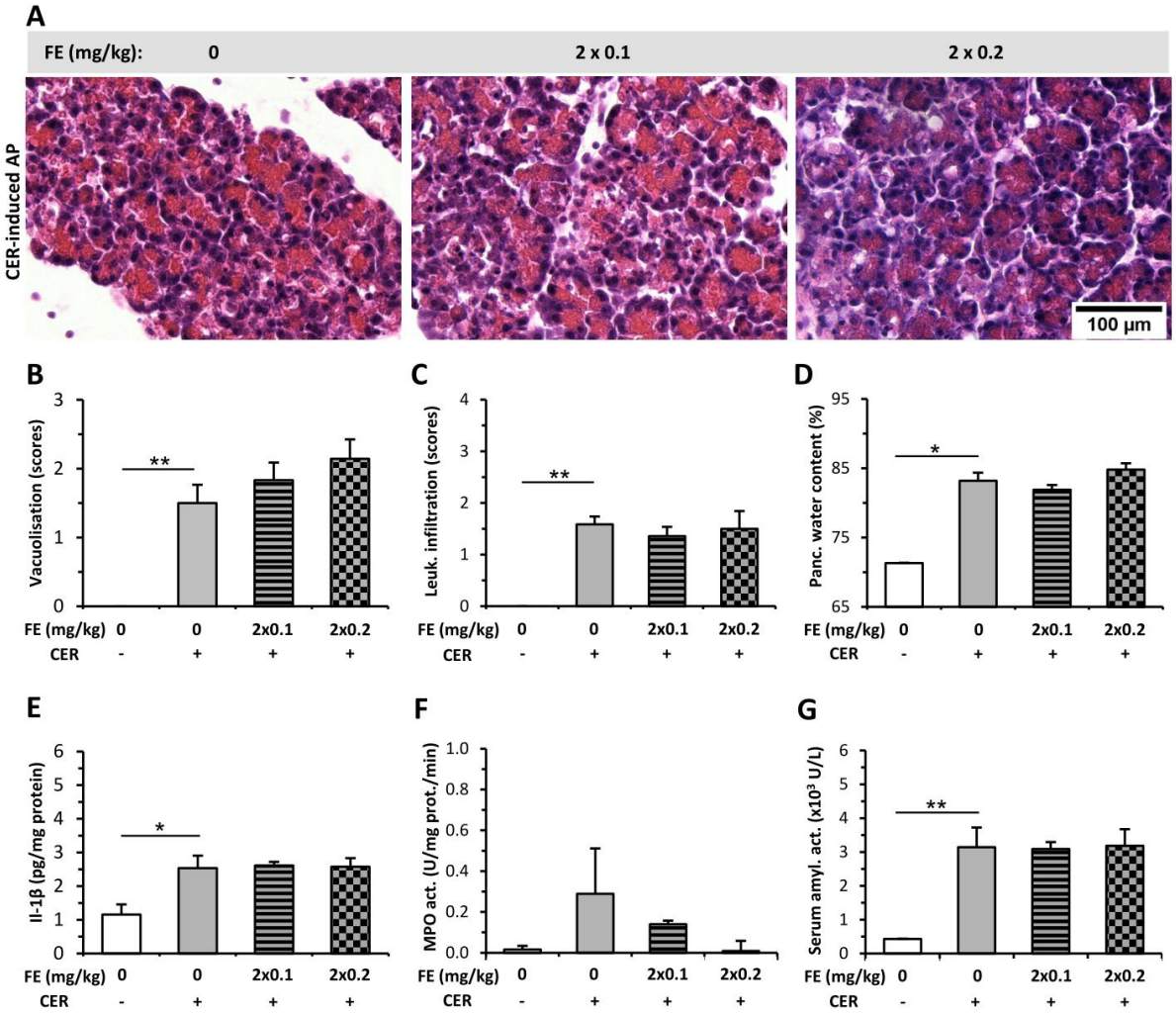


Figure 5. Fentanyl (FE) treatment started before induction of acute pancreatitis with caerulein (CER) did not affect disease severity.
A) Representative histopathological images of pancreatic tissues of the treatment groups. Bar charts show the extent of pancreatic B) vacuolisation, C) leukocyte infiltration, D) water content, E) IL-1β concentration, F) MPO activity, and G) serum amylase activity measurements. Values represent means ± standard error, n=5-7. *: $p<0.05$; ** $p<0.001$.

IV.1.2. The effect of fentanyl post-treatment on acute pancreatitis

In contrast to FE pre-treatment, both doses of FE post-treatment decreased the extent of histopathological changes (leukocyte infiltration and pancreatic tissue necrosis) caused by LO-induced AP (Figure 6A-C). On the other hand, FE administration did not alter pancreatic water content in the AP groups (Figure 6D). LO-induced AP increased serum amylase and pancreatic MPO activities, which were decreased by both FE doses tested (Figure 6F-G). Pancreatic IL-1β levels only decreased significantly in case of the LO + 3 x 0.2 mg/kg FE group (Figure 6E).

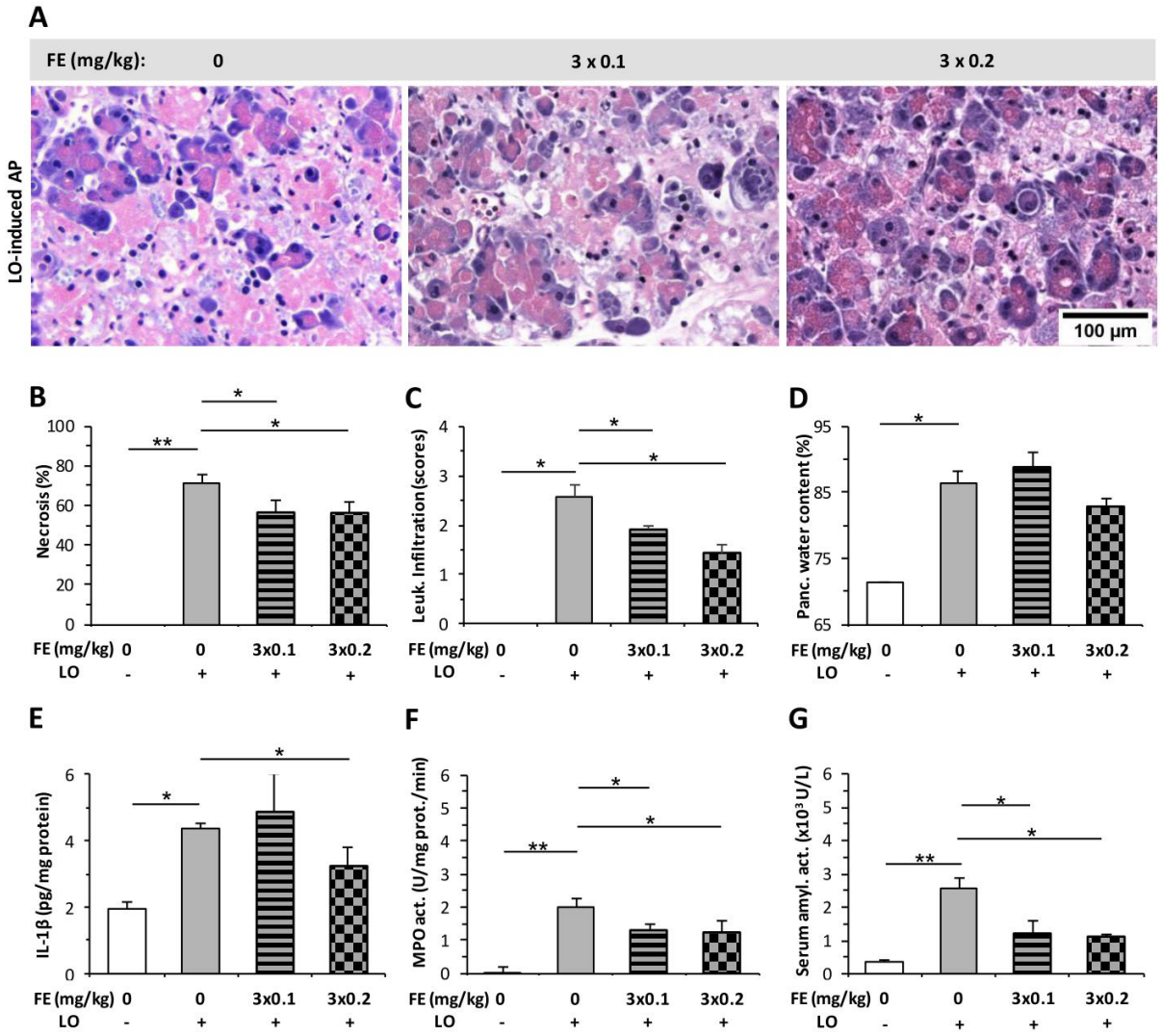


Figure 6. Fentanyl (FE) treatment started 1 hour after induction of L-ornithine (LO) acute pancreatitis reduced disease severity.
A) Representative histopathological images of pancreatic tissues of the treatment groups. Bar charts show the extent of pancreatic B) necrosis, C) leukocyte infiltration, D) water content, E) IL-1β concentration, F) MPO activity, and G) serum amylase activity measurements. Values represent mean ± standard error, n=10-18. *: $p<0.05$; ** $p<0.001$.

I.p. injections of CER increased the extent of pancreatic vacuolisation, leukocyte infiltration, and tissue water content causing mild oedematous AP (Figure 7A-D). FE treatment did not affect either histological parameters (tissue necrosis, leukocyte infiltration) or pancreatic water content (Figure 7A-D). The elevated amylase and MPO activities during AP were unaffected by FE post-treatment (Figure 7F-G). Interestingly, the smaller dose of FE (0.1 mg/kg) further increased the elevated serum IL-1β during AP, while the higher dose of FE had no effect (Figure 7E).

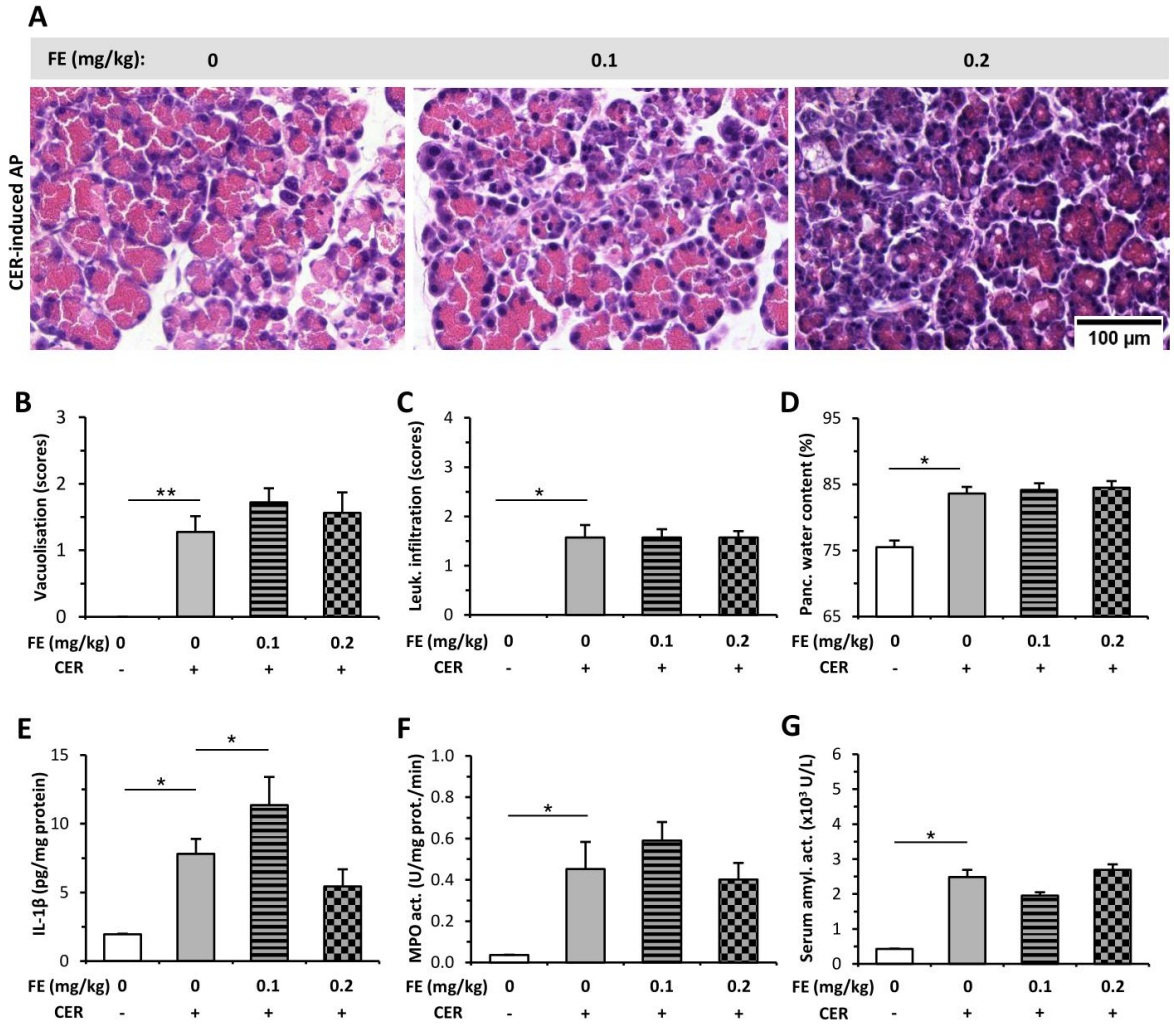


Figure 7. Fentanyl (FE) treatment started after induction of acute pancreatitis with caerulein (CER) did not affect disease severity.
A) Representative histopathological images of pancreatic tissues of the treatment groups. Bar charts show the extent of pancreatic B) vacuolisation, C) leukocyte infiltration, D) water content, E) IL-1β concentration, F) MPO activity, and G) serum amylase activity measurements. Values represent mean ± standard error, n=6. *: $p<0.05$; ** $p<0.001$.

IV.1.3. Morphine administration did not affect the severity of acute pancreatitis

The effect of MO was investigated on the severity of LO-induced AP by using doses of 9x10 and 8x5 mg/kg of the drug (pre- and post-treatment, respectively; Figure 2B). MO at the tested doses did not induce any structural changes in pancreatic tissues of rats, and no inflammatory cell infiltration could be observed in histological sections (results not shown). Treatment with LO-induced AP resulted in marked pancreatic damage (tissue necrosis, leukocyte infiltration and increased pancreatic water content Figure 8A-D). MO did not significantly alter the value of these parameters during AP. Furthermore, MO did not influence serum amylase or pancreatic MPO activity in the AP groups either (Figure 8E-F).

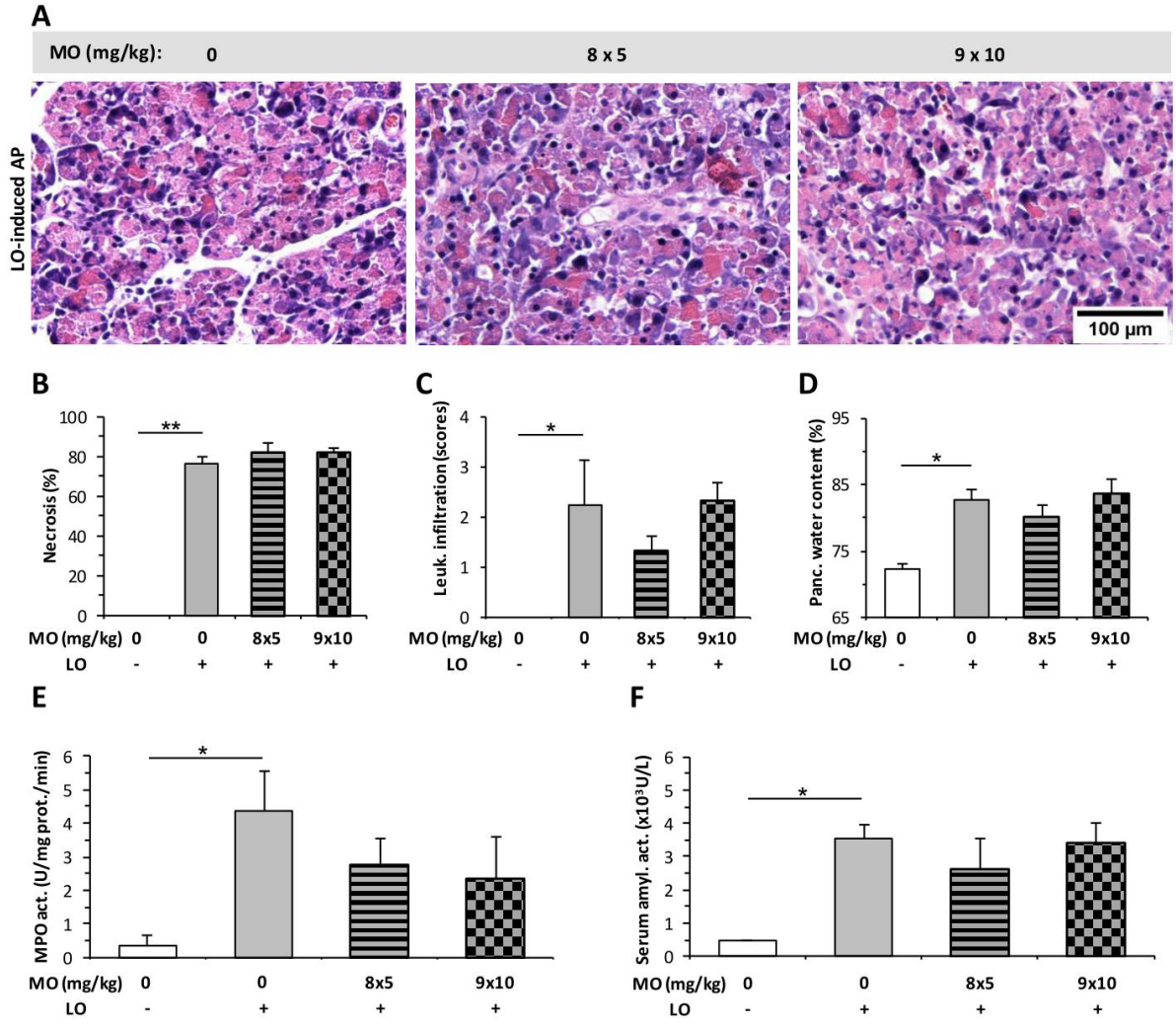


Figure 8. Morphine (MO) treatment did not affect the severity of L-ornithine (LO)-induced acute pancreatitis.
A) Representative histopathological images of pancreatic tissues of the treatment groups. Bar charts show the extent of pancreatic B) necrosis, C) leukocyte infiltration, D) water content, E) MPO activity, and F) serum amylase activity measurements. Values represent mean ± standard error, n=6. *: $p<0.05$; ** $p<0.001$.

The effect of 4x5 mg/kg MO was tested in a CER-induced AP model. Due to the shorter duration of AP in case of CER (12 h) compared to the LO model (24 h), the number of MO injections was reduced from eight (applied in LO-induced AP, Figure 8) to four. MO was administered simultaneously with CER. In the CER-induced oedematous AP MO significantly reduced vacuolisation (Figure 9A-B), but it had no effect on leukocyte infiltration or pancreatic water content (Figure 9C-D). Serum amylase activity was significantly elevated after AP induction and MO had no further effect on it (Figure 9F). However, AP did not induce any significant increase in pancreatic MPO activity (Figure 9E). Both of these parameters showed

negligible increase indicating mild AP. MO had no additional effect on MPO activity during AP (Figure 9E).

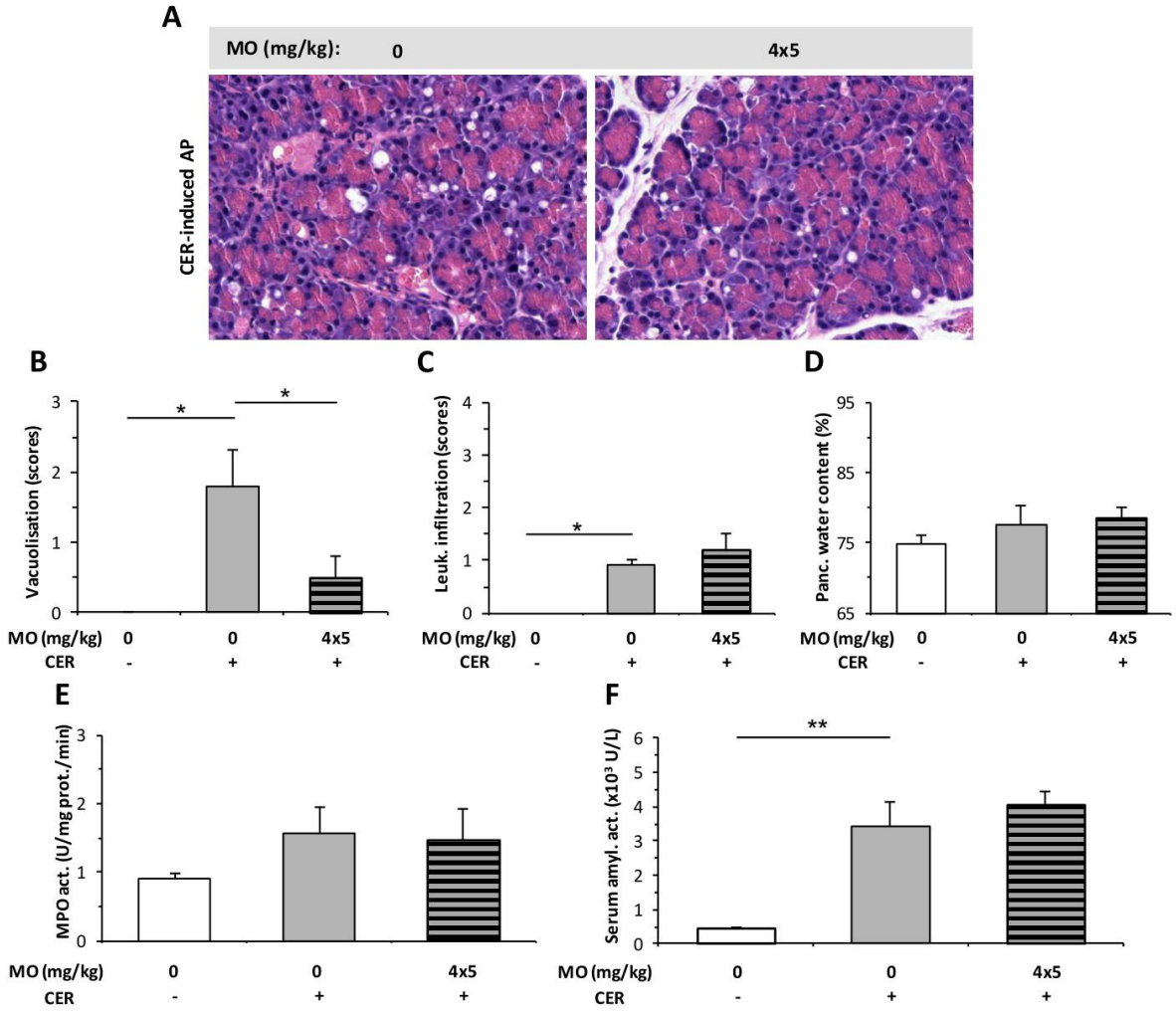


Figure 9. Morphine (MO) treatment did not affect the severity of caerulein (CER)-induced acute pancreatitis.
A) Representative histopathological images of pancreatic tissues of the treatment groups. Bar charts show the extent of pancreatic B) vacuolisation, C) leukocyte infiltration, D) water content, E) MPO activity, and F) serum amylase activity measurements. Values represent mean ± standard error, n=6. *: $p<0.05$; ** $p<0.001$.

IV.1.4. NIM811 has a protective effect against EtOH+FA-induced acute pancreatitis

The i.p. administration of EtOH+FA significantly increased serum amylase activity and the scores of necrosis, oedema and leukocyte infiltration compared to the absolute control group. The higher dose (10 mg/kg) of NIM811 pre-treatment significantly reduced most parametres of AP examined by us (elevated serum amylase levels, necrosis and leukocyte infiltration), whereas oedema was not influenced by it (Figure 11A-E). Furthermore, the 5 mg/kg dose had no effect on the severity of AP compared to the control AP group.

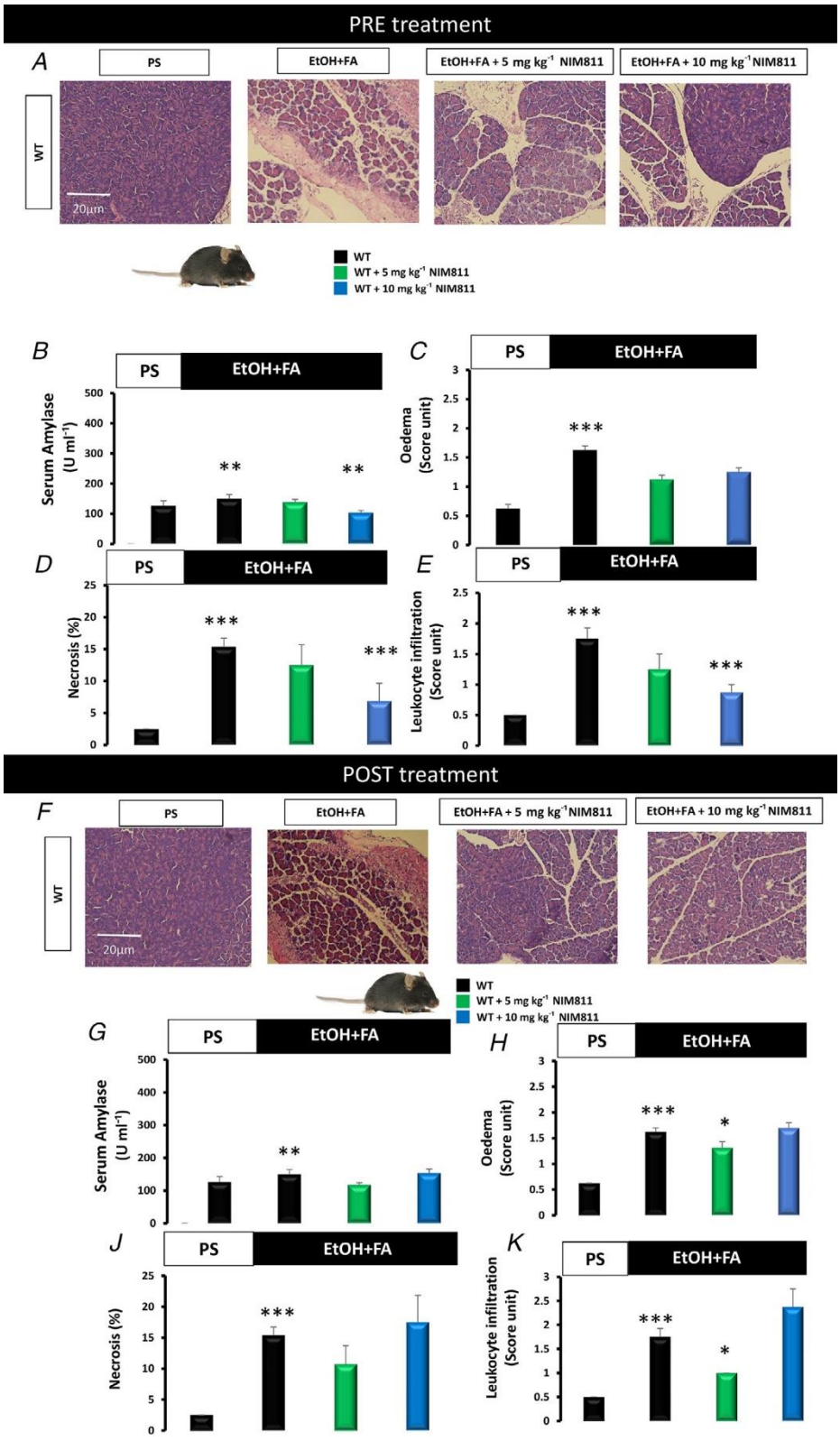


Figure 10. NIM811 treatment improves the severity of ethanol+fatty acid (EtOH+FA)-induced AP.
A-E NIM811 pre-treatment: A) Representative histopathological images of pancreatic tissues of the treatment groups. Bar charts show the extent of B) serum amylase activity, **: p: < 0.002 EtOH+FA vs. pre 10 mg/kg NIM811 + EtOH+FA, PS vs. EtOH+FA C) oedema, ***: p < 0.001 PS vs. EtOH+FA, D) necrosis, ***: p: < 0.001 EtOH+FA vs. pre 10 mg/kg NIM811 + EtOH+FA, PS vs. EtOH+FA, E) leukocyte infiltration, ***: p: < 0.001 EtOH+FA vs. pre 10 mg/kg NIM811 + EtOH+FA, PS vs. EtOH+FA.
F-K NIM811 post-treatment: F) Representative histopathological images of pancreatic tissues of the treatment groups. Bar charts show the extent of G) serum amylase activity, **: p < 0.002 PS vs. EtOH+FA, H) oedema, *: p < 0.05 EtOH+FA vs. post 5 mg/kg NIM811 + EtOH+FA, ***: p: < 0.001 PS vs. EtOH+FA, J) necrosis, ***: p: < 0.001 PS vs. EtOH+FA, K) leukocyte infiltration, *: p < 0.05 EtOH+FA vs. post 5 mg/kg NIM811 + EtOH+FA, ***: p: < 0.001 PS vs. EtOH+FA. Values represent mean ± standard error, n=6.

In case of NIM811 post-treatment, the lower dose (5 mg/kg) improved leukocyte infiltration and oedema, while serum amylase activity or necrosis levels did not differ significantly (Figure 10 F-K). The 10 mg/kg dose did not affect any of the examined parameters in AP compared to the AP group not receiving NIM811.

IV.2. Meta-analysis

IV.2.1. Study selection

The search strategy identified 11,288 records (Figure 11). After removing duplicates 7,733 articles were retrieved. Out of these, 456 records seemed to be relevant to the study question based on screening by title or abstract. After assessing the articles in full text, 329 records had to be excluded with different reasons (see details in Figure 11). Finally, 127 publications fulfilled the eligibility criteria.

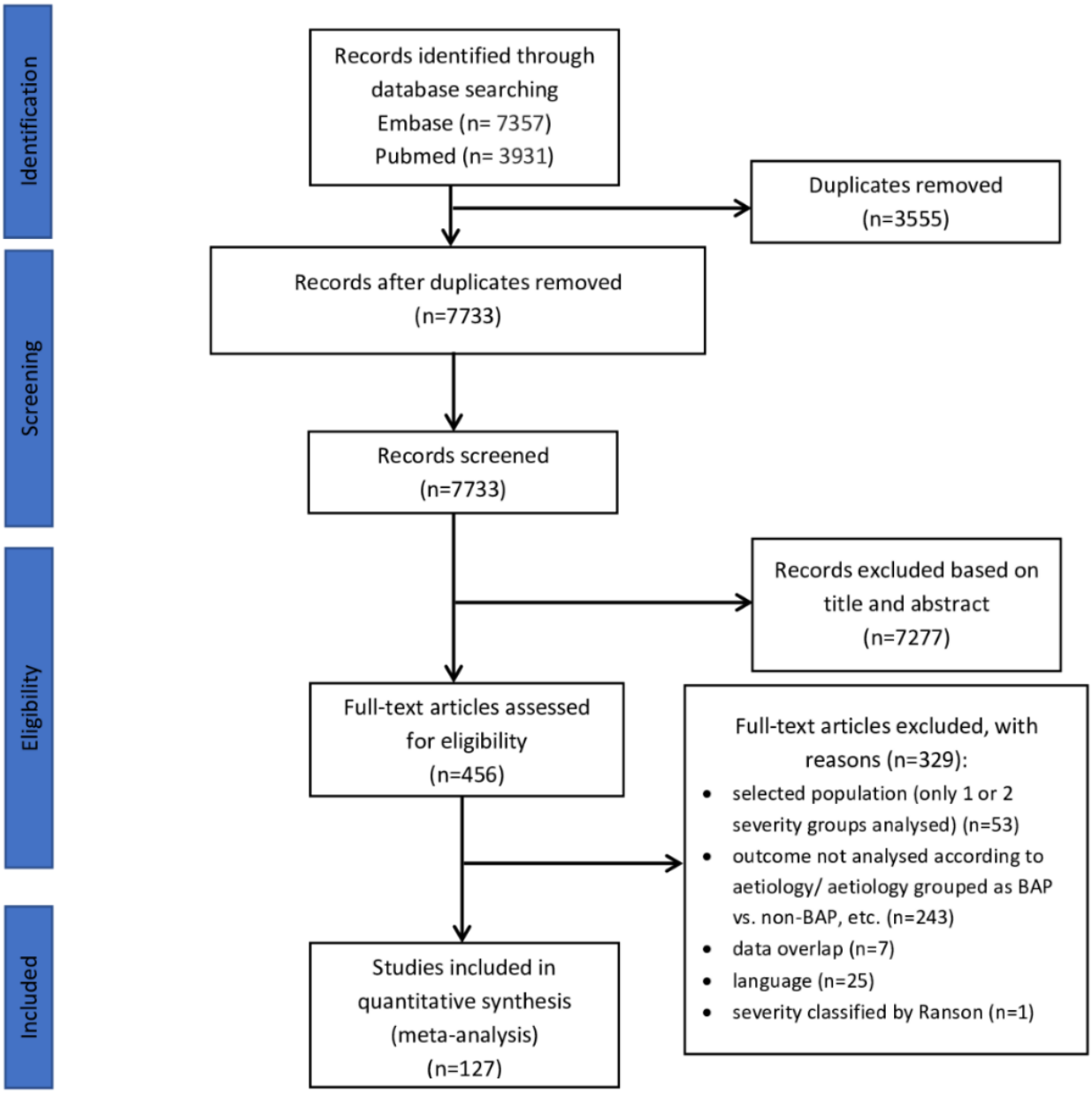


Figure 11. PRISMA 2009 flow diagram for identification of relevant articles

IV.2.2. Characteristics of studies included

The majority of the included cohort studies (108 out of 127) collected data from the 2010's. Our meta-analysis contained 102 single[60–161] and 23 multicentre studies[162–184]. In two cases, there were no relevant data regarding the number of centres involved[185,186]. Sample sizes ranged from 11 to 1,165,777. Only the data of the four types of AP (AAP, BAP, HTG-AP, PAP) were analysed in the article, of which the data of PAP are not included in the thesis. Due to the limitations of the thesis, the detailed characteristics of the included studies or the quality assessment are not included here. Please find the details in our Scientific Reports publication.

IV.2.3. Clinical outcomes

IV.2.3.1. Severity

HTG proved to induce non-mild AP in a significantly higher number of cases than the other aetiological factors (Figure 12). ORs of non-mild cases in HTG-AP were 1.35 [CI: 1.12-1.63] and 1.35 [CI: 1.13-1.62] vs. AAP and BAP, respectively (Figure 12). Alcoholic aetiology significantly increased AP severity compared to biliary-related events (Figure 13; OR: 1.36 [CI: 1.15-1.60]). We found heterogeneity in comparison of AAP vs. BAP (Figure 13). No signs of small-study effect could be detected in comparisons of AAP vs. BAP, AAP vs. HTG-AP, BAP vs. HTG-AP.

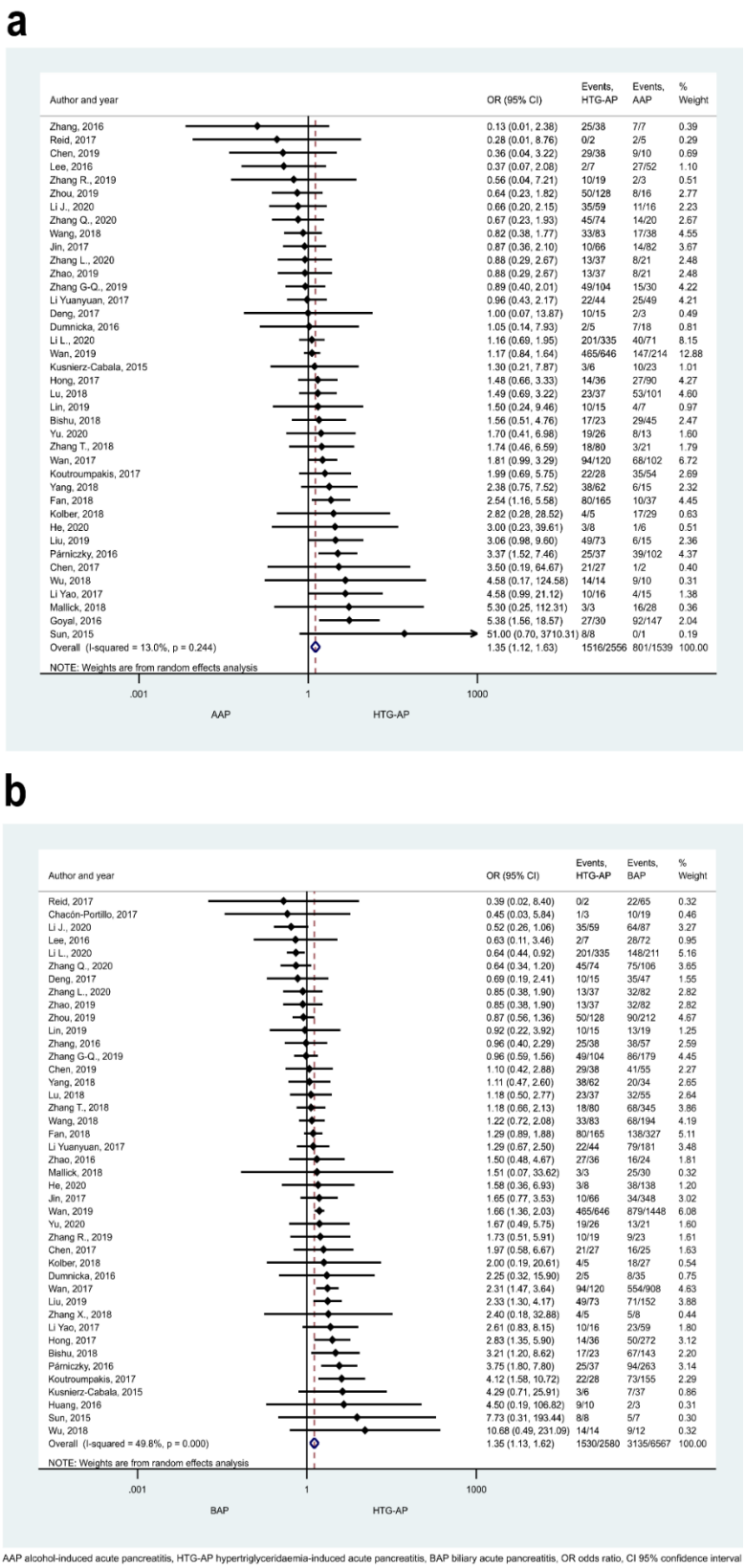
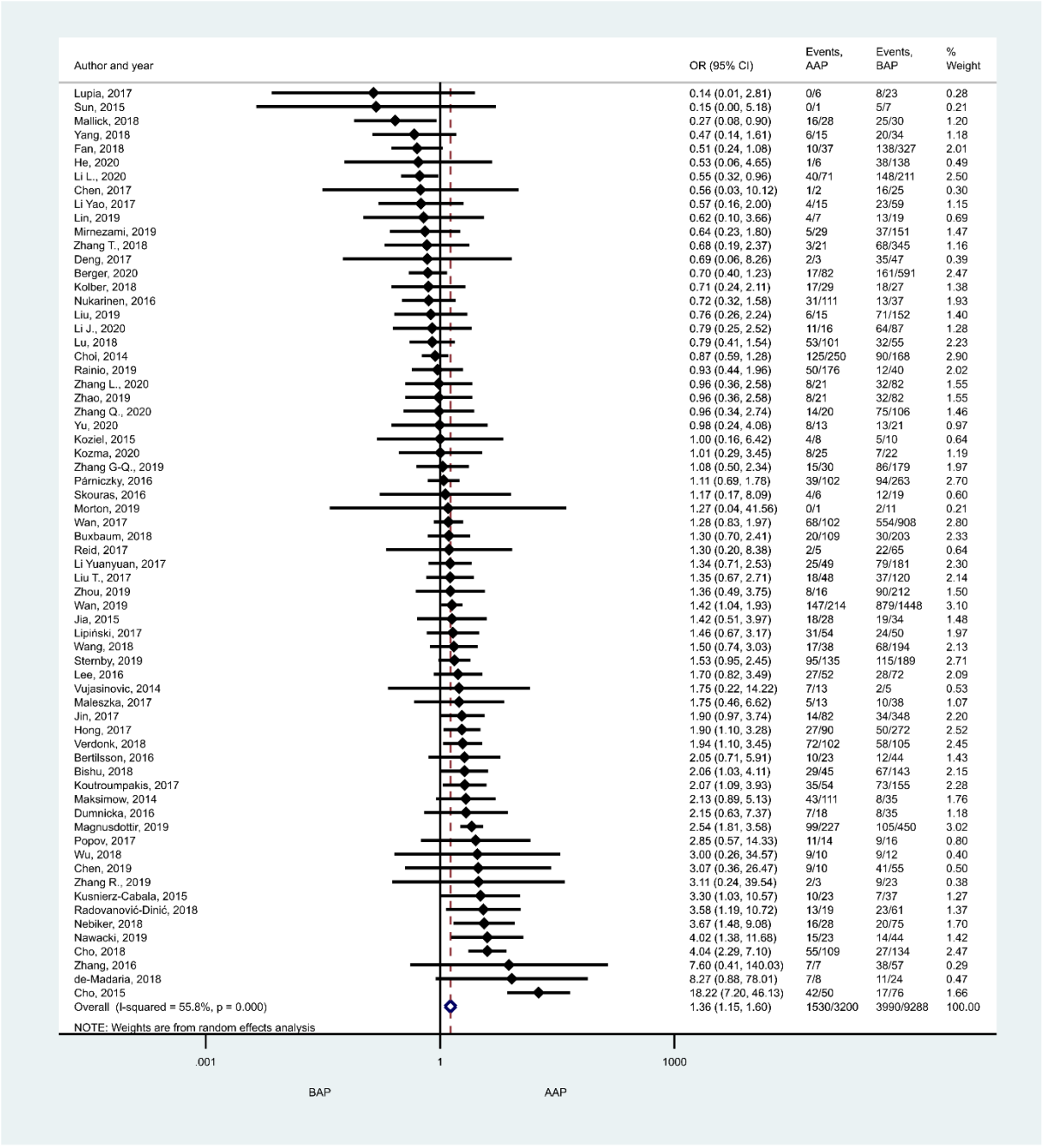


Figure 12. Forest plot showing the comparison of disease severity in (A) hypertrigliceridaemia-induced acute pancreatitis (HTG-AP) and alcoholic acute pancreatitis AAP, **(B) HTG-AP and biliary acute pancreatitis (BAP)**. Filled diamonds represent the ORs derived from the articles analysed. Horizontal bars represent CI. Empty diamond shows the overall OR (middle of the diamond and CIs are the edges) for non-mild (moderately severe and severe groups based on the Revised Atlanta Classification) disease, p=0.001. Heterogeneity of the results was presented by I-square and p value.



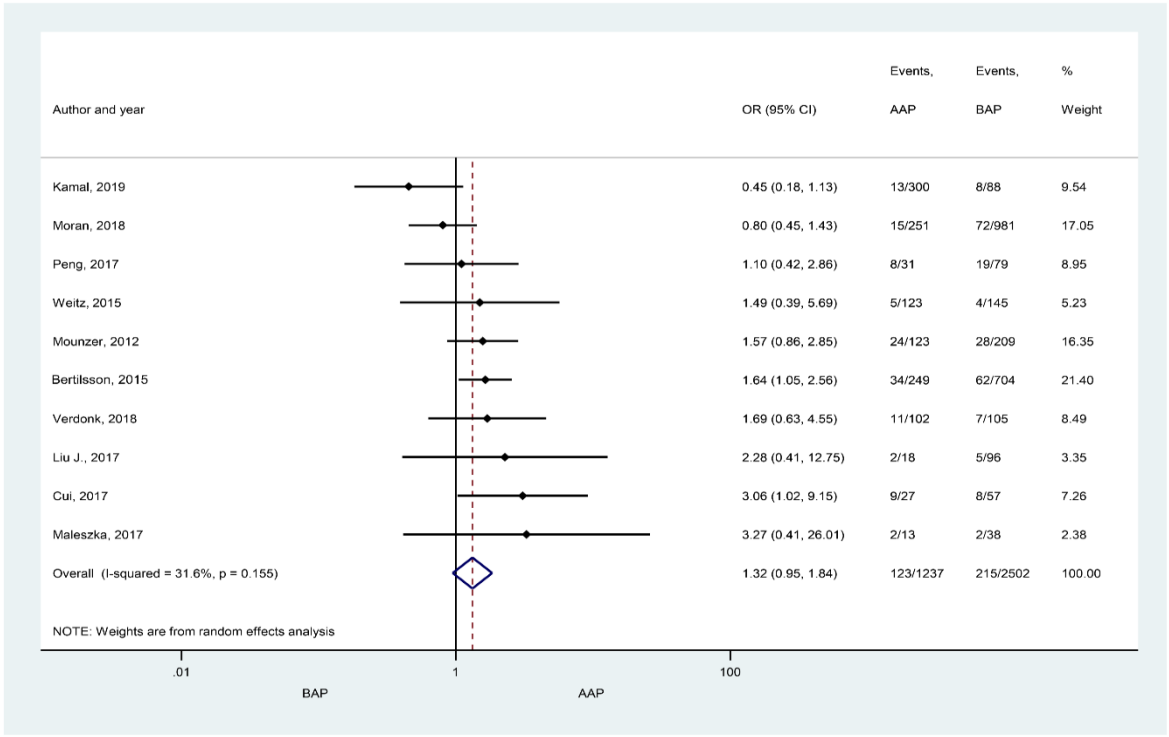
AAP alcohol-induced acute pancreatitis, BAP biliary acute pancreatitis, OR odds ratio, CI 95% confidence interval

Figure 13. Forest plot showing the comparison of disease severity in alcoholic acute pancreatitis (AAP) and biliary acute pancreatitis (BAP). Filled diamonds represent the ORs derived from the articles analysed. Horizontal bars represent CI. Empty diamond shows the overall OR (middle of the diamond, CIs are the edges) for non-mild disease, p<0.001.

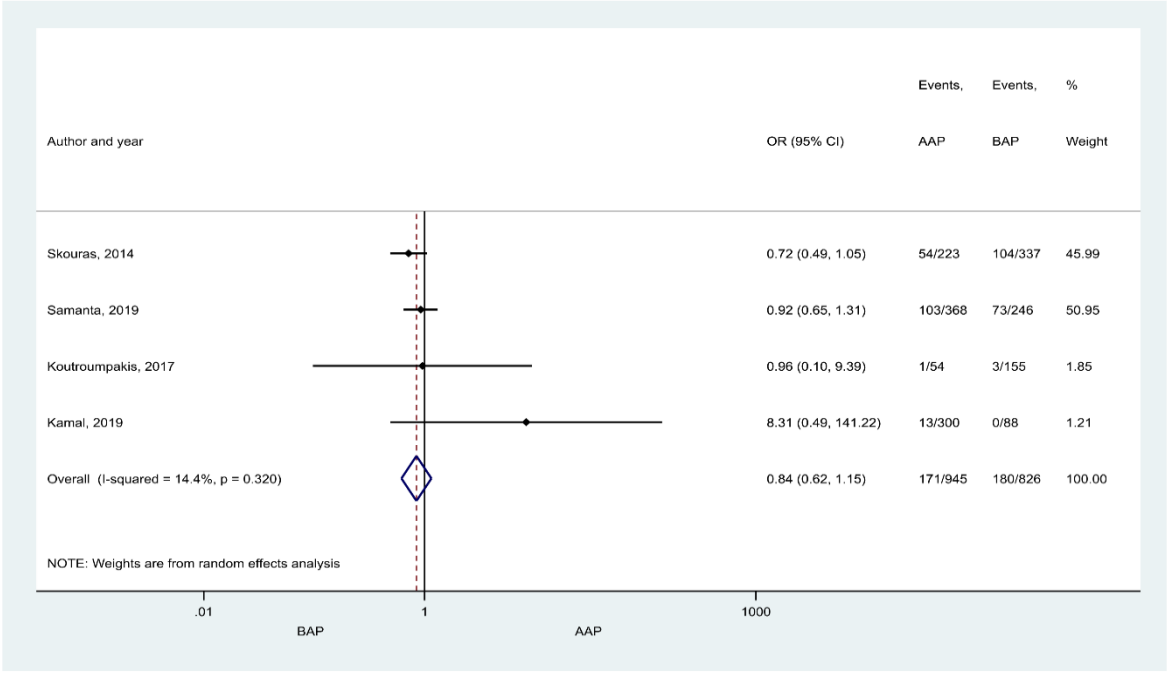
IV.2.3.2. Organ failures and intensive care unit admission

There was no significant difference in the occurrence of POF or MOF between AAP and BAP (Figure 14). The frequency of ICU admission was also similar in AAP and BAP patients (Figure 15). Heterogeneity was found in case of ICU admission (Figure 15).

a

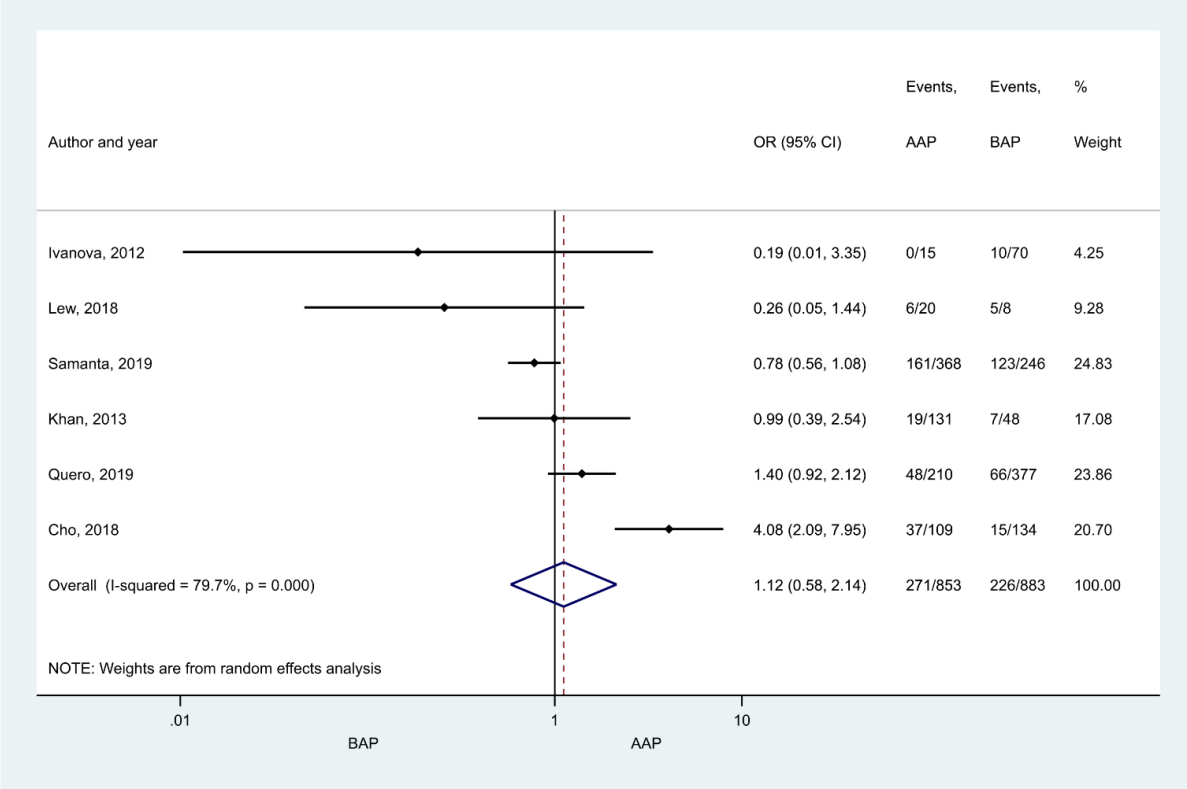


b



AAP alcohol-induced acute pancreatitis, BAP biliary acute pancreatitis, POF persistent organ failure, MOF multiple organ failure, OR odds ratio, CI 95% confidence interval

Figure 14. Forest plot showing the effect of different disease aetiologies on persistent organ failure (POF) and multiple organ failure (MOF). The effects of BAP vs. AAP on (A) POF, p=0.102; and (B) MOF, p=0.284. Filled diamonds represent the ORs derived from the articles analysed. Horizontal bars represent CI. Empty diamond shows the overall OR (the middle of the diamond, CIs are the edges).



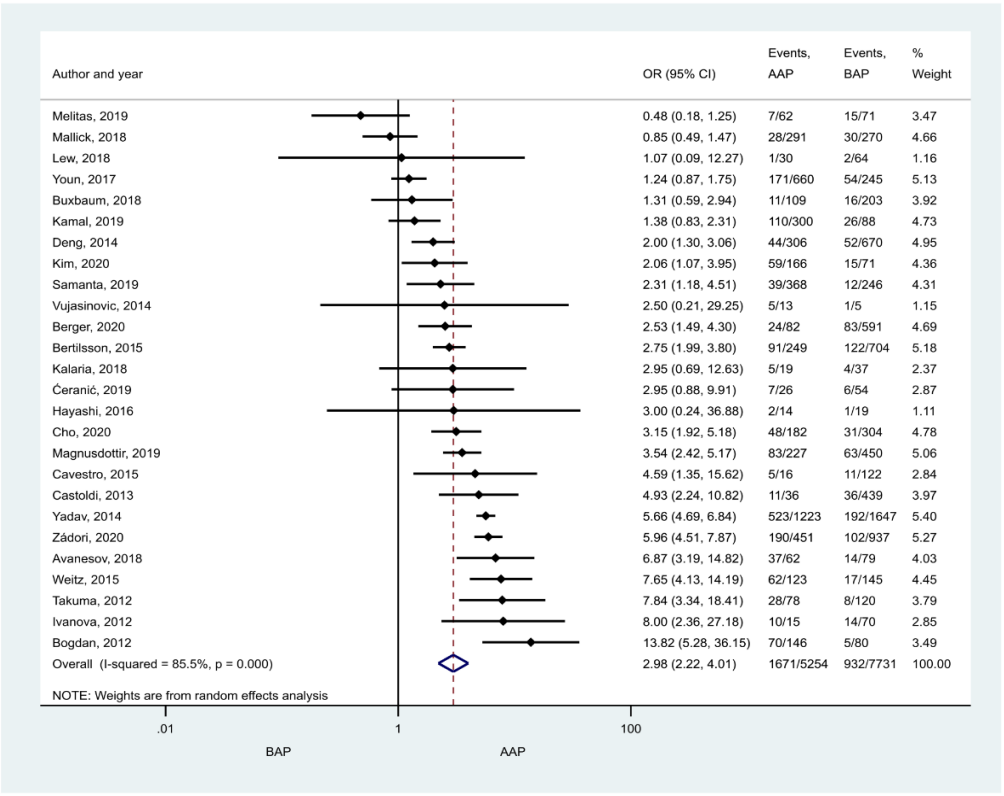
AAP: alcohol-induced acute pancreatitis, BAP: biliary acute pancreatitis, ICU: intensive care unit, OR: odds ratio, CI: 95% confidence interval

Figure 15. Forest plot showing the effect of alcoholic acute pancreatitis (AAP) and biliary acute pancreatitis (BAP) on intensive care unit (ICU) admission. Filled rhombuses represent the ORs derived from the articles analysed. Horizontal bars represent CI. Empty rhombus shows the overall OR (the middle of the rhombus, CIs are the edges), p=0.742.

IV.2.3.3. Recurrence rate

Recurrence rate was significantly higher in AAP vs. BAP patients (Figure 16A; OR: 2.98 [CI: 2.22-4.01]) and in HTG-AP vs. BAP patients (Figure 16B; OR: 2.69 [CI: 1.55-4.65]). However, AP did not reoccur more frequently due to alcoholic aetiology than HTG or post-ERCP (Figure 17). Heterogeneity was found in all comparisons except for the comparison between HTG-AP and AAP. No signs of small-study effect could be detected when recurrence rate was compared in AAP and BAP groups.

a



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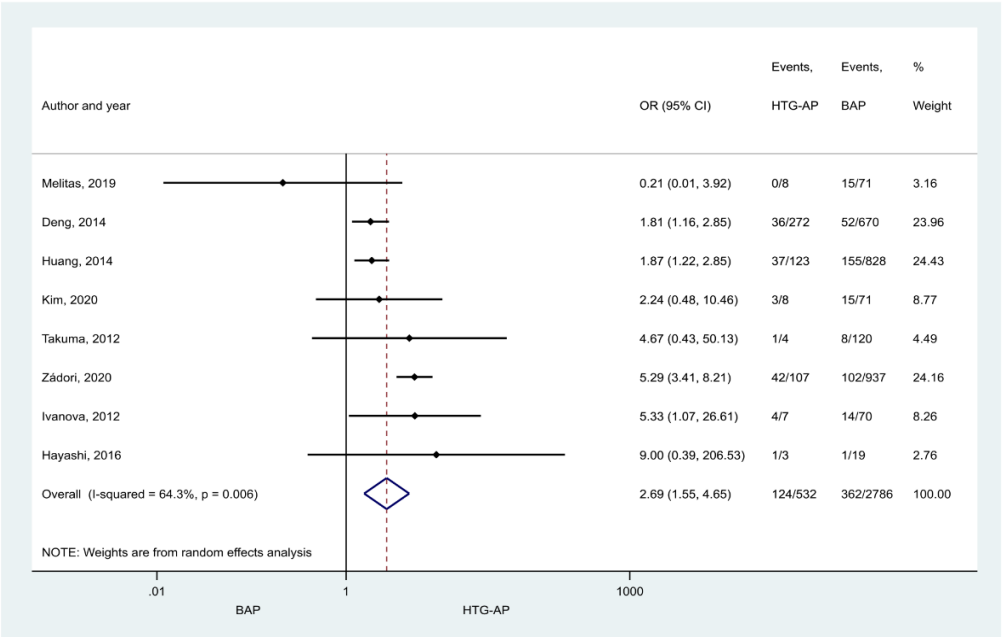
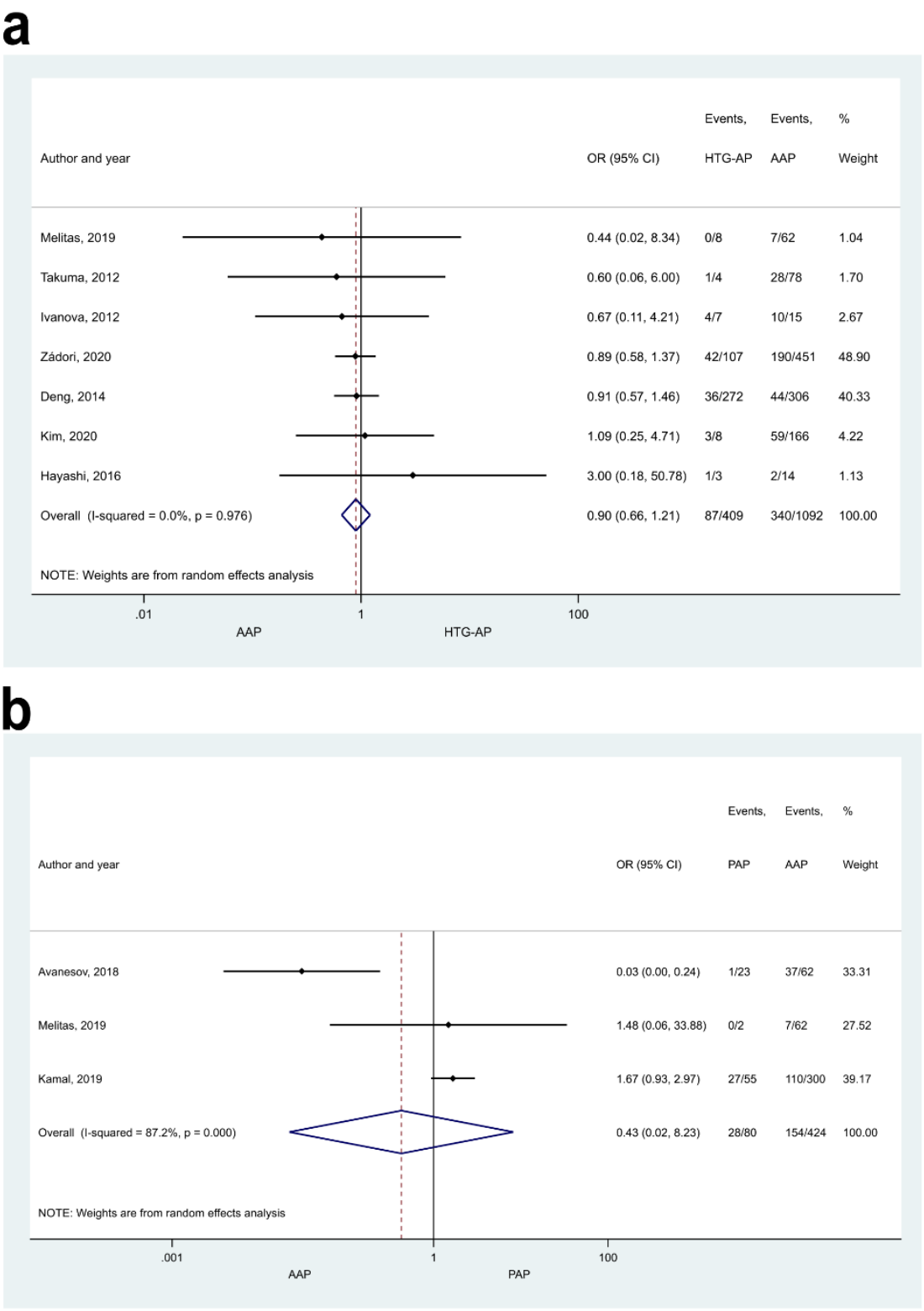


AAP alcohol-induced acute pancreatitis, BAP biliary acute pancreatitis, HTG-AP hypertriglyceridaemia-induced acute pancreatitis, OR odds ratio, CI 95% confidence interval

Figure 16. Forest plot showing the effect of different disease aetiologies on recurrence rate. The effects of **(A) alcoholic acute pancreatitis (AAP) and biliary acute pancreatitis (BAP)**, p<0.001; (**B) hypertrigliceridaemia-induced acute pancreatitis (HTG-AP) and BAP**, p<0.001. Filled rhombuses represent the ORs derived from the articles analysed. Horizontal bars represent CI. Empty rhombus shows the overall OR (the middle of the rhombus, CIs are the edges).



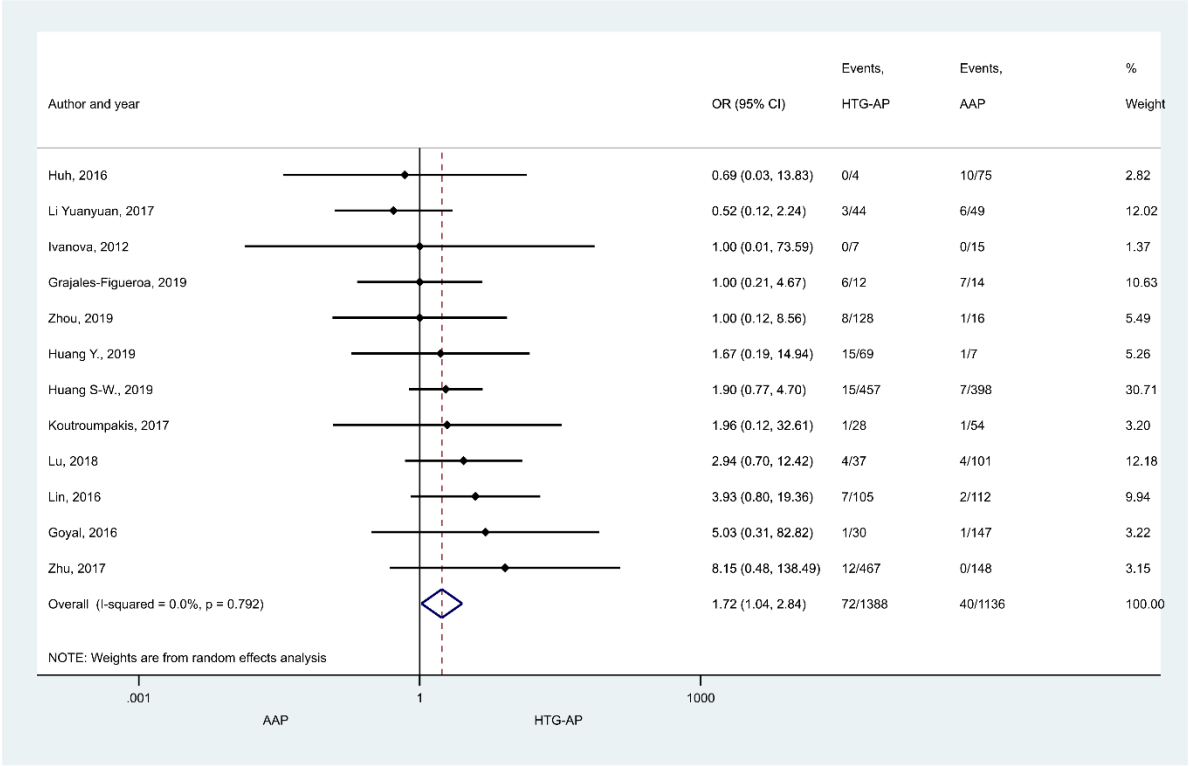
AAP alcohol-induced acute pancreatitis, HTG-AP hypertrygliceridaemia-induced acute pancreatitis,
PAP post-endoscopic retrograde cholangiopancreatography- induced acute pancreatitis, OR odds ratio, CI 95% confidence interval

Figure 17. Forest plot showing the effect of different disease aetiologies on recurrence rate. The effects of **(A) hypertrigliceridaemia-induced acute pancreatitis (HTG-AP) and alcoholic acute pancreatitis (AAP)**, p=0.477; **(B) AAP and post-endoscopic retrograde cholangiopancreatography-induced acute pancreatitis (PAP)**, p=0.572; Filled rhombuses represent the ORs derived from the articles analysed. Horizontal bars represent CI. Empty rhombus shows the overall OR (the middle of the rhombus, CIs are the edges).

IV.2.3.2. Mortality and pancreatic necrosis

Mortality rate proved to be significantly higher in HTG-AP than in AAP (Figure 18; OR: 1.72 [CI: 1.04-2.84]), but no statistical difference was found between any other patient groups (Figure 19). In the comparison of AAP and BAP a large proportion of patients came from one study contributing 1,165,777 subjects (accounting for 12.76% weight, Figure 19). However, sensitivity analysis showed that the results remained similar when this study was excluded (OR=0.96 [CI: 0.75-1.23]; Figure 20).

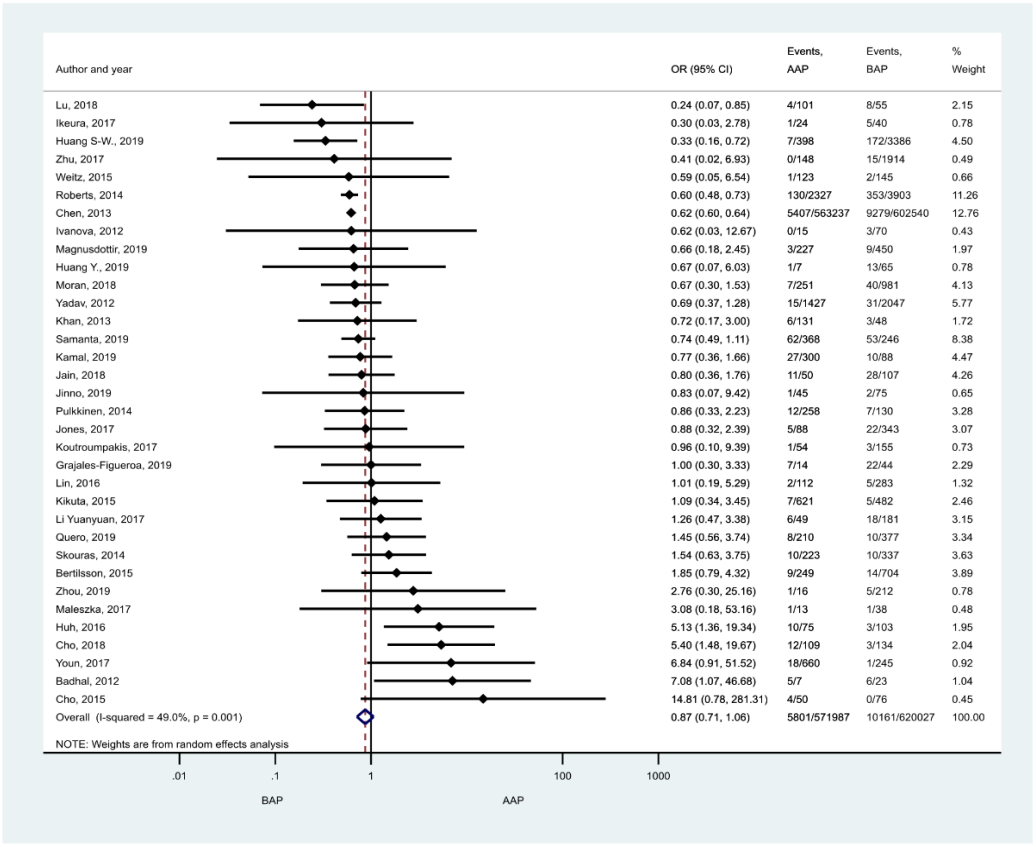
Pancreatic necrosis was reported more often in AAP than BAP patients (Figure 21A, OR=1.58 [CI: 1.08-2.30]). No significant difference was detected in any other comparisons regarding necrosis (Figure 21B-C). Heterogeneity was found in the comparison of mortality rate between BAP and HTG-AP, AAP and BAP (Figure 19), and in case of necrosis when AAP and BAP groups were compared (Figure 21A).



AAP alcohol-induced acute pancreatitis, HTG-AP hypertriglyceridaemia-induced acute pancreatitis, OR odds ratio, CI 95% confidence interval

Figure 18. Forest plot showing the effect of hypertrigliceridaemia-induced acute pancreatitis (HTG-AP) and alcoholic acute pancreatitis (AAP) on mortality. Filled diamonds represent the ORs derived from the articles analysed. Horizontal bars represent CI. Empty diamond shows the overall OR (the middle of the diamond, CIs are the edges), p=0.034.

a



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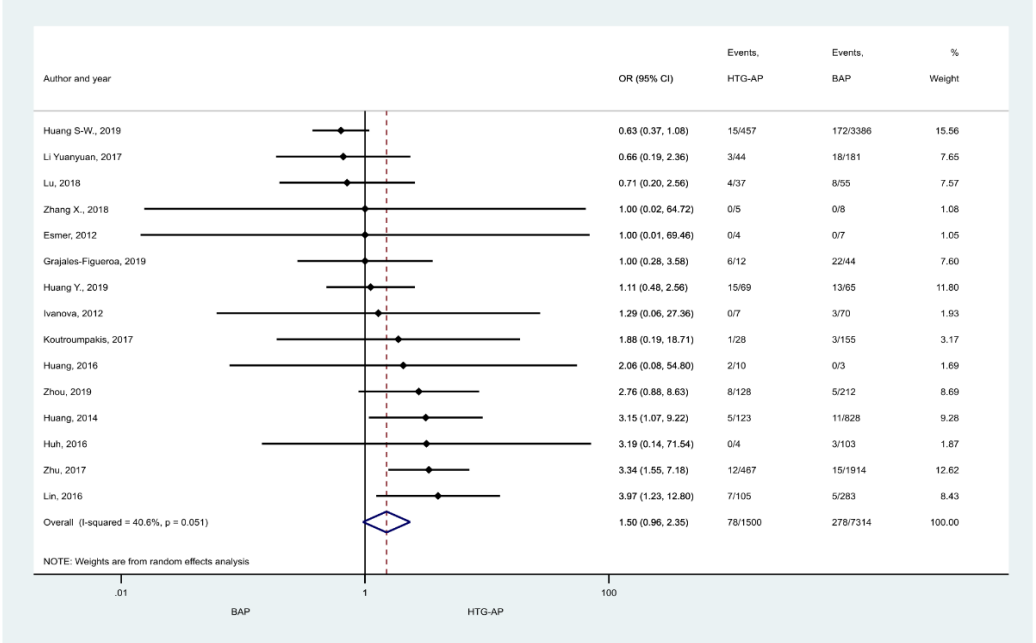
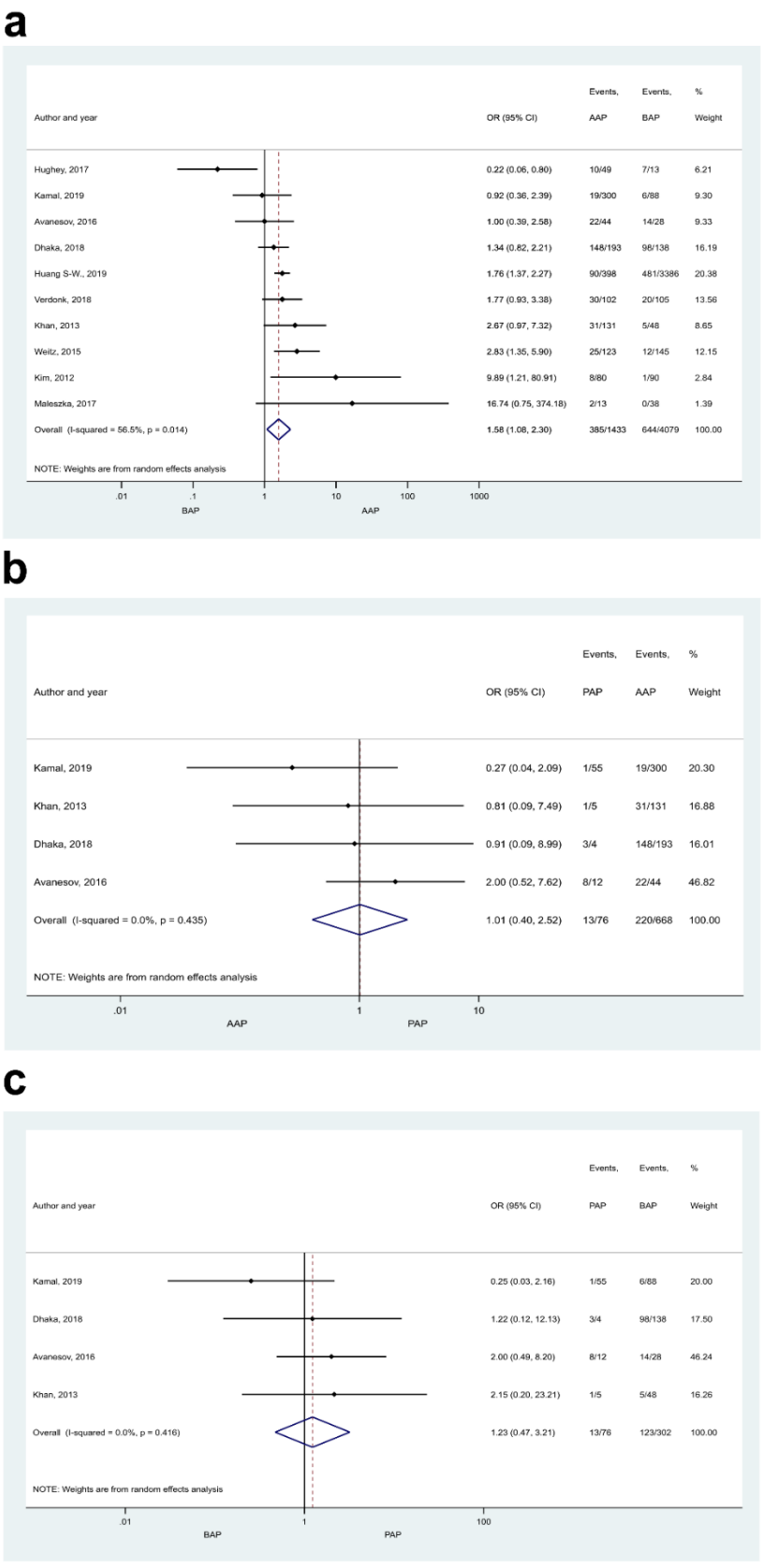


AAP alcohol-induced acute pancreatitis, BAP biliary acute pancreatitis, HTG-AP hypertriglyceridaemia-induced acute pancreatitis, OR odds ratio, CI 95% confidence interval

Figure 19. Forest plot showing the effect of different disease aetiologies on mortality. The effects of **(A) alcoholic acute pancreatitits (AAP) and biliary acute pancreatitis (BAP)**, p=0.175; **(B) hypertrigliceridaemia-induced acute pancreatitis (HTG-AP) and BAP**, p=0.074. Filled rhombuses represent the ORs derived from the articles analysed. Horizontal bars represent CI. Empty rhombus shows the overall OR (the middle of the rhombus, CIs are the edges).

Study omitted	Estimate	[95% Conf.	Interval]
Lu, 2018	0.89259994	0.72928458	1.0924881
Ikeura, 2017	0.87899673	0.71754813	1.0767714
Huang S-W., 2019	0.90859193	0.74022543	1.1152539
Zhu, 2017	0.87560093	0.7147072	1.0727148
Weitz, 2015	0.87493253	0.71392518	1.072251
Roberts, 2014	0.95173234	0.74705201	1.2124918
Chen, 2013	0.95645046	0.74642336	1.2255746
Ivanova, 2012	0.87380737	0.71321142	1.0705651
Magnusdottir, 2019	0.87825656	0.71539879	1.0781883
Huang Y., 2019	0.87452191	0.71347725	1.0719173
Moran, 2018	0.88428497	0.7180829	1.0889549
Yadav, 2012	0.88867122	0.7196914	1.0973266
Khan, 2013	0.87594867	0.71379721	1.0749356
Samanta, 2019	0.89362723	0.71985841	1.1093426
Kamal, 2019	0.87927032	0.71388412	1.0829717
Jain, 2018	0.87746894	0.7127251	1.0802927
Jinno, 2019	0.87277251	0.71221918	1.0695189
Pulkkinen, 2014	0.87350643	0.71063322	1.0737094
Jones, 2017	0.87256646	0.71011847	1.0721763
Koutroumpakis, 2017	0.8717351	0.7113806	1.0682356
Grajales-Figueroa, 2019	0.86918569	0.70828968	1.0666311
Lin, 2016	0.87039781	0.7099359	1.0671278
Kikuta, 2015	0.86653262	0.70627856	1.0631483
Li Yuanyuan, 2017	0.85875916	0.70037854	1.0529554
Quero, 2019	0.85149056	0.69542015	1.0425873
Skouras, 2014	0.84655708	0.69199455	1.0356425
Bertilsson, 2015	0.83310246	0.68335605	1.0156635
Zhou, 2019	0.86085904	0.70408529	1.0525404
Maleszka, 2017	0.86447799	0.70676577	1.0573832
Huh, 2016	0.82032442	0.67868459	0.99152416
Cho, 2018	0.81530166	0.67576832	0.98364604
Youn, 2017	0.84386343	0.69394869	1.0261644
Badhal, 2012	0.83910716	0.69102049	1.0189291
Cho, 2015	0.85124594	0.69935167	1.0361305
Combined	.87006749	0.71154741	1.063903

Figure 20. Sensitivity analysis related to the Forest plot of mortality, alcoholic acute pancreatitis (AAP) vs. biliary acute pancreatitis (BAP).



AAP alcohol-induced acute pancreatitis, BAP biliary acute pancreatitis, PAP post-endoscopic retrograde cholangiopancreatography-induced acute pancreatitis, OR odds ratio, CI 95% confidence interval

Figure 21. Forest plot showing the effects of different disease aetiologies on pancreatic necrosis. The effects of **(A) biliary acute pancreatitis (BAP) vs. alcoholic acute pancreatitis (AAP)**, p=0.019 **(B) AAP vs. post-endoscopic retrograde cholangiopancreatography-induced acute pancreatitis (PAP)**, p=0.982; (C) BAP vs. PAP, p=0.674. Filled diamonds represent the ORs derived from the articles analysed. Horizontal bars represent CI. Empty diamond shows the overall OR (the middle of the diamond, CIs are the edges).

V. DISCUSSION

V.1. Animal experiments

V.1.1. The effect of opioids on the severity of acute pancreatitis

Opioids are commonly used for pain control in AP patients. It has been speculated that these analgesics (such as MO) may affect AP progression. Therefore, we comprehensively investigated the effects of FE on the severity of experimental AP, and this research was further supplemented with the examination of the effects of MO. It is important to note, that measurements were performed when experimental AP reached its maximal severity.

I.p. FE pre-treatment significantly increased the severity of necrotizing AP induced by LO, but it had no effect on oedematous AP evoked by CER. Interestingly, the clinically more relevant post-treatment with FE either decreased or had no effect on the various parameters of AP severity in different models. Wang and Chen[187] also tested the effect of FE on NaTc-induced AP. They administered FE i.v. 23-23.5 h after AP induction, and sacrificed the animals 24 h after the induction of the disease. Surprisingly, FE exerted anti-inflammatory effects on the pancreas and AP-induced myocardial damage within that really short time (30-60 min). In clinical settings, Stevens *et al.*[188] showed that FE did not have any side effects compared to the placebo control group (intramuscular Demerol containing pethidine). Some studies draw attention on the importance of administration site of FE, especially into the epidural site. The use of FE in epidural anaesthesia partially restored the decrease of microcirculatory flow caused by AP and prevented the development of tissue necrosis and systemic complications[189,190].

MO pre- or post-treatment did not affect the severity of the disease in case of LO-induced necrotising AP. Furthermore, simultaneous administration of MO and CER had no remarkable effect on disease progression either, except for vacuolisation, which was decreased by MO. In a recent study, Barlass *et al.*[45] also investigated MO in two necrotising mouse AP models. They concluded that MO application delayed AP resolution, reduced intestinal motility, which increased the risk for bacterial translocation. MO also delayed macrophage migration and caused persistence of inflammation. Their findings related to macrophages are in accordance with earlier studies showing mononuclear cell suppression and chemokine receptor transdeactivation after MO treatment[191,192]. Our study focused on the early-mid events of AP and showed no adverse effects of MO, while Barlass *et al.*[45] investigated the later effects of MO (at 48, 72, or 120 h). However, our results do not rule out the possibility of later side effects that were shown by Barlass *et al.*[45]. Marked differences in the results can be explained by

species differences, the latter study used mice while in the present study rats were investigated. Moreover, one randomized clinical trial[193] and two related reviews[40,43] did not find any significant difference in the effects of MO vs. the non-opioid metamizole. It should be noted that relatively low number of patients (8 per group) were included in this randomized clinical trial. Based on these observations, we conclude that MO does not affect the severity of the AP at the early-mid stage of the disease, but later side effects may appear according to literature data.

Opioids exert their effects primarily through mu, kappa, or delta opioid receptors which are expressed mainly by neuronal or immune cells. The effects can differ depending on their affinity or specificity to certain receptors. Publications showed that MO has immunosuppressant properties through full mu receptor agonism. MO treatment resulted in inhibition of cytokine production, NK cell activity, cellular responses to mitogens, antibody production, cell growth, and decreased phagocytic activity[194,195]. FE, an 80 times potent MO analogue[196] is a full mu receptor agonist, similarly to MO. Therefore, it can also suppress the immune system[42]. MO and FE can also cause Sphincter of Oddi spasm, what could further aggravate AP severity[197]. Only FE pre-treatment resulted in increased AP severity. The early immunosuppression by FE may cause this adverse effect, while FE post-treatment was beneficial for AP outcome. However, the later consequences were not investigated by this work. For all clinically applied opioids, including FE and MO, these effects should be considered and investigated in future studies. Moreover, the timing of opioid administration can be critical, especially in case of FE.

We detected MOR mRNA and protein expression in the pancreas and brain of control rats (results not shown in this thesis). The expression of MOR in the brain was not altered by AP, while its functional activity was reduced by FE stimulation. This suggests that the effect of AP on MOR activity is independent of changes in protein expression. To the best of our knowledge, we have shown for the first time that AP diminishes the function of opioid receptors not only in the pancreas but also in the brain.

In the clinical setting, there are no guidelines or recommendations suggesting the best opioid to use in AP. However, the application of effective and strong analgesics is necessary in the treatment of this disease. In light of the results discussed above, post-treatments (e.g. FE, MO) do not increase disease severity, but some of the opioids (e.g. MO) may affect the resolution of AP. Therefore, the latter may not be the best treatment option in this severe disease. Our results showed that FE post- treatments have promising effects besides pain relief; therefore, its use could also be beneficial on AP severity. Overall, this research contributes to

the better understanding of the opioid effect in AP and can help in planning further clinical trials which will be necessary to choose the most appropriate opiate for treatment of this potentially lethal disease.

Although pre-treatment with analgesics in AP is clinically less relevant, but in case of ERCP, rectal administration of non-steroid anti-inflammatory drugs (e.g. indomethacin or diclofenac) is indicated[198]. These agents reduce the development of PAP. In this case, opiates should be used with caution.

The present study has limitations as well. The long-term consequences of opiates on AP were not investigated as it was performed by Barlass *et al.*[45]. Furthermore, the above mentioned and beneficial epidural administration route[189,190] was not investigated by our group.

Based on our findings, we conclude that FE post-treatment improved, while FE pre-treatment exacerbated disease severity in experimental necrotizing AP. However, FE did not affect the outcome of oedematous AP. MO administration had minimal effects in both pre- and post-treatments including cellular vacuolisation, pancreatic water content and leukocyte infiltration. In conclusion, FE post-treatment proved to be beneficial in AP. Our results suggest that type, dosing, administration route and timing of opioid treatment can determine the effects on AP outcome. Clinical studies are needed to determine which opioid(s) is the best in AP.

V.1.2. The mitochondrial transition pore as potential therapeutic target in acute pancreatitis

Mitochondrial injury is one of the most important pathophysiological events in the early phase of AP[51]. Over the past decade, both genetic and pharmacologic inhibition of mPTP has been shown to reduce bile acid- or EtOH+FA-induced acinar cell damage and alleviate AP severity[21,22,24]. Numerous mPTP inhibitors have been experimented with, none of which proved beneficial. Due to its immunosuppressive activities, the use of CYA is excluded in circumstances where mPTP is targeted. E.g. it is likely to adversely affect the infective outcomes of AP[25]. Clinical testing of non-immunosuppressive CYA derivatives Debio025 and TRO40303 was also terminated before reaching ‘proof of concept’ phase 2 clinical trials in AP due to unfavourable effects (see Introduction). Other novel mPTP inhibitors have been also tested in experimental studies without breakthrough success. E.g. cinnamic anilides were reported to improve myocardial infarction[199], however, later they turned out to have age-related toxicity[200].

Contrary to the compounds mentioned before, NIM811 has been found to be advantageous in several diseases without adverse effects. *Per os* administration of 5 or 10 mg/kg NIM811

significantly alleviated the severity of experimental AP. However, it is interesting that the higher dose of NIM811 was effective in pre-treatment, while the lower dose proved beneficial in post-treatment. Further investigations are needed to find out the underlying mechanisms. These results suggest that mitochondrial function and restoration of cellular energy are key features in AP[51] In this study, our team was the first to confirm that NIM811 is a potential compound to be tested in clinical trials for AP. Phase 2 clinical trials should be set up to test the utility of this promising drug candidate in human medicine.

V.2. Meta-analysis

Our meta-analysis is the first detailed study investigating the relationship between different aetiologies (alcohol abuse, biliary, HTG, post-ERCP) and the course of AP. Our study revealed that the prevalence of severe and moderately severe (non-mild) disease forms was highest in case of HTG-AP which was followed by AAP and BAP and PAP (Table 1). Due to the large number of included articles and patients, our results have strong evidence in case of the severity outcome. These are also in accordance with our earlier observations[201] and the data of Wang *et al.*[202]. However, previously we compared the severe disease category to moderately severe and mild groups, which has less relevance than merging the severe and moderately severe groups as we did in the current study. Furthermore, our previous study and that of Wang *et al.*[202] compared the characteristics and outcome of HTG-AP to non-HTG-AP patients, without classifying non-HTG-AP group any further according to aetiology. Importantly, non-HTG-AP group is rather heterogenous and it cannot be decided whether all aetiological subgroups included in non-HTG-AP are less severe than HTG-AP or some of the subgroups are basically more severe than HTG-AP but due to other much milder subtypes, HTG-AP turns out to be the most severe disease form. In addition, non-HTG-AP included all aetiologies ("idiopathic" and "other" as well) except HTG-AP, while we only analysed well-defined and clear aetiologies. Therefore, the current meta-analysis provides a more refined picture of the outcomes. Wang *et al.*[202] evaluated AP severity using the APACHE-II scoring system, but this does not specifically define AP severity.

In our study, no difference could be observed between any aetiological groups in POF (Table 1). Although HTG-AP and AAP exhibited the most severe forms of AP from an aetiological point of view, the data of POF does not support it. This can be explained by the fact that POF associated with AP was assessed in HTG-AP patients only in three of the articles included in our meta-analysis[72,115,130].

Severity	HTG-AP > AAP > BAP
POF	AAP ⋦ BAP
MOF	AAP ⋦BAP
ICU admission	AAP ⋦ BAP
Recurrence rate	[HTG-AP/AAP] > BAP HTG-AP ⋦ AAP PAP ⋦ AAP
Mortality	HTG-AP > AAP [HTG-AP/AAP] ⋦ BAP
Necrosis	AAP > BAP PAP ⋦ [AAP/BAP]

Table 1. Summary of the results of our study. Statistically significant difference ($p<0.05$) was presented with >; ⋦ shows no significant difference. AAP: alcohol-induced acute pancreatitis, BAP: biliary acute pancreatitis, HTG-AP: Hypertriglyceridaemia-induced acute pancreatitis, ICU: intensive care unit, MOF: multiple organ failure, PAP: post endoscopic retrograde cholangiopancreatography-induced acute pancreatitis, POF: persistent organ failure.

In the study of Wang *et al.*[202] POF was most commonly observed in HTG-AP, which is in accordance with our results for severity. Although several articles evaluate characteristic features of severe AP such as POF, they focus exclusively on severe AP patients. For this reason, these could not be utilized in our analysis. MOF is another distinctive feature of severe and moderately severe AP. In the current study, no significant difference could be detected in MOF between any of the analysed groups. Similarly, in a previous study, we did not find differences in MOF among HTG-AP vs. non-HTG-AP patients[201]. Tai *et al.*[203] also found a higher risk for the severe form of AP in HTG-AP patients compared to BAP. They diagnosed MOF more frequently in BAP patients, however, there was no difference in single OFs (renal, heart, pulmonary).

AP patients with systemic complications eventually end up in ICU. In case of this outcome, only one comparison could be performed: no significant difference was found between AAP and BAP (Table 1), which is supported by our previous findings[201]. The 27% recurrence rate of AP in the 1990s[204] has nowadays decreased to about 20%[205], which could be explained by better diagnosis and treatment after the first attack. In our study, alcoholic and hypertriglyceridaemic aetiologies caused more AP recurrence than biliary, while the repeated hospitalization for AAP and HTG-AP patients was similar. Tai *et al.* also found higher recurrence rate of HTG-AP than BAP[203]. Other studies drew the conclusion that alcohol is the most frequent aetiological factor for recurrent AP[204,205,]. Suchsland *et al.*[205] analysed the risk factors for readmittance in AP, most of which were related to alcohol abuse, so these patients have a higher risk for disease

recurrence after discharge. In case of BAP, delayed cholecystectomy could be responsible for recurrence[206,207].

Our study has shown that HTG-AP led to significantly higher mortality rate than AAP. However, no significant difference could be detected between the other aetiological groups. BAP used to have a higher mortality than AAP; however, this rate has decreased in the last decade due to improved supportive care[208]. Several studies have reported that mortality rate was not influenced by aetiological factors[209,210]. Other studies stated that HTG-AP did not cause significantly higher mortality rate, even though it led to higher severity and complication rates compared to other aetiological factors[211,212]. Wang *et al.*[202] concluded that HTG-AP caused higher mortality rate than non-HTG-AP, while Kiss *et al.*[201] did not find significant difference in this respect. Based on the current study, there is no strong relationship between aetiology of AP and mortality.

HTG carried the greatest risk for non-mild (moderately severe and severe) AP, which was followed by AAP; the least severe disease forms were observed in BAP and PAP. One of the possible pathomechanisms is that lipotoxicity mediated by unsaturated FAs contributes to necrosis, OF (e.g. cardiovascular diseases) and mortality[213]. Experimental studies also demonstrated that HTG exacerbates the severity of AP[213,214]. FA administration resulted in elevated intracellular Ca^{2+} concentration in pancreatic acinar cells and impaired mitochondrial function[17,215]. HTG-AP is often accompanied by one or more secondary factors (alcoholism, medications, uncontrolled diabetes mellitus, physical inactivity), which can further aggravate the severity of the disease[216–219]. Furthermore, elevated serum chylomicron concentration during HTG increases viscosity, causing reduced blood flow in microvessels and resulting in ischemic conditions. This could be an additional risk factor for a severe form of AP[17,215].

Determining the exact aetiology of AP may be challenging in some cases. For example, alcohol is not only known as an independent risk factor for AP but can also increase serum TG concentrations, as mentioned before. In addition, mild-to-moderate elevation in TG concentrations can be observed in the early phase of AP, regardless of aetiology[220]. Since TG concentrations can rapidly decrease during fasting state after the diagnosis of AP, the measurement of TG concentrations on (or shortly after) admission is crucial.

The number of events, which refer to positive outcomes in certain aetiologies were relatively high in case of severity (1,516 severe events occurred out of 2,556 HTG-AP patients in Figure 12A) and partly in mortality (10,161 events/620,027 BAP patients in Figure 19A) and recurrence rate outcomes (1,671 events/ 5,254 AAP patients in Figure 16). Smaller number of events (9-97) could be included in the analysis of other outcomes (Table 1). Low event rates

can have detrimental influence on the reliability of the results[221,222]. Based on the studies mentioned above, the results of all severity comparisons, mortality and recurrence rates in comparisons of AAP vs. BAP are strongly reliable. Most of the other calculations have lower reliability but there is no precedent to contradict the results of severity.

Our meta-analysis has strengths and limitations that should be noted. The major strengths are the following: we included a large number of articles. Four major aetiologies were analysed, leaving out miscellaneous or idiopathic backgrounds. For the analysis of severity, we only included articles where severity was defined according to the RAC, which provided a clear and consistent base for the comparisons. In addition, we compared mild to moderately severe and severe ("non-mild") AP groups, which further refined our analysis.

The quality of the involved articles determines the value of pooled data. There has been high variability in methodology of the studies which may have unintended effects on the final results and interpretation, study populations were diverse in age and gender, which might cause heterogeneity in aetiological distribution. Aetiologies were not necessarily defined the same way. Certain outcomes (e.g. necrosis) were only evaluated by a limited number of studies, especially in case of HTG-AP, which may be the reason that no statistically significant difference could be detected between HTG-AP and other aetiologies or no statistical analysis could be performed. One article analysed data from 1975 to 2010, which was only applied for the assessment of recurrence rate. Another article contributed 1,165,777 patients to the analysis, which was only used for the evaluation of mortality. In addition, only articles published in English or Hungarian were included.

AP is a complex disorder mediated by metabolic, environmental and genetic factors, which can lead to death in the most severe forms. Therefore, clinicians should be more alert for a severe disease course in the at-risk patients. Our observations highlight the importance of disease aetiology. We found association between aetiology and the development and course of AP. HTG proved to carry the highest risk for non-mild (moderately severe and severe) AP, which was followed by AAP; the least severe disease forms were observed in BAP and PAP. It is essential to determine the cause of the disease in time to apply the most appropriate therapy. Based on the results, greater emphasis should be placed on determining aetiology on admission, especially in case of HTG-AP.

VI. SUMMARIES

VI.1. Summary of the thesis

Introduction: The main causes of acute pancreatitis (AP) are biliary disease, alcohol consumption and hypertriglyceridaemia (HTG). Mitochondrial dysfunction plays a crucial role in the development of acute pancreatitis (AP); however, no specific compound is currently available for the treatment of mitochondrial dysfunction with clinically acceptable effectiveness and safety. Opioids are widely used for the management of pain associated with AP, but their impact on the progression of the disease is unclear. Therefore, our **aims** were to evaluate the effects of the above-mentioned aetiological factors, to investigate the impact of a novel mitochondrial transition pore inhibitor, N-methyl-4-isoleucine cyclosporin (NIM811) and the effects of clinically relevant opioids on the severity and outcome of AP.

Methods: Various doses of fentanyl (FE, intraperitoneal-i.p.) and morphine (MO, i.p. or intravenous) were administered as pre- and/or post-treatment in Wistar rats. Necrotizing AP was induced by i.p. injection of large doses of L-ornithine-HCl, while i.p. caerulein administration resulted in oedematous AP. The effects of NIM811 were tested in an AP model induced by ethanol+fatty acid in C57Bl/6 J mice. NIM811 was gavaged orally before or after AP induction. Disease severity was determined by laboratory and histological measurements. To assess the impact of aetiological factors, Pubmed and Embase were searched for articles published from 01/01/2012 to 31/05/2020. Included articles involved adult alcoholic, biliary, HTG- or post-ERCP AP (PAP) patients. Primary outcome was severity, secondary outcomes were organ failures, intensive care unit admission, recurrence rate, pancreatic necrosis, mortality, length of hospital stay, pseudocyst, fluid collection and systematic inflammatory response syndrome.

Results: FE post-treatment improved necrotizing AP severity in a dose dependent manner, while pre-treatment exacerbated it. FE did not affect the outcome of oedematous AP. The overall effects of MO on disease severity was negligible. Administration of NIM811 had no toxic effects. *Per os* administration of NIM811 had a protective effect in AP in both pre- and post-treatments. The risk for non-mild (moderately severe and severe) condition was the highest in HTG-induced AP (HTG-AP) followed by alcoholic AP (AAP), biliary AP (BAP) and PAP. Recurrence rate was significantly lower among BAP vs. HTG-AP or AAP patients. Mortality rate was significantly greater in HTG-AP vs. AAP or BAP, pancreatic necrosis occurred more frequently in AAP than BAP patients.

Conclusions: Type, dosing, administration route and timing of opioid treatment can determine the effects of opioids on experimental AP severity. FE post-treatment proved to be beneficial in AP. Clinical studies are needed to determine which opioids are best in AP. The novel mitochondrial transition pore inhibitor NIM811 thus seems to be an exceptionally good candidate compound for clinical trials in AP. As for the effects of aetiological factors, there is a potential association between aetiology and the development and course of AP. HTG-AP is associated with the highest number of complications. Furthermore, AAP is likely to be more severe than BAP. Greater emphasis should be placed on determining aetiology on admission.

VI.2. Summary of new findings

- FE post-treatment diminished, while pre-treatment exacerbated the severity of experimental acute necrotizing pancreatitis. Therefore, the timing of FE administration has significant impact on the disease.
- Morphine had no significant effect on the severity of experimental AP in pre/post-treatments.
- NIM811 is a highly potential compound to be tested in clinical trials for AP.
- HTG proved to carry the highest risk for non-mild (moderately severe and severe) AP.

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”It always seems impossible until it’s done.” — Nelson Mandela

IX. APPENDIX

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Article

Fentanyl but Not Morphine or Buprenorphine Improves the Severity of Necrotizing Acute Pancreatitis in Rats

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Abstract: Opioids are widely used for the pain management of acute pancreatitis (AP), but their impact on disease progression is unclear. Therefore, our aim was to study the effects of clinically relevant opioids on the severity of experimental AP. Various doses of fentanyl, morphine, or buprenorphine were administered as pre- and/or post-treatments in rats. Necrotizing AP was induced by the intraperitoneal injection of L-ornithine-HCl or intra-ductal injection of Na-taurocholate, while intraperitoneal caerulein administration caused edematous AP. Disease severity was determined by laboratory and histological measurements. Mu opioid receptor (MOR) expression and function was assessed in control and AP animals. MOR was expressed in both the pancreas and brain. The pancreatic expression and function of MOR were reduced in AP. Fentanyl post-treatment reduced necrotizing AP severity, whereas pre-treatment exacerbated it. Fentanyl did not affect the outcome of edematous AP. Morphine decreased vacuolization in edematous AP, while buprenorphine pre-treatment increased pancreatic edema during AP. The overall effects of morphine on disease severity were negligible. In conclusion, the type, dosing, administration route, and timing of opioid treatment can influence the effects of opioids on AP severity. Fentanyl post-treatment proved to be beneficial in AP. Clinical studies are needed to determine which opioids are best in AP.

Keywords: acute pancreatitis; fentanyl; morphine; buprenorphine; opioids; analgesia

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1. Introduction

Acute pancreatitis (AP) is one of the most common causes for hospitalization within gastrointestinal diseases [1], which has an overall mortality of about 2% [2]. This death proportion in severe cases can increase to 30%. The incidence of the disease is more than 30 per 100,000 population in Europe, and this number has increased over time [3,4].

Excessive alcohol consumption and gallstone diseases account for approximately 70% of cases [2,5]. AP can present in mild, moderately severe, and severe forms based on the Revised Atlanta Classification [6]. The pathomechanism of AP is rather complex, and our understanding of the disease is far from complete, but it involves toxic cellular Ca^{2+} overload causing NF-κB activation, impaired autophagy, mitochondrial dysfunction, and the early intra-acinar and intra-ductal activation of digestive enzymes [7–10]. The clinical symptoms of AP include severe abdominal pain (which can radiate to the back), fever, nausea, and vomiting. The diagnostic criteria for AP include the presentation at least two of the following: (i) upper abdominal pain, (ii) >3× elevated serum amylase or lipase, and/or (iii) imaging (CT, MRI, ultrasonography) [6,11]. Notably, pain is present in 95% of AP patients [5]. The therapy of AP is only supportive, and there is no specific drug against this disease. Recent AP management guidelines highlight the importance of (a) early intravenous (i.v.) fluid resuscitation; (b) analgesics; (c) enteral nutrition [12–15].

As pain is the most prominent symptom of AP, its relief is a priority in clinical settings. Unfortunately, recent guidelines for AP treatment do not have clear recommendations for the types of analgesics to be used [12,13,16]. Most commonly, the WHO pain management guideline is utilized, and treatment ranges from nonsteroidal anti-inflammatory drugs (NSAID) to high potent opioids. The latter are applied in cases of severe AP and include fentanyl (FE), buprenorphine (BQ), pethidine, pentazocine, morphine (MO), etc. [17]. Although opioids are the most effective pain killers, which makes them valuable in clinical settings, there is a scientific debate on their use due to their side effects such as constipation or immunosuppression [18,19]. Actually, Meng et al. (2013) attempted to collect all randomized controlled trials that investigated the side effects of analgesics (opioids and non-opioids) in AP, but the included studies were of low quality, without clear outcome. However, the use of MO is often not preferred in humans due to the spasm of sphincter of Oddi, which might worsen the outcome of AP [20]. Even more importantly, Barlass et al. [21] have also shown the drawbacks of MO use in AP and the pathological processes of its side effects in a mouse model.

Despite the dubious benefits of opioid use, their impact on the progression of AP is unclear. Therefore, our aim was to investigate opioid receptor function, the effects of FE, MO, and BQ on the severity of AP in rats. We utilized different AP models with opioid pre- and/or post-treatments.

2. Results

2.1. The Effect of Fentanyl Pre-Treatment on AP Severity

The pancreata of the control group displayed normal morphology (Figure 1A), and intraperitoneal (i.p.) FE alone did not induce any structural changes in the pancreas (Figure S1A). L-ornithine (LO)-induced AP resulted in about 60% pancreatic necrosis and intensive leukocyte infiltration (Figure 1A–C). These signs even worsened due to FE pre-treatment. The extent of tissue necrosis significantly increased when the higher dose (3 × 0.2 mg/kg) of FE was applied, whereas the level of leukocyte infiltration was higher in the 3 × 0.1 mg/kg FE and AP group compared to the AP group not receiving FE. FE treatment did not cause any change in pancreatic water content in the AP groups (Figure 1D). Serum amylase activity markedly increased in the AP groups versus the control group (Figure 1G). Importantly, 3 × 0.1 mg/kg FE significantly increased serum amylase activity during AP. MPO activity was greatly elevated in the AP groups compared to the control group (Figure 1F), and the dose of 3 × 0.2 mg/kg FE further increased MPO activity in AP. Interestingly, the concentration of pancreatic IL-1β significantly decreased due to 3 × 0.1 mg/kg FE in the AP group.

I.p. injections of CER induced mild AP and increased the extent of pancreatic vacuolization, leukocyte infiltration, and water content (Figure 2A–D) compared to the control group (histology of control is shown in Figure S1). FE pre-treatment did not cause any change during AP progression in histological parameters or water content (Figure 2A–D). CER-induced AP resulted in elevated pancreatic IL-1β content and serum amylase activity,

whereas it did not significantly affect MPO activity (Figure 2E–G). FE pre-treatment did not alter IL-1β level, MPO, or serum amylase activity in the AP groups.

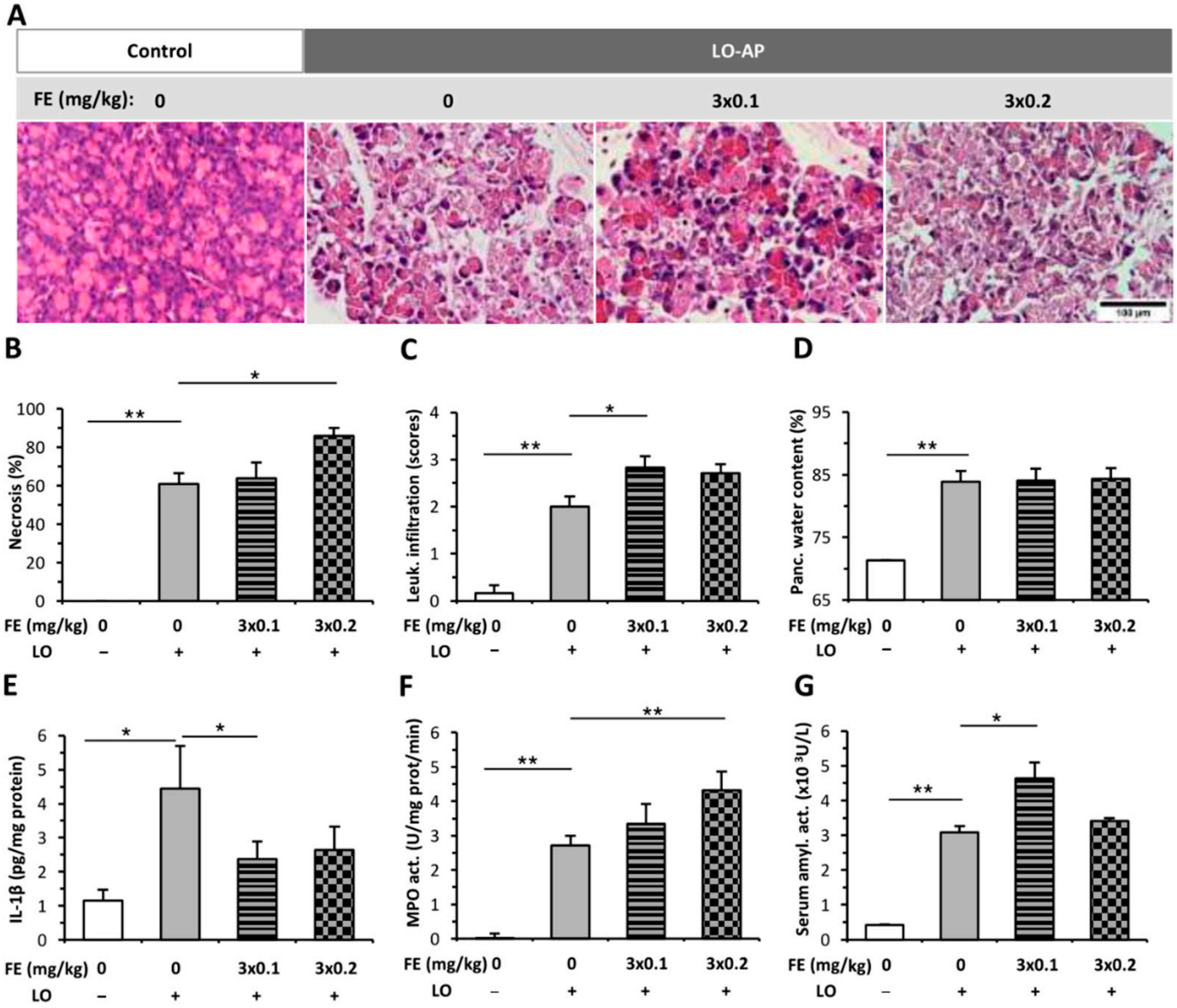

Figure 1. Fentanyl (FE) pre-treatment in L-ornithine (LO)-induced necrotizing acute pancreatitis (AP). Rats were treated with 3 × 0.1 or 3 × 0.2 mg/kg FE intraperitoneally (i.p.), whereas i.p. injection with 3 g/kg LO-HCl (LO +) was used to induce AP. Control animals received physiological saline instead of LO (LO −) or FE (0 mg/kg). Animals were sacrificed at 24 h after LO or physiological saline injection. (**A**) Representative histopathological images of pancreatic tissues of the treatment groups. Bar charts show the extent of pancreatic (**B**) necrosis, (**C**) leukocyte infiltration, (**D**) water content, (**E**) interleukin-1β (IL-1β) concentration, (**F**) myeloperoxidase (MPO) activity, and (**G**) serum amylase activity measurements. Values represent means with standard error, n = 9–11. Two-way ANOVA was performed followed by the Holm–Sidak post hoc test. * $p < 0.05$; ** $p < 0.001$.

2.2. The Effect of Fentanyl Post-Treatment on AP

In contrast to FE pre-treatment (Figure 1), both doses of FE post-treatment decreased the extent of histopathological changes (pancreatic tissue necrosis and leukocyte infiltration) caused by LO-induced AP (Figure 3A–C). On the other hand, FE administration did not alter pancreatic water content in the AP groups (Figure 3D). LO-induced AP increased pancreatic MPO and serum amylase activities, which were decreased by both FE doses

tested (Figure 3F,G). Pancreatic IL-1β levels only decreased significantly in case of the LO + 3 × 0.2 mg/kg FE group (Figure 3E).

Intra-ductal (i.d.) infusion of sodium taurocholate (NaTc) induced necrotizing AP in the head but not in the tail of the pancreas (not shown), which is in accord with the finding of others [22]. Therefore, only the pancreatic heads were used for analysis. NaTc also elevated the extent of pancreatic necrosis, leukocyte infiltration, and edema (Figure 4A–D). Both necrosis and immune cell infiltration were decreased by the higher dose of FE (0.2 mg/kg, Figure 4B,C), while the score of edema did not change in the AP groups after FE treatment (Figure 4D). Serum amylase activity also decreased in the NaTc + 3 × 0.2 mg/kg FE group versus the AP group without FE treatment (Figure 4E).

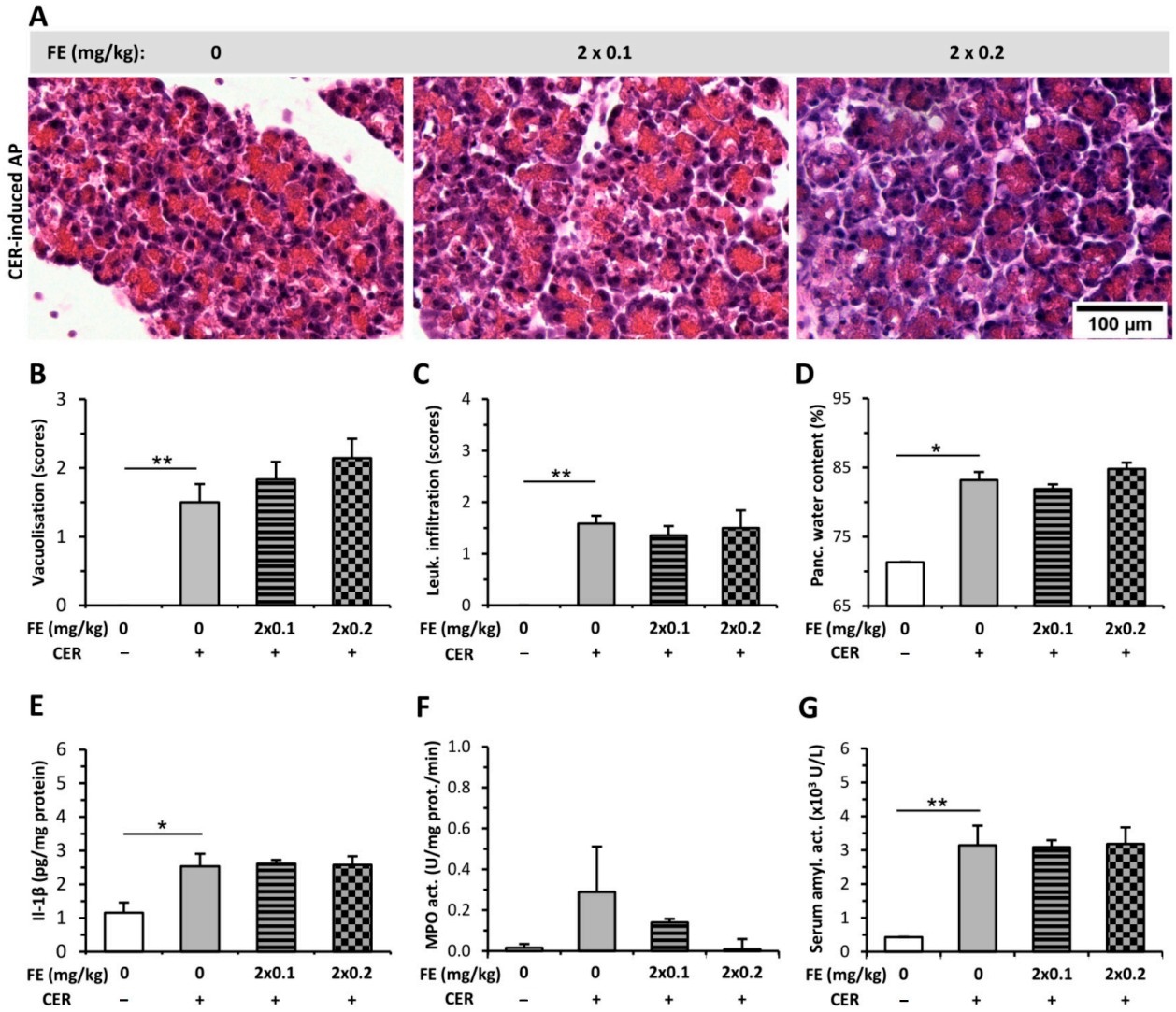

Figure 2. Fentanyl (FE) treatment started before the induction of mild acute pancreatitis (AP) with cerulein (CER) does not affect disease severity. Rats were treated with 2 × 0.1 or 2 × 0.2 mg/kg FE i.p., whereas i.p. injection with 4 × 20 µg/kg CER (CER +) was used to induce AP. Control animals received physiological saline instead of CER (CER −) or FE (0 mg/kg). Animals were sacrificed at 12 h after the first CER or physiological saline injection. (**A**) Representative histopathological images of pancreatic tissues of the treatment groups. Bar charts show the extent of pancreatic (**B**) vacuolization, (**C**) leukocyte infiltration, (**D**) water content, (**E**) interleukin-1β (IL-1β) concentration, (**F**) myeloperoxidase (MPO) activity, and (**G**) serum amylase activity measurements. Values represent means with standard error, n = 5–7. Two-way ANOVA was performed followed by the Holm–Sidak post hoc test. * $p < 0.05$; ** $p < 0.001$.

I.p. injections of CER increased the extent of pancreatic vacuolization, leukocyte infiltration, and tissue water content causing mild edematous AP (Figure 5A–D). FE treatment did not affect either histological parameters (tissue necrosis, leukocyte infiltration) or pancreatic water content (Figure 5A–D). The elevated amylase and MPO activities during AP were unaffected by FE post-treatment (Figure 5E,F). Interestingly, the smaller dose of FE (0.1 mg/kg) further increased the elevated serum IL-1β during AP, while the higher dose of FE had no effect (Figure 5G).

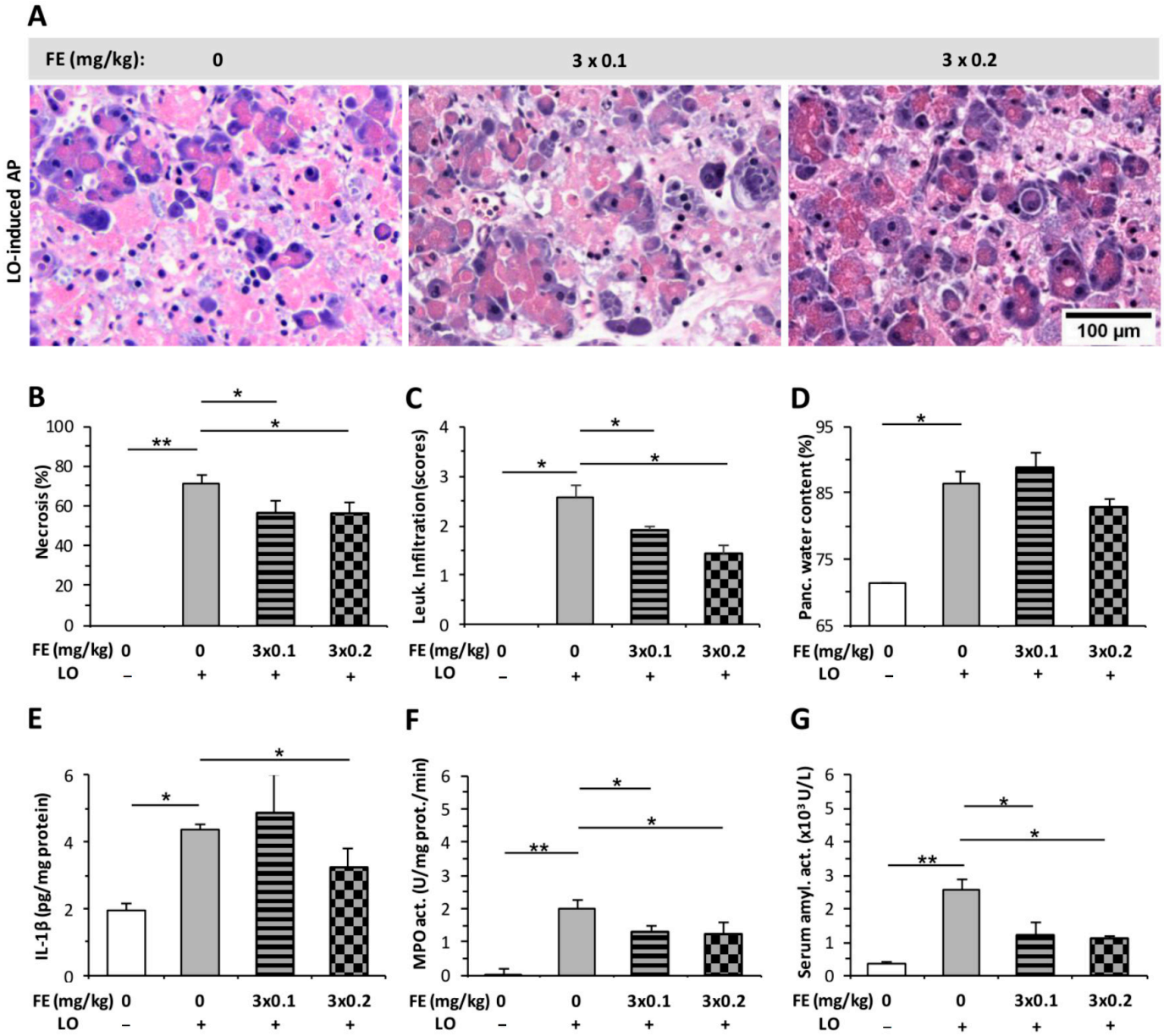

Figure 3. Fentanyl (FE) treatment started after the induction of L-ornithine (LO) acute pancreatitis (AP) reduces disease severity. (**A**) Representative histopathological images of pancreatic tissues of the treatment groups. Bar charts show the extent of pancreatic (**B**) necrosis, (**C**) leukocyte infiltration, (**D**) water content, (**E**) interleukin-1β (IL-1β) concentration, (**F**) myeloperoxidase (MPO) activity, and (**G**) serum amylase activity measurements. Values represent mean with standard error, n = 10–18. Two-way ANOVA was performed followed by the Holm–Sidak post hoc test. * $p < 0.05$; ** $p < 0.001$.

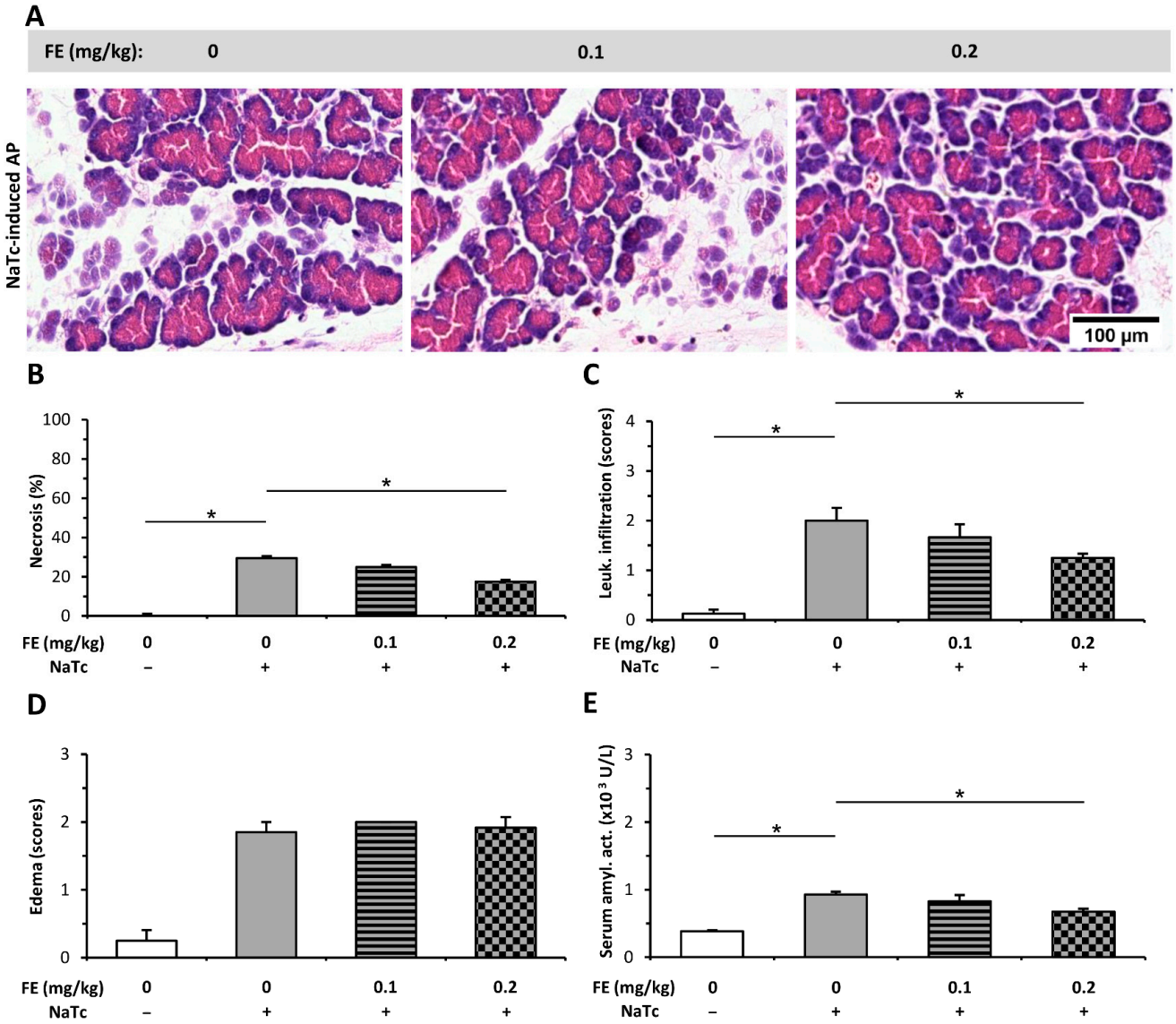

Figure 4. Fentanyl (FE) treatment started after the induction of necrotizing acute pancreatitis (AP) with sodium taurocholate (NaTc) reduces disease severity. Rats were treated with 0.1 or 0.2 mg/kg FE i.p., whereas the intra-ductal injection of 40 mg/kg NaTc (NaTc +) was used to induce AP. Control animals received physiological saline instead of NaTc (NaTc −) or FE (0 mg/kg). Animals were sacrificed at 16–24 h after the NaTc or physiological saline injection. (**A**) Representative histopathological images of pancreatic tissues of the treatment groups. Bar charts show the extent of pancreatic (**B**) necrosis, (**C**) leukocyte infiltration, (**D**) edema, and (**E**) serum amylase activity measurements. Values represent mean with standard error, n = 9–12. Two-way ANOVA was performed followed by the Holm–Sidak post hoc test. * $p < 0.05$.

2.3. Morphine Administration Does Not Affect the Severity of AP

The effect of MO on the severity of AP was investigated by using different doses of the drug: 8 × 5, 9 × 10, and 4 × 5 mg/kg. MO at the tested doses did not induce any structural changes in the pancreatic tissues of rats, and no inflammatory cell infiltration could be observed in histological sections (Figure S1B). Treatment with LO induced AP and resulted in marked pancreatic damage (tissue necrosis, leukocyte infiltration, and increased pancreatic water content Figure 6A–D). MO did not significantly alter the value of these parameters during AP. Furthermore, MO did not influence pancreatic MPO or serum amylase activity in the AP groups, either (Figure 6E,F).

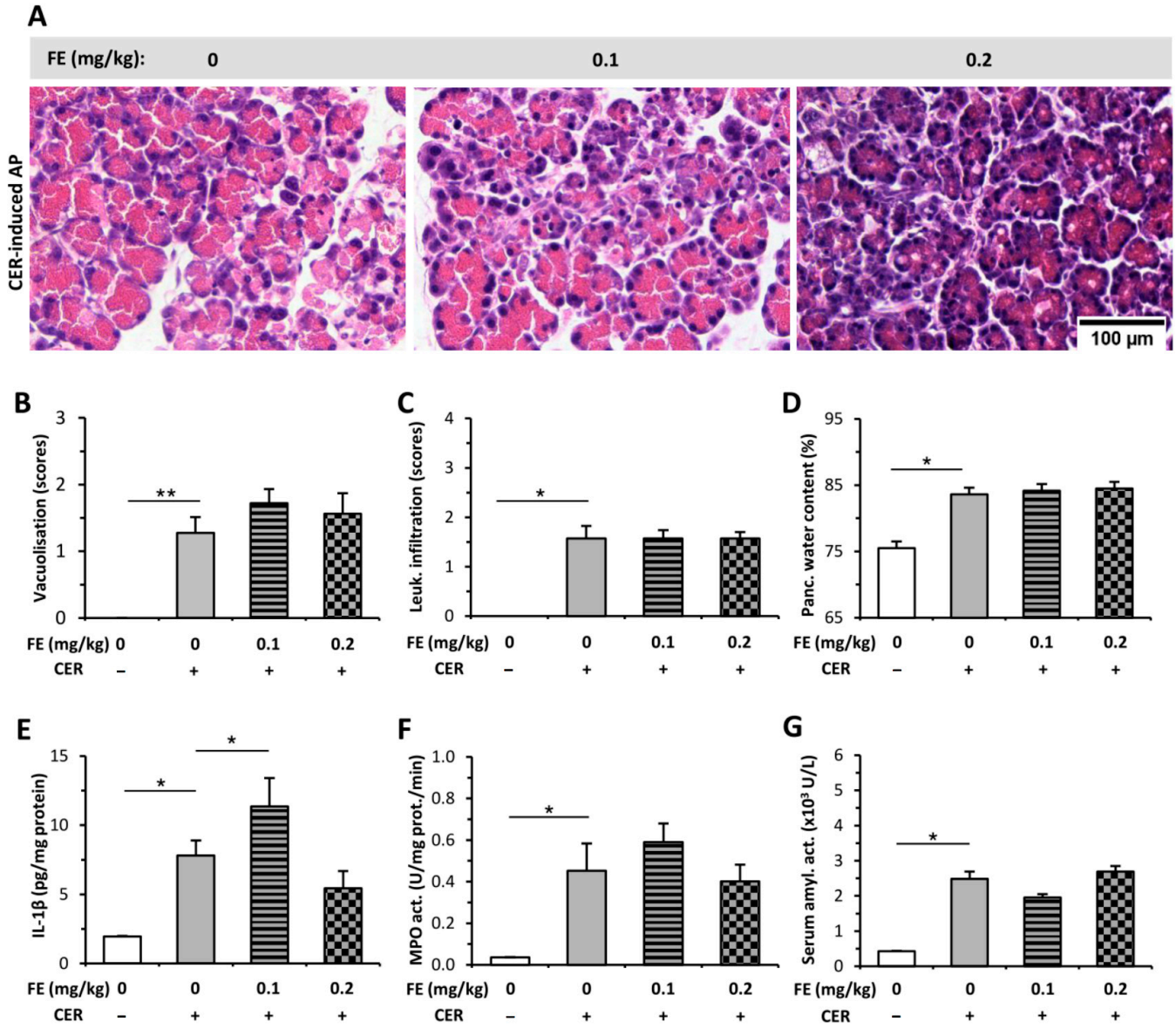

Figure 5. Fentanyl (FE) treatment started after the induction of acute pancreatitis (AP) with cerulein (CER) does not affect disease severity. (**A**) Representative histopathological images of pancreatic tissues of the treatment groups. Bar charts show the extent of pancreatic (**B**) vacuolization, (**C**) leukocyte infiltration, (**D**) water content, (**E**) interleukin-1β (IL-1β) concentration, (**F**) myeloperoxidase (MPO) activity, and (**G**) serum amylase activity measurements. Values represent mean with standard error, $n = 6$. Two-way ANOVA was performed followed by the Holm–Sidak post hoc test. * $p < 0.05$; ** $p < 0.001$.

The effect of 4 × 5 mg/kg MO was tested in a CER-induced AP model. Due to the shorter duration of AP in case of CER (12 h) compared to the LO model (24 h), the number of MO injections was reduced from eight (applied in LO-induced AP, Figure 6) to four. In the CER-induced edematous AP, MO significantly reduced vacuolization (Figure 7A,B), but it had no effect on leukocyte infiltration or pancreatic water content (Figure 7C,D). Serum amylase activity was significantly elevated after AP induction, and MO had no further effect on it (Figure 7F). However, AP did not induce any significant increase in pancreatic MPO activity (Figure 7E). MO had no additional effect on MPO activity during AP (Figure 7E).

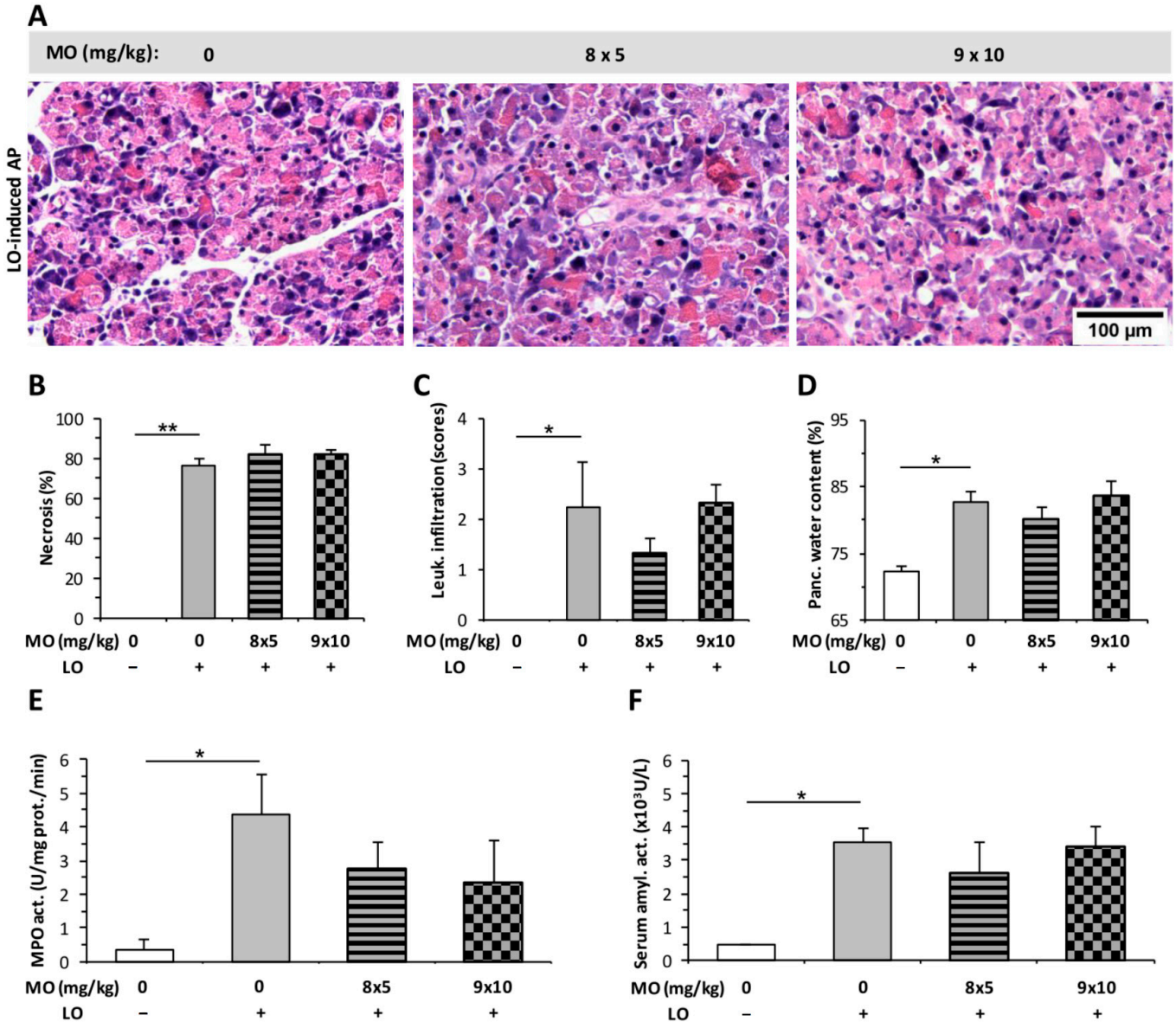

Figure 6. Morphine (MO) treatment does not affect the severity of L-ornithine (LO)-induced acute pancreatitis (AP). Rats were treated with 8 × 5 or 9 × 10 mg/kg MO i.p., whereas 3 g/kg LO-HCl (LO +) was used i.p. to induce AP. Control animals received physiological saline instead of LO (LO −) or MO (0 mg/kg). Animals were sacrificed at 24 h after LO or physiological saline injection. (**A**) Representative histopathological images of pancreatic tissues of the treatment groups. Bar charts show the extent of pancreatic (**B**) necrosis, (**C**) leukocyte infiltration, (**D**) water content, (**E**) myeloperoxidase (MPO) activity, and (**F**) serum amylase activity measurements. Values represent mean with standard error, $n = 6$. Two-way ANOVA was performed followed by the Holm–Sidak post hoc test. * $p < 0.05$; ** $p < 0.001$.

2.4. Buprenorphine Has No Effect on the Severity of LO-Induced AP

The effect of BQ was tested by i.p. and i.t. administrations. BQ alone did not induce any changes in pancreatic tissues (Figure S1C). The tested i.p. doses (2 × 0.1; 2 × 0.5; 2 × 1 mg/kg) of BQ did not affect the LO-induced pancreatic necrosis, leukocyte infiltration, or serum amylase activity (Figure 8A–C,E). However, 2 × 1 mg/kg BQ slightly enhanced the pancreatic water content in AP (Figure 8D).

Intrathecal (i.t.) administration of BQ was also tested on rats during AP (Figure 9). The dose of 3 × 3 µg/kg BQ had no effect on any parameters of AP, while the 3 × 6 µg/kg dose significantly decreased the extent of leukocyte infiltration in AP (Figure 9C).

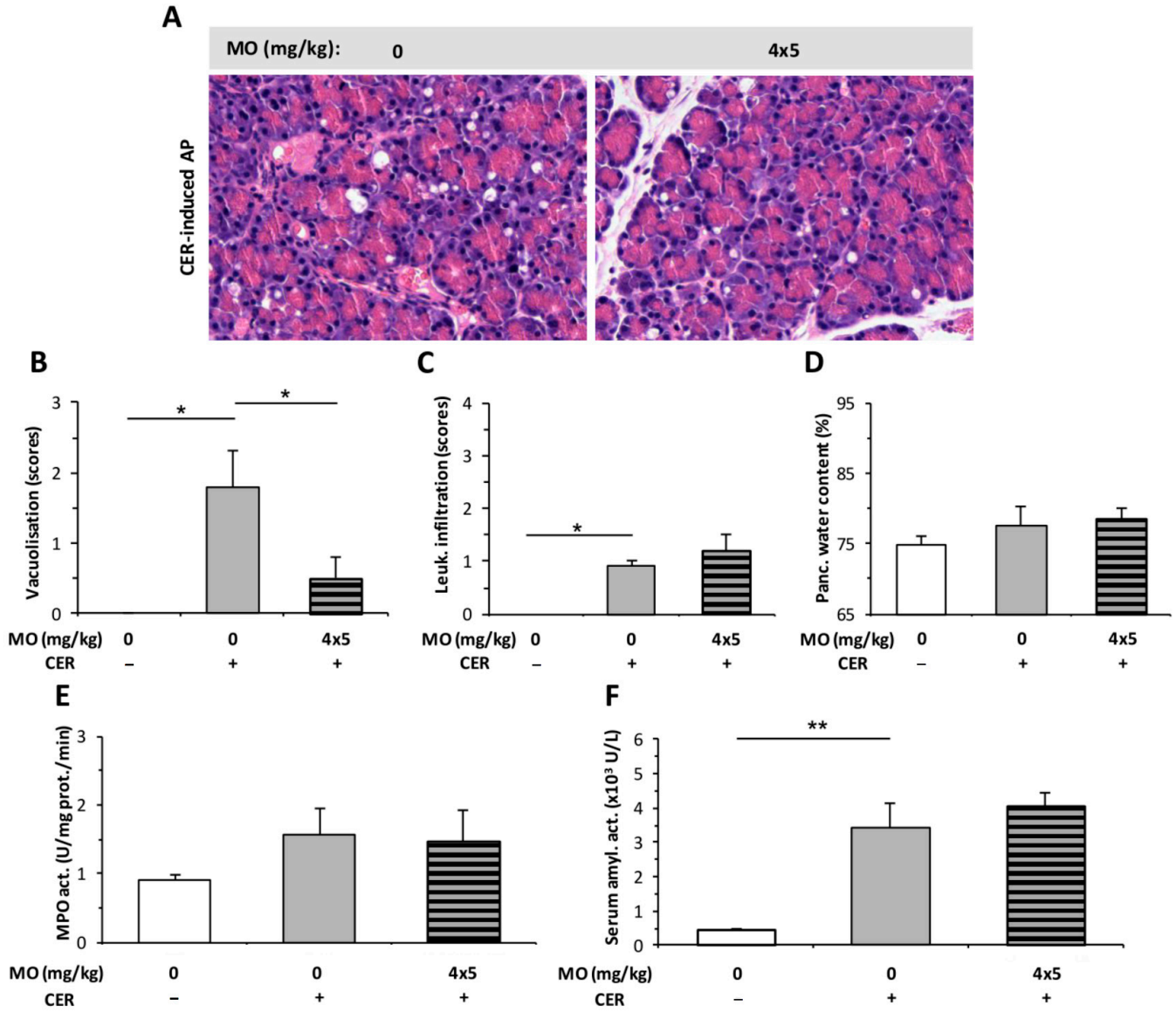

Figure 7. Morphine (MO) treatment does not affect the severity of cerulein (CER)-induced acute pancreatitis (AP). Rats were treated with 4 × 5 mg/kg MO i.p., whereas 4 × 20 µg/kg CER (CER +) was used i.p. to induce AP. Control animals received physiological saline instead of CER (CER −) or MO (0 mg/kg). Animals were sacrificed at 12 h after the first CER or physiological saline injection. (**A**) Representative histopathological images of pancreatic tissues of the treatment groups. Bar charts show the extent of pancreatic (**B**) vacuolization, (**C**) leukocyte infiltration, (**D**) water content, (**E**) myeloperoxidase (MPO) activity, and (**F**) serum amylase activity measurements. Values represent mean with standard error, $n = 6$. Two-way ANOVA was performed followed by the Holm–Sidak post hoc test. * $p < 0.05$; ** $p < 0.001$. Scale bar.

2.5. Pancreatic mu Opioid Receptor Expression Is Decreased in LO-Induced AP

The mRNA and protein expression of mu opioid receptor (MOR) were investigated in the pancreas and brain (Figure 10). In the brain, MOR was detected in control animals, and AP did not influence the amount of MOR after 24 h (Figure 10A,C). In case of the pancreas, control animals also expressed MOR, but the induction of AP significantly reduced the presence of the receptor (Figure 10B,D).

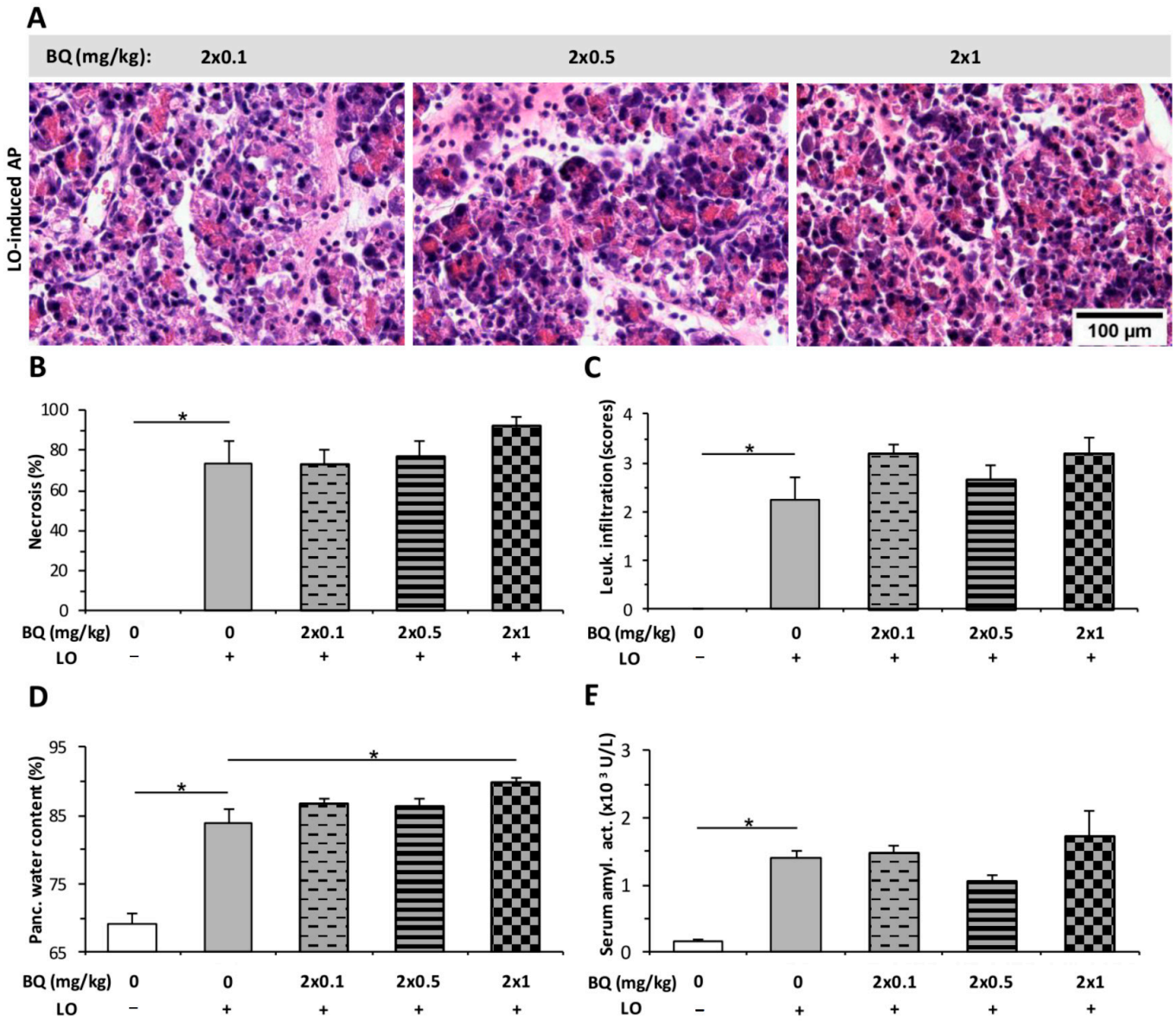

Figure 8. Intraperitoneal (i.p.) buprenorphine (BQ) treatment does not affect the severity of L-ornithine (LO)-induced acute pancreatitis (AP). Rats were treated with 2 × 0.1, 2 × 0.5, or 2 × 1 mg/kg BQ i.p., whereas i.p. injection with 3 g/kg LO (LO +) was used to induce AP. Control animals received physiological saline instead of LO (LO −) or BQ (0 mg/kg). Animals were sacrificed at 24 h after the first CER or physiological saline injection. (**A**) Representative histopathological images of pancreatic tissues of the treatment groups. Bar charts show the extent of pancreatic (**B**) necrosis, (**C**) leukocyte infiltration, (**D**) water content, and (**E**) serum amylase activity measurements. Values represent mean with standard error, $n = 6$. Two-way ANOVA was performed followed by the Holm–Sidak post hoc test. * $p < 0.05$.

2.6. Pancreatic and Brain mu Opioid Receptor Functions Are Reduced in AP

The functional activity of opioid receptors in pancreatic and brain-derived cell membrane homogenates were studied by receptor mediated in vitro G-protein stimulation (Figure 11). The G-protein activating effect of three well-known MOR agonists (FE, MO, and the highly selective synthetic opioid peptide Tyr-D-Ala-Gly-$_{(NMe)}$Phe-Gly-ol—DAMGO) was measured at a concentration above the saturation level of the receptor (10 µM). The involvement of opioid receptors in G-protein activation was demonstrated by the inhibition with the well-known opioid receptor specific antagonist naloxone at equimolar concentration. In our experiments, brain and pancreatic preparations were investigated in animals with or

without AP. All three tested agonists efficiently activated Gi/o proteins in guanosine-5′-[^{35}S] thiophosphate ([^{35}S]GTPγS) binding experiments. The level of pancreatic activation was lower than the corresponding values found in the brain samples (statistics were not performed in that comparison). In the pancreas, the rank order of efficacy of the activating agonist ligands was fentanyl > morphine ≅ DAMGO. The activation of G proteins was virtually eliminated in samples from AP compared to the control group (Figure 11).

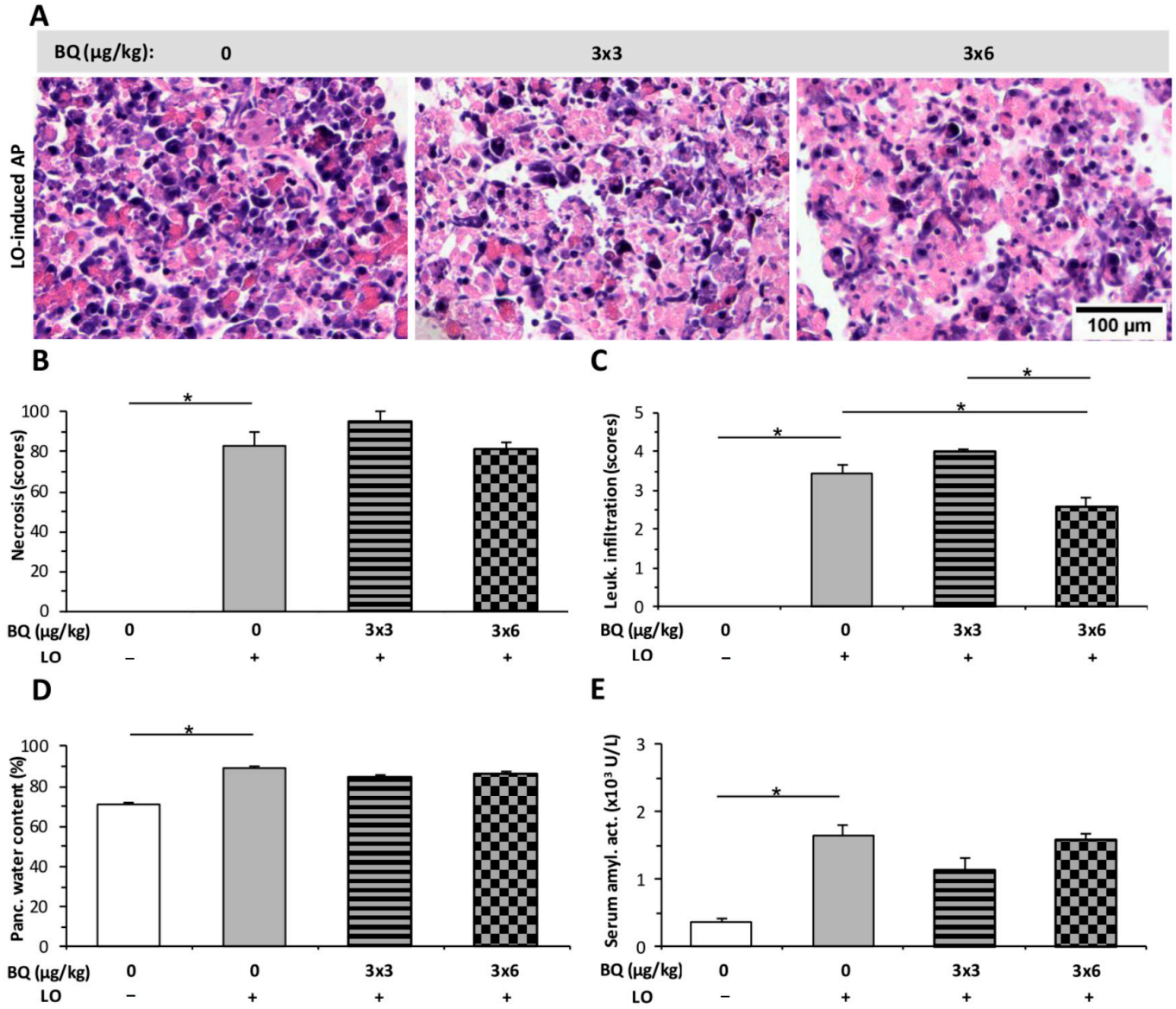

Figure 9. Intrathecal (i.t.) buprenorphine (BQ) treatment does not affect the severity of L-ornithine (LO)-induced acute pancreatitis (AP). Rats were treated with 3 × 3 or 3 × 6 mg/kg BQ i.t., whereas i.p. injection with 3 g/kg LO (LO +) was used to induce AP. Control animals received physiological saline instead of LO (LO −) or BQ (0 mg/kg). Animals were sacrificed at 24 h after the first CER or physiological saline injection. (**A**) Representative histopathological images of pancreatic tissues of the treatment groups. Bar charts show the extent of pancreatic (**B**) necrosis, (**C**) leukocyte infiltration, (**D**) water content, and (**E**) serum amylase activity measurements. Values represent mean with standard error, $n = 6$. ANOVA was performed followed by the Holm–Sidak post hoc test. * $p < 0.05$.

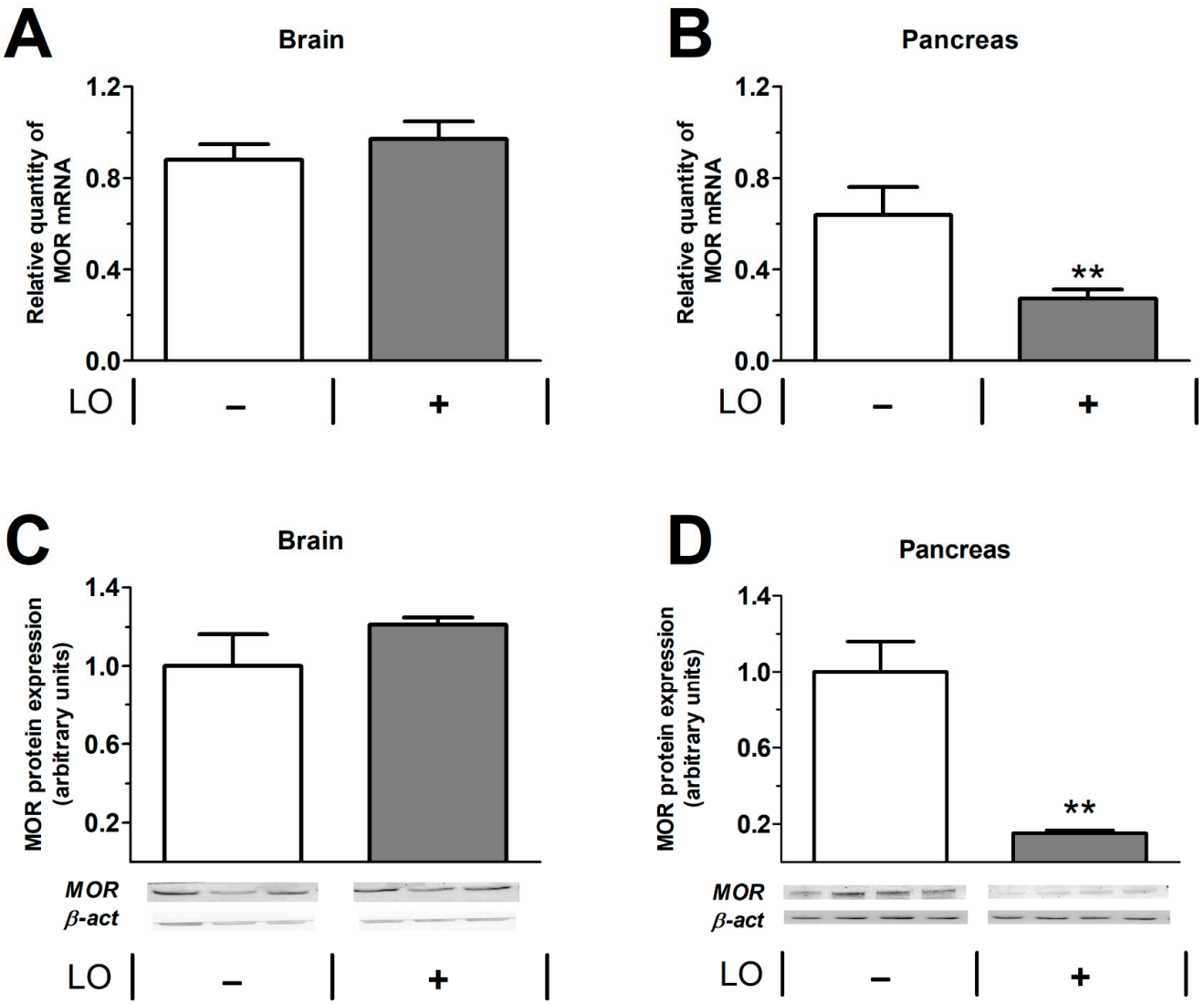

Figure 10. Expression of mu opioid receptor (MOR) in the brain and pancreas in control and LO-induced AP. Rats were treated with vehicle or 3 g/kg LO-HCl and were sacrificed at 24 h. MOR mRNA (**A**,**B**) and protein (**C**,**D**) expression levels were determined in the brain (**A**,**C**) and pancreas (**B**,**D**). In (**C**,**D**), the bar charts show the quantitative analysis of Western blot images. Values represent mean with standard error, n = 13–17 (RT-PCR), n = 3–4 (Western blot analysis). Student's t test was performed, ** $p < 0.01$. Abbreviations: LO, L-ornithine-induced acute pancreatitis; β-act, β-actin; MOR, mu opioid receptor.

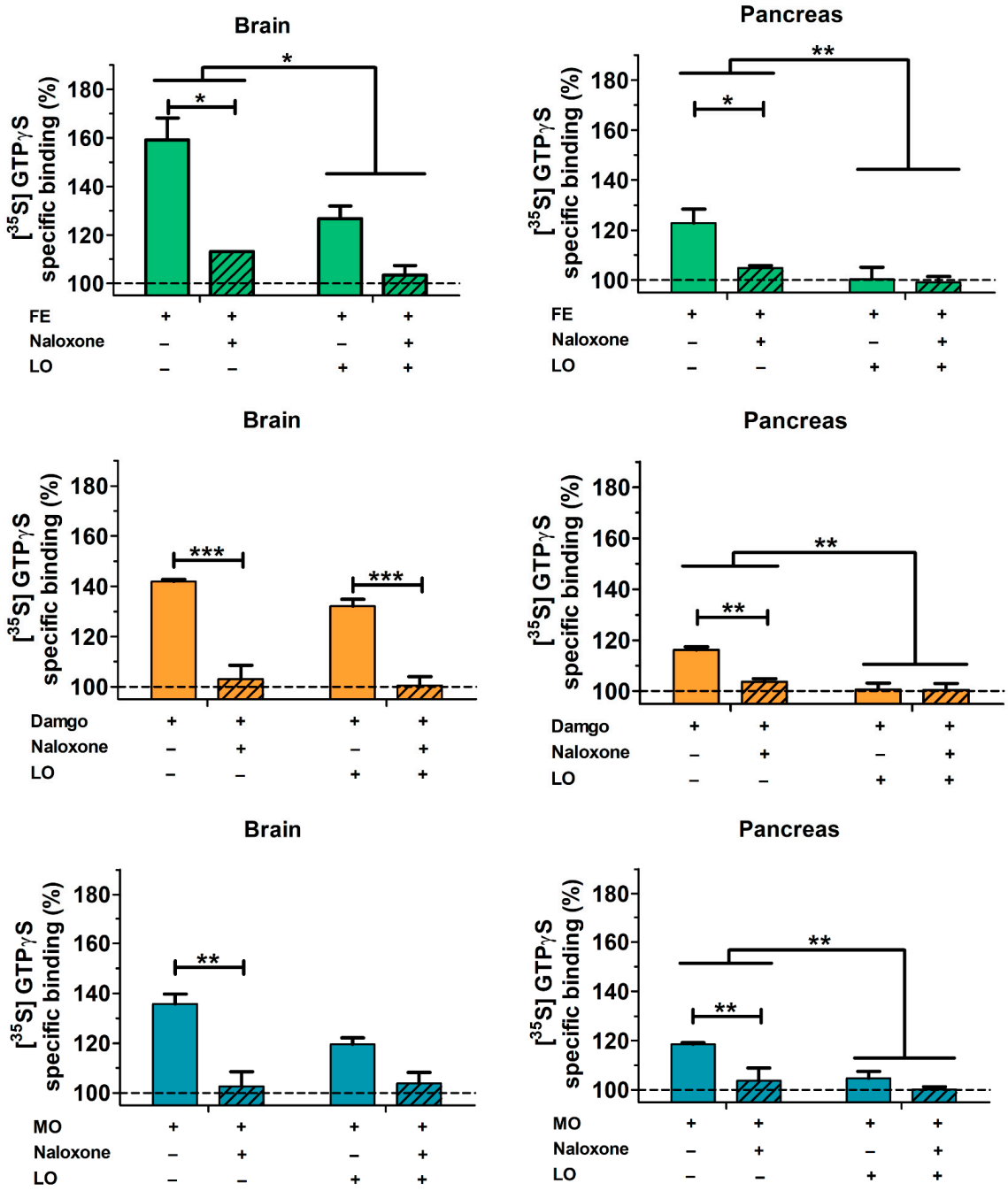

Figure 11. Stimulation of G-protein activation in rat brain and pancreas membrane homogenates. Tissue samples were derived from control and AP animals. Treatments of pancreatic homogenates were as follows: 10 μM FE; 10 μM DAMGO; 10 μM MO. Striped bars represent the combined treatment with mu receptor ligands (FE, MO, DAMGO) and equimolar naloxone. Values represent mean with standard error, $n = 6$. Two-way ANOVA was performed followed by Bonferroni post hoc test. * $p < 0.05$, ** $p < 0.01$; *** $p < 0.001$.

3. Discussion

Opioids are commonly used for pain control in AP patients. It has been speculated that these analgesics (such as morphine) may affect AP progression. Therefore, we comprehensively investigated the effects of FE on the severity of experimental AP, and this research was further supplemented with the examination of the effects of MO and BQ. It is important to note that measurements were performed when the experimental AP reached its maximal severity.

I.p. FE pre-treatment significantly increased the severity of necrotizing AP induced by LO, but it had no effect on edematous AP evoked by CER. Interestingly, the clinically more relevant post-treatment with FE either decreased or had no effect on the various parameters of AP severity in different models. Wang and Chen [23] also tested the effect of FE on NaTc-induced AP. They administered FE i.v. 23–23.5 h after AP induction and sacrificed the animals 24 h after the induction of the disease. Surprisingly, FE exerted anti-inflammatory effects on the pancreas and AP-induced myocardial damage within that really short time (30–60 min). In clinical settings, Stevens et al. [24] showed that FE did not have any side effects compared to the placebo control group (intramuscular Demerol containing pethidine). Some studies draw attention to the importance of the administration site of FE, especially into the epidural site. The use of FE in epidural anesthesia partially restored the decrease in microcirculatory flow caused by AP and prevented the development of tissue necrosis and systemic complications [25,26].

MO pre- or post-treatment did not affect the severity of the disease in case of LO-induced necrotizing AP. Furthermore, the simultaneous administration of MO and CER had no remarkable effect on disease progression either, except for vacuolization, which was decreased by MO. In a recent study, Barlass et al. [21] also investigated MO in two necrotizing mouse AP models. They concluded that MO application delayed AP resolution and reduced intestinal motility, which increased the risk for bacterial translocation. MO also delayed macrophage migration and caused a persistence of inflammation. Their findings related to macrophages are in accordance with earlier studies showing mononuclear cell suppression and chemokine receptor transdeactivation after MO treatment [27,28]. Our study focused on the early-mid events of AP and showed no adverse effects of MO, while Barlass et al. [21] investigated the later effects of MO (at 48, 72, or 120 h). However, our results do not rule out the possibility of later side effects that were shown by Barlass et al. [21]. Marked differences in the results can be explained by species differences, the latter study used mice, while in the present study, rats were investigated. Moreover, one randomized clinical trial [29] and two related reviews [17,30] did not find any significant difference in the effects of MO vs. the non-opioid metamizole. It should be noted that a relatively low number of patients (eight per group) were included in this randomized clinical trial. Based on these observations, we conclude that MO does not affect the severity of the AP at the early-mid stage of the disease, but later side effects may appear according to literature data.

The partial opioid receptor agonist BQ did not cause any adverse effects during AP in i.p. pre-treatment; only tissue water content was increased by the highest dose. I.t injection of the smaller dose of BQ did not affect any other aspects of disease severity measured in our experiments. However, the higher dose significantly decreased immune cell infiltration. Based on this, i.t. administration could be more beneficial during experimental AP. Furthermore, we demonstrated first the effects of BQ on AP at the spinal level. Literature data showed that in an NaTc-induced AP rat model, i.v. BQ administration did not influence disease severity [31]. In a CER-AP model, subcutaneous 0.5 mg/kg BQ reduced the zymogen content and protein synthesis of acinar cells [32]. These results strengthen the beneficial effect of BQ during AP.

Opioids exert their effects primarily through mu, kappa, or delta opioid receptors, which are expressed mainly by neuronal or immune cells. The effects can differ depending on their affinity or specificity to certain receptors. Publications showed that MO has immunosuppressant properties through full mu receptor agonism. MO treatment resulted in the inhibition of cytokine production, NK cell activity, cellular responses to mitogens, antibody production, cell growth, and decreased phagocytic activity [33,34]. FE is 80 times more potent than MO and is a highly selective full MOR agonist ligand [35]. Therefore, it can also suppress the immune system [19]. MO and FE can also cause a sphincter of Oddi spasm, which could further aggravate AP severity [36]. In contrast to MO and FE, BQ is a partial agonist of the mu receptor, while it is an antagonist of kappa and delta opioid receptors [19]. Therefore, BQ has a different pharmacological profile than the other

opioids, and it does not inhibit NK cells, T cells, phagocytosis of macrophages, or cytokine production [19], and it has no morphine-like effect on the sphincter of Oddi [37]. These effects of opioids on cellular processes or on the sphincter of Oddi may explain the changes observed during AP in our experiments. Only FE pre-treatment resulted in increased AP severity. The early immunosuppression by FE may cause this adverse effect, while FE post-treatment was beneficial for AP outcome. However, the later consequences were not investigated by this work. For all clinically applied opioids, including FE, MO, or BQ, these effects should be considered and investigated in future studies. Moreover, the timing of opioid administration can be critical, especially in case of FE.

We demonstrated MOR mRNA and protein expression in the control rat pancreas and brain. It is well known that the brain expresses large amounts of opioid receptors [38,39]; in case of the pancreas, other research groups have also shown MOR expression in rats [40], sheep [41], and humans [42]. Pancreatic islet cells express MOR [43], which influences glucose homeostasis and insulin secretion. There is no direct evidence on opioid receptor expression in exocrine pancreatic cells. However, it has been demonstrated that enkephalin and MO inhibit pancreatic bicarbonate and protein secretion during endogenous or exogenous stimulation (secretin or cholecystokinin-octapeptide) in dogs [44], which may indicate the presence of MOR in both acinar and ductal cells. Other opioid receptors (nociception/orphanin FQ and delta opioid receptors) also play a role in regulating exocrine pancreatic secretion [45]. Furthermore, pancreatic cholinergic neurons have opiate receptors as well [46].

The efficiency of G-protein stimulation by mu opioid agonists was markedly higher in the rat brain than in pancreatic preparations. Transmembrane signaling mediated by opioid agonists was almost completely eliminated in the pancreatic cell membrane preparations of AP animals at 24 h. This can be explained by the dramatic decrease in pancreatic MOR mRNA and protein expression. In case of brain tissue, no reduction in MOR protein and mRNA levels could be observed. At 24 h, pancreatic tissue necrosis is extensive, which can contribute to the reduction of different receptors such as MOR, while there is no tissue necrosis in the brain; therefore, MOR expression remained unaltered. To the best of our knowledge, we demonstrated for the first time that AP reduced the function of opioid receptors not only in the pancreas but also in the brain. Notably, other groups have shown that mu opioid receptor expression is upregulated in hind paw or intestinal inflammatory animal models [47,48]. However, tissue acidification induced by injury or inflammation impaired MOR signaling [49]. Since the extent of AP severity is influenced by FE acting via opioid receptors (predominantly on MOR), we wanted to check their expression in the pancreas and brain and their functional activity in cell membrane fractions prepared from both tissues. The expression of MOR in the brain was unchanged in response to AP, whereas its functional activity was decreased during FE stimulation. This means that AP may affect MOR activity independently of changes in protein expression. The increase in serum pro-inflammatory cytokine (interleukin 1β) concentration has been shown to reduce central opioid neurotransmitter function [50]. Furthermore, there is a crosstalk between chemokines and opioid receptors, since certain chemokines (e.g., CCR2, CCR5, CCR7, CXCR4) can desensitize opioid receptors [34]. The most prominent symptom of AP is pain. During the disease, endogenous opioids (such as enkephalins, endorphins, and dynorphins) are released [51]. These substances may cause MOR desensitization [52,53], which could also contribute to the observed reduction in MOR activity. Moreover, high amounts of MOR are expressed in the spinal cord, which modulates pain sensation via the descending pain pathway system [54]. It is known that chronic pancreatitis causes chronic pain, which will result in epigenetic modulations of pain-related genes [55]. The latter is mediated by increased histone deacetylase 2 activity during chronic pancreatitis in the spinal cord. Consequently, there will be a reduction of MOR expression within some weeks. AP lasts for a shorter period, but due to the persistent pain, MOR expression can also be affected in the spinal cord. Further studies could investigate MOR not just in the pancreas and brain but also in the spinal cord. Overall, the mechanisms by which AP affects opioid

receptor activity is partly unknown, but we must infer a very likely interaction between the biochemical processes of opioid ligand binding and G-protein-mediated transmembrane signaling and organ inflammation.

In the clinical setting, there are no guidelines or recommendations suggesting which is the best opioid to use in AP. However, the application of effective and strong analgesics is necessary in the treatment of this disease. In light of the results discussed above, post-treatments (e.g., FE, MO) do not increase disease severity, but some of the opioids (e.g., MO) may affect the resolution of AP. Therefore, the latter may not be the best treatment option in this severe disease. Our results showed that FE post- and BQ pre-treatments have promising effects besides pain relief; therefore, the use of these opioids could also be beneficial for AP severity. Overall, this research contributes to a better understanding of the opioid effect in AP and can help design further clinical trials that will be necessary to select the most appropriate opiate to treat this potentially lethal disease.

Although pre-treatment with analgesics in AP is clinically less relevant, rectal administration of nonsteroidal anti-inflammatory drugs (NSAIDs, e.g., indomethacin or diclofenac) is indicated for endoscopic retrograde cholangiopancreatography (ERCP) [56]. These agents reduce the development of post-ERCP-related AP. In this case, the use of opiates could be also tested.

The present study has limitations as well. The long-term consequences of opiates on AP were not investigated as it was performed by Barlass et al. [21]. Furthermore, the above-mentioned and beneficial epidural administration route [25,26] was not investigated by our group.

In conclusion, we showed for the first time that AP reduced the transmembrane signaling of mu opioid receptors in both the pancreas and the brain. We demonstrated that FE post-treatment improved, while FE pre-treatment exacerbated disease severity in necrotizing AP. However, FE did not affect the outcome of edematous AP. MO administration had minimal effects in both pre- and post-treatments including cellular vacuolization, pancreatic water content, and leukocyte infiltration. I.t. administration of BQ showed slight benefit over i.p. injection. FE post-treatment proved to be beneficial in AP. Finally, our results suggest that type, dosing, administration route, and timing of opioid treatment can determine the effects on AP outcome. Clinical studies are needed to determine which opioid(s) is the best in AP.

4. Materials and Methods

4.1. Animals

Female Wistar rats weighing 200–250 g were used for experiments. The animals were kept at a constant room temperature of 24 °C with a 12 h light–dark cycle and were allowed free access to water and standard laboratory chow (Biofarm, Zagyvaszántó, Hungary).

4.2. Materials

All chemicals were purchased from Sigma-Aldrich (Budapest, Hungary) unless indicated otherwise.

4.3. In Vivo Experiments: Acute Pancreatitis Induction, Opiate Treatments, and Tissue Collection

Three different models of AP were applied (Figure 12). Necrotizing AP was induced by (a) single i.p. injection of 3 g/kg L-ornithine-HCl (LO, 30%, pH = 7.4); (b) intra-ductal administration of 1 mL/kg Na-taurocholate solution (NaTc; 40 mg/mL) as described previously [9,22]. Edematous AP was induced by hourly i.p. injections of 20 µg/kg cerulein (CER, 50 µg/mL) four times. Briefly, in case of NaTc-induced AP, abdominal surgery was performed on anesthetized rats (with 70 mg/kg ketamine and 14 mg/kg xylazine i.p.—purchased from CP-Pharma-Handelsgesellschaft MBH (Burgdorf, Germany)). Then, a cannula was placed into the pancreatic duct, and the biliary duct was transiently occluded via a microvessel clip. The NaTc solution was injected at a speed of 50 µL/min. At the end of the procedure, rats were placed on a heating pad for 40 min or until they woke up.

Thereafter, rats were placed back into their cages for 16–24 h. Control groups were given physiological saline (0.9% NaCl) solution instead of LO/CER/NaTc, respectively. Animals were sacrificed at 24 h in the LO-induced experimental pancreatitis model, between 16 and 24 h in case of the NaTc model, and at 12 h in case of the CER model. In case of NaTc-induced AP, rats were extensively monitored, and when body temperature decreased below 30 °C, they were humanely sacrificed by deep anesthesia induced by 85 mg/kg i.p. pentobarbital injection (Bimeda MTC, Cambridge, ON, Canada).

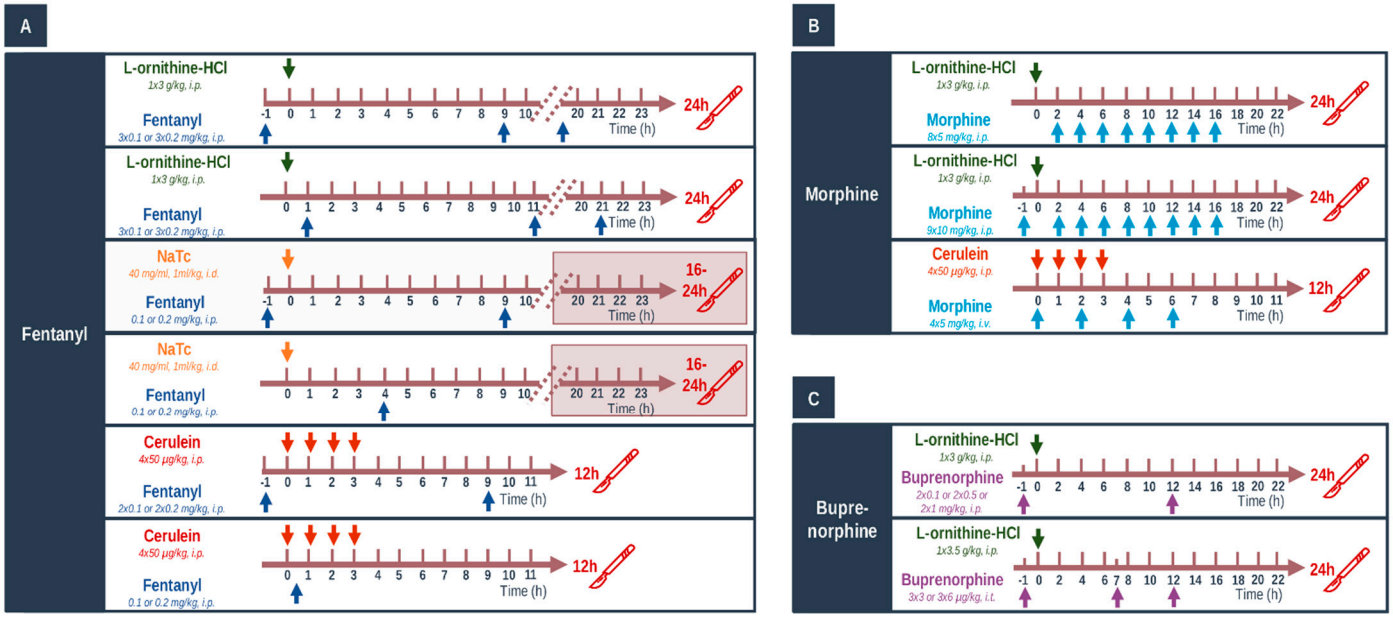

Figure 12. (**A**–**C**) Schematic view of experimental setup. Treatment arrangements for acute pancreatitis (AP) induction and opioid administration in Wistar rats. Arrows above or below the timeline show the injections. Control animals were injected with physiological saline. Abbreviations: i.p.—intraperitoneal; i.t.—intrathecal; i.v.—intravenous.

FE was administered i.p. at doses of 0.1 and 0.2 mg/kg based on the literature data [57]. Different timing arrangements were applied for FE in various AP models; repeated injections were performed when the analgesic effect of FE was decreased (this was determined in preliminary experiments or by literature data). In addition, FE was used as pre- or post-treatment. In the pre-treatment groups, the first FE injection was given 1 h prior to the induction of AP, and it was repeated every 11 h in LO- and every 10 h in NaTc- or CER-induced AP, respectively (Figure 12A). In preliminary experiments, FE pre-treatment was also tested in NaTc-induced AP, but the condition of animals was critical; therefore, humane termination was performed, and these investigations were stopped. In the post-treatment setup, animals received the first FE injection 1 h after AP induction in case of the LO model or 0.5 h after AP induction in case of the CER model. Since FE depresses respiration [58], it could not be administered within 3 h after surgery; therefore, FE was injected 4 h after the beginning of surgery in case of the NaTc model of AP (Figure 12A).

In the post-treatment setup, 5 mg/kg MO was administered i.p. 8 times every 2 h in case of the LO model (Figure 12B). The dose and timing of MO was chosen based on literature data; repeated injections were performed when the analgesic effect of MO was decreased [59]. During pre-treatment, 10 mg/kg MO was injected i.p. 9 times every 2 h (Figure 12B). When AP was induced by CER, 4 × 5 mg/kg dose of MO was used i.v. every 2 h, and analgesia started simultaneously with AP induction (Figure 12B). Animals were sacrificed 24 or 12 h after AP induction with LO or CER, respectively.

BQ has prolonged analgesic effects, and its recommended dosing intervals are between 8 and 12 h [60]. Instead of testing BQ in different AP models, it was administered via two

routes: i.p and intrathecally (i.t., Figure 12C). For i.t. administration, rats were anesthetized with a mixture of ketamine hydrochloride and xylazine (72 and 8 mg/kg i.p, respectively). An i.t. catheter (PE-10 tubing Intramedic, Clay Adams; Becton Dickinson; Parsippany, NJ, USA; I.D. 0.28 mm; O.D. 0.61 mm) was inserted via the cistern magna and passed 8.5 cm caudally into the subarachnoid space [61], which served to place the catheter tip between vertebrae Th12 and L2 vertebrae, corresponding to the spinal segments that innervate the hind paws [62]. After surgery, animals were injected by gentamycin (10 mg/kg, subcutaneously) to prevent infection and were housed individually. Rats exhibiting postoperative neurologic deficits, or those ones that did not show paralysis of one of the hindpaws after the administration of 100 µg lidocaine were excluded (about 10%) [62]. The drugs were applied at least after 4 days of recovery. I.p. injections of 0.1, 0.5, and 1 mg/kg BQ were given 1 h before and 12 h after the beginning of AP induction. I.t. injections of 3 and 6 µg/kg BQ were administered 1 h before AP induction and were repeated at 7 and 12 h after AP induction with LO. BQ was injected over 120 s in a volume of 10 µL, which was followed by a 10 µL flush of physiological saline. These BQ doses are in accordance with literature data [63,64].

At the end of experiments/treatments, deep anesthesia was induced by 85 mg/kg i.p. pentobarbital injection. Blood was collected through cardiac puncture; then, the pancreas was rapidly removed. Pancreata were cleaned from fat and lymph nodes on ice and then cut into pieces. Two parts of the pancreatic tissue were immediately frozen in liquid nitrogen and stored at −80 °C until biochemical assays or dry–wet weight measurements were performed. The third part of the pancreas was fixed in 8% neutral formaldehyde solution for histological analysis. In case of the NaTc model, pancreata were stored only for histological analysis due to the heterogeneity of AP induction. Blood samples were centrifuged at 2500 RCF for 15 min at 4 °C, and the sera were stored at −20 °C until use. Brains were also rapidly collected from rats, and the whole tissues were used for [^{35}S]GTPγS functional binding assay, whereas the cortex was used for PCR and Western blots. Brain samples were stored at −80 °C until further processing.

4.4. Laboratory Measurements

Serum amylase activity was measured on a Fluorostar Optima plate reader (BMG Labtech, Ortenberg, Germany) with a colorimetric kinetic method using a commercial kit purchased from Diagnosticum Zrt. (Budapest, Hungary). To evaluate the pancreatic water content, the wet weight (WW) of the pancreata was measured; then, the tissues were dried for 24 h at 100 °C, and the dry weight (DW) was also measured. The wet/dry weight ratio was calculated as follows: [(WW−DW)/WW] × 100. Pancreatic myeloperoxidase (MPO) activity is a hallmark of leukocytic infiltration and was measured according to Kuebler et al. [65]. MPO activities were normalized to total protein content as measured by the Lowry method [66]. To determine the extent of inflammatory response in the pancreata, we measured interleukin (IL)-1β levels by a commercial ELISA kit from R&D Systems (Minneapolis, MN, USA), as described by the manufacturer.

4.5. Histological Examination

Formalin-fixed pancreatic tissues were sectioned to 3 µm. These sections were prepared and stained with hematoxylin and eosin and were analyzed and scored by two independent experts blinded to the experimental protocol. Five different random areas were observed and scored per section per researcher. Edema was scored between 0 and 3 points (0: none; 1: patchy interlobular; 2: diffuse interlobular; 3: diffuse interlobular and intra-acinar), leukocytic infiltration between 0 and 4 points (0: none; 1: diffuse/mild; 2: diffuse/moderate; 3: diffuse/severe; 4: diffuse/very severe), vacuolization between 0 and 3 points (0: none; 1: mild; 2: moderate; 3: severe); the percentage of acinar cell damage was also evaluated.

4.6. Total RNA Preparation from Tissue

A small piece of pancreas or brain cortex was placed on ice in 1 mL of TRIzol reagent in a 13 mL centrifuge tube and was homogenized immediately with IKA Ultra Turrax (Type: TP18/10; Janke and Kunkel IKA, Staufen im Breisgau, Germany). Then, the tissue homogenate was instantly placed on liquid nitrogen and stored at −80 °C until use (for a maximum of 1 or 2 days). Total RNA purification was performed in three steps. In the first step, phase separation was performed by adding 200 µL of chloroform to the samples and shaking vigorously for 15 min, allowing to stand, and then centrifuging at 12,000 *g* for 15 min at 4 °C. From the resulting three phases, the top aqueous phase was aspirated into an empty Eppendorf tube, and 500 µL of isopropanol was added. Then, this was vortexed and allowed to stand for a few minutes, and after that, it was centrifuged at 12,000 *g* for 10 min at 4 °C. RNA precipitated in the Eppendorf tubes. The supernatant was removed, and 1 mL of 75% alcohol was added. It was vortexed and centrifuged at 7500 *g* for 5 min at 4 °C. After removal of the supernatant, the excess ethanol was evaporated briefly, and then, the RNA was redissolved in 70 µL of RNAse-free water. RNA was stored at −80 °C until further use.

RNA concentration was measured using a NanoDrop instrument from Thermo Fisher Scientific. We considered the optimal ranges for RNA to be A260/A280: 1.9–2.1 and A260/A230: 1.8–2.5. RNA integrity was examined after agarose gel electrophoresis.

4.7. Real-Time Quantitative Reverse Transcription-PCR (RT-PCR)

Reverse transcription and amplification of the PCR products were performed by using the TaqMan RNA-to-CT-Step One Kit (Thermo Fisher Scientific, Budapest, Hungary) and an ABI StepOne Real-Time cycler (Applied Biosystems, Thermo Fisher Scientific). Reverse-transcriptase PCR amplifications were performed as follows: at 48 °C for 15 min and at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and at 60 °C for 1 min. The generation of specific PCR products was confirmed by melting curve analysis. The following primers were used: assay ID Rn01430371_m1 for Oprm1 and Rn00667869_m1 for β-actin as endogenous control (Thermo Fisher Scientific). Each sample was run in triplicates. The fluorescence intensities of the probes were plotted against PCR cycle number. The amplification cycle displaying the first significant increase in the fluorescence signal was defined as the threshold cycle (Ct). Relative quantity of MOR mRNA expression was calculated by using the $2^{-\Delta\Delta Ct}$ method.

4.8. Western Blot Analysis

Pancreatic and brain tissues were homogenized using a Micro-Dismembrator (Sartorius AG, Göttingen, Germany) and centrifuged at 5000 g for 15 min at 4 °C in RIPA Lysis and Extraction Buffer (Thermo Fisher Scientific) with a protease and phosphatase inhibitor cocktail (10 mM Na-HEPES, 1 µM $MgCl_2$, 10 mM KCl, 1 mM DL-dithiothreitol, 5 mM iodoacetamide, 4 mM benzamidine-HCl, 1 mM phenylmethyl sulfonylfluoride). Total protein amounts from supernatant were determined with spectrophotometry (BioSpec-nano, Shimadzu, Kyoto, Japan).

Then, 25 µg of protein per well was subjected to electrophoresis on 4–12% NuPAGE Bis-Tris Gel in XCell SureLock Mini-Cell Units (Thermo Fisher Scientific). Proteins were transferred from gels to nitrocellulose membranes, using the iBlot Gel Transfer System (Thermo Fisher Scientific). Antibody binding was detected with the WesternBreeze Chromogenic Western blot immunodetection kit (Thermo Fisher Scientific). The blots were incubated on a shaker with OPRM1 (1:200, cat. no.: AOR-011, Alomone Labs, Jerusalem, Israel) and β-actin (cat. no.: bs-0061R, 1:200, Bioss Antibody, Woburn, MA, USA) polyclonal antibodies in the blocking buffer. Images were captured with the EDAS290 imaging system (Kodak Ltd., Rochester, NY, USA), and the optical density of each immunoreactive band was determined with Kodak 1D Images analysis software. Optical densities were calculated as arbitrary units after local area background subtraction. MOR expression was corrected for β-actin levels. Values were normalized to control groups.

4.9. Preparation of Brain and Pancreas Samples for Binding Assays

Frozen rat brain and pancreas samples from LO or physiological saline-treated animals were prepared for membrane preparation according to Szűcs et al. [67]. Briefly, tissue samples were homogenized in 30 volumes (*v*/*w*) of ice-cold 50 mM Tris-HCl pH 7.4 buffer (containing 4 mM benzamidine hydrochloride hydrate, 1 mM phenylmethyl sulfonylfluoride (Serva Electrophoresis GmbH, Heidelberg, Germany), 5 mM iodoacetamide, and 1 mM DL-dithiothreitol (Fluka Honeywell Research Chemicals, Charlotte, NC, USA)) with a Teflon-glass Braun homogenizer operating at 1500 rpm. The homogenate was centrifuged at 40,000 rcm for 20 min at 4 °C, after which the pellet was taken up in the original volume of Tris-HCl buffer. The homogenate was incubated at 37 °C for 30 min in a shaking water-bath. Then, centrifugation was repeated as described before. The final pellet was suspended in 5 volumes of TEM buffer (50 mM Tris-HCl, 1 mM EGTA, 5 mM $MgCl_2$, pH 7.4) and stored at −80 °C.

4.10. [^{35}S]GTPγS Functional Binding Assay

The functional [^{35}S]GTPγS binding experiments were performed as previously described [68]. Briefly, the membrane proteins (≈10 μg/mL) were incubated at 30 °C for 60 min with [^{35}S]GTPγS (20 MBq/0.05 cm^3; 0.05 nM; Perkin Elmer, Boston, MA, USA) and with 10 μM FE, DAMGO (Bachem Holding AG, Bubendorf, Switzerland) or MO in Tris-EGTA buffer (containing 30 μM GDP, 1 mM EGTA, 5 mM $MgCl_2$, 100 mM NaCl, and 50 mM Tris-HCl, pH 7.4) in a final volume of 1 mL/reaction tube. The non-selective opioid receptor antagonist naloxone (Endo Laboratories DuPont de Nemours, Wilmington, DE, USA) was used to detect receptor specificity. Non-specific binding was determined with 10 μM unlabeled GTPγS and subtracted from total binding. Basal activity (was defined as 100%) indicates constitutive G-protein activity level in the absence of any stimulating ligand. Bound and free [^{35}S]GTPγS were separated by vacuum (Brandel M24R Cell Harvester) filtration through Whatman GF/B glass fiber filters washed three times with 5 mL of ice-cold 50 mM Tris-HCl (pH 7.4) buffer. The results were performed in triplicates and repeated at least three times.

4.11. Statistical Analysis

The sufficient animal number per group was estimated by power analysis before each experiment, using the G*Power (3.1.9.2., Heinrich-Heine-Universität Düsseldorf, Germany) software [69] and setting the effect size to 0.8. Data are presented as means ± SEM. Experiments were evaluated by Student's *t*-test or by one- or two-way ANOVA followed by Holm–Sidak or Bonferroni post hoc tests (SPSS, IBM, Armonk, NY, USA). $p < 0.05$ was accepted as statistically significant.

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Data Availability Statement: The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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The Journal of Physiology

Novel mitochondrial transition pore inhibitor *N*-methyl-4-isoleucine cyclosporin is a new therapeutic option in acute pancreatitis

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Key points

- Bile acids, ethanol and fatty acids affect pancreatic ductal fluid and bicarbonate secretion via mitochondrial damage, ATP depletion and calcium overload.
- Pancreatitis-inducing factors open the membrane transition pore (mPTP) channel via cyclophilin D activation in acinar cells, causing calcium overload and cell death; genetic or pharmacological inhibition of mPTP improves the outcome of acute pancreatitis in animal models.
- Here we show that genetic and pharmacological inhibition of mPTP protects mitochondrial homeostasis and cell function evoked by pancreatitis-inducing factors in pancreatic ductal cells.
- The results also show that the novel cyclosporin A derivative NIM811 protects mitochondrial function in acinar and ductal cells, and it preserves bicarbonate transport mechanisms in pancreatic ductal cells.
- We found that NIM811 is highly effective in different experimental pancreatitis models and has no side-effects. NIM811 is a highly suitable compound to be tested in clinical trials.

Abstract Mitochondrial dysfunction plays a crucial role in the development of acute pancreatitis (AP); however, no compound is currently available with clinically acceptable effectiveness and

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safety. In this study, we investigated the effects of a novel mitochondrial transition pore inhibitor, *N*-methyl-4-isoleucine cyclosporin (NIM811), in AP. Pancreatic ductal and acinar cells were isolated by enzymatic digestion from Bl/6 mice. *In vitro* measurements were performed by confocal microscopy and microfluorometry. Preventative effects of pharmacological [cylosporin A (2 μM), NIM811 (2 μM)] or genetic (Ppif$^{-/-}$/Cyp D KO) inhibition of the mitochondrial transition pore (mPTP) during the administration of either bile acids (BA) or ethanol + fatty acids (EtOH+FA) were examined. Toxicity of mPTP inhibition was investigated by detecting apoptosis and necrosis. *In vivo* effects of the most promising compound, NIM811 (5 or 10 mg kg^{-1} per os), were checked in three different AP models induced by either caerulein (10 × 50 μg kg^{-1}), EtOH+FA (1.75 g kg^{-1} ethanol and 750 mg kg^{-1} palmitic acid) or 4% taurocholic acid (2 ml kg^{-1}). Both genetic and pharmacological inhibition of Cyp D significantly prevented the toxic effects of BA and EtOH+FA by restoring mitochondrial membrane potential ($\Delta\psi$) and preventing the loss of mitochondrial mass. *In vivo* experiments revealed that per os administration of NIM811 has a protective effect in AP by reducing oedema, necrosis, leukocyte infiltration and serum amylase level in AP models. Administration of NIM811 had no toxic effects. The novel mitochondrial transition pore inhibitor NIM811 thus seems to be an exceptionally good candidate compound for clinical trials in AP.

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Introduction

Acute pancreatitis (AP) is among the most common gastrointestinal disorders requiring hospitalization in the United States (Fagenholz *et al.* 2007*a*; Peery *et al.* 2012). Although the disease is generally mild, the mortality rate in its severe form is still unacceptably high (Parniczky *et al.* 2016). In recent years, our understanding of the mechanisms that play a crucial role in the development of the disease has improved (Abu-El-Haija *et al.* 2018). Impaired autophagy, trypsinogen activation, excessive Ca^{2+} influx, calcineurin activation, mitochondrial dysfunction and inhibition of the cystic fibrosis transmembrane conductance regulator (CFTR) were shown to have considerable impact in the early phase of AP. Therefore, targeting one of these mechanisms may lead to the first specific therapy in AP.

Among the mechanisms noted above, one of the earliest events in AP is mitochondrial dysfunction (Sah & Saluja, 2011; Maleth *et al.* 2013; Abu-El-Haija *et al.* 2018; Biczo *et al.* 2018). It has been shown in acinar cells that bile acids (BAs) and ethanol and fatty acids (EtOH+FA) open the membrane transition pore (mPTP) channel via cyclophilin D (Cyp D) activation, keeping the channel continuously opened and thus resulting in mitochondrial depolarization, lower ATP synthesis and cell necrosis (Shalbueva *et al.* 2013; Mukherjee *et al.* 2016; Abu-El-Haija *et al.* 2018). Although it remains unknown how the pancreatitis-inducing factors noted above modify mPTP channel activity in pancreatic ductal epithelial cells (PDECs), it still seems to be one of the most promising drug targets and calls for further investigation.

Until now, cyclosporin A (CYA) is the only licensed compound used experimentally to inhibit mPTP (via Cyp D) (Javed *et al.* 2018); however, its clinical usefulness is highly questionable for several reasons. A pilot study found that CYA could reduce the size and damage of myocardial infarction, but larger studies showed no beneficial effects (Piot *et al.* 2008; Cung *et al.* 2015; Javed *et al.* 2018). Even efforts to decrease its immunosuppressive activity have not been successful. Moreover, the CYA derivative Debio025 (Alispovirir, Debiopharm, Lausanne, Switzerland) has been found to be effective against the hepatitis C virus (HCV), but it had serious side-effects. Surprisingly, some of the patients developed pancreatitis, resulting in a clinical hold on the global Debio025 trial programme (Zeuzem *et al.* 2015; Stanciu *et al.* 2019). Another derivative, TRO40303 (3,5-seco-4-nor-cholestan-5-one oxime-3-*o*, TROPHOS, Roche, Indianapolis, IN, USA), was not beneficial in a phase 2 trial of cardiac preservation following acute myocardial infarction, suggesting that this compound has low or no effectivity (Atar *et al.* 2015). Indeed it has recently been shown that TRO40303 does not even bind to Cyp D directly (Sileikyte & Forte, 2016; Javed *et al.* 2018). With regard to AP, both Debio025 and TRO40303 have been shown to be beneficial in animal models, but neither of them has reached 'proof of concept' clinical trials in AP, probably due to the clinical failures noted above. New compounds are therefore crucially needed.

A novel CYA derivative, *N*-methyl-4-isoleucine cyclosporin (NIM811), was found to be highly beneficial in different experimental and clinical studies. NIM811 was effective in animal models of CNS injury (Readnower *et al.* 2011), allergic encephalomyelitis (Huang *et al.* 2017), ischaemic-reperfusion injury after surgical intervention (Garbaisz *et al.* 2014), hepatitis C (Arai *et al.* 2014),

Table 1. Solutions used in the present study

	Hepes (Standard) (mM)	HCO_3^- (Standard) (mM)	NH_4Cl^- HCO_3^- (mM)	1× TBS (mM)	HBSS (Standard) (mM)
NaCl	140	115	95	150	0.137
KCl	5	5	5	–	5.4
$CaCl_2$	1	1	1	–	0.3
$MgCl_2$	1	1	1	–	–
Glucose	10	10	10	–	6
Hepes	10	–	–	–	–
$NaHCO_3^-$	–	25	25	–	4.2
NH_4Cl^-	–	–	20	–	–
Trisma base	–	–	–	50	–
Na_2HPO_4	–	–	–	–	0.25
KH_2PO_4	–	–	–	–	0.44
$MgSO_4$	-	-	-	-	1.03

liver transplantation (Rehman *et al.* 2011) and pulmonary injury during liver transplantation (Liu *et al.* 2012). Importantly, none of the studies reported side-effects. NIM811 had no severe or serious adverse effects in a phase 2 clinical trial on HCV-infected patients, suggesting that it has no toxic immunosuppressant activity either (Lawitz *et al.* 2011).

In this study, we show in several *in vitro* and *in vivo* experiments that either pharmacological or genetic inhibition of Cyp D restores mitochondrial function not only in acinar cells, but also in ductal cells, highlighting the general importance of mPTP in AP. Moreover, we provide evidence that NIM811 is highly effective in different experimental pancreatitis models and that it has no side-effects.

Materials and methods

Ethical approval

The animal experiments were performed in compliance with European Union Directive 2010/63/EU and Hungarian Government Decree 40/2013 (II.14.). Experiments were approved by local ethics committees for investigations involving animals at the University of Szeged (XII/4988/2015). In our study all animals were killed via 200 mg kg^{-1} pentobarbital I.P. (Bimeda MTC, Cambridge, Canada).

Animals

Seventy wild type (WT) and Cyp D knockout (Cyp D KO, (B6;129-Ppiftm1Maf/J) mice were used. Cyp D KO mice were generated by targeted disruption of the Ppif gene (which encodes the *Cyp D* that is a component of the mPTP) (Baines *et al.* 2005). Cyp D KO animals were provided for by the Department of Medical Biochemistry, Semmelweis University, Budapest, Hungary. WT and Cyp D-deficient littermate mice (of C57Bl/6J background, either sex, aged between 20 and 45 days) were housed in a room maintained at 20–22°C on a 12 h light–dark cycle with food and water available ad libitum. To ensure a homologous genetic background, mice were backcrossed with C57Bl6/J mice for at least eight generations.

Solutions and chemicals

Chemicals were obtained from Sigma-Aldrich (Budapest, Hungary), unless otherwise stated: 2.7-bis-(2-carboxyethyl)-5-(and-6-) carboxyfluorescein-acetoxymethylester (BCECF-AM) and tetramethylrhodamine-methylester (TMRM) were purchased from ThermoFisher Scientific (Waltham, MA, USA); NIM811 was purchased from MedChem Express Europe (Sollentuna, Sweden). CYA, caerulein (CER), NIM811, carbonyl cyanide 3-chlorophenylhydrazone (CCCP) and fluorescence dies were diluted in DMSO. Table 1 describes the constitution of solutions that we used during the study. In this study 500 μM chenodeoxycholic acid (BA) or 100 mM ethanol (EtOH) + 200 μM palmitoleic acid (FA) were used during the fluorescence, confocal microscopy and immunostaining measurements, to evaluate the effect of bile acids or the alcohol and fatty acid induced damage on the mitochondrial and cell function during the genetic or pharmacological inhibition of the mPTP in pancreatic ducts or acinar cells. CCCP at 100 μM was used in the mitochondrional measurements as a positive control for mitochondrial damage.

CYA (2 μM) and NIM811 (2 μM) were used to pharmacologically inhibit mPTP. Prior to the fluorescence and confocal microscopy, and immunostaining, the cells (duct and acinar cells as well) from the CYA- or NIM811-treated groups were pretreated for 25–30 min with the compounds (CYA or NIM811).

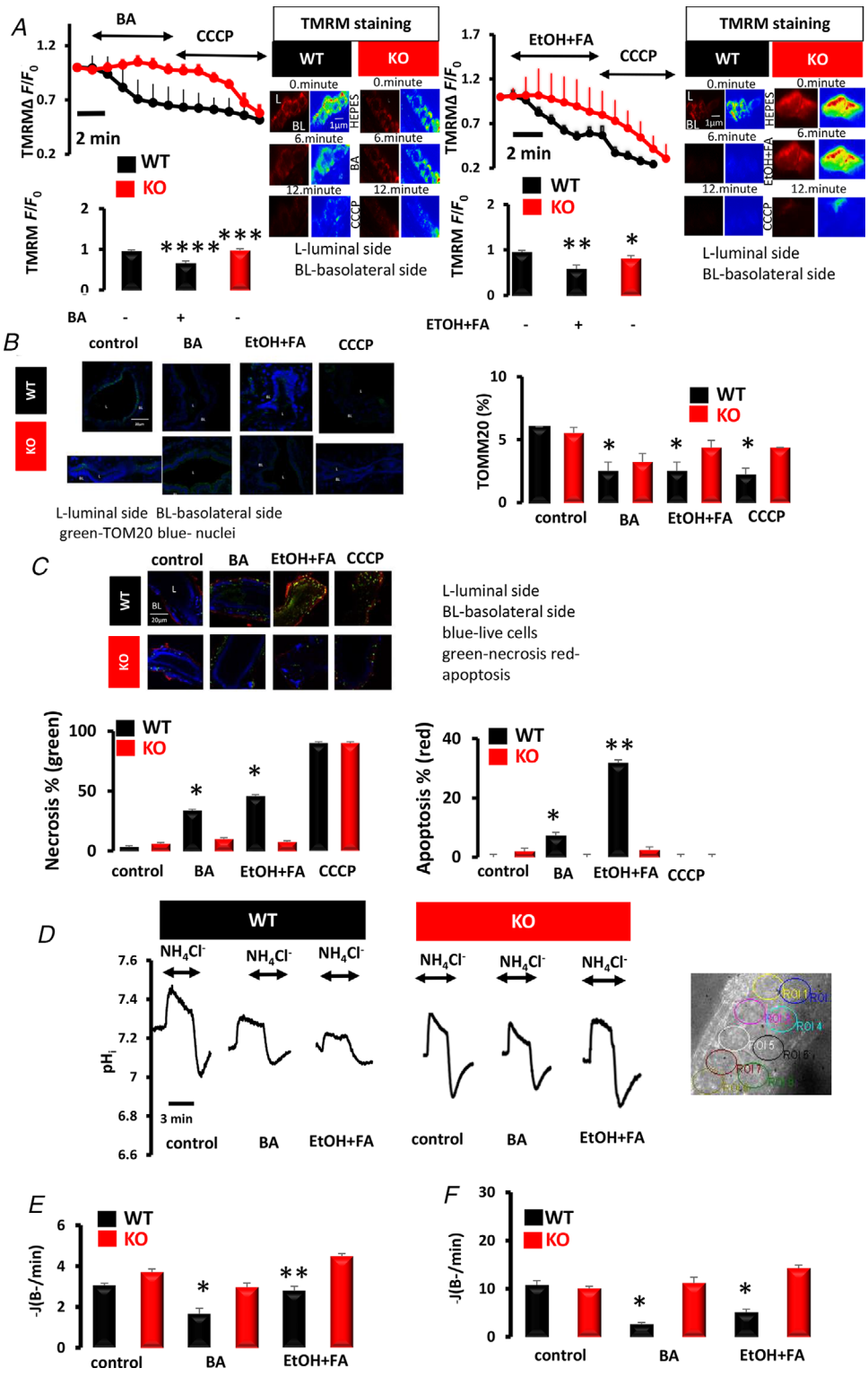

Figure 1. Genetic inhibition of Cyp D reduces the severity of bile acid- or ethanol and fatty acid-induced damage in PDECs

A, mitochondrial membrane potencial measurements revealed that genetic inhibition of mPTP significantly reduces the mitochondrial membrane potencial loss compared to WT controls during the administration of bile acid (500 μM CDC) or ethanol (100 mM) and fatty acid (200 μM FA) treatment (WT control *vs.* WT BA ***$P < 0.001$, WT BA *vs.* Cyp D KO BA **$P < 0.002$, WT control *vs.* Cyp D KO BA $p = 0.712$, WT control *vs.* WT EtOH + FA $P < 0.01$, WT EtOH + FA *vs.* KO EtOH + FA *$P < 0.05$, WT control *vs.* Cyp D KO EtOH + FA $p = 0.145$); $n = 4$–6 experiments

Table 2. Oligonucleotide primers used in genotyping

Primer	Sequence
F-null2	TTCTCACCAGTGCATAGGGCTCTG
LoxP1f	AAACTTCTCAGTCAGCTGTTGCCTCTG
CyPuP2	GCTTTGTTATCCCAGCTGGCG

Methods

Genotypes of Cyp D deficient mice were identified by PCR (typical PCR, analyses from tail genomic DNA). The PCR-mix contained: Taq DNA pol 5 U and 10× Taq Buffer (Abgene, Portsmouth, NH, USA), $MgCl_2$ 1.5 mM, dNTP 2.5 mM, F-null2/LoxP1f /CyPuP2 primers (20–20 μM), dH_2O and template DNA sample. The total reaction mix volume was 25 μl. The wild type allele was detected using LoxP1f, 5′-AAACTTCTCAGTCAGCTGTTGCCTCTG-3′, as a forward primer and F-null2, 5′-GCTTTGTTATC CCAGCTGGCGC-3′, as a reverse primer. For genotyping of the mutant Cyp D deficient allele, F-null2, 5′-TTCTCACCAGTGCATAGGGCTCTG-3′ was used as a forward primer with the reverse primer for WT (Table 2). DNA was denatured at 95°C for 2 min, followed by 30 cycles of amplification: 94°C for 30 s, 60°C for 30 s, 72°C for 45 s and a final primer extension step at 72°C for 7 min. Bands of 270 and 470 bp were amplified for WT and Cyp D KO mice, respectively.

Pancreatic ducts and acinar cells were isolated by micro-dissection and enzymatic digestion as described previously (Argent *et al.* 1986; Gout *et al.* 2013).

The mitochondrial membrane potential (Ψ) was determined by using a Zeiss LSM 880 confocal laser scanning microscope (Carl Zeiss Technika Kft, Budaörs, Hungary). BA or EtOH + FA were used to induce mitochondrial damage. Isolated pancreatic ducts or acinar cells were incubated in standard Hepes solution and loaded with TMRM (100 nmol l^{-1}).

To monitor apoptotic and necrotic cells in isolated pancreatic ducts or acinar cells an apoptosis/necrosis kit was used (ab176750, Abcam, Cambridge, MA, USA). To determinate live, necrotic or apoptotic cells, CytoCalcein Violet 450 fluorescent, Apopxin Deep Red Indicator and Nuclear Green DCS1 fluorescence dies (ab176750, Abcam) were used. Samples were incubated in the mixture of the above stated fluorescence dyes at room temperature for 30–35 min (after 25 min of treatment with BA/EtOH + FA/CYA/NIM811) in the dark prior to the confocal microscopy measurements. For CYA- or NIM811-treated ducts or acinar cells, incubation with these compounds were performed before staining with the fluorescence dyes. Stainings were analysed using a Zeiss LSM 880 confocal laser scanning microscope. Live, necrotic or apoptotic cells were counted and summarized as a percentage of each sample, and data were then averaged and statistical analysis was performed.

Microfluorometry was used to measure pancreatic ductal HCO_3^- secretion as described earlier (Hegyi *et al.* 2013; Hegyi *et al.* 2004) by using BCECF-AM (1.5 mmol l^{-1}).

Functionally active mitochondria were detected with immunofluorescent staining (TOM20 mitochondrial marker EPR15581-39, Abcam). To determine mitochondrial localization in isolated pancreatic ductal or acinar cells we labelled the mitochondria by the using TOM20 primary antibody (Abcam, EPR15581-39). TOM20 is the central unit of the receptor TOM complex in the mitochondrial outer membrane and its role is to recognize and translocate cytosolically synthetized mitochondrial preproteins (Schatz *et al.* 1996; Pfanner, 1998; Rapaport, 2002). Isolated pancreatic ducts were frozen in cryomold at 20°C. The cryosections (thickness 7 μm) of the isolated pancreatic ducts from WT and Cyp D KO mice were cut via a Leica Cryostat. Sections were fixed in 4% paraformaldehyde. Washing periods were administered with 1× Tris-buffered saline (TBS) solution. Antigen retrieval was performed with 10 mM sodium citrate solution at pH 6 at 95°C for 15 min. Blocking was obtained for 1 h with 1% goat serum in 5% bovine serum albumin (BSA)-TBS solution. These sections were then incubated with TOM20 rabbit monoclonal antibody (dilution 1:400, Abcam) overnight at 4°C. The following day the samples were incubated with goat anti-rabbit secondary antibody (Alexa fluor 488, Thermo Fisher) for 2 h in the dark in room temperature. Nuclei were counterstained with Hoechst 33342 (Thermo Fisher). Immunofluorescence staining of the isolated pancreatic acinar cells was performed immediately after the isolation procedure with the same conditions as stated above (except: cells were fixed in 2% paraformaldehyde and dilution of the primary antibody was 1:200). Both ductal

per group; data are means ± SEM. *B*, immunostaining revealed a significant decrease of the TOM20 stainings in BA-, EtOH + FA- or CCCP-treated WT ducts; results were compared to Cyp D KO stainings (*$P < 0.05$). *C*, genetic inhibition of mPTP also decreased the necrosis and apoptosis levels during bile acid; ethanol and fatty acid or CCCP treatment (*$P < 0.05$) *D*, representative traces from the pancreatic ductal HCO_3^- secretion measurements. *E* and *F*, the data revealed that levels of alkalosis recovery were significantly lower due to BA or EtOH + FA administration (*$P < 0.05$) compared to the results from Cyp D KO ducts. Levels of alkalosis recovery were significantly lower in the WT ducts due to the treatment with BA or EtOH + FA (*$P < 0.05$), while in Cyp D KO ducts these levels were significantly higher (*$P < 0.05$). n = 5–7 experiments per group; data are means ± SEM. *y* axis=base flux [–J(B–/min)] was calculated from the 1pH/1t obtained by linear regression analysis of pHi (HCO_3-secretion) measurements. [Colour figure can be viewed at wileyonlinelibrary.com]

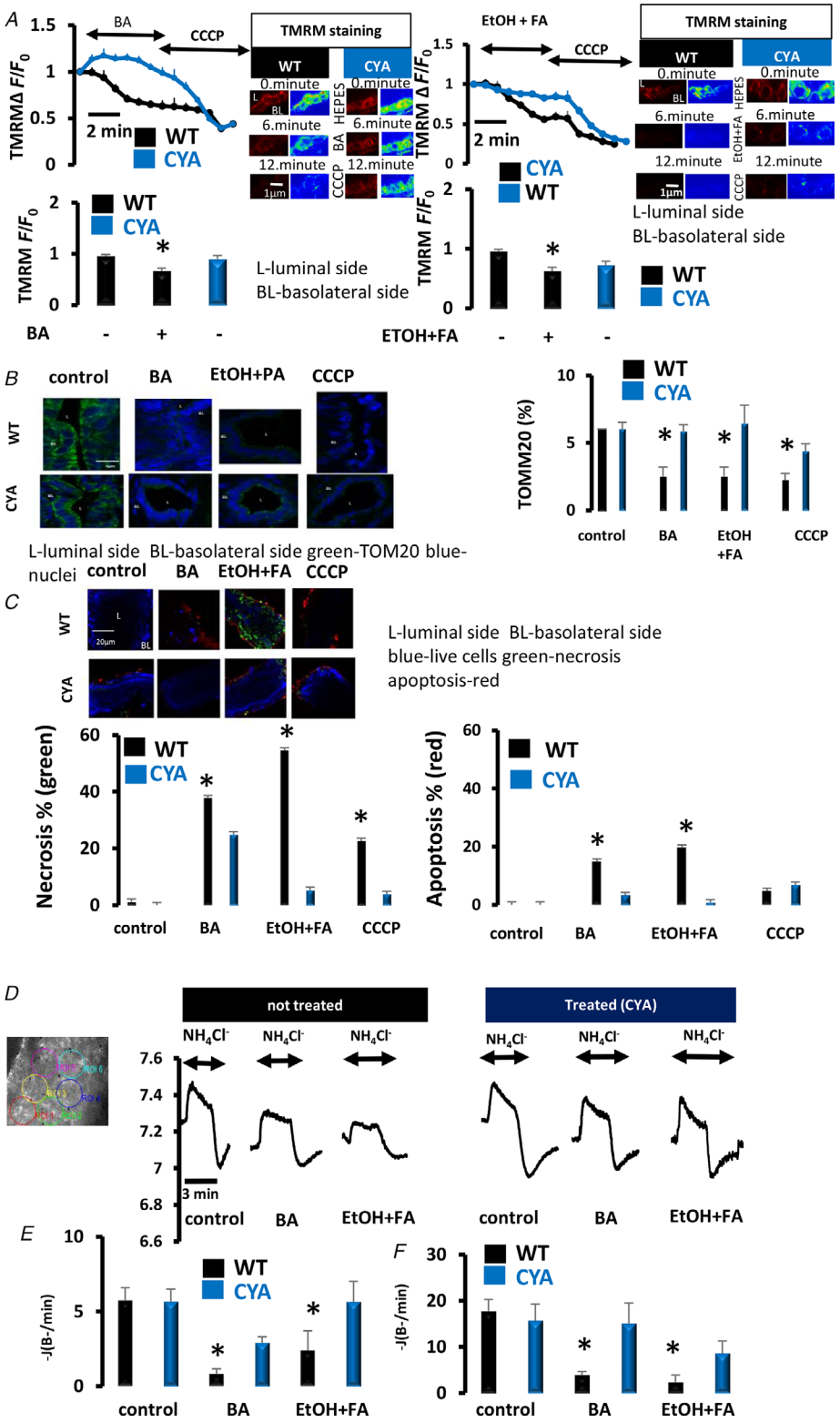

Figure 2. CYA reduces the severity of bile acid- or ethanol and fatty acid-induced pancreatic ductal damage

A, treatment with 2 μM CYA reduced the drop of mitochondrial membrane potencial loss which accured due to the BA or EtOH + FA treatment (WT *vs.* CYA). In WT ducts BA or EtOH + FA treatment resulted in significantly reduced mitochondrial membrane potencial (WT control *vs.* WT BA $^*P < 0.05$, WT control *vs.* WT EtOH + FA $P < 0.05$), while between WT control groups compared to CYA-treated BA or EtOH + FA there was no significant

and acinar cell samples were mounted with Fluoromount and then analysed using a Zeiss LSM 880 confocal laser scanning microscope. To quantify TOM20 positively stained area, five or six representative images from each group were taken by with the Zeiss LSM 880 microscope. Image J software was used to convert images to grey scale (16 bit), and threshold function was used to select the positively stained area. The fluorescence signal was calculated by the software [arbitrary scale from 0-negative (white) to 255-maximal staining (black)] (Venglovecz *et al.* 2018). Fluorescence intensity of the images was then normalized to the total ductal or acinar area of the samples, which were measured in arbitrary units. Fluorescence intensity was given as a percentage, normalized to the total ductal or acinar total area.

AP was induced by CER (10×50 μg kg^{-1}), 4% sodium taurocholate (TAU, 2 ml kg^{-1}, 4%) (Niederau *et al.* 1985; Ding *et al.* 2003; Perides *et al.* 2010; Pallagi & Balla *et al.* 2014) or alcohol and fatty acid (I.P. injection of 1.75 g kg^{-1} ethanol and 750 mg kg^{-1} palmitic acid, EtOH + FA) as described previously (Huang, 2014; Maleth *et al.* 2016). All control groups received physiological saline in the same amount as the CER, EtOH + FA or the TAU solutions respectively. Pre-treatment of the animals by NIM811 was performed and mice were gavaged orally once 1 h prior to the induction of AP (concentrations of NIM811 were 10 or 5 mg kg^{-1}). The dose of NIM811 was chosen according to a previous study in which NIM811 was effective against mitochondrial damage in liver transplantation (Rehman *et al.* 2011). Oral gavage treatment were performed by the use of plastic feeding tubes (20 gauge × 38 mm, Instech Laboratories, Plymouth Meeting, PA, USA). NIM811 were solubilized in a vehicle which contained 8.3% polyoxyl 40 hydrogenated castor oil and 8.3% ethanol (Rehman *et al.* 2011).

NIM811 was used as a post-AP treatment as well. NIM811 was administered 12 h after the induction of AP in the TAU- or EtOH + FA-induced experimental pancreatitis models. Concerning the CER-induced AP, NIM811 was administered after the third injection of CER. The method for retrograde intraductal infusion of TAU has been described by Perides *et al.* (2010). The surgery was performed on anaesthetized mice (with ketamine–xylazine, dosage: 87.5 mg kg^{-1} ketamine/12.5 mg kg^{-1} xylazine). At the end of the procedure the mice were placed on a heating pad for 40 min and received buprenorphine I.P. (0.075 mg kg^{-1}) immediately to reduce pain. Following these mice were replaced into their cages for 24 h. They had free access to food and water. Twenty-four hours after the TAU- or EtOH + FA-induced AP the mice were killed via I.P. 200 mg kg^{-1} pentobarbital (Bimeda MTC, Cambridge, Canada). During the CER-induced AP mice were killed with I.P. 200 mg kg^{-1} pentobarbital (Bimeda MTC) 2 h after the last injections of CER. Mice were exsanguinated through cardiac puncture and the pancreas was removed. Blood from the cardiac puncture was placed on ice, then centrifuged with at 2500 *g* for 15 min at 4°C. Blood serum was collected from the pellet and stored at −20°C until use. Pancreas samples were placed into 8% neutral formaldehyde solution and stored at −4°C until the haematoxylin–eosin staining was performed. A colorimetric kit was used to measure serum amylase activity (Diagnosticum, Budapest, Hungary). Absorbance of the samples was detected at 405 nm with the use of a FLUOstar OPTIMA (BMG Labtech, Budapest, Hungary) microplate reader. Formaldehyde-fixed pancreas samples were embedded in paraffin and were cut into 3 μm thick sections and stained for haematoxylin–eosin by using a standard laboratory method. To quantify oedema, necrosis and leukocyte infiltration grades a semiquantitative scoring system was used according to Kui *et al.* (2015).

In vitro pancreatic ductal fluid secretion (luminal swelling) assays were developed by Fernández-Salazar *et al.* (2004) performed by videomicroscopy as described by Balázs *et al.* (2018). Briefly, stimulation of pancreatic ductal fluid secretion was induced by 5 μM forskolin and 100 μM 3-isobutyl-1-methylxanthine (IBMX), and quantification were performed using ImageJ software (Balázs *et al.* 2018). *In vivo* fluid secretion measurements were performed on anaesthetized (I.P. 87.5 mg kg^{-1} ketamine/12.5 mg kg^{-1} xylazine) mice after CER- or EtOH + FA-induced AP before the animals were killed. Animals were placed on warm pads (37°C) to maintain body temperature. Briefly, the abdomen was opened and

decrease. *B*, TOM20 levels were significantly reduced in BA, EtOH + FA or CCCP control (not CYA treated) ducts, while in the CYA-treated groups the percentage of TOM20-stained area was significantly higher (*$P < 0.05$). Between the control groups (WT control or only CYA-treated samples) we found no significant alterations in the stainings. *C*, necrosis was much higher in BA- or EtOH-treated groups in WT ducts but not in CYA-treated groups. Apoptosis levels were significantly higher as well in the non-CYA-treated groups compared to the CYA-treated groups. Measurements of HCO_3^- secretion levels revealed a significant difference in WT and CYA-treated ducts during administration of BA ($P < 0.05$ WT BA *vs.* CYA BA) or EtOH + FA (*$P < 0.05$). *E* and *F*, in WT ducts the levels of base flux [-J(B-/min)] grades were significantly decreased due to BA (WT *vs.* WT BA $P < 0.05$) or EtOH + FA (WT *vs.* WT EtOH + FA $P < 0.05$) treatment. Recovery from alkalosis (*E*) and recovery from acidosis (*F*) values are presented ± SEM. Comparison within CYA-treated groups revealed no significant difference (CYA control *vs.* CYA BA $p = 0.644$). *y* axis=base flux [–J(B–/min)] was calculated from the 1pH/1t obtained by linear regression analysis of pHi (HCO_3-secretion) measurements. [Colour figure can be viewed at wileyonlinelibrary.com]

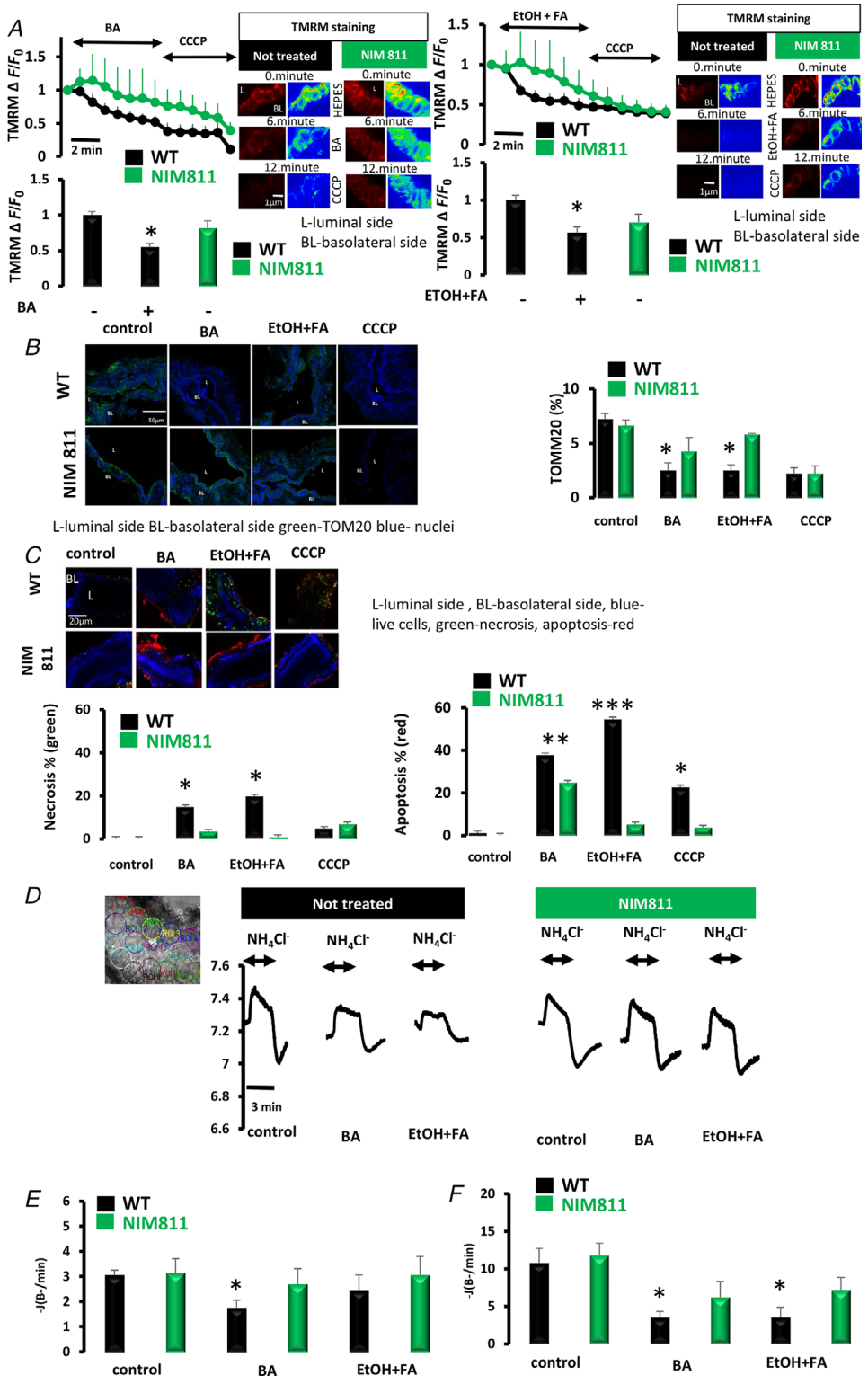

Figure 3. NIM811 protects mitochondrial and cell function in PDECs
A, NIM811-treated ducts revealed a significantly consolidated loss of mitochondrial membrane potential during the BA (WT BA *vs.* NIM811 BA $^*P < 0.05$) or EtOH + FA (WT EtOH + FA *vs.* NIM811 EtOH + FA $^*P < 0.05$) treatment. In NIM811-treated ducts the percentage of fluorescence intensity was significantly higher compared to non-NIM811-treated ducts during BA or EtOH + FA administration. *B*, in CCCP-treated ducts we found no significant difference in the amount of TOM20 staining in NIM811-treated or untreated groups. NIM811 itself did not alter the level of TOM20 staining compared to the WT control samples. *C*, NIM811 decreased the numbers of

apoptotic and necrotic cells during bile acid or ethanol and fatty acid treatment (WT BA *vs.* NIM811 BA $^*P < 0.05$, WT EtOH + FA *vs.* NIM811 $^*P < 0.05$). During the administration of CCCP the apoptosis and necrosis grades were not significantly different in the comparative groups. *D–F*, NIM811 treatment did not decrease the HCO_3^- secretion grade (control), while during the administration of BA or EtOH + FA it had a protective effect against the reduction of HCO_3^- secretory levels (*E*, *F*) (WT BA *vs.* NIM811 BA $^*P < 0.05$, WT EtOH + FA *vs.* NIM811 EtOH + FA $^*P < 0.05$). Regarding recovery levels from alkali load during EtOH and FA treatment, differences were not significant in WT EtOH + FA- compared to the NIM811 and EtOH + FA-treated groups (*E*). [Colour figure can be viewed at wileyonlinelibrary.com]

cannulation of the lumen of the common biliopancreatic duct was performed with a 30-gauge needle (Maléth *et al.* 2016). The proximal end of the common duct was closed by a microvessel clip (Braun-Aesculap, Tuttlingen, Germany) to prevent contamination with bile, and the pancreatic juice was collected in a PE-10 tube for 15 min. *In vivo* secretion was induced by I.P. administration of 0.75 CU kg^{-1} secretin (Maléth *et al.* 2016).

Statistical analysis

All data are expressed as means ± SEM. Data were compared by either one- or two-way ANOVA or

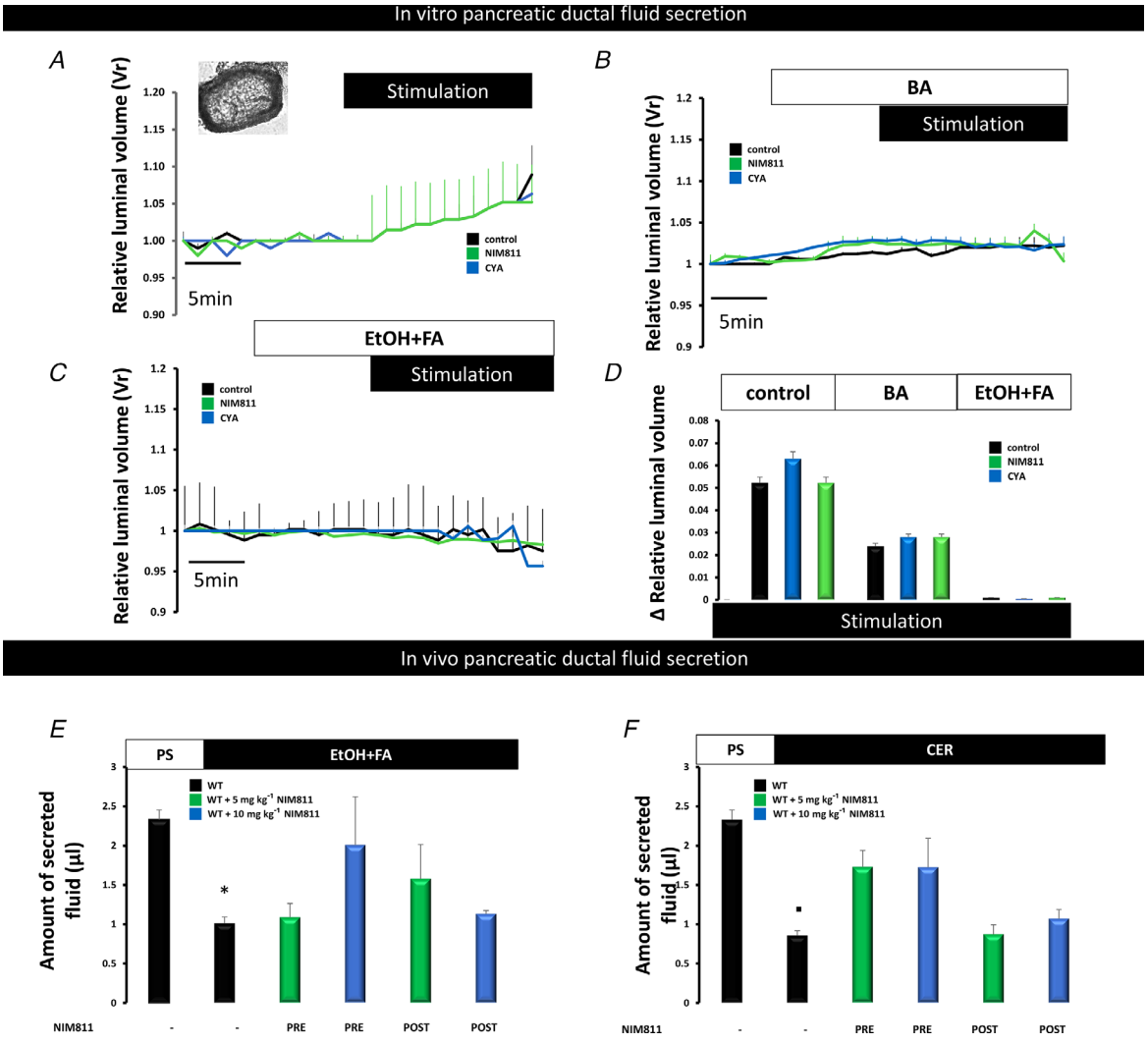

Figure 4. Pancreatic ductal fluid secretion is not altered by NIM811 or CYA treatment
A, *in vitro* fluid secretion was stimulated by 5 µM forskolin and 100 µM IBMX (stimulation). *B* and *C*, BA or EtOH + PA treatment inhibited luminal swelling. *D*, relative luminal volume changes during forskolin and IBMX stimulation. Means ± SEM; $n = 5$–10 ducts per group. *E* and *F*, *in vivo* fluid secretion measurements were performed after CER- or EtOH + FA-induced AP. These experiments confirmed that pancreatic ductal fluid secretion is not affected by NIM811 or CYA. $^*P < 0.05$ WT PS *vs.* WT EtOH + FA, ▪ $P < 0.05$ WT PS *vs.* WT CER $n = 4$–7 animals per group. [Colour figure can be viewed at wileyonlinelibrary.com]

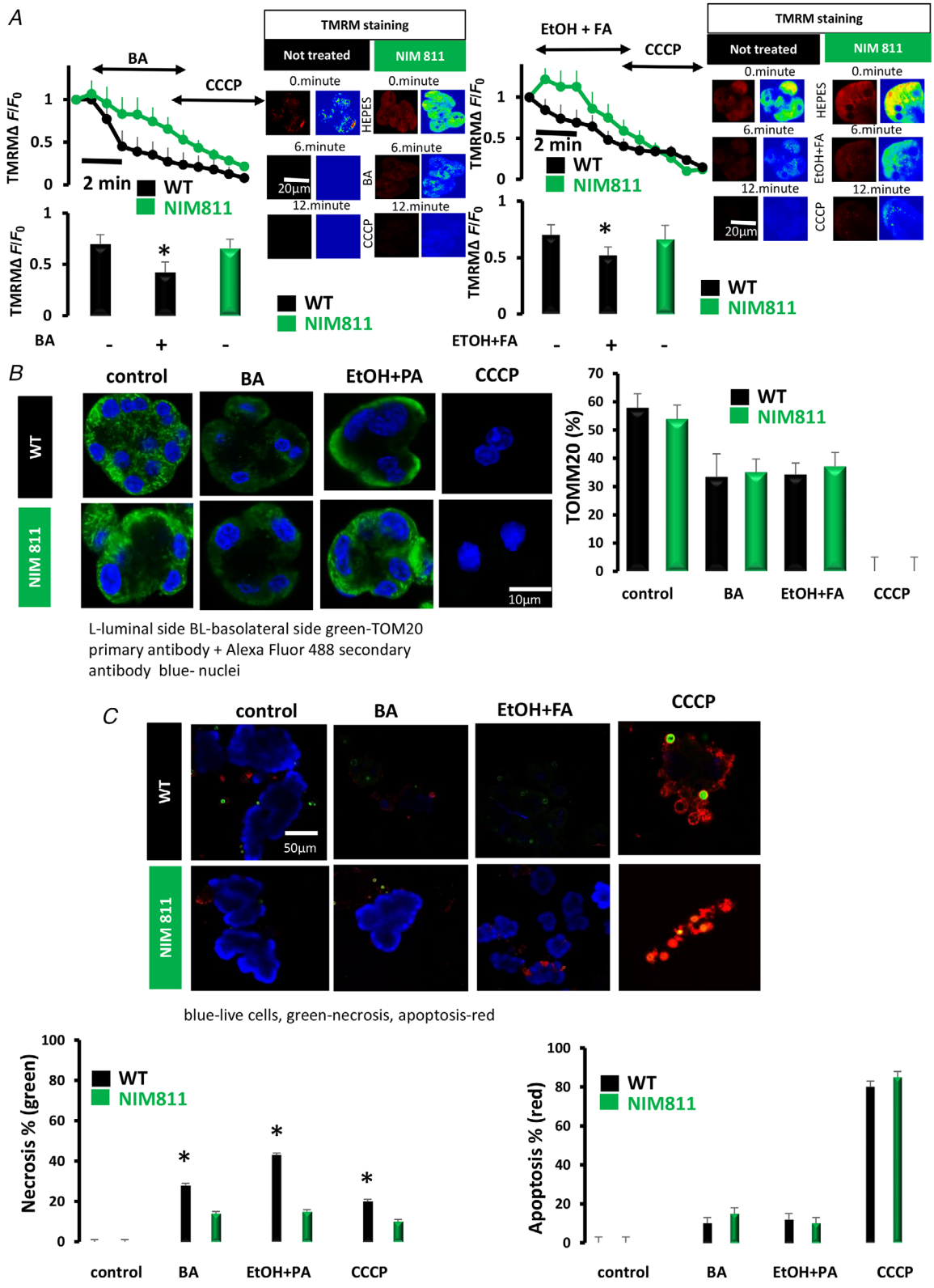

Figure 5. NIM811 treatment protects mitochondrial function in pancreatic acinar cells
A, mitochondrial membrane potential measurements revealed a significant difference between WT untreated and NIM811-treated acinar cell response due to bile acid or ethanol and fatty acid treatment (WT BA *vs.* NIM811 BA $^*P < 0.05$; WT EtOH + FA *vs.* NIM811 EtOH + FA $^*P < 0.05$). A significant difference was detected between the NIM811-treated acinar cells and the groups which were not treated with NIM811 during BA or EtOH + FA

Kruskal–Wallis tests followed by the Holm–Sidak method as appropriate (Sigma Plot). The effects were considered significant at $p < 0.05$.

Results

Genetic inhibition of mPTP protects mitochondrial homeostasis and cell function evoked by pancreatitis-inducing factors in PDECs

First, we measured the effects of the most relevant pancreatitis-inducing factors on mitochondria in primary intact ducts isolated from $Ppif^{-/-}$ and WT mice. Experiments with TMRM and TOM20 revealed that genetic inhibition of mPTP decreased both the loss of $\Delta\psi$ (Fig. 1*A*) and mitochondrial mass (Fig. 1*B*) caused by 500 μM chenodeoxycholic acid (CDC; BA) or co-administration of 100 mM ethanol and 200 μM palmitoleic acid (EtOH + FA). Co-staining the pancreatic ducts with CytoCalcein Violet, Apopxin Deep Red and Nuclear Green showed that genetic inhibition of mPTP also decreased the extent of necrosis and apoptosis during the administration of BA or EtOH + FA (Fig. 1*C*), suggesting that genetic inhibition of Cyp D has a protective effect on PDECs. Next, we investigated how the genetically preserved mitochondrial function affects the cellular function of PDECs (Fig. 1*D*). We used the NH_4Cl pulse technique, which is uniquely suited to characterizing both HCO_3^- influx and efflux mechanisms. Our experiments demonstrated that the inhibitory effects of BA and EtOH + FA on Cl^-/HCO_3^- exchangers (HCO_3^- efflux) and on Na^+/HCO_3^- co-transporters (HCO_3^- influx) were totally blocked in $Ppif^{-/-}$ *vs.* WT mice, suggesting that inhibition of mPTP can preserve ductal function and thus has therapeutic benefits (Fig. 1*D–F*).

Pharmacological inhibition of mPTP by CYA effectively prevents mitochondrial damage evoked by pancreatitis-inducing factors in PDECs

Both BA and EtOH + FA significantly decreased the ψ of PDECs (Fig. 2*A*). Importantly, 2 μM CYA effectively blocked the toxic effects of the BA- and EtOH + FA-preserving function of mitochondria during the presence of pancreatitis-inducing factors. As regards the quantity of mitochondria, CYA effectively inhibited loss, as observed during the genetic inhibition of mPTP (Fig. 2*B*). CYA at 2 μM decreased the extent of necrosis and apoptosis during the administration of BA or EtOH + FA in PDECs (Fig. 2*C*). Finally, we provided strong evidence of the beneficial effects of CYA on mPTP noted above, mitochondrial mass and cell death, resulting in preserved HCO_3^- efflux and influx mechanisms during BA or EtOH + FA administration (Fig. 2*D–F*).

NIM811 treatment protects mitochondrial function and preserves bicarbonate transport mechanisms in PDECs

Next, we investigated the effects of the novel CYA derivative NIM811 on mitochondrial function and of bicarbonate secretion on isolated pancreatic ducts. According to our data, NIM811 reduces the BA- or EtOH + FA-induced damage to mitochondrial function and morphology in isolated pancreatic ducts (Fig. 3*A*, *B*). Experiments using CytoCalcein Violet, Apopxin Deep Red and Nuclear Green showed that NIM811 alone has no toxic effects on PDECs. Furthermore, it strongly decreases BA- or EtOH-FA-evoked necrosis and apoptosis (Fig. 3*C*). NH_4Cl^- experiments revealed that the inhibitory effects of BA and EtOH + FA on Cl^-/HCO_3^- exchangers (HCO_3^- efflux) and on Na^+/HCO_3^- co-transporters (HCO_3^- influx) were significantly reduced in the NIM811-treated groups compared to the controls, showing a protective effect of NIM811 on PDECs (Fig. 3*D*).

NIM811 and CYA have no effects on pancreatic ductal fluid secretion

Both *in vivo* and *in vitro* measurements revealed that NIM811 or CYA treatment did not prevent BA- or EtOH + FA-induced fluid secretiory damage in isolated ducts (Fig. 4*A–D* and *E*, *F*).

NIM811 treatment protects mitochondrial function in acinar cells

In vitro measurements of freshly isolated pancreatic acinar cells showed that NIM811 treatment decreased the BA- and EtOH-FA-induced loss of ψ as effectively as seen in PDECs (Fig. 3*A*). However, results obtained from TOM20 staining suggest that NIM811 has no effect on mitochondrial mass in acinar cells (Fig. 5*B*). Microfluorometric measurements demonstrated that NIM811 alone has no toxic effects on acinar cells and has no effect on BA- or EtOH-FA-induced apoptosis, but is protective against BA- or EtOH-FA-induced necrosis (Fig. 5*C*).

treatment. *B*, mitochondrial protein TOM20 levels did not show a difference in the NIM811-treated or untreated groups after BA, EtOH + FA or CCCP treatment ($P > 0.05$). *C*, a significant difference in necrosis levels was found between NIM811-treated and untreated groups in BA or EtOH + FA (*$P < 0.05$). However, no difference was found for the CCCP-treated groups. Apoptosis levels were not altered significantly by NIM811 during BA or EtOH + FA treatment. [Colour figure can be viewed at wileyonlinelibrary.com]

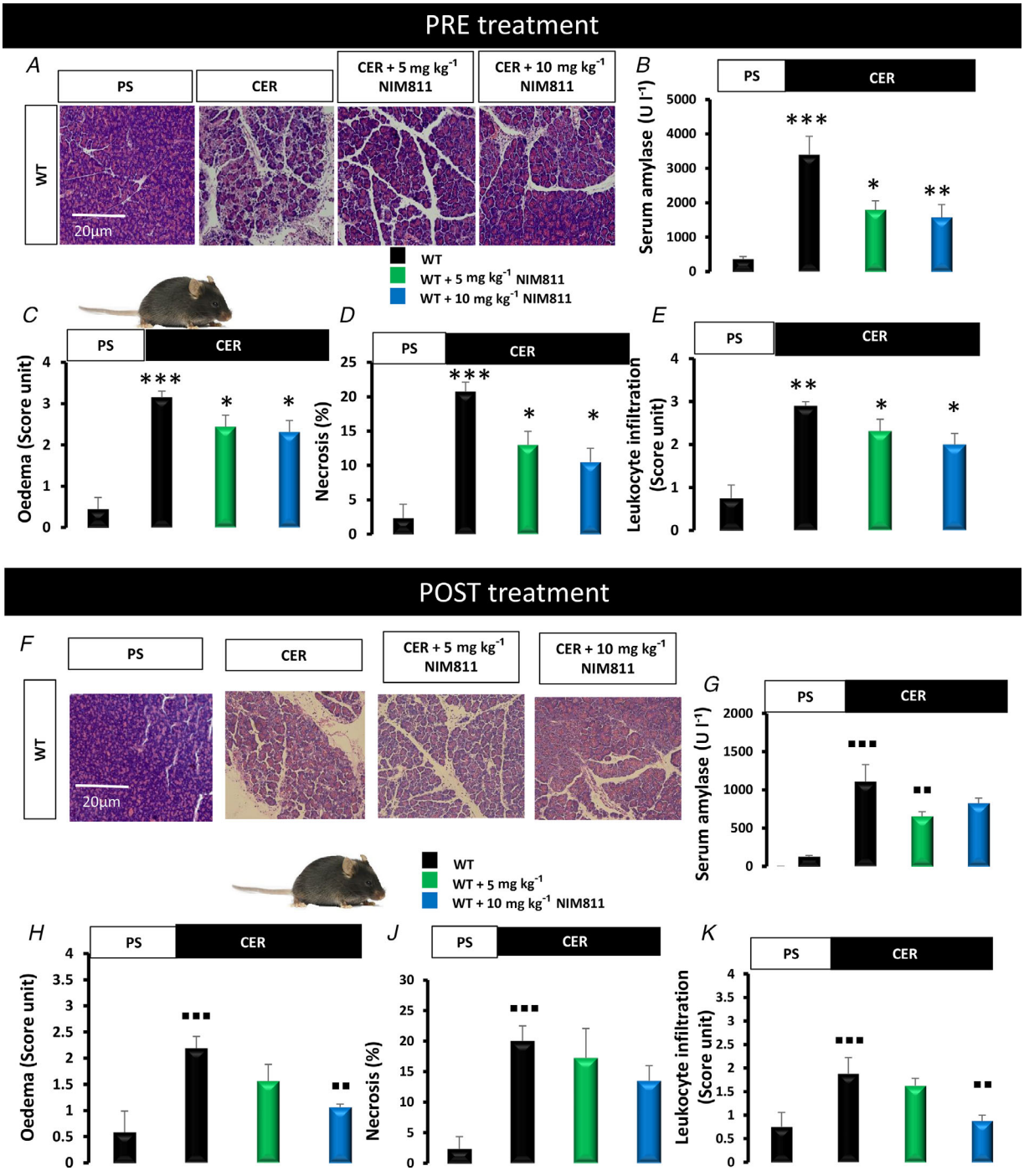

Figure 6. NIM811 reduces the severity of CER induced AP
A, representative images of pancreas sections. *B*, serum amylase levels were elevated in the CER-treated groups and NIM811 treatment resulted in a reduced serum amylase levels during CER-induced AP compared to the WT CER group (***$P < 0.01$ WT PS *vs.* WT CER, **$P < 0.02$ WT CER *vs.* pre 10 mg kg^{-1} NIM811 CER, *$P < 0.05$ WT CER *vs.* pre 5 mg kg^{-1} NIM811 CER, $p = 0.717$ CER + pre 5 mg kg^{-1} NIM811 *vs.* CER + pre 10 mg kg^{-1} NIM811). *A–E*, for CER-induced pancreatitis both 5 mg kg^{-1} body weight NIM811 ($P < 0.05$ WT CER *vs.* pre 5 mg kg^{-1} NIM811 CER) and pre 10 mg kg^{-1} NIM811 ($P < 0.05$ WT CER *vs.* pre 10 mg kg^{-1} NIM811 CER) treatment reduced the CER-induced damage. *F–K*, post 5 mg kg^{-1} NIM811 treatment significantly reduced serum amylase levels compared to WT CER (▪▪$P < 0.05$, ▪▪▪$P < 0.001$ WT PS *vs.* WT CER. *H*, post-insult administration of 10 mg kg^{-1} NIM811 significantly reduced oedema and leukocyte infiltration levels compared to WT CER-treated groups (▪▪$P < 0.05$, n = 8–10 animals per group, data are means ± SEM). [Colour figure can be viewed at wileyonlinelibrary.com]

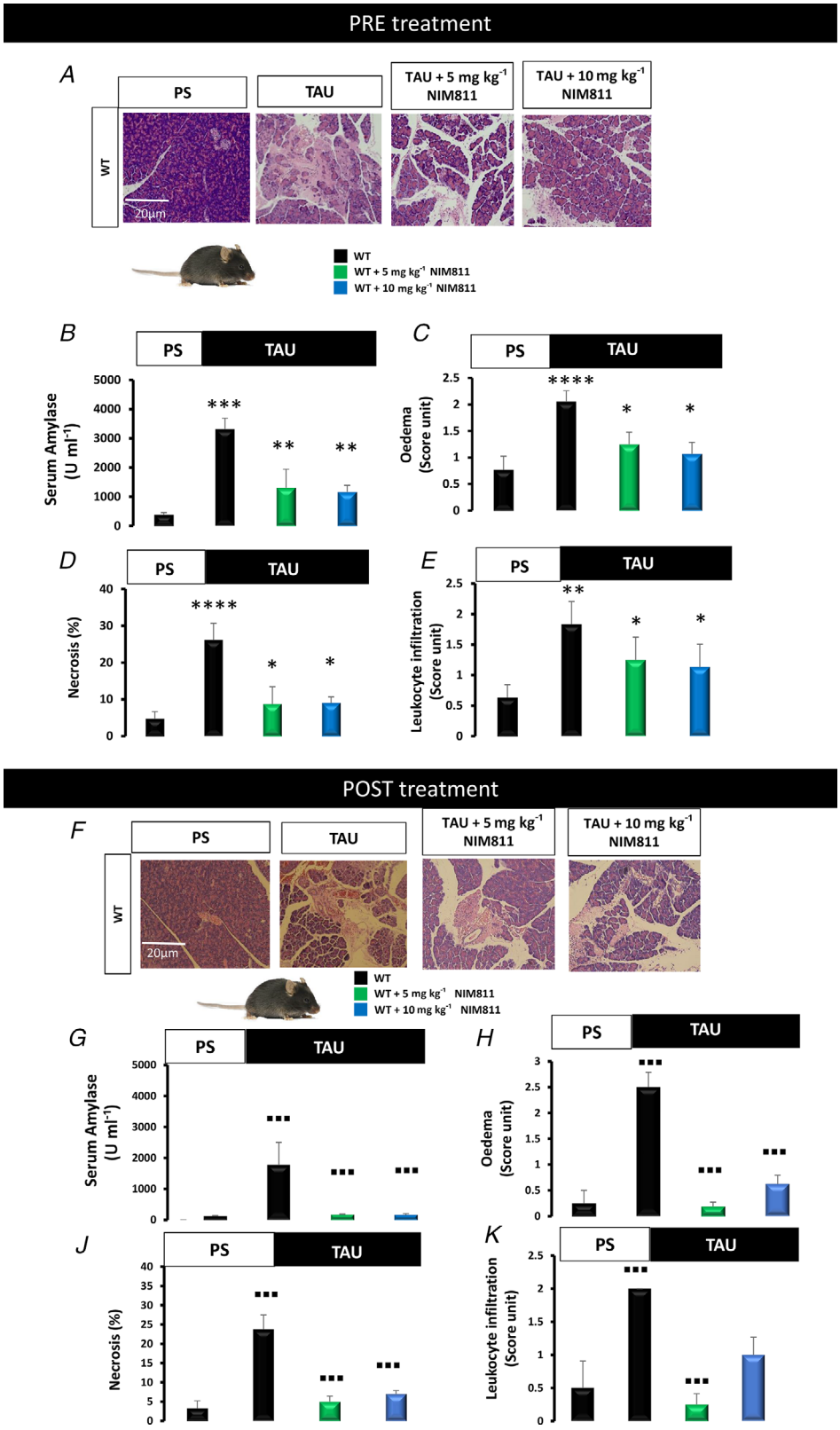

Figure 7. NIM811 reduces the severity of TAU induced AP in mice
A–K, in TAU-induced pancreatitis, serum amylase measurements revealed that retrograde infusion of TAU led to elevated serum amylase levels [***$P < 0.01$ WT PS *vs.* WT TAU (*B*), ■■■$P < 0.001$ WT PS *vs.* WT TAU (*G*)], but 5 or 10 mg kg^{-1} body weight NIM811 treatment significantly reduced the enzyme levels both before and after treatment (*B*: **$P < 0.02$ WT TAU *vs.* pre 5 mg kg^{-1} NIM811 + TAU, **$P < 0.02$ WT TAU *vs.* pre 10 mg kg^{-1}

NIM811 + TAU, ▪▪▪$P < 0.001$ WT TAU *vs.* post 5 mg kg^{-1} NIM811 TAU, ▪▪▪$P < 0.001$ WT TAU *vs.* post 10 mg kg^{-1} NIM811 + TAU), and serum amylase levels were reduced compared to WT TAU-treated groups (*B* and *G*: *$P < 0.01$ WT TAU *vs.* WT 5 mg kg^{-1} NIM811 TAU and *$P < 0.01$ WT TAU *vs.* WT 10 mg kg^{-1} NIM811 TAU). During pre-NIM811 treatment oedema, necrosis and leukocyte infiltration scores were significantly decreased compared to the only TAU-treated groups (*A*, *C*, *D*, *E*: $P < 0.05$ WT TAU *vs.* pre 5 mg kg^{-1} NIM811 TAU/10 mg kg^{-1} NIM811 TAU). Post-insult administration of NIM811 decreased oedema, leukocyte infiltration and necrosis levels in the TAU group (*G–K*: ▪▪▪$P < 0.001$). $n = 4$–6 animals per group; data are means ± SEM. [Colour figure can be viewed at wileyonlinelibrary.com]

NIM811 has therapeutic benefits in CER-, TAU- and EtOH-FA-induced AP

First, we confirmed that per os administration of either 5 or 10 mg kg^{-1} NIM811 alone has no toxic effect on the pancreas (Fig. 9). Second, we tested the compound in three different experimental AP models: the CER-, EtOH + FA- and TAU-induced models (Niederau *et al.* 1985; Perides *et al.* 2010; Huang, 2014). Importantly, pre-treatment with both 5 and 10 mg kg^{-1} NIM811 significantly reduced the elevation of serum amlylase activity, as well as pancreatic oedema, necrosis and leukoctye infiltration in experimental AP models (Figs 6–8). We also confirmed that subsequent treatment with 5 or 10 mg kg^{-1} NIM811 has protective effects against pancreatic damage (Figs 6–8).

Discussion

AP is a multifactorial disease (Hegyi & Petersen, 2003; Sahin-Toth & Hegyi, 2017) involving several types of cell, including acinar and ductal cells. None of the therapeutic efforts targeting only one of them has been successful. Intravenous administration of secretin, which targeted ductal cells only, was found to be either slightly beneficial or neutral in AP (Lankisch *et al.* 1983; Renner *et al.* 1983; Keim *et al.* 1985). By contrast, neither gabexate mesilate nor trasylol, which effectively inhibit trypsin activity, had beneficial effects in AP (Imrie *et al.* 1978; Buchler *et al.* 1993). Therefore, we need to find common targets which can restore both acinar and ductal cell functions in AP.

Mitochondrial damage is one of the key pathophysiological events in the early phase of AP in both types of cell (Hegyi & Petersen, 2003; Maleth *et al.* 2013; Maleth & Hegyi, 2015). It decreases ATP production, causing an elevation of intracellular calcium concentration; moreover, it negatively influences ATP-dependent Cl^-/HCO_3^- exchangers, CFTR Cl^- channels in ductal cells and enzyme secretory processes in acinar cells (Maleth *et al.* 2011, 2013, 2015; Judak *et al.* 2014; Maleth & Hegyi, 2015; Mukherjee *et al.* 2016; Biczo *et al.* 2018; Katona *et al.* 2016). In addition, mitochondrial damage is the main factor in determining cell death pathway necrosis and apoptosis. Release of mitochondrial cytochrome c into the cytosol causes apoptosis, whereas mitochondrial depolarization leads to necrosis (Odinokova *et al.* 2008). Generally, the standard apoptotic pathway involves mitochondrial outer membrane permeabilization, which causes apoptotic factors such as cytochrome c to be released from the inner membrane to the cytosol (Tait & Green, 2010; Maleth & Hegyi, 2015). On the other hand, opening of the mPTP leads to loss of ψ ATP depletion, increased inner membrane permeability, mitochondrial swelling and necrotic cell death (Golstein & Kroemer, 2007; Halestrap *et al.* 2009; Maleth & Hegyi, 2015). Uniquely, inhibition of mPTP could prevent both cell death mechanisms in PDECs, which is different from that seen in acinar cells, where only necrosis could have been prevented. Inhibition of mPTP thus seems to be highly beneficial in both cell types. In the last decade, it has been shown that genetic or pharmacological inhibition of mPTP reduces BA- or EtOH + FA-induced acinar cell damage as well as augmenting the severity of AP (Sah & Saluja, 2011; Gukovskaya *et al.* 2016; Mukherjee *et al.* 2016; Biczo *et al.* 2018). As regards ductal cells, we have shown earlier that both BA and EtOH + FA induce inhibition of HCO_3^- secretion via severe mitochondrial damage in PDECs (Maleth *et al.* 2011, 2016). Now, we have continued our experiments investigating the role of mPTP and its inhibition in this type of epithelial cell. First, we characterized the role of mPTP (both genetic and pharmacological CYA) inhibition in PDECs and found that its inhibition has a strong protective effect against the toxic effects of BA or EtOH + FA in ductal cells, suggesting that targeting mPTP may have general benefits. Although many mPTP inhibitors have been tested, none of them has been successful. CYA itself inhibits calcineurin, which leads to immunosuppressant activity and thus could negatively affect the treatment of patients due to hazardous infections. Clinical testing of non-immunosuppressive CYA derivatives Debio025 and TRO40303 was also stopped before reaching 'proof of concept' phase 2 clinical trials in AP because of its inconsistent behaviour in other trials (see Introduction). Recently, other new mPTP inhibitors have been introduced in experimental studies. Isoxazoles had inconsistent effects in myocardial infarction (Sileikyte & Forte, 2016). Benzamides resulted in impaired ATP generation (Sileikyte & Forte, 2016; Javed *et al.* 2018). Cinnamic anilides were shown to be effective in myocardial infarction (Fancelli *et al.* 2014); however, it has since been shown that it has an age-related toxicity (Fang *et al.* 2019). In contrast, NIM811 seemed

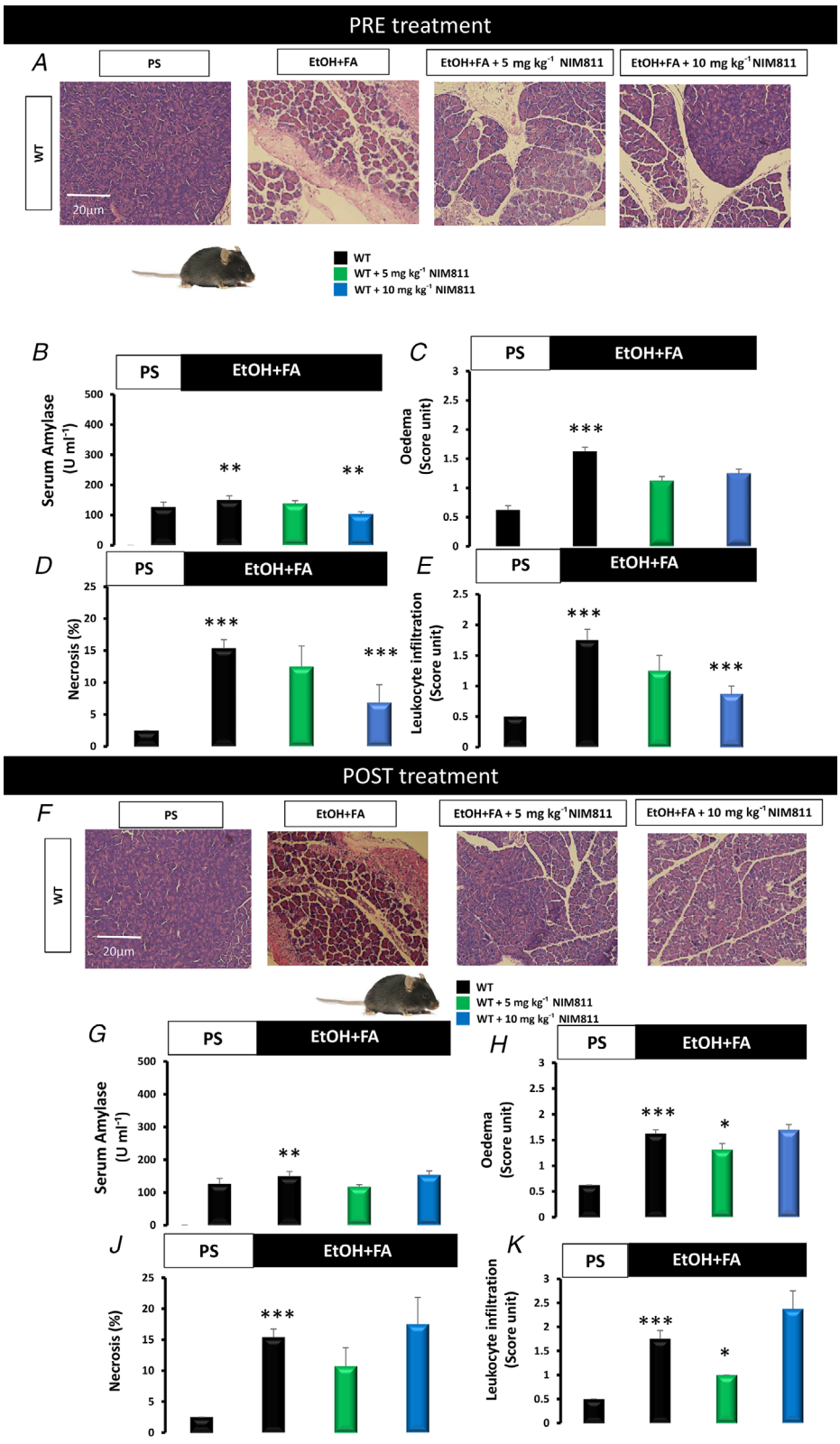

Figure 8. NIM811 has a protective effect against EtOH + FA induced pancreatic damage
A–K, in EtOH + FA-induced pancreatitis, serum amylase measurements revealed that in pretreatment with 10 mg kg^{-1} NIM811 significantly reduced serum amylase levels (*B*: $^{**}P < 0.002$ WT EtOH + FA *vs.* pre 10 mg kg^{-1} NIM811 + EtOH + FA; *B* and *G*: $^{**}P < 0.002$ WT PS *vs.* WT EtOH + FA), whereas with post-NIM811 treatment

serum amylase levels did not differ significantly compared to its EtOH + FA control (*G*). With pre 10 mg kg^{-1} NIM811 treatment leukocyte infiltration (***P < 0.001 WT EtOH + FA *vs.* 10 mg kg^{-1} NIM811) and necrosis levels (***P < 0.001 WT EtOH + FA *vs.* 10 mg kg^{-1} NIM811) were significantly reduced compared to EtOH + FA AP group (*D–E*). *C–E*: ***P < 0.001 WT PS *vs.* Wt EtOH + FA. Oedema and leukocyte infiltration levels were significantly reduced in post 5 mg kg^{-1} NIM811-treated groups compared to WT EtOH + FA groups (*H* and *K*: *P < 0.05 WT EtOH + FA *vs.* post 5 mg kg^{-1} NIM811). n = 4–7 animals per group; data are means ± SEM. [Colour figure can be viewed at wileyonlinelibrary.com]

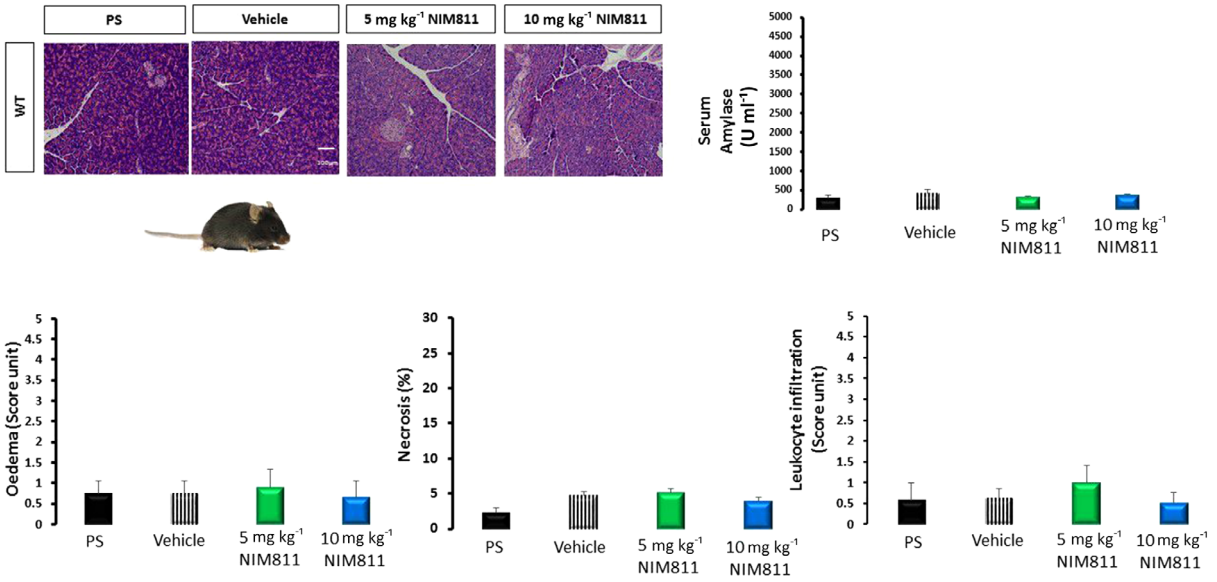

Figure 9. NIM811 itself does not induce pancreatic damage
No significant difference was found between the NIM811-treated - (8.3% polyoxyl 40 hydrogenated castor oil, 8.3% EtOH) *vs.* the control groups. n = 4–5 animals per group. [Colour figure can be viewed at wileyonlinelibrary.com]

to be a perfect choice. It has been shown to be protective in several diseases, and until now no toxic effects have been demonstrated. Therefore, we continued our study by testing the effects of NIM811 on both ductal and acinar cells *in vitro*. We found that NIM811 reduces the mitochondrial damage caused by BA or EtOH + FA. Importantly, NIM811 decreased apoptosis levels during BA or EtOH + FA treatment in ductal cells, but not in acinar cells, a result which could be due to the observation that ductal cells have more mitochondria than acinar cells (Maleth *et al.* 2013). Surprisingly, inhibition of mPTP protected pancreatic ductal bicarbonate but not fluid secretion during BA or EtOH + FA treatment. These data suggest that rescuing intracellular ATP levels and the activity of Na^+/K^+-ATPase do not result in overall protection alone and other fluid transport mechanisms such as aquaporins may remain diminished (Venglovecz *et al.* 2018). Per os administration of 5 or 10 mg kg^{-1} NIM811 alone had no toxic effect, but significantly reduced the severity of AP. We found that NIM811 treatment was more beneficial in the TAU- than in the EtOH + FA-induced AP model. One explanation could be that besides the direct toxic effect of EtOH and FA, the non-oxidative metabolites of FA (fatty acid ethyl esters) have even higher toxicity on the mitochondria in both acinar and ductal cells (Criddle *et al.* 2006; Petersen *et al.* 2009).

Taken together, mitochondrial function and bioenergetics play a crucial role in the development of AP; however, translation of these results to a patient benefit remains lacking (Maleth *et al.* 2013; Gukovskaya *et al.* 2016; Maleth & Hegyi, 2015; Mukherjee *et al.* 2016; Biczo *et al.* 2018). In this study, we have confirmed that the mPTP inhibitor NIM811 is a highly suitable compound to be tested in clinical trials. As a next step, phase 2 clinical trials are needed with the use of this novel and promising drug candidate.

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Additional information

Competing interest

The authors have no conflicts of interest to disclose.

Author contributions

PH had the original idea, initiated the study, obtained funding and supervised the experimental procedures. Most of the protocols were designed by ET, JM, JF, VV, PP, ZR and PH. ET, NZ, AG and RE performed the experiments. Experiments were performed at the Laboratory of Cell Physiology, First Department of Medicine, University of Szeged, or Institute for Translational Medicine and First Department of Medicine, University of Pécs, Pécs, Hungary. ERB contributed to the quantification of the histological samples. LT and GH provided the Ppif$^{-/-}$ mice and were involved in data interpretation. ET, NZ and PH evaluated the statistical analysis. JF, JM, PP, ERB and VV provided conceptual advice on the experimental protocols (JF: isolation procedure for pancreatic acinar cells; JM: confocal microscopy and study design; ERB: histological quantification; PP and VV: fluorescence microscopy). ET and PH wrote the paper. JM, NZ, JF, AG, RE, PP, LT, GH, ERB, ZR and VV reviewed and contributed to the manuscript. All authors approved the final manuscript.

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Keywords

acute pancreatitis, cyclophilin D, mitochondrial transition pore, NIM811

Translational perspective

Acute pancreatitis (AP) is a severe disorder with high morbidity, mortality and no specific treatment. It is generally accepted that one of the earliest events in initiation of the disease is mitochondrial dysfunction and ATP depletion. It has been shown that the pancreatitis-inducing factors ethanol, fatty acids and bile acids open the membrane transition pore (mPTP) channel, and keep it continuously open, resulting in mitochondrial depolarization, lower ATP synthesis and cell necrosis both in pancreatic acinar and ductal cells. In this study, we provided strong evidence that one of the mPTP inhibitors, namely NIM811, is highly effective in different experimental pancreatitis models. Since NIM811 had no side-effects and passed the important phase 1 stage in the clinical trial process, phase 2 clinical trials are needed with the use of this novel and promising drug candidate.

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Assessment of the course of acute pancreatitis in the light of aetiology: a systematic review and meta-analysis

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The main causes of acute pancreatitis (AP) are biliary disease, alcohol consumption, hypertriglyceridaemia (HTG) and endoscopic retrograde cholangiopancreatography (ERCP). The aim of this meta-analysis was to evaluate the effects of these aetiological factors on the severity and outcome of AP. Pubmed and Embase were searched between 01/01/2012 and 31/05/2020. Included articles involved adult alcoholic, biliary, HTG- or post-ERCP AP (PAP) patients. Primary outcome was severity, secondary outcomes were organ failures, intensive care unit admission, recurrence rate, pancreatic necrosis, mortality, length of hospital stay, pseudocyst, fluid collection and systematic inflammatory response syndrome. Data were analysed from 127 eligible studies. The risk for non-mild (moderately severe and severe) condition was the highest in HTG-induced AP (HTG-AP) followed by alcoholic AP (AAP), biliary AP (BAP) and PAP. Recurrence rate was significantly lower among BAP vs. HTG-AP or AAP patients (OR = 2.69 and 2.98, 95% CI 1.55–4.65 and 2.22–4.01, respectively). Mortality rate was significantly greater in HTG-AP vs. AAP or BAP (OR = 1.72 and 1.50, 95% CI 1.04–2.84 and 0.96–2.35, respectively), pancreatic necrosis occurred more frequently in AAP than BAP patients (OR = 1.58, 95% CI 1.08–2.30). Overall, there is a potential association between aetiology and the development and course of AP. HTG-AP is associated with the highest number of complications. Furthermore, AAP is likely to be more severe than BAP or PAP. Greater emphasis should be placed on determining aetiology on admission.

Abbreviations

AP	Acute pancreatitis
AAP	Alcohol-induced/alcoholic acute pancreatitis
BAP	Biliary acute pancreatitis
CI	95% Confidence interval
ERCP	Endoscopic retrograde cholangiopancreatography
HTG-AP	Hypertriglyceridaemia-induced acute pancreatitis
HTG	Hypertriglyceridaemia
ICU	Intensive care unit
LOS	Length of hospital stay
MOF	Multiple organ failure
OF	Organ failure
OR	Odds ratio
PAP	Post-endoscopic retrograde cholangiopancreatography-induced acute pancreatitis
POF	Persistent organ failure

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PUF	Pulmonary failure
RAC	Revised Atlanta Classification
SIRS	Systematic inflammatory response syndrome
TOF	Transient organ failure

Acute pancreatitis (AP) is a sudden inflammatory disease of the pancreas. In the last 20 years, the incidence of the disease has increased by more than 20%[1,2]. Nowadays, AP is one of the most common reasons for hospitalization in case of gastrointestinal diseases[3].

Gallstones represent the main aetiological background of AP globally (42%), which are diagnosed by imaging techniques and liver function tests[4]. Gallstone-related or biliary AP (BAP) occurs twice as often as alcohol-induced AP (AAP)[4]. AAP is caused by regular, excessive alcohol consumption usually with a clinical history of > 5 years and > 50–100 g/day[5]. Hypertriglyceridaemia (HTG) with serum triglyceride concentrations > 11.3 mM is the third most common (9%) known aetiological factor of the disease[6–8]. Less frequent causes of AP include endoscopic retrograde cholangiopancreatography (ERCP), hypercalcaemia, pancreas divisum, tumours, genetic polymorphisms and drugs[9]. To date, no standardized diagnostic criteria exist for post-ERCP AP (PAP). The guidelines recommended by Cotton et al.[10] are most commonly applied, which suggest PAP to be diagnosed if pancreatitis develops within 24 h after the procedure.

Based on the Revised Atlanta Classification (RAC), AP severity can be categorized into three groups: mild, moderately severe and severe[11]. Although the majority of cases are mild with a self-limiting course[11], the mortality rate of severe AP can reach 30% which underlies the desperate need of finding proper treatment[12]. Organ failure (OF) is the most important determinant of this classification system[11]. Patients with mild AP have no organ dysfunction and usually recover within a week. Moderately severe AP resolves slower and might require interventions because of the presence of transient organ failure (TOF, < 48 h). Severe AP results in persistent organ failure (POF) which lasts > 48 h. Multiple organ failure (MOF) is defined as failure of two or more organ systems, which can be transient or persistent[13]. The three extrapancreatic organs most commonly affected by AP are the lungs, the heart and the kidneys[11]. Approximately 25% of AP patients develop severe complications and have to be admitted to an intensive care unit (ICU)[14]. Local complications can also occur in cases of moderately severe and severe AP, which include acute peripancreatic fluid collections, pancreatic pseudocysts, acute necrotic collections and walled-off necrosis[11]. About 20% of patients experience recurrent AP (RAP), which refers to a clinical condition defined by repeated episodes of AP[15]. 10% of AP patients with a single episode and 36% with RAP progress to chronic pancreatitis (CP)[15]. The risk of progression to CP increases with excessive alcohol consumption, smoking and male gender. 5% of CP patients develop pancreatic cancer[16].

Although there are several risk factors, it is difficult to predict which patient will develop mild, moderately severe or severe AP. To date, numerous clinical studies have investigated the effect of aetiology on AP progression. However, to the best of our knowledge, there have been no efforts to summarize clinical data on how various aetiological backgrounds affect the severity and course of AP. Consequently, this study was undertaken to reveal the impact of the above-mentioned aetiologies (HTG-AP, AAP, BAP, PAP) by performing thorough literature search and meta-analysis on available clinical data.

Methods

This systematic review and meta-analysis followed the recommendations of Stroup et al.[17] and was conducted in line with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses guidelines[18] (Supplementary Table S1). The analysis was based on the Problem, Intervention, Comparison intervention and Outcome (PICO) model[18] as follows: AP patients with alcoholic, biliary, hypertriglyceridaemic and post-ERCP aetiologies were compared in order to examine the effect of aetiology on disease outcomes. Primary outcome was severity, secondary outcomes were POF, MOF, TOF, ICU admission, recurrence rate, mortality, pancreatic necrosis, pulmonary failure (PUF), renal failure, length of hospital stay (LOS), pseudocyst, fluid collection, and systematic inflammatory response syndrome (SIRS).

The protocol for the meta-analysis was registered in the PROSPERO database on 15/05/2018 (https://www.crd.york.ac.uk/PROSPERO/, ID: CRD42018093574).

Search strategy. Literature search was conducted in the electronic databases Embase and Pubmed from publication date 01/01/2012 to 31/05/2020. The reason for the start date is that the RAC was introduced in 2012, which provides the most accepted and widespread criteria for determining AP severity. The following search query was used for Embase: (alcohol* OR ethanol* OR biliary OR gallstone OR cholelithiasis OR 'post-ercp' OR 'post ercp' OR idiopathic OR triglyceride OR hypertriglyceridemia OR hyperlipidemia OR severity OR severe OR mild OR moderate) AND acute AND pancreatitis NOT ('conference abstract'/it OR 'review'/it)) AND (2012:py OR 2013:py OR 2014:py OR 2015:py OR 2016:py OR 2017:py OR 2018:py OR 2019:py OR 2020:py). In Pubmed, the following search terms were applied: (alcohol* OR ethanol* OR biliary OR gallstone OR cholelithiasis OR "post-ercp" OR "post ercp" OR idiopathic OR triglyceride OR hypertriglyceridemia OR hyperlipidemia OR severity OR severe OR mild OR moderate) AND acute AND pancreatitis NOT Review[ptyp] NOT Case Reports[ptyp]. The search was restricted to studies written in English or in Hungarian.

Eligibility criteria. All randomised trials, retrospective and prospective cohort studies were included that involved adult patients with AP and relevant data (primary and secondary outcomes) are categorized according to the aetiology of the disease. Four major disease backgrounds were included: alcohol abuse, HTG, biliary disease and post-ERCP. Articles that studied only one aetiological group or compared one aetiological group with another group called others or non-… (e.g. alcohol vs. non-alcohol) were excluded. Non-human studies or arti-

cles with data from patients younger than 18 years of age were not included. In case of cohort overlap between studies, only the most recent study was included unless a prior study had higher quality.

When assessing AP severity, only studies were included where severity was defined according to the RAC, because in this case it was crucial to present a consistent and clear definition for the analysis. Articles were also excluded if only one or two of the three severity groups were analysed. Both local complications and OFs could lead to serious conditions and death which are characteristic features of moderately severe and severe AP. Therefore, these two groups were combined in our study, and are referred to as "non-mild" disease forms and compared to the mild group. In cases of outcomes other than severity, using only the RAC was not in the criteria.

Study selection and data extraction. Titles and abstracts of publications were screened independently by two review authors (E.R.B. and G.F.) to identify studies that potentially meet inclusion criteria. The full texts of these potentially eligible studies were independently assessed for eligibility by the same two review authors. Disagreement between reviewers was resolved by discussion with other two colleagues (L.K. and Z.R.). E.R.B. and G.F. independently extracted study characteristics (author, title, journal, study location, inclusion period, number of centres involved, type of study, number of participants) and outcome data (severity, POF, MOF, TOF, ICU admission, recurrence rate, mortality, pancreatic necrosis, PUF, renal failure, LOS, pseudocyst, fluid collection, SIRS), which were recorded on a standardized Microsoft Excel spreadsheet. Discrepancies were resolved by discussion.

Quality assessment. Methodological quality of the articles was assessed by applying the Quality In Prognosis Studies (QUIPS) tool[19] (Supplementary Table S2). This considers the following domains: study participation, study attrition, prognostic factor measurement, outcome measurement, study confounding, and statistical analysis and reporting. All domains were scored by three individual researchers (E.R.B., G.F., L.K., each article was assessed by at least two of them). The overall risk of bias was considered:

- low if < 3 domains were rated a moderate risk of bias and all others were rated a low risk of bias,
- moderate if ≥ 3 domains were rated a moderate risk of bias and all others were rated a low risk of bias,
- high if ≥ 1 domain was rated a high risk of bias, irrespective of all other domains.

Consensus was reached after classification by the individual researchers.

Data analyses. Statistical analysis was performed with Stata 11 SE (StataCorp LLC, College Station, TX, USA). The investigated aetiologies were analysed pairwise. Odds ratios (ORs) calculated from patient numbers were used to compare outcomes in different aetiologic groups. ORs were pooled using the random effects model with the DerSimonien–Laird estimation and displayed on forest plots. Summary OR estimation, p value and 95% confidence interval (CI) were calculated. $P < 0.05$ was considered as significant difference from summary OR = 1. BAP was defined as primary reference group, the other aetiologies were ranked in the following order: AAP, HTG-AP, PAP.

Statistical heterogeneity was analysed using the I^2 statistic and the chi-square test to acquire probability values; $p < 0.1$ was defined to indicate significant heterogeneity. The small-study effect (in case of comparisons with at least 10 articles) was visually investigated on funnel plots and was also confirmed by Egger's test. Sensitivity analysis was performed to examine the robustness of our results.

Results

Study selection. The search strategy identified 11,288 records. After removing duplicates 7733 articles were retrieved. Out of these, 456 records seemed to be relevant to the study question based on screening by title or abstract. After assessing the articles in full text, 328 records had to be excluded with different reasons (see details in Fig. 1). Finally, 127 publications fulfilled the eligibility criteria.

Characteristics of studies included. The majority of the included cohort studies (108 out of 128) collected data from the 2010's. Our meta-analysis contains 102 single[20–121] and 23 multicentre studies[122–144]. In two cases, there were no relevant data regarding the number of centres involved[145,146]. Sample sizes ranged from 11 to 1,165,777. Only the data of the four types of AP (AAP, BAP, HTG-AP, PAP) were analysed. Detailed characteristics of the included studies are provided in Supplementary Table S3. During quality assessment, we evaluated patient selection, comparability of the groups, and outcome data, which are presented in Supplementary Table S4.

Risk of bias assessment. According to the QUIPS checklist, most of the included studies had an overall moderate risk of bias (80, 63%; Supplementary Figure S1). 30 studies (23.4%) had low and 17 (13.3%) had high risk of bias. High risk was mainly due to the confounding factors which showed significant difference between the analysed aetiological groups. Moderate risk of bias resulted mainly from „Study confounding" and „Statistical Analysis and Reporting", furthermore „Prognostic factor measurement" was also missing in a relatively high number (66.9%) of included studies. A detailed analysis can be found in Supplementary Table S4.

Clinical outcomes. *Severity.* HTG proved to induce non-mild AP in a significantly higher number of cases than the other aetiological factors (Figs. 2, 3). ORs of non-mild cases in HTG-AP were 1.35 [CI 1.12–1.63] and 1.35 [CI 1.13–1.62] vs. AAP and BAP, respectively (Fig. 2a, b). PAP also appeared to be significantly less

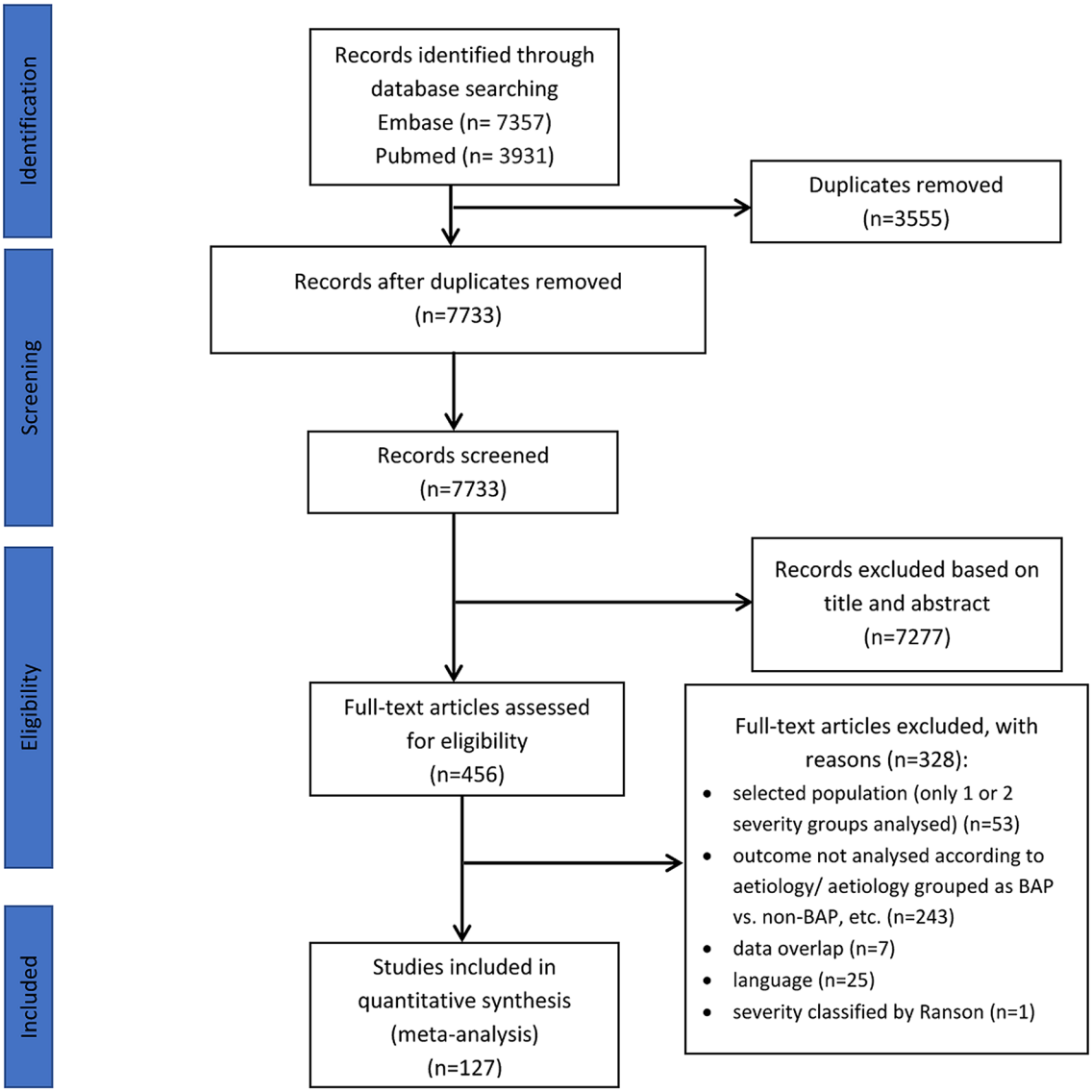
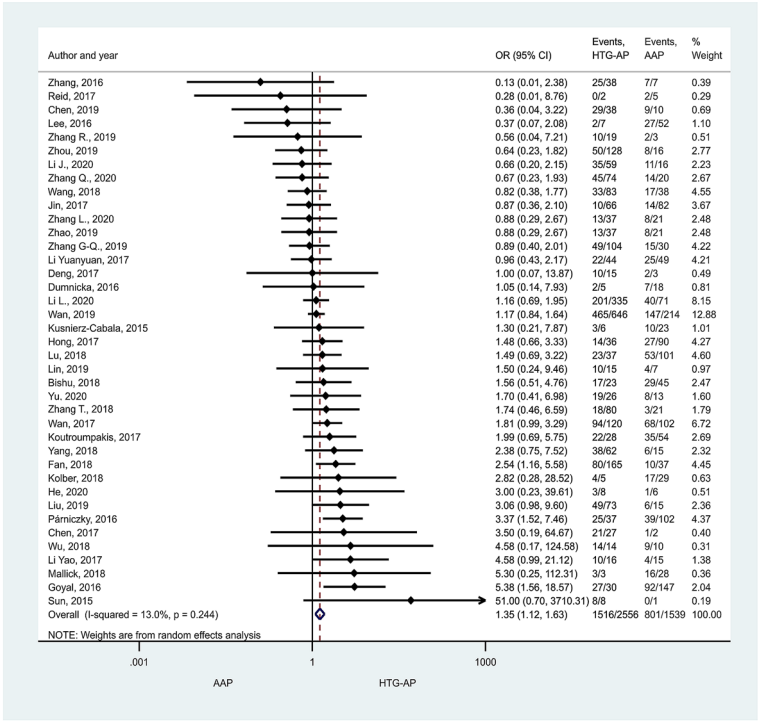


Figure 1. PRISMA 2009 flow diagram for identification of relevant articles.

severe compared to HTG-AP (Fig. 3a; OR: 0.38 [CI 0.15–0.98]) or AAP (Fig. 3b; OR: 0.43 [CI 0.25–0.74]), while no significant difference could be detected between the severities of BAP and PAP (Supplementary Figure S2]). Alcoholic aetiology significantly increased AP severity compared to biliary-related events (Fig. 4; OR: 1.36 [CI 1.15–1.60]). We found heterogeneity in the comparison of HTG-AP vs. BAP, HTG-AP vs. PAP, AAP vs. BAP, PAP vs. AAP, BAP vs. PAP (Figs. 2b, 3, 4 and Supplementary Figure S2). No signs of small-study effect were detected in any comparison (Supplementary Figure S3).

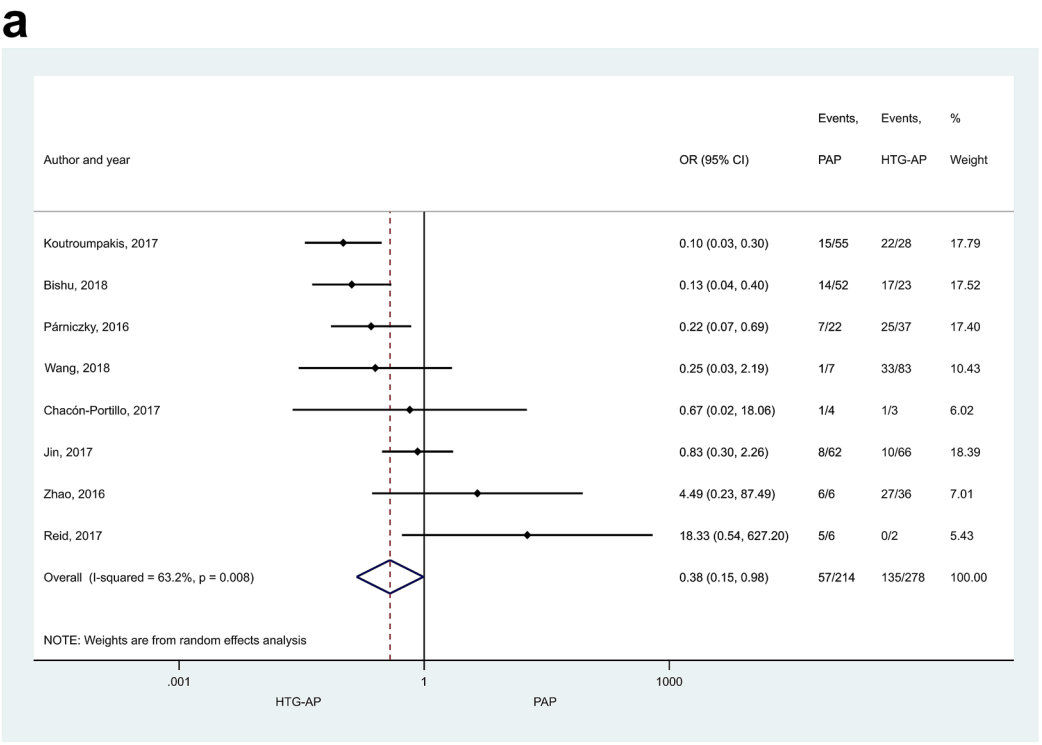
Organ failures, intensive care unit admission, and systematic inflammatory response syndrome. No significant difference was found in POF between any aetiological groups (AAP vs. BAP, HTG-AP vs. AAP, HTG-AP vs. BAP; Fig. 5a; Supplementary Figure S4). No signs of small-study effect were found in POF in the comparison of AAP vs. BAP (Supplementary Figure S5a). There were no significant differences in the occurrences of MOF, TOF or renal failure between AAP and BAP (Fig. 5b and Supplementary Figures S6a,b, respectively). PUF occurred more frequently in HTG-AP patients compared to BAP (Supplementary Figure S7a; OR: 2.39 [CI 1.06–5.39]), while AAP and BAP patients did not differ in this respect (Supplementary Figure S7b). The frequency of ICU admission was similar in AAP and BAP patients (Supplementary Figure S8). More AAP patients developed SIRS than PAP patients (Supplementary Figure S9a, OR: 0.40 [CI 0.21–0.77]. The rate of SIRS did not differ when comparing other patient groups (Supplementary Figure S9b,c). Heterogeneity was found in the comparison of renal failure, PUF and ICU admission between AAP and BAP (Supplementary Figures S6b, S7b, S8).

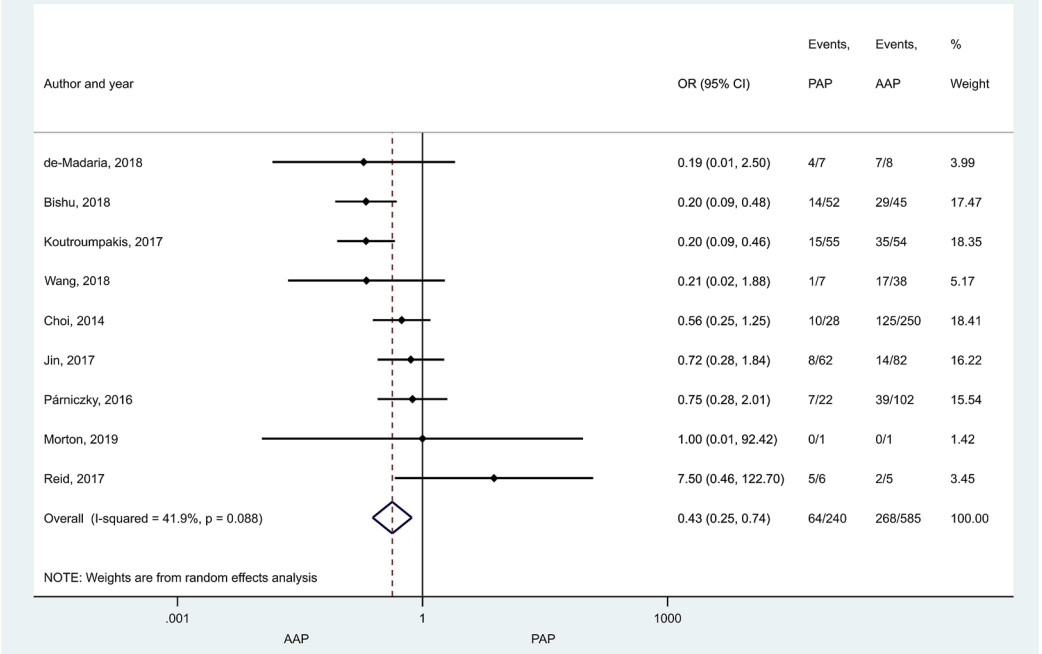
Recurrence rate and length of hospital stay. Recurrence rate was significantly higher in AAP vs. BAP patients (Supplementary Figure S10a; OR: 2.98 [CI 2.22–4.01]) and in HTG-AP vs. BAP patients (Supplementary Figure S10b; OR: 2.69 [CI 1.55–4.65]). However, AP did not reoccur more frequently due to alcoholic aetiology than



AAP alcohol-induced acute pancreatitis, HTG-AP hypertriglyceridaemia-induced acute pancreatitis, BAP biliary acute pancreatitis, OR odds ratio, CI 95% confidence interval

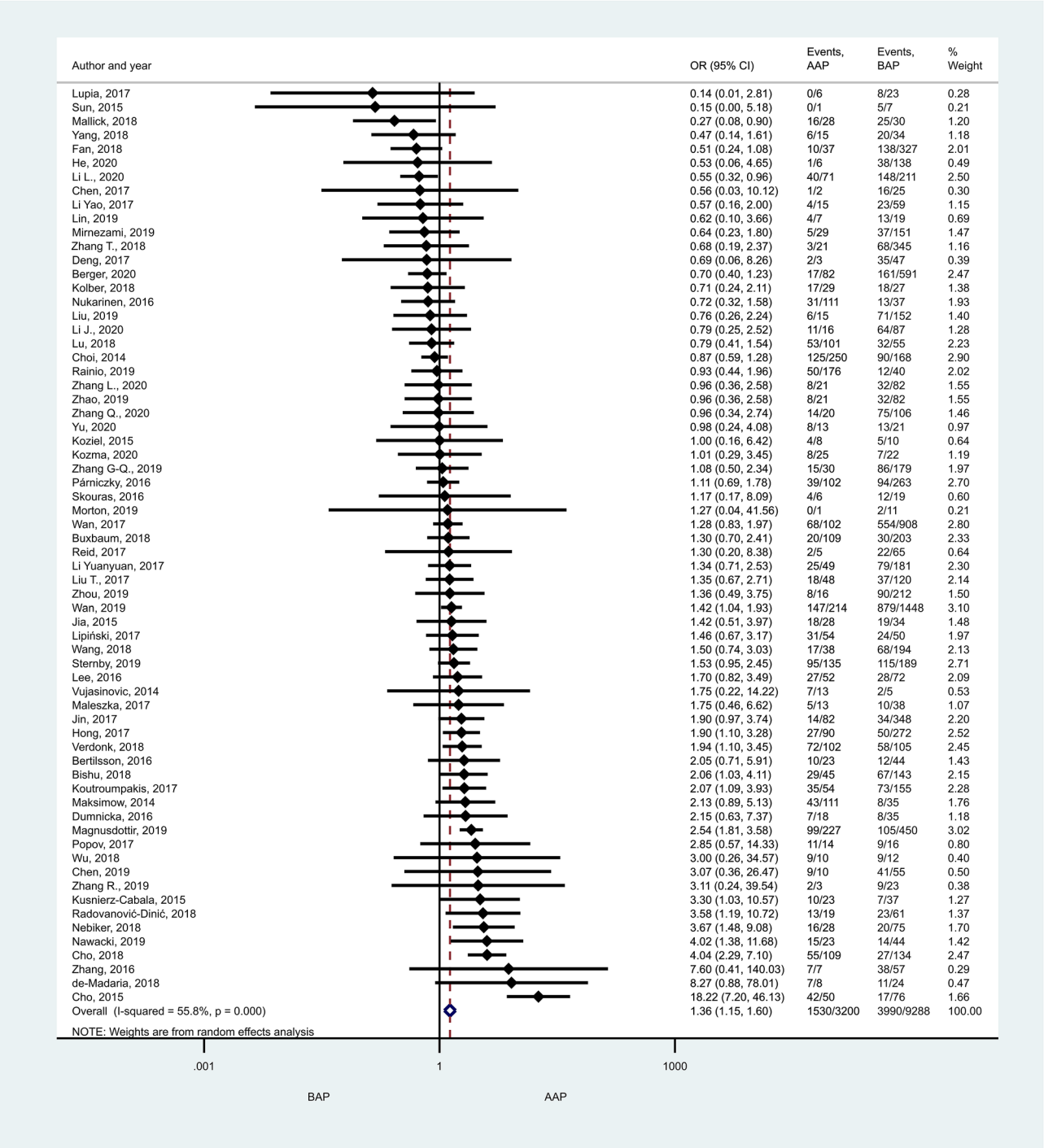
Figure 2. Forest plot showing the comparison of disease severity in (**A**) HTG-AP and AAP, p = 0.001; (**B**) HTG-AP and BAP, p = 0.001. Filled diamonds represent the ORs derived from the articles analysed. Horizontal bars represent CI. Empty diamond shows the overall OR (middle of the diamond and CIs are the edges) for non-mild (moderately severe and severe groups based on the Revised Atlanta Classification) disease. Heterogeneity of the results was presented by I-square and p value.





AAP alcohol-induced acute pancreatitis, HTG-AP hypertriglyceridaemia-induced acute pancreatitis, PAP post-endoscopic retrograde cholangiopancreatography-induced acute pancreatitis, OR odds ratio, CI 95% confidence interval,

Figure 3. Forest plot showing the comparison of disease severity in (**A**) HTG-AP and PAP, p = 0.045; (**B**) AAP and PAP, p = 0.002. Filled diamonds represent the ORs derived from the articles analysed. Horizontal bars represent CI. Empty diamond shows the overall OR (the middle of the diamond, CIs are the edges) for non-mild disease.



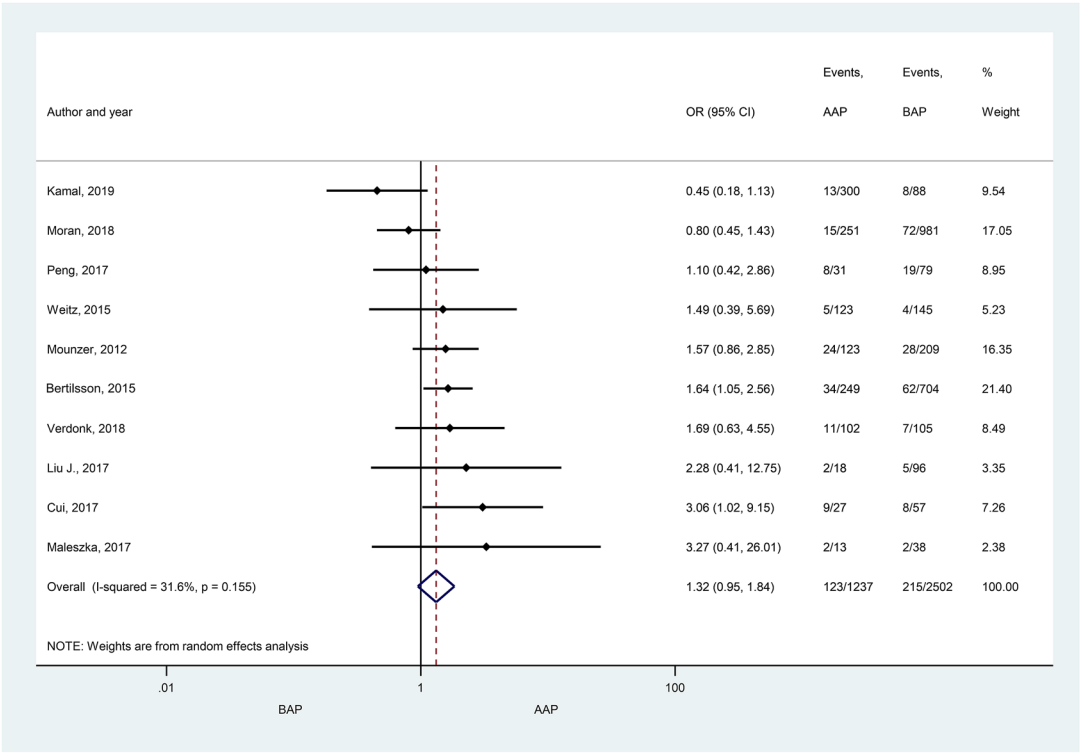
AAP alcohol-induced acute pancreatitis, BAP biliary acute pancreatitis, OR odds ratio, CI 95% confidence interval

Figure 4. Forest plot showing the comparison of disease severity in AAP and BAP, $p < 0.001$. Filled diamonds represent the ORs derived from the articles analysed. Horizontal bars represent CI. Empty diamond shows the overall OR (middle of the diamond, CIs are the edges) for non-mild disease.

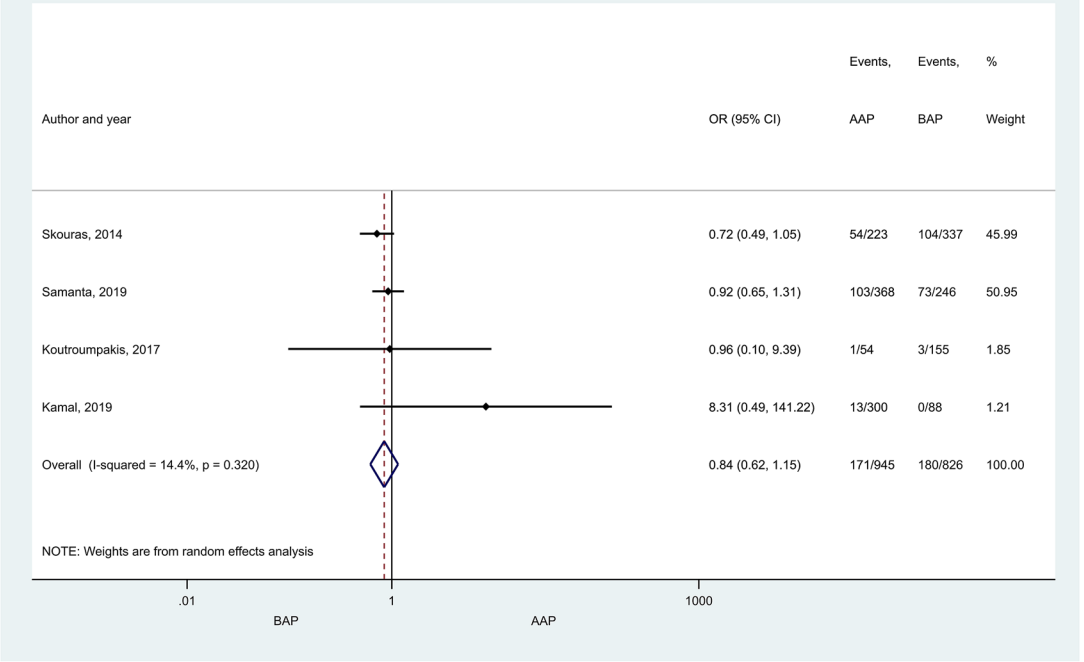
HTG or post-ERCP (Supplementary Figure S11a,b). Recurrence rate was also similar in BAP and PAP patients (Supplementary Figure S11c). Patients of the analysed aetiologies were hospitalized for a similar length of time (Supplementary Figure S12a,b). We found heterogeneity in the comparison of all cases of LOS and all cases of recurrence rate, except for the comparison between AAP and HTG-AP (Supplementary Figure S11a). No signs of small-study effect could be detected in case of recurrence rate or LOS (Supplementary Figure S5b,c).

Mortality and pancreatic necrosis. Mortality rate proved to be significantly higher in HTG-AP than in AAP (Fig. 6; OR: 1.72 [CI 1.04–2.84]), but no statistical difference was found between any other patient groups (Sup-

a

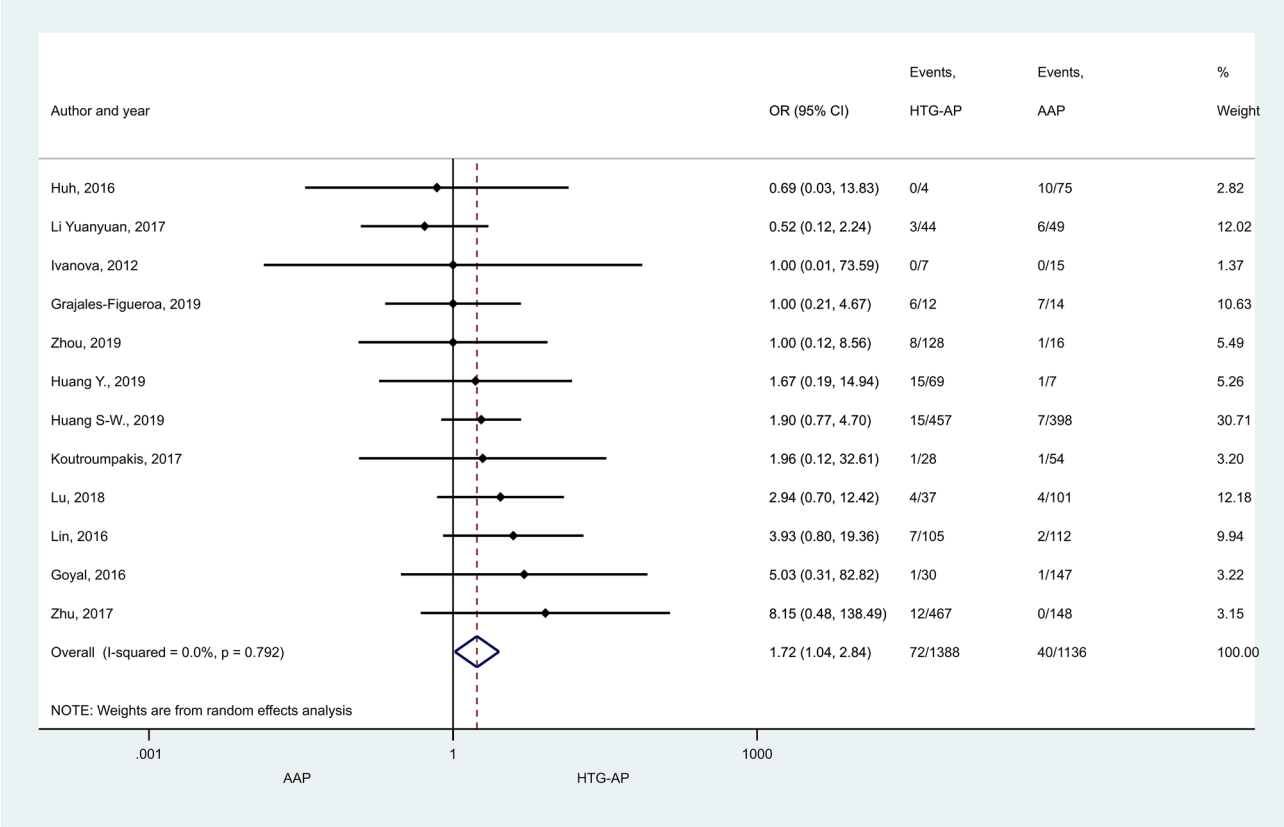


b



AAP alcohol-induced acute pancreatitis, BAP biliary acute pancreatitis, POF persistent organ failure, MOF multiple organ failure, OR odds ratio, CI 95% confidence interval

Figure 5. Forest plot showing the effect of different disease aetiologies on POF and MOF. The effects of BAP vs. AAP on (**A**) POF, p = 0.102; and (**B**) MOF, p = 0.284. Filled diamonds represent the ORs derived from the articles analysed. Horizontal bars represent CI. Empty diamond shows the overall OR (the middle of the diamond, CIs are the edges).



AAP alcohol-induced acute pancreatitis, HTG-AP hypertriglyceridaemia-induced acute pancreatitis, OR odds ratio, CI 95% confidence interval

Figure 6. Forest plot showing the effect of HTG-AP and AAP on mortality, p = 0.034. Filled diamonds represent the ORs derived from the articles analysed. Horizontal bars represent CI. Empty diamond shows the overall OR (the middle of the diamond, CIs are the edges).

plementary Figures S13 and S14). In the comparison of AAP and BAP a large proportion of patients came from one study contributing 1,165,777 subjects (accounting for 12.76% weight, Supplementary Figure S13a). However, sensitivity analysis showed that the results remained similar when this study was excluded (OR = 0.96 [CI 0.75–1.23]; Supplementary Figure S15). Pancreatic necrosis was reported more often in AAP than BAP patients (Fig. 7a, OR = 1.58 [CI 1.08–2.30]). No significant difference was detected in any other comparisons regarding necrosis (Fig. 7b,c). Heterogeneity was found in the comparison of mortality rate between BAP and HTG-AP, AAP and BAP (Supplementary Figure S13), and in case of necrosis when AAP and BAP groups were compared (Fig. 7a). We found signs of the small-study effect in case of mortality in the comparison of AAP and BAP (Supplementary Figure S16).

Pseudocyst, fluid collection. There was no significant difference in the presence of fluid collection or pseudocysts in the available comparisons (Supplementary Figure S17). We found heterogeneity in case of fluid collection (AAP vs. BAP) and pseudocyst (HTG-AP vs BAP).

Discussion

This is the first detailed meta-analysis investigating the relationship between different aetiologies (alcohol abuse, biliary, HTG, post-ERCP) and the course of AP. Our study revealed that the prevalence of severe and moderately severe (non-mild) disease forms was highest in case of HTG-AP which was followed by AAP, BAP and PAP (Table 1). Due to the large number of included articles and patients, our results have strong evidence in case of the severity outcome. These are also in accordance with our earlier observations[147] and the data of Wang et al.[148]. However, previously we compared the severe disease category to moderately severe and mild groups, which has less relevance than merging the severe and moderately severe groups as we did in the current study. Furthermore, our previous study and that of Wang et al.[148] compared the characteristics and outcome of HTG-AP to non-HTG-AP patients, without classifying non-HTG-AP group any further according to aetiology. Importantly, non-HTG-AP group is rather heterogenous and it cannot be decided whether all aetiological subgroups included in non-HTG-AP are less severe than HTG-AP or some of the subgroups are basically more severe than HTG-AP but due to other much milder subtypes, HTG-AP turns out to be the most severe disease form. In addition, non-HTG-AP included all aetiologies ("idiopathic" and "other" as well) except HTG-AP, while we only analysed well-defined and clear aetiologies. Therefore, the current meta-analysis provides a more refined

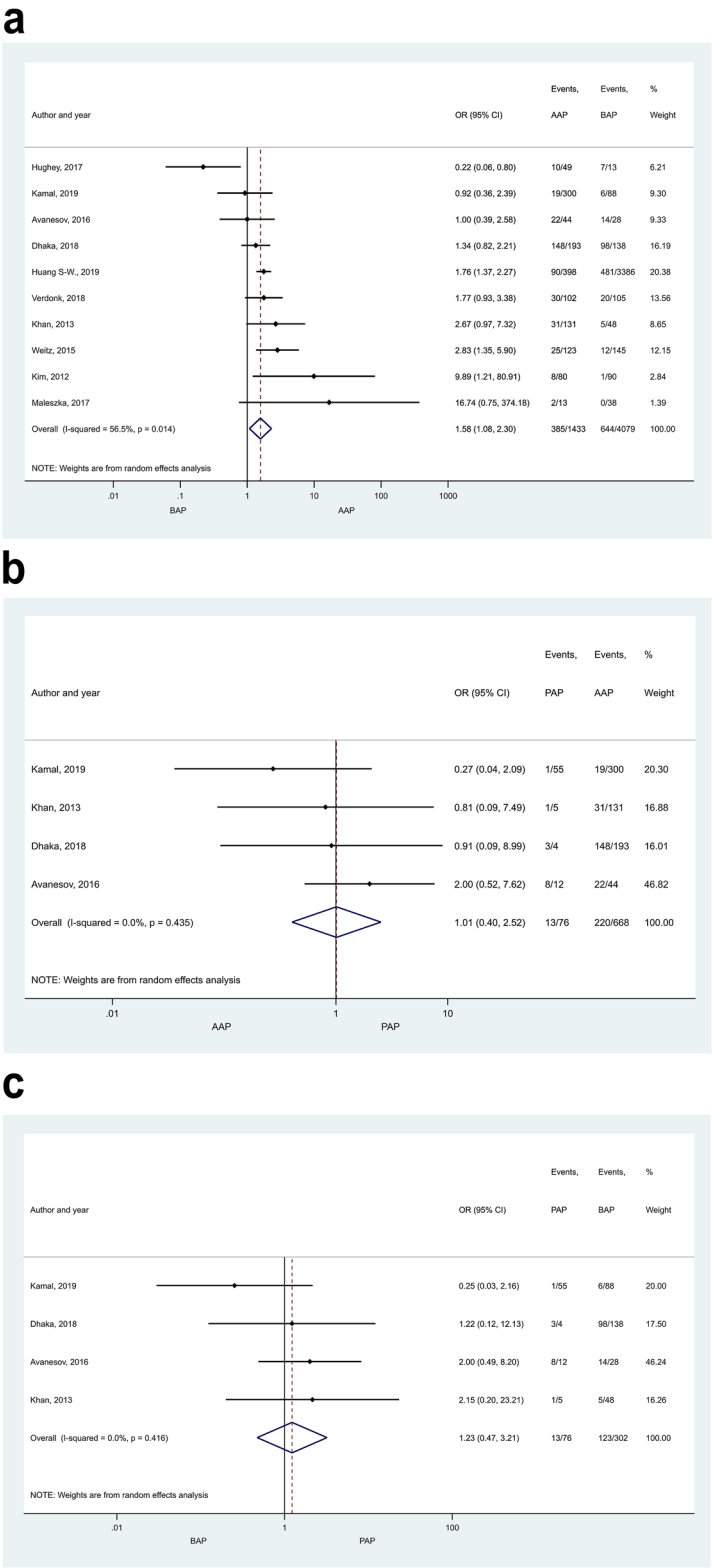


Figure 7. Forest plot showing the effects of different disease aetiologies on pancreatic necrosis. The effects of (**A**) BAP vs. AAP, p = 0.019; (**B**) AAP vs. PAP, p = 0.982; (**C**) BAP vs. PAP, p = 0.674. Filled diamonds represent the ORs derived from the articles analysed. Horizontal bars represent CI. Empty diamond shows the overall OR (the middle of the diamond, CIs are the edges).

Severity	HTG-AP > AAP > BAP ⫹ PAP
POF	AAP ⫹ BAP HTG-AP ⫹ [AAP/BAP]
MOF	AAP ⫹BAP
TOF	AAP ⫹BAP
PUF	HTG-AP > BAP ⫹ AAP
Renal failure	AAP ⫹BAP
ICU admission	AAP ⫹ BAP
SIRS	BAP ⫹ AAP > PAP BAP ⫹ PAP
Recurrence rate	[HTG-AP/AAP] > BAP HTG-AP ⫹ AAP PAP ⫹ [AAP/ BAP]
Mortality	HTG-AP > AAP [HTG-AP/AAP/PAP] ⫹ BAP AAP ⫹ PAP
Necrosis	AAP > BAP PAP ⫹ [AAP/BAP]
Pseudocyst	HTG-AP ⫹ BAP AAP ⫹ BAP
LOS	[HTG-AP/AAP] ⫹ BAP

Table 1. Summary of the results of our study. *AAP* alcohol-induced acute pancreatitis, *BAP* biliary acute pancreatitis, *HTG-AP* hypertriglyceridaemia-induced acute pancreatitis, *ICU* intensive care unit, *LOS* length of hospital stay, *MOF* multiple organ failure, *PAP* post endoscopic retrograde cholangiopancreatography-induced acute pancreatitis, *POF* persistent organ failure, *PUF* pulmonary failure, *SIRS* systematic inflammatory response syndrome, *TOF* transient organ failure. Statistically siginificant difference ($p < 0.05$) was presented with <; ⫹ shows no significant difference.

picture of the outcomes. Wang et al.[148] evaluated AP severity using the APACHE-II scoring system, but this does not specifically define AP severity.

In our study, no difference could be observed between any aetiological groups in POF (Table 1). Although HTG-AP and AAP exhibited the most severe forms of AP from an aetiologic point of view, the data of POF does not support it. This can be explained by the fact that POF associated with AP was assessed in HTG-AP patients only in three of the articles included in our meta-analysis[38,77,90]. In the study of Wang et al.[148] POF was most commonly observed in HTG-AP, which is in accordance with our results for severity. Although several articles evaluate characteristic features of severe AP such as POF, they focus exclusively on severe AP patients. For this reason, these could not be utilized in our analysis.

MOF is another distinctive feature of severe and moderately severe AP. In the current study, no significant difference could be detected in MOF between any of the analysed groups. Similarly, in a previous study, we did not find differences in MOF among HTG-AP vs. non-HTG-AP patients[147]. Tai et al.[149] also found a higher risk for the severe form of AP in HTG-AP patients compared to BAP. They diagnosed MOF more frequently in BAP patients, however, there was no difference in single OFs (renal, heart, pulmonary).

AP patients with systemic complications eventually end up in ICU. In case of this outcome, only one comparison could be performed: no significant difference was found between AAP and BAP (Table 1), which is supported by our previous findings[147].

The 27% recurrence rate of AP in the 1990s[150] has nowadays decreased to about 20%[151], which could be explained by better diagnosis and treatment after the first attack. In our study, alcoholic and hypertriglyceridaemic aetiologies caused more AP recurrence than biliary, while the repeated hospitalization for AAP and HTG-AP patients was similar. Tai et al. also found higher recurrence rate of HTG-AP than BAP[149]. Other studies drew the conclusion that alcohol is the most frequent aetiological factor for recurrent AP[150,151]. Suchsland et al.[151] analysed the risk factors for readmittance in AP, most of which were related to alcohol abuse, so these patients have a higher risk for disease recurrence after discharge. In case of BAP, delayed cholecystectomy could be responsible for recurrence[152,153].

Our study has shown that HTG-AP led to significantly higher mortality rate than AAP. However, no significant difference could be detected between the other aetiological groups. BAP used to have a higher mortality than AAP; however, this rate has decreased in the last decade due to improved supportive care[154]. Several studies have reported that mortality rate was not influenced by aetiological factors[155,156]. Other studies stated that HTG-AP did not cause significantly higher mortality rate, even though it led to higher severity and complication rates compared to other aetiological factors[157,158]. Wang et al.[148] concluded that HTG-AP caused higher mortality rate than non-HTG-AP, while Kiss et al.[147] did not find significant difference in this respect. Based on the current study, there is no strong relationship between aetiology of AP and mortality.

HTG carried the greatest risk for non-mild (moderately severe and severe) AP, which was followed by AAP; the least severe disease forms were observed in BAP and PAP. One of the possible pathomechanisms is that lipotoxicity mediated by unsaturated fatty acids contributes to necrosis, OF (eg. cardiovascular diseases) and mortality[159]. Experimental studies also demonstrated that HTG exacerbates the severity of AP[159,160]. Fatty acid

administration resulted in elevated intracellular Ca^{2+} levels in pancreatic acinar cells and impaired mitochondrial function[161,162]. HTG-AP is often accompanied by one or more secondary factors (alcoholism, medications, uncontrolled diabetes mellitus, physical inactivity), which can further aggravate the severity of the disease[163–166]. Furthermore, elevated serum chylomicron concentration during HTG increases viscosity, causing reduced blood flow in microvessels and resulting in ischemic conditions. This could be an additional risk factor for a severe form of AP[161,162].

Determining the exact aetiology of AP may be challenging in some cases. For example, alcohol is not only known as an independent risk factor for AP but can also increase serum TG concentrations, as mentioned before. In addition, mild-to-moderate elevation in TG concentrations can be observed in the early phase of AP, regardless of aetiology[167]. Since TG concentrations can rapidly decrease during fasting state after the diagnosis of AP, the measurement of TG concentrations on (or shortly after) admission is crucial.

The number of events, which refer to positive outcomes in certain aetiologies were relatively high in case of severity (1516 severe events occurred out of 2556 HTG-AP patients in Fig. 2a) and partly in mortality (10,161 events/620,027 BAP patients in Supplementary Figure S13a) and recurrence rate outcomes (1671 events/5254 AAP patients in Supplementary Figure S10a). Smaller number of events (9–97) could be included in the analysis of other outcomes (Table 1). Low event rates can have detrimental influence on the reliability of the results[168,169]. Based on the studies mentioned above, the results of all severity comparisons, mortality and recurrence rates in comparisons of AAP vs. BAP are strongly reliable. Most of the other calculations have lower reliability but there is no precedent to contradict the results of severity.

The current meta-analysis has strengths and limitations that should be noted. The major strengths are the following: we included a large number of articles. Four major aetiologies were analysed, leaving out miscellaneous or idiopathic backgrounds. For the analysis of severity, we only included articles where severity was defined according to the RAC, which provided a clear and consistent base for the comparisons. In addition, we compared mild to moderately severe and severe ("non-mild") AP groups, which further refined our analysis. The quality of the involved articles determines the value of pooled data. There has been high variability in methodology of the studies which may have unintended effects on the final results and interpretation, study populations were diverse in age and gender, which might cause heterogeneity in aetiologic distribution. Aetiologies were not necessarily defined the same way. Certain outcomes (e.g. necrosis) were only evaluated by a limited number of studies, especially in case of HTG-AP, which may be the reason that no statistically significant difference could be detected between HTG-AP and other aetiologies or no statistical analysis could be performed. One article analysed data from 1975 to 2010, which was only applied for the assessment of recurrence rate. Another article contributed 1,165,777 patients to the analysis, which was only used for the evaluation of mortality. In addition, only articles published in English or Hungarian were included.

Conclusions

AP is a complex disorder mediated by metabolic, environmental and genetic factors, which can lead to death in the most severe forms. Therefore, clinicians should be more alert for a severe disease course in the at-risk patients. Our observations highlight the importance of disease aetiology. We found association between aetiology and the development and course of AP. HTG proved to carry the highest risk for non-mild (moderately severe and severe) AP, which was followed by AAP; the least severe disease forms were observed in BAP and PAP. It is essential to determine the cause of the disease in time to apply the most appropriate therapy. Based on the results, greater emphasis should be placed on determining aetiology on admission, especially in case of HTG-AP.

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Author contributions

E.R.B.: study planning, data collection, quality assessment, data interpretation, drafting manuscript, critical revision of the manuscript, G.F.: data collection, quality assessment, L.K.: planning and conduct of the study, critical revision of the manuscript, D.I.N., A.S.: statistical analysis, P.H.: conduct of the study, critical revision of the manuscript, Zs.Sz.: coordination of quality assessment, critical revision of the manuscript, B.T., P.V., Á.V., B.E., J.C., Z.Sz., G.V.: data interpretation, critical revision of the manuscript, Á.V.: data interpretation, critical revision of the manuscript, Z.R.: study concept, planning and conduct of the study, data interpretation, critical revision of the manuscript. All authors approved the final draft submitted.

Competing interests

The authors declare no competing interests.

Additional information

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