IMPORTANT THERAPEUTIC TARGETS IN ACUTE PANCREATITIS

Ph.D. Thesis

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I. PUBLICATIONS

I.1. Publications related to the subject:

Publication No.1.;

Emese Tóth, József Maléth, Noémi Závogyán,Júlia Fanczal, Anna Grassalkovich, Réka Erdős, Petra Pallagi, Gergő Horváth, László Tretter, Emese Réka Bálint, Zoltán Rakonczay Jr., Viktória Venglovecz, Péter Hegyi "Novel mitochondrial transition pore inhibitor N-methyl-4-isoleucine cyclosporin is a new therapeutic option in acute pancreatitis"

The Journal of Physiology (2019 in press)

Original publication , **IF**: **4.98, Q1**

Publication No.2.;

Viktória Venglovecz, Petra Pallagi, Lajos V. Kemény, Anita Balázs, Zsolt Balla, Eszter Becskeházi, Eleonóra Gál**, Emese Tóth**, Ágnes Zvara, László G. Puskás, Katalin Borka, Matthias Sendler, Markus M. Lerch, Julia Mayerle, Jens-Peter Kühn, Zoltán Rakonczay, Jr., and Péter Hegyi "The Importance of Aquaporin 1 in Pancreatitis and Its Relation to the CFTR Cl- Channel. "

Frontiers in physiology (2018) doi: 10.3389/fphys.2018.00854

Original publication, **IF: 3.394, Q2**

Publication No.3.;

Zoltan Rumbus*, **Emese Toth*,** Laszlo Poto, Aron Vincze, Gabor Veres, Laszlo Czako, Emoke Olah, Katalin Marta, Alexandra Miko, Zoltan Rakonczay, Jr., Zsolt Balla, Jozsef Kaszaki, Imre Foldesi, Jozsef Maleth, Peter Hegyi▪, and Andras Garami▪ "Bidirectional Relationship Between Reduced Blood pH and Acute Pancreatitis: A Translational Study of Their Noxious Combination"

Frontiers in physiology (2018) doi: 10.3389/fphys.2018.01360

Original publication, **IF: 3.394, Q2**

*****Authors share a co-authorship of this article, ▪ Authors share a co- last authorship of this article

I.2. Publication not related to the subject;

Andrea Szentesi, **Emese Tóth**, Emese Bálint, Júlia Fanczal, Tamara Madácsy, Dorottya Laczkó, Imre Ignáth, Anita Balázs, Petra Pallagi, József Maléth, Zoltán Rakonczay, Jr, Balázs Kui, Dóra Illés, Katalin Márta, Ágnes Blaskó, Alexandra Demcsák, Andrea Párniczky, Gabriella Pár, Szilárd Gódi, Dóra Mosztbacher, Ákos Szücs, Adrienn Halász, Ferenc Izbéki, Nelli Farkas, Péter Hegyi, and Hungarian Pancreatic Study Group¶ "Analysis of Research Activity in Gastroenterology: Pancreatitis Is in Real Danger"

Plos One (2016) doi: 10.1371/journal.pone.0165244

Original publication, **IF: 3.057, D1**

I.3. Scientific metrics:

Number of publications:	**4 (2 first authors)**
Cummulative impact factor:	**14.825**
Number of total citations (Google Scholar) https://scholar.google.com/citations?hl=hu&user=esjTpS , **Hirsch index**	**24** **2**
Number of total citations (MTMT2) https://m2.mtmt.hu/gui2/?type=authors&mode=browse&sel**, Hirsch index**	**19** **2**

II. LIST OF ABBREVATIONS

AP- acute pancreatitis,

NIM811- N-metil-izoleucine cyclosporine,

mPTP- mitochondrial transition pore, mitochondrial membrane potencial- ψ,

CFTR- cystic fibrosis transmembrane conductance regulator,

PDEC-pancreatic ductal epithelial cells,

Cyclophylin D- Cyp D,

CYA- cylosporin A,

Hepatitis C virus-HCV,

Debio025- Alispovirir,

Tro40303-3,5-seco-4-nor-cholestan-5-one-oxime-3-o,

TMRM- Tetramethylrhodamine Methyl Ester Perchlorate,

TOM20- Mitochondrial import receptor subunit**,**

FA- fatty acid (palmitoliec acid),

FAEE- Fatty acid ethyl ester,

ETOH- ethanol,

BA- CDC- chenodeoxycholic acid,

BCECF-AM (2',7'-Bis-(2-Carboxyethyl)-5-(and-6)-Carboxyfluorescein, Acetoxymethyl Ester),

CER-caerulein,

TAU- sodium taurocholate,

TBS- Tris Buffered Solution,

BSA- Bovine Serum Albumin,

HBSS- Hank1s Stock Solution,

CBD- Common Pancreatic Biliary Duct,

CCCP- Carbonyl cyanide 3-chlorophenylhydrazone

CP-Chronic panreatitis

AQP-aquaporin channel

MA-metabolic acidosis

MAP- mild acute pancreatitis

SAP- severe acute pancreatitis

III. INTRODUCTION

III.1. The physiology of the pancreas

The pancreas is a heterocrine gland which has endocrine and digestive exocrine function as well. It is a major organ of the digestive system, it functions as an exocrine gland by secreting pancreatic juice into the duodenum. The endocrine function of the gland is that , it regulates blood sugar levels, by secreting hormones such as insulin, glucagon, somatostatin and pancreatic polypeptide. The exocrine part of the pancreas include the acinar and ductal system. As an exocrine organ, the pancreas secretes 1.5–2 l of fluid daily[1, 2] which contains the inactive form of digestive enzymes and is high in HCO_3 – which is produced by the pancreatic ductal cells [2]. The main functions of this high in HCO_3 − fluid are:

- to provide an alkaline environment in the duodenum for the optimal function of digestive enzymes [3]
- to wash out the toxic factors (such as bile acids) from the ducts [4] to prevent the premature activation of zymogens[5]

III.2. Pathophysiology of acute pancreatitis

One of the leading gastrointestinal disorders which requires hospitalization in the United States is acute pancreatitis (AP) [6-8] . AP is generally mild, but in severe forms the mortality rate is unacceptably high (28%) [9]. Specific treatment of the patients is still unsolved, the current clinical therapy relies on fluid replacement, jejunal, or parenteral nutrition and intensive care in severe cases. **AP is one of the most challenging gastrointestinal disorders for several reasons**:

- its development is not fully understood [10, 11]
- it has no specific therapy [10, 11]
- the incidence rate of the disease is continuously increasing [12]
- The mortality of the disease is extremely high [9]

Henceforth, recently several investigations have been published concerning **the risk factors of AP and chronic pancreatitis (CP)**;

- obesity, hyperlipidemia and hypertension are independent risk factors for a few complications in AP [13]
- timing and optimization of ERCP indication in biliary AP patients is critical [14]
- pancreatitis-associated mortality is more common with advanced age [15]

- Comorbidities determine mortality whereas both comorbidities and aging predict severity of AP [16]
- Body-mass index correlates with severity and mortality in AP[17]
- The presence of hypertriglyceridemia worsens the course and outcome of AP [18]
- Preexisting diabetes elevates risk of local and systemic complications in AP[19]
- Misfolding cationic trypsinogen variant p.L104P causes hereditary pancreatitis [20]

Also, there have been some recent studies which were focusing on the details of AP treatment ; centralized care for acute pancreatitis significantly improves outcomes of the disease[21], C-reactive protein (CRP) within 24 h from the onset of pain is an inclusion criterion elevates event rates and reduces the number of patients required in trials on AP[22] and patients with suspected infection because of fever has no benefit from antibiotic therapy [23]. Moreover, the Goulash plus trial will examine risk and influencing factors leading to CP and identify the most useful measurable parameters. [24]

However, much less attention have been made on new therapeutic targets. AP is a multifactorial disease [10, 11] involving several types of cells, including acinar and ductal cells. **Concerning the molecular mechanism involved in the disease, it is known that the followings have important roles:**

- trypsinogen activation [11, 25]
- impaired autophagy [11, 25]
- excessive Ca2+ influx [11, 25, 26]
- calcineurin activation[11, 25]
- mitochondrial dysfunction[11, 25]
- cystic fibrosis transmembrane conductance regulator (CFTR) inhibition[11, 25]

Therefore, targeting one of these could lead to the first specific therapy in AP.

III.3. Targeting the mitochondrial transition pore as potential therapeutic target in AP

Mitochondrial dysfunction is one of the earliest events in the disease [27-30]. It has been revealed, that in acinar cells bile acids (BA) and ethanol and fatty acids (EtOH+FA) open the membrane transition pore (mPTP) channel via the cyclophilin D (Cyp D) subunit, and by keeping the channel opened mitochondrial depolarization, lower ATP synthesis and cell necrosis occur [29, 31, 32]. Yet, it is still a mystery how pancreatic ductal epithelial cells (PDEC) are affected. Nowadays, to experimentally inhibit mPTP (via Cyp D) cyclosporin A (CyA) is the only licenced compound [33] . However, the clinical use of CyA is questionable:

- A trial found that CyA could reduce the size and damage of myocardial infarction, but larger studies showed no beneficial effects [33-35].
- Debio025 (a CyA derivative, Alispovirir, Debiopharm) has been found useful against hepatitis C virus (HCV), but surprisingly, some of the patients developed pancreatitis, which ended up in a clinical hold on the global Debio025 trials [36, 37].
- TRO40303 (3,5-seco-4-nor-cholestan-5-one oxime-3-o, TROPHOS, Roche) is another mPTP inhibitor and it was not beneficial in a phase 2 trial of cardiac preservation following acute myocardial infarction, questioning its effectivity [33, 38, 39].
- Both Debio025 and TRO40303 have been described as useful in experimental models, but due to the clinical failures they did not reached higher levels of clinical trials in AP.
- Recently, a novel CyA A derivative; *N*-methyl-4-isoleucine cyclosporin (NIM811), was shown to be greatly beneficial in different experimental and clinical studies[40-45]. No toxicity or severe or serious adverse effects have been reported in the studies in which NIM811 were used, suggesting that it does not have severe immunosuppressant activity either [46].

III.4. Importance of pancreatic ductal fluid secretion

As mentioned before, clinical and experimental studies indicate that impaired ductal HCO_3^- secretion makes the pancreas more susceptible to inflammatory diseases such as AP or chronic pancreatitis (CP) [25, 47-50]. Interestingly, the available data about the pancreatic ductal water transport processes are much less than what is known about pancreatic ductal HCO_3- secretion, except the general fact that the movement of electrolytes is osmotically coupled to water flow. It is assumed by numerous studies that there is a physical interaction between the CFTR Cl^- channel and certain aquaporin (AQP) isoforms [51-53] Henceforth, colocalization of this two channel has been revealed in the human pancreas [54]. AQP1 is the major water channel of human red blood cells and in the digestive system the main result of AQP1 deletion is manifested in serum hypotriglyceridemia and steatorrhea with higher stool trygliceride concentration and increased lipase activity [55, 56].In the peritoneum the lack of AQP1 ends up in significantly reduced osmotical water transport. [55, 57-59] Howewer, there is only a few data available about AQPs in the pancreas and how these channels interact with other channels of the pancreatic ducts. During our study we aimed to characterize the pathophysiological and pathological role of AQPs in the pancreatic ductal secretion, one part of my dissertation focuses on the expression and possible interaction between CFTR and AQP1 channels in pancreatic ducts.

III.5. Alteration between acid-base balance and AP

AP is often co-occurred by alterations in the acid-base balance, however, how changes of blood pH influences the outcome of AP is still unknown. Acidosis is often considered as a marker of disease severity[60]. It is known that when pancreatic bicarbonate production is altered by local or systemic acid load (metabolic acidosis, MA), the resulting lower pH can trigger pancreatic enzyme activation and deteriorate cell damage [61]. Moreover, injection of acidic contrast solution into the pancreatic duct increased the severity of experimental AP in rats [62, 63]. Takács et al. have shown that in patients with AP the luminal pH of the main pancreatic duct was also lower compared to control human samples [48].

These suggest that may the development of AP is coupled with the decrease of local pH. It is important to mention, that there are some mechanisms which can take place during AP and can result in MA, for example during the occurrence of drainage or pancreatic fistula the HCO_3-rich pancreatic juice could be lost [64], or lactic acidosis could be manifested.[65] Lactic acidosis could happen during AP for example because of shock, sepsis, cardiovascular failure, or upper gastrointestinal bleeding[65] . Sadly, the interaction between AP and systemic pH is still not fully clarified. During our study we developed a new mouse model of chronic metabolic acidosis (MA) and induced mild (MAP) or severe (SAP) AP in the mice to study the alterations between the diseases. During our study, we measured laboratory parameters and quantified the extent of pancreatic edema, necrosis, and leukocyte. The discovery of how the metabolic acidosis affect the outcome of AP in animals could open new therapeutic ways in the treatment of AP. [66]

IV. AIMS

I. (Publication No.1.):

a.) Pancreatitis inducing factors open the membrane transition pore (mPTP) channel via cyclophilin D activation in acinar cells causing calcium overload and cell death. Notably, there is still no available data from how pancreatic ductal epithelial cells are affected by mPTP inhibition. **Therefore, we aimed to investigate how genetic and pharmacological inhibition of mPTP affects the function of pancreatic ductal epithelial cells.**

b.) Genetic or pharmacological inhibition of mPTP improves the outcome of acute pancreatitis in animal models. Howewer, clinical testing of different mPTP inhibitors were stopped before reaching the "proof of concept" phase 2 clinical trials due to severe problems of their effectiveness and/or safety. **Thereby, we aimed to test the novel Cyclosporin A derivative NIM811 during in vivo animal experiments.**

II. (Publication No.2.):

Decreased pancreatic ductal fluid secretion plays a critical role in AP. Therefore, our aim was to study the mechanisms and function of aquaporins which are involved in transepithelial water flow movements in epithelial fluid secretion in several types of tissues.

Specific aim: **To investigate the presence of AQP1 water and CFTR ion channels in mouse pancreatic tissue slices**.

III. (Publication No.3.):

Acid-base abnormality is common in acute pancreatitis (AP). Lowering extracellular pH deteriorates the manifestation of AP in rats and decrease of luminal pH in the pancreas contributes to the tissue damage in AP in mice. **Hence, our aim was to study effect of metabolic acidosis during the manifestation of AP in mice.**

Specific aim I.: **To develop a mouse model of metabolic acidosis in mice**

Specific aim II: **To study the effect of metabolic acidosis on experimental AP.**

V. MATERIALS AND METHODS

V.1. Ethics (Publication No.1.-3.)

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 . In our studies all animals were euthanized by 200 mg/kg pentobarbital i.p. (Bimeda MTC, Cambridge, Canada).

V.2. Solution and chemicals (Publication No.1.-3.)

All 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 Termofischer Scientific. NIM811 were purchased from MedChem Express Europe (Sweden). Cyclosporin A (CYA), caerulein (CER) , NIM811, CCCP and fluorescence dies were diluted in dimethyl sulfoxide (DMSO) . Table 1 describes the constitution of solutions that we used during the study.

V.3. Statistical analysis (Publication No.1.-3.)

All data are expressed as means ± SEM. Analysis were performed by either one- or two-way analysis of variance (ANOVA) or Kruskal–Wallis tests followed by the Holm–Sidak Method as appropriate (Sigma Plot). The effects were considered significant when $p < 0.05$.

V.4. Materials and methods used in publication No.1.

V.4.1. Animals

A total of 70 wild type (WT) and cyclophilin D knockout (Cyp D KO, (B6;129-Ppiftm1Maf/J) mice were sacrificed. *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). [67] Cyp D KO animals were provided for us by the Department of Medical Biochemistry, Semmelweis University, Budapest, Hungary. Wild type and Cyp D-deficient littermate mice (of C57Bl/6 J 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.

V.4.2. Chemicals

In this study 500μM Chenodeoxycholic acid (bile acid,BA) or 100mM ethanol (EtOH) + 200μM palmitoleic acid (fatty acid, FA) was 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 pharmachological inhibition of the mPTP in pancreatic ducts or acinar cells. 100 μM of Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) were used in the mitochondrional measurements as a positive control for mitochondrial damage. 2 μM CYA and 2 μM NIM811 were used to pharmacologically inhibit mPTP. Prior to the fluorescence and confocal microscopy, immunostainings, the cells (ducts and acinar cells as well) from the CYA- or NIM811- treated groups were pretreated for 25-30 minutes with the compounds (CYA or NIM811).

V.4.3. Isolation

Isolation of pancreatic ducts and acinar cells were performed by microdissection and enzymatic digestion as described earlier. [68, 69] Briefly, intra/interlobular ducts were isolated by enzymatic digestion and microdissection from the pancreas and cultured overnight.

V.4.4. Confocal microscopy

Mitochondrial membrane potential (Ψ) were determined by 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 (Tetramethylrhodamine Methyl Ester Perchlorate ,100 nmol/L).

In order to monitor apoptotic and necrotic cells in isolated pancreatic ducts or acinar cells an apoptosis/necrosis kit was used (ab176750, Abcam). To determinate live, necrotic or apoptotic cells, CytoCalcein Violet 450 fluorescent, Apopxin Deep Red Indicator and Nuclear Green DCS1 fluorecence dies (ab176750, Abcam) were used. Samples were incubated in the mixture of the above stated fluorescence dyes at room temperature for 30-35 mins (after 25 min treatment of with BA/ETOH+FA/CYA/NIM811) in dark prior to the confocal microscopy measuremets. In case of CYA or NIM811 treated ducts or acinar cells, the incubation with these compounds were performed before staining with the fluorescence dyes. Stainings were analyzed using a Zeiss LSM 880 confocal laser scanning microscope (Carl Zeiss Technika Kft., Budaörs, Hungary). Live, necrotic or apoptotic cells were counted and summarized in

percentage of each sample, then data were summarized to average and statistical analysis was performed.

V.4.5. Fluorescent microscopy

Microfluorometry was used to measure pancreatic ductal HCO_3^- secretion as described earlier [70, 71] by using BCECF-AM (2',7'-Bis-(2-Carboxyethyl)-5-(and-6)-Carboxyfluorescein, Acetoxymethyl Ester, 1.5 mmol/L).

V.4.6. Videomicroscopy

In vitro pancreatic ductal fluid secretion (luminal swelling) assays were developed by Fernández-Salazar et al, [72] performed by videomicroscopy as described earlier [73]. Briefly, stimulaton of pancreatic ductal fluid secretion was induced by 5 μM forskolin and 100 μM 3-isobutyl-1-methylxanthine (IBMX) , quantification were performed by Image J Software [73]. In vivo fluid secretion measurements were performed on anesthetized (by i.p. 87.5 mg/kg ketamine-12.5 mg/kg xylazine) mice after CER or EtOH+FA induced AP prior to euthanasia. Animals were placed on warm pads (37◦ C) to maintain the body temperature. Briefly, the abdomen of the mice were opened and cannucaltion of the lumen of the common biliopancreatic duct was performed by a 30-gauge needle[50] . Then 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 PE-10 tube for 15 min. In vivo secretion was induced by i.p. administration of 0.75CU/kg secretin [50].

V.4.7. Immunfluorescent staining

Mitochondria were detected with immunofluorescent staining (TOM20 mitochondrial marker, (EPR15581-39, Abcam)). In order to determine mitochondrial localization in isolated pancreatic ductal or acinar cells we labeled the mitochondria by the using of TOM20 primary antibody (Abcam, EPR15581-39). TOM20 is the central unit of the receptor TOM complex in the mitochondrial outer membrane and the role of it is to recognize and translocate cytosolically synthetized mitochondrial preproteins [74-76] 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 by Leica Cryostat. Sections were fixed in 4% paraformaldehyde. Washing periods were administered with 1xTBS solution. Antigen retrieval was performed with 10 mM Sodium –Citrate solution at the pH of 6 at 95 ˚C for 15 minutes. Blocking was obtained for 1h with 1% goat serum in 5% BSA-TBS solution. After these sections were incubated with TOM20 rabbit monoclonal antibody (dilution 1:400, Abcam) overnight incubation at 4˚C. The

following day the samples were incubated with goat anti rabbit secondary antibody (Alexa fluor 488, Thermo Fisher, Rockford, IL, United States) for 2 hours at dark in room temperature. The nuclei were counterstained with Hoechst 33342 (Termofischer, Rockford,IL,United States) . Immunofluorescence staining of the isolated pancreatic acinar cells were performed freshly after the isolation procedure with the same conditions as stated above, (except two parameters ; cells were fixed in 2% paraformaldehyde and dilution fo the primary antibody was 1:200) as stated above. Both ductal and acinar cell samples were mounted with Fluoromount and then analyzed using a Zeiss LSM 880 confocal laser scanning microscope (Carl Zeiss Technika Kft., Budaörs, Hungary). To quantify TOM20 positively stained area, 5-6 representative images from each group were taken by Zeiss LSM 880 Confocal Scannig Microscope (Carl Zeiss Technika Kft., Budaörs, Hungary). Image J software was used to convert images to gray scale (16 bit), threshold function was used to select the positively stained area. The fluorescence signal were calculated by the software (arbitary scale from 0-negative (white) to 255-maximal staining (black) [77]. Fluorescence intensity of the images were then normalized to the own total ductal or acinar area of the samples, which were measured in arbitary units. Fluorescence intensity was given in %, normalized to the total ductal or acinar total area.

V.4.8. In vivo measurements

V.4.8.1. Induction of acute pancreatitis

AP was induced by caerulein (CER,10x50µg/kg) and 4% sodium taurocholate (TAU, 2ml/kg,4%)[49, 78-80]. We also performed alcohol and fatty acid (intraperitonal injection of 1.75 g/kg ethanol and 750 mg/kg palmitic acid , EtOH+FA) induced AP as described earlier [50, 81], however it is not part of this dissertation. All control groups received physiological saline in the same amount as the CER, EtOH+FA or the TAU solutions respectively.

V.4.8.2. Oral gavage treatment of the mice

Oral gavage treatment was performed using plastic feeding tubes (20ga x 38mm, Instech Laboratories, USA). NIM811 were solubilized in a vehicle which contained 8.3% polyoxyl 40 hydrogenated castor oil and 8.3% ethanol [43]. Pre-treatment of the animals by NIM811 was performed and mice were gavaged orally once 1 h prior to the induction AP, concentrations of NIM811 were 10 mg/kg or 5mg/kg. Dosage of NIM811 was chosen according to a previous study in which NIM811 was effective against mitochondrial damage in liver transplantation [43].

Besides the pretreatment, NIM811 was used as a post-AP treatment as well. NIM811 was administered 12 hours 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 3rd injection of CER. The method for retrograde intraductal infusion of TAU has been described by Perides et al [80]. The surgery was performed on anesthetized mice (with ketamine-xylazine, dosage: 87.5 mg/kg ketamine-12.5 mg/kg xylazine). At the end of the procedure the mice were placed on a heating pad for 40 minutes and received buprenorphine i.p. injection (0.075 mg/kg) at once to reduce their occurrent pain. Following these mice were replaced into their cages for 24hours. They had free access to food and water. 24 hours after the TAU or EtOH +FA induced AP the mice were euthanized by 200 mg/kg pentobarbital i.p. (Bimeda MTC, Cambridge, Canada). During the CER induced AP mice were euthanized with 200 mg/ kg pentobarbital i.p. (Bimeda MTC, Cambridge, and Canada) 2 hours after the last injections of CER. Mice were exsanguinated through cardiac puncture and the pancreas were removed.

V.4.9. Serum amylase measurements

We collected blood from the mice by cardiac puncture, blood was immediately placed on ice, then centrifuged with 2500 RCF for 15 mins 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 hematoxylin –eosin staining was performed. A colorimetric kit was used to measure serum amylase activity (Diagnosticum, Budapest, Hungary). Absorbance of the samples were detected at 405 nm with the use of FLUOstar OPTIMA (BMG Labtech, Budapest, Hungary) microplate reader.

V.4.10. Histological analysis

Formaldehyde-fixed pancreas samples were embedded in paraffin, then were cut into sections (3 μm) and hematoxylin-eosin staining were performed by using a standard laboratory method. To quantify histological differences a semiquantitative scoring system was used as Kui et al described previously [82].

V.5.Materials and methods used in publication No.2.

V.5.1. Animals

CFTR knock out (KO) (background FVB/N) mice were kindly provided by Dr. Ursula Seidler (Hannover Medical School,Hannover,Germany). AQP1 KO (background CD4) (mice were

supplied by Dr. Alan Verkman (University of Carolina, CA,Unites States) and Dr. Alastair Poole (University of Bristol,United Kingdom). Animals were kept in a room maintained at 23±1°C on a 12 h light–dark cycle with food and water available ad libitum. CFTR specific laboratory chow were administered to CFTR KO animals. Experiments were performed on 12-16 weeks old mice (sex ratio 1:1) from litter-matched groups.

V.5.2. Immunfluorescent stainings and detection of AQP1 and CFTR channels in mouse pancreas

7 μm thick cryosections from WT, AQP1, and CFTR KO mice pancreas were fixed in 2% paraformaldehyde. Permeabilisation of the slices occured in 10% Tween 20-sodium citrate, they were blocked with 5% goat serum. Immunofluorescent double staining for AQP1 mouse monoclonal antibody (1:500 dilutions; Thermo Fisher, Rockford, IL, United States) and CFTR rabbit polyclonal antibody (1:100 dilutions; Alomone Labs, Jerusalem, Israel) were performed by overnight incubation at 4◦C. After the washing periods, slices were incubated with secondary antibodies goat-anti-mouse (Alexa fluor 488, Thermo Fisher, Rockford, IL, United States) and goat-anti-rabbit (Alexa fluor 568, Thermo Fisher, Rockford, IL, United States) for 120 minutes at room temperature in the dark. Nuclei staining were performed with the use of DAPI fluorescent dye. Results of the immunostaining were then analyzed using a Zeiss LSM 880 confocal laser scanning microscope (Carl Zeiss Technika Kft., 10–12 representative pictures were taken from the mice (WT, AQP1 KO and CFTR KO) pancreas sections. Digital images were taken at 40x magnification. Samples were excited at 405 (DAPI), 488 (Alexa fluor 488) and 568 (Alexa fluor 568) nm and emissions were collected at 453, 516, and 603 nm, respectively. Following this step, Image J software were used and images were then converted to gray scale (16-bit) and thresholded to select the positively stained area, using the ImageJ software. The fluorescence intensity (mice pancreas) were arbitrary scaled from 0 to 255, where 0 were the negative staining (white pixels) and 255 were the maximal staining (black pixels). In the mice pancreas slices, the ductal area was selected and the total fluorescence intensity was calculated and summarized which was then normalized to the ductal area (μm2) and expressed in arbitrary units/μm2 .[77]

V.6. Methods used in Publication No.3.

V.6.1. Animals

We performed our experiments on female FVB/N mice (Charles Rivers Laboratories, Wilmington, MA, USA). The mice were housed in standard plastic cages which were kept in

room temperature of 23-24 °C and kept in 12 h light-dark cycle in the animal facility of First Department of Medicine, University of Szeged. Animals could free access of water and standard laboratory chow (Biofarm, Zagyvaszántó, Hungary).

V.6.2. Development of the new model of MA in mice

To develop a mouse model of chronic MA, the mice were randomly divided into the following 4 groups for a 12-day treatment:

- ammonium chloride (NH_4Cl^-) administration with drinking water (8.2 ± 0.5 ml/day/mouse) as described earlier [83, 84]
- intraperitoneal (i.p.) injections of NH_4Cl^- (0.5 ml, 0.28 M) on days 1 and 6;
- administration of NH_4Cl^- with drinking water (as in group 1) and i.p. injections (as in group 2);
- and controls, receiving NH_4Cl^-free tap water and 2 i.p. injections of saline on days 1 and 6.

V.6.3. Induction of AP

Severe AP (SAP) was induced by caerulein (CER,10x50µg/kg), CER was administered i.p.[78]. Mild AP was induced by alcohol and fatty acid (i.p. of 1.75 g/kg ethanol and 750 mg/kg palmitic acid , EtOH+FA) as described previously [50, 81]. During the experimental model of MA, MAP and SAP were induced on day 12 of the acidifying treatment.

V.6.4. Measurement and histological analysis

Laboratory parameters from blood serum and urine were performed by standard methods at the Institute of Laboratory Medicine, University of Szeged. Serum amylase measurement and histological analysis were performed as described in the previous chapters respectively. For blood gas pH measurements, samples of arterial blood (170µl) were collected from the mice in heparin and lithium treated and sealed plastic capillaries. Analysis of the arterial blood was performed by blood gas analyser (Cobas 221, Roche Ltd., Basel,Switzerland) within 1 minute after the blood collection (at room temperature 22ºC).

VI. RESULTS

VI.1. Results of publication No.1.

VI.1.1. Genetic inhibition of mPTP protects mitochondrial homeostasis and cell function evoked by pancreatitis-inducing factors in PDEC

As a beginning, we studied the effects of the most relevant pancreatitis-inducing factors on mitochondria in primary intact ducts isolated from *Ppif*$^{-/-}$ and WT mice. Experiments performed with TMRM and TOM20 revealed that genetic inhibition of mPTP decreased both the loss of $\Delta\psi$ (Fig. 1A) and mitochondrial mass (Fig. 1B) caused by BA or co-administration of EtOH+FA. Genetic inhibition of mPTP also decreased the extent of necrosis and apoptosis during the administration of BA or EtOH+FA (Fig. 1C), suggesting that genetic inhibition of Cyp D has a protective effect on PDEC. Next, we investigated how the genetically preserved mitochondrial function affects the cellular function of PDEC (Fig. 1D). 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) are totally blocked in *Ppif*$^{-/-}$ vs WT mice, suggesting that inhibition of mPTP can preserve ductal function and thus has therapeutic benefits (Fig. 1D–F).

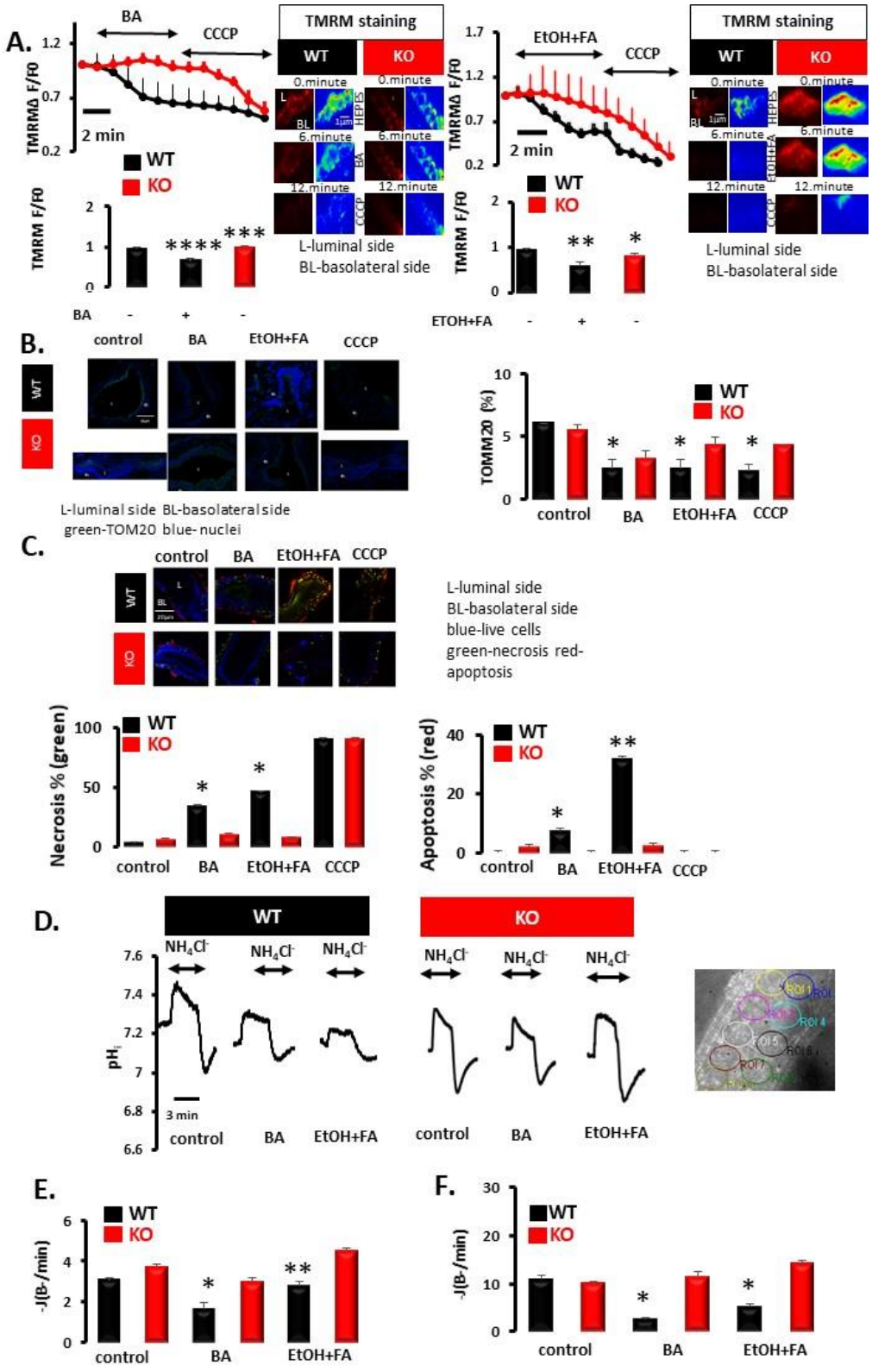

Figure1. Genetic inhibition of Cyp D reduces the severity of bile acid or ethanol and fatty acid induced damage in PDEC

Genetic inhibition of mPTP significantly reduces the mitochondrial membrane potencial loss compared to WT controls during the administration of BA or EtOH+FA (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=07.12,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/group, data means ±SEM. Results from the immunostainings revealed a significant decrease of the TOM20 stainings in BA; EtOH+PA or CCCP treated WT ducts , results were compared to Cyp D KO stainings . (Fig1.B) (*p<0.05). Genetic inhibition of mPTP also decreased the necrosis and apoptosis levels during bile acid; ethanol or fatty acid or CCCP treatment (Fig1.C). (*p<0.05) Representative traces from the pancreatic ductal HCO_3- secretion measurements (Fig.1.D) Our data revealed that recovery from the alkalosis grades were significantly lower due to BA or ETOH+FA administration (*p<0.05) compared to the results from Cyp D KO ducts (Fig1.E). Recovery from the acidosis grades were significantly lower in the WT ducts due to the treatment with BA or EtOH and FA (*p<0.05), while in Cyp D KO ducts these grades were significantly higher (*p<0.05) . n=5-7 experiments/group, data means ±SEM.

VI.1.2. Pharmacological inhibition of mPTP by CyA effectively prevents mitochondrial damage evoked by pancreatitis-inducing factors in PDEC.

BA and EtOH+FA significantly decreased the ψ of PDEC (Fig. 2A). Importantly, 2μM 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 we could see during the genetic inhibition of mPTP (Fig. 2B). 2μM CYA decreased the extent of necrosis and apoptosis during the administration of BA or EtOH+FA in PDEC (Fig. 2C). 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. 2D–F).

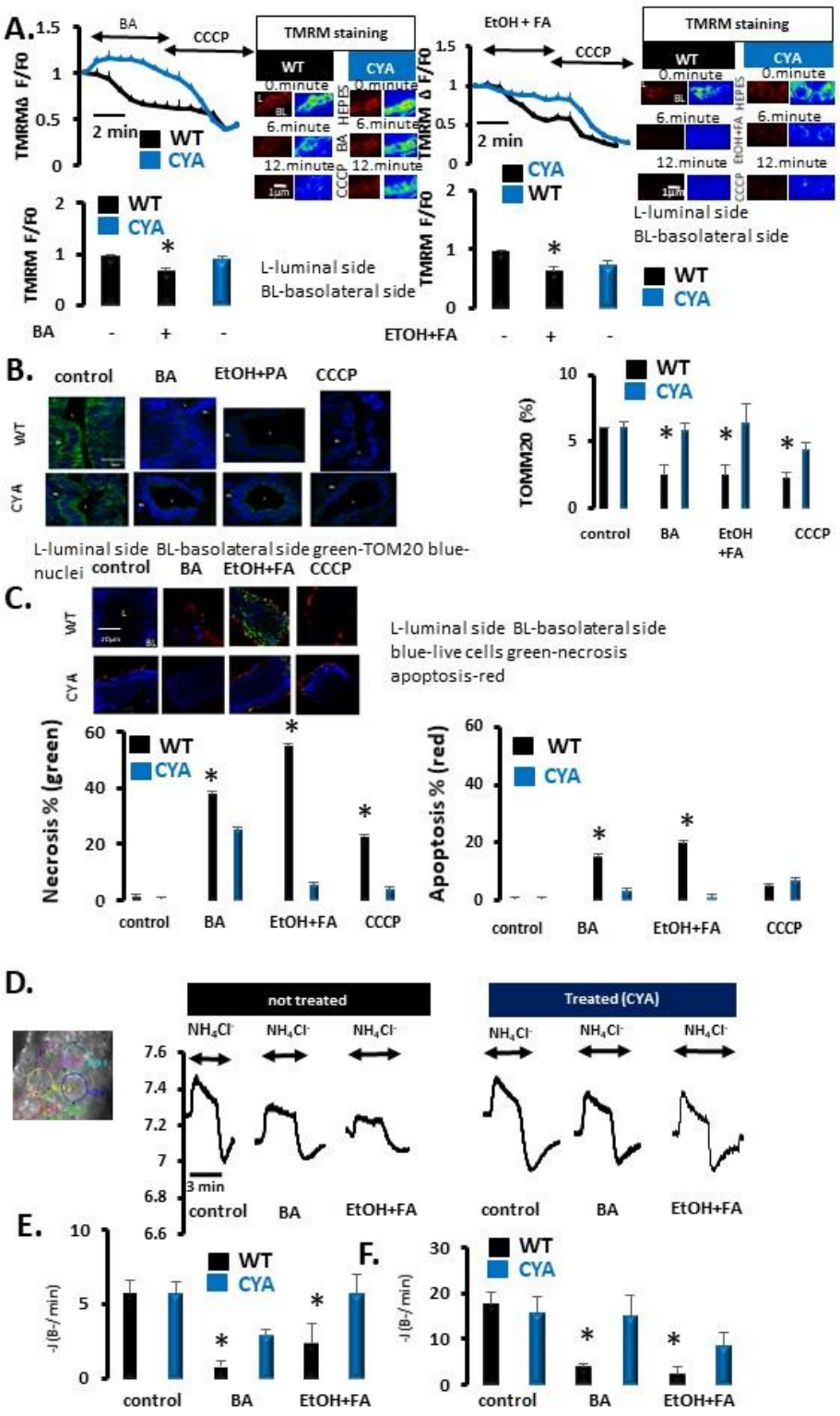

Figure2. CYA reduces the severity of bile acid or ethanol and fatty acid induced pancreatic ductal damage Treatment of the ducts with CYA reduced the drop of mitochondrial membrane potencial loss which accured due to the BA or ETOH+FA treatment. (WT vs. CYA) (Fig.2.A). In WT ducts BA or ETOH+FA treatment 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 were no significant decrease. 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 were significantly higher (Fig2.B) *p<0.05. Between the control groups (WT control or only CYA treated samples) we found no significant alterations in the stainings. Necrosis levels were intensively elevated in BA or EtOH treated groups in WT ducts but not in CYA treated groups (Fig.2.C). Apoptosis levels were significantly higher as well in the not CYA treated groups compared to the CYA treated groups (Fig2. C). Measurements of HCO_3^- secretion levels revealed a significant difference in WT and CYA treated ducts during the administration of BA ($p<0.05$ WT BA vs CYA BA) or EtOH+FA (*$p<0.05$). In WT ducts the levels of base flux (-J(B-/min) grades were significantly decreased (Fig2.E,F) due to BA (WT vs WT BA $p<0.05$) or ETOH+FA (WT vs WT EtOH+PA $p<0.05$) treatment (Fig2 E,F). Recovery from alkalosis (Figure 2. E) and recovery from acidosis values are presented in base flux ((-J(B-/min) grades respectively, with ±SEM. Comparison within CYA treated groups revealed no significant difference (CYA control vs CYA BA $p=0.644$).

VI.1.3. NIM811 treatment protects mitochondrial function and bicarbonate transport mechanisms in PDEC

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. 3A–B). NIM811 alone has no toxic effects on PDEC. Furthermore, it can strongly decrease BA- or EtOH-FA-evoked necrosis and apoptosis (Fig. 3C). 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 PDEC (Fig. 3D).

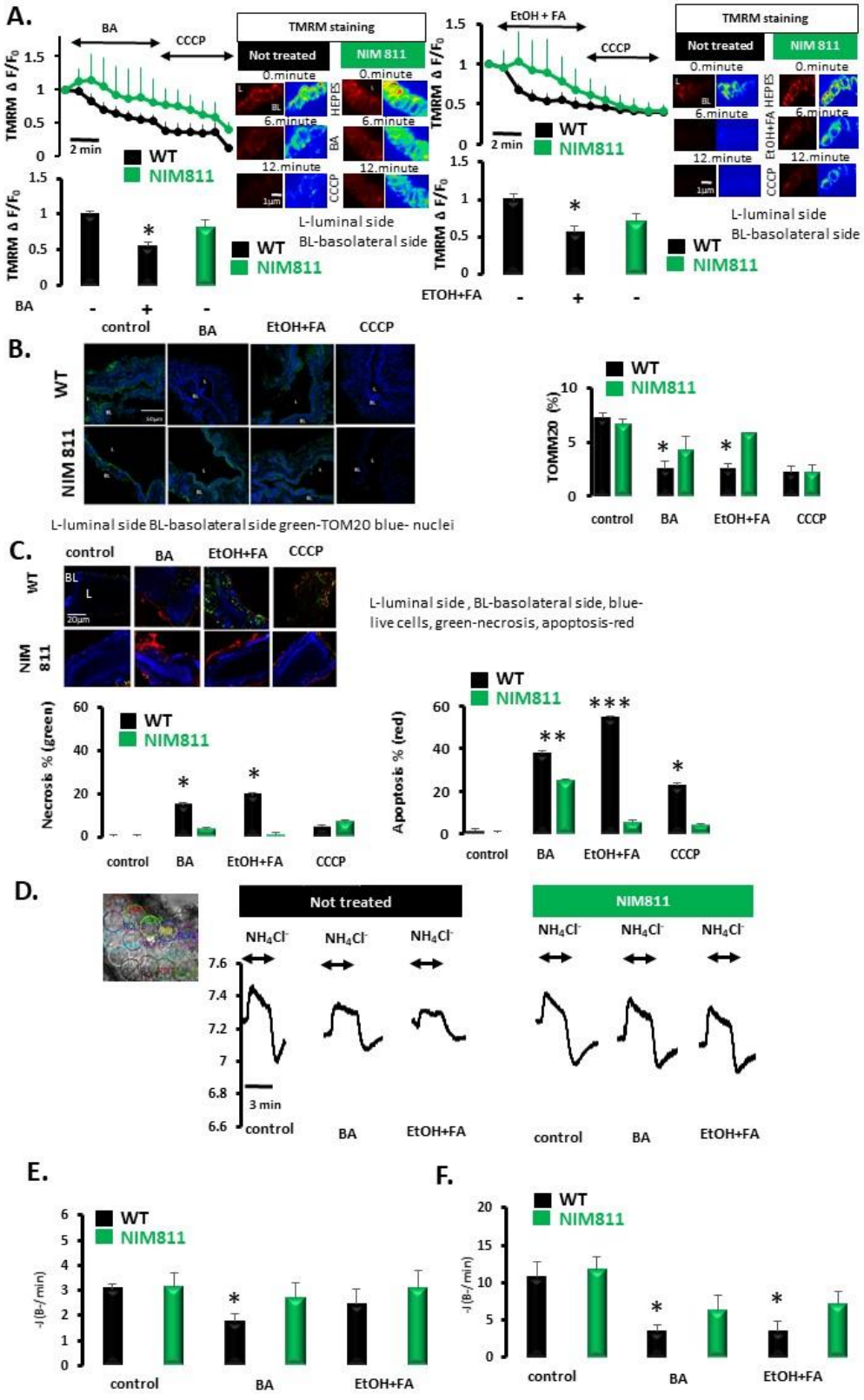

Figure3. NIM811 protects mitochondrial and cell function in PDEC. NIM811 treated ducts revealed a significantly consolidated loss of mitochondrial membrane potencial during the BA

(WT BA vs NIM811 BA *p<0.05) or ETOH+FA (WT ETOH+FA vs NIM811 ETOH+FA *p<0.05) treatment (Fig.3A) . In NIM811 treated ducts the percentage of fluorescence intensity were significantly higher compared to not NIM811 treated ducts during BA or ETOH+FA administration. In CCCP treated ducts we found no significant difference in the amount of TOM20 stainings in the aspect of NIM811 treated or not treated groups. NIM811 itself did not alter the value of TOM20 stainings compared to the WT control samples (Fig.3B). NIM811 decreased the numbers of apoptotic and necrotic cells during bile acid or ethanol and fatty acid treatment (Fig.3C) (WT BA vs NIM811 BA *p<0.05, WT EtOH+FA vs NIM811 *p<0.05). While during the administration of CCCP the apoptosis and necrosis grades were not significantly different in the comparative groups (Fig.3.C). NIM811 treatment did not decreased the HCO_3^- secretion grades (control, Fig.3 D,E,F) , while during the adminsitration of BA or ETOH+FA treatment it had a protective effect against the reduction of HCO_3^- secretory levels (Fig.3E/F) (WT BA vs NIM811 BA *p<0.05, WT EtOH+FA vs NIM811 EtOH+FA *p<0.05). In the aspect of recovery levels from alkali load during EtOH and FA treatment , the difference were not sigfnificant in WT EtOH+FA compared to the NIM811 and EtOH+FA treated groups (Fig.3E).

VI.1.4. Pharmachological inhibition of mPTP have no effects on pancreatic ductal fluid secretion

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

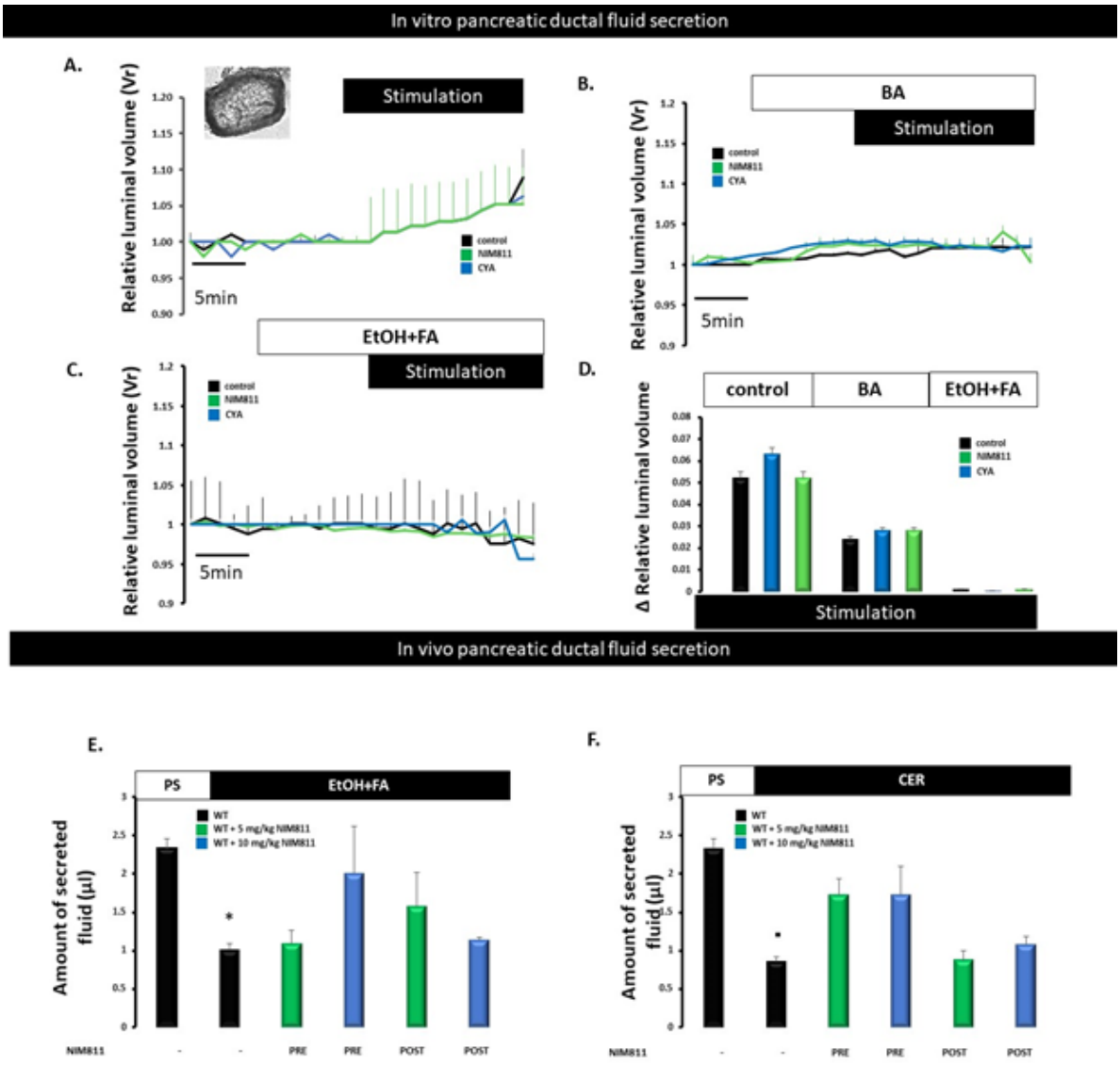

Figure 4. Pancreatic ductal fluid secretion is not altered by NIM811 or CYA treatment . In vitro fluid secretion was stimulated by 5µM forskolin and 100µM IBMX (stimulation). BA or EtOH+PA treatment inhibited the luminal swelling (Fig.4.B-C). Figure 4D represents the relative luminal volume changes during forskolin and IBMX stimulation (Figure4.D). Means ±SEM. n= 5-10 ducts/group. In vivo fluid secretion measurements were performed after the induction of CER or EtOH+FA induced AP (Fig.4.E-F.). These experiments confirmed that pancreatic ductal fluid secretion is not affected by NIM811 or CyA. (Fig.4.E-F). *p<0.05 WT PS vs. WT EtOH+FA, ▪p<0.05 WT PS vs. WT CER n=4-7 animal/group

VI.1.5. 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 we have seen in PDEC (Fig. 4A). However, results obtained from TOM20 staining suggest that NIM811 has no effect

on mitochondrial mass in acinar cells (Fig. 5B). 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. 5C).

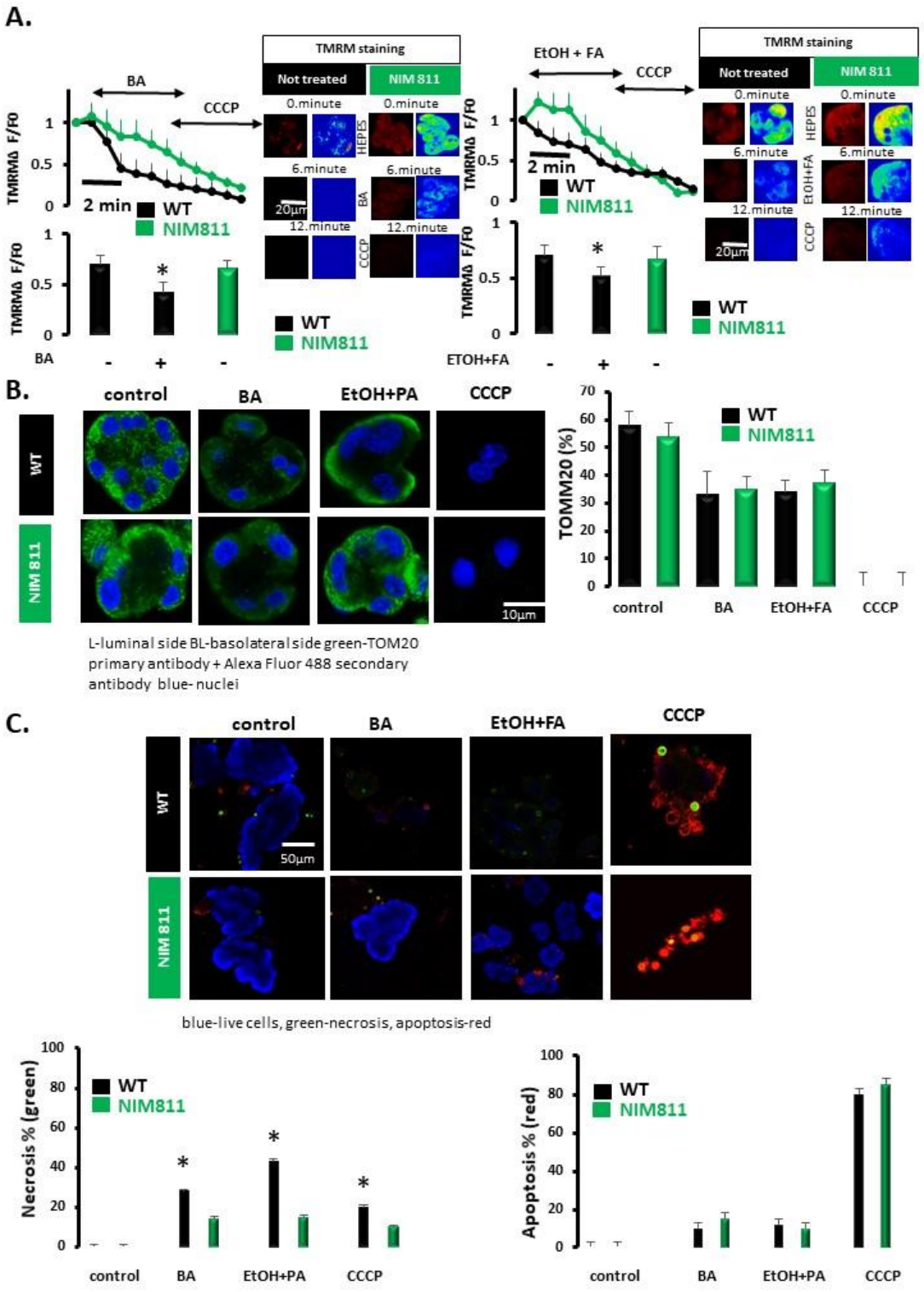

Figure5. NIM811 treatment protects mitochondrial function in pancreatic acinar cells

Mitochondrial membrane potencial measurements revealed a significant difference between WT not NIM811 treated and the NIM811 treated acinar cell response due to bile acid or ethanol and fatty acid treatment (Fig.5A) (WT BA vs NIM811 BA *p<0.05; WT EtOH+FA vs NIm811 ETOH+FA * p<0.05). Significant difference was detected between the NIM811 treated acinar cells and the groups which were not treated with NIM811 (Fig.5A) during BA or ETOH+FA treatment. Mitochondrial protein TOM20 levels did not show difference in the NIM811 treated or not treated groups after BA, ETOH+FA or CCCP treatment (Fig.5B) (p>0.05). In necrosis levels we found significant difference between NIM811 treated and not treated groups in BA or ETOH+FA (Fig.5C) (*p<0.05). However, in CCCP treated groups we found no difference (Fig.5C). Apoptosis levels were not altered significantly by NIM811 during BA or ETOH+FA treatment.

VI.1.6. NIM811 reduces the severity of AP in mice

Firstly, we confirmed that per os administration of either 5 or 10mg/kg NIM811 alone has no toxic effect on the pancreas (Fig 6.) .

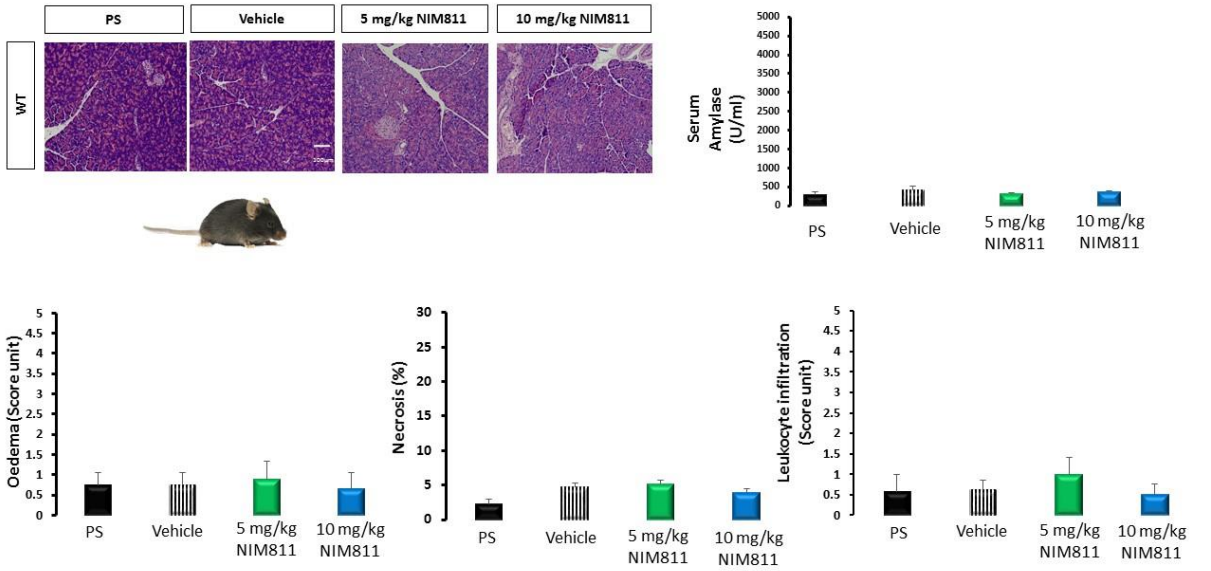

Figure6. NIM811 itself does not induce pancreatic damage . No significant difference was found between the NIM811-treated - (8.3% Polyoxyl 40 hydrogenated castor oil, 8.3% EtOH) vs. the control groups. n=4-5 animal/group

Secondly, we tested the compound in two different experimental AP models, the caerulein (CER) and the taurocholic (TAU)-induced ones (Niederau et al,1985; Huang et al, 2014) . Importantly, both pretreatment 5 or 10mg/kg NIM811 significantly reduced the elevation of

serum amlylase activity, as well as pancreatic oedema, necrosis and leukoctye infiltration in experimental AP models (Figs. 7–8). In our study we also confirmed, that post treatment of 5mg/kg or 10 mg/kg NIM811 has protective effects against pancreatic damage (Figs. 7-8.).

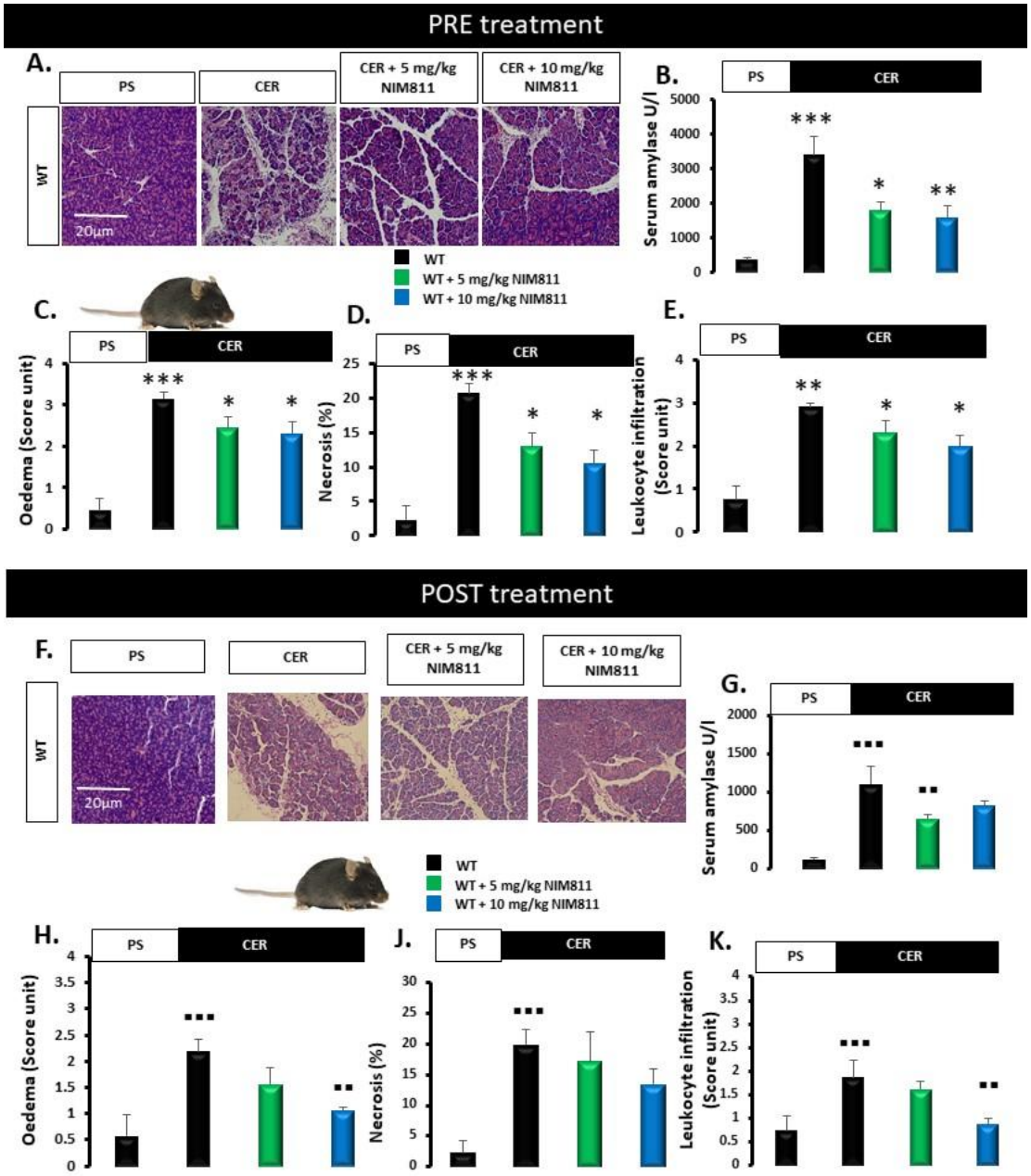

Figure 7. NIM811 reduces the severity of CER induced AP

Serum amylase levels were increased in the CER treated groups and NIM811 treatment resulted in a reduced serum amylase levels during CER induced AP compared to WT CER group (Fig. 7B ***p<0.01 WT PS vs WT CER, **p<0.02 WT CER vs pre10mg/kg NIM811 CER, *p<0.05

WT CER vs pre 5mg/kg NIM811 CER, p=0.717 CER+ pre 5mg/kg NIM811 vs CER + pre 10mg/kg NIM811). In CER induced pancreatitis both 5 mg/bwkg NIM811 (Fig.7 A-F, $p<0.05$ WT CER vs. pre 5mg/bwkg NIM811 CER) and pre 10 mg/bwkg NIM811 (Fig.7 A-F, $p<0.05$ WT CER vs. Pre 10mg/bwkg NIM811 CER)) treatment reduced the CER-induced damage. Post 5mg/kg NIM811 treatment significantly reduced serum amylase levels compared to WT CER ▪▪$p<0.05$, ▪▪▪$p<0.001$ WT PS vs WT CER (Fig.7G-E). Post insult administration of 10mg/kg NIM811 significantly reduced oedema and leukocyte infiltration levels compared to WT CER treated groups ▪▪$p<0.05$ (Fig.7H), n=8-10 animals per group, data means ±SEM).

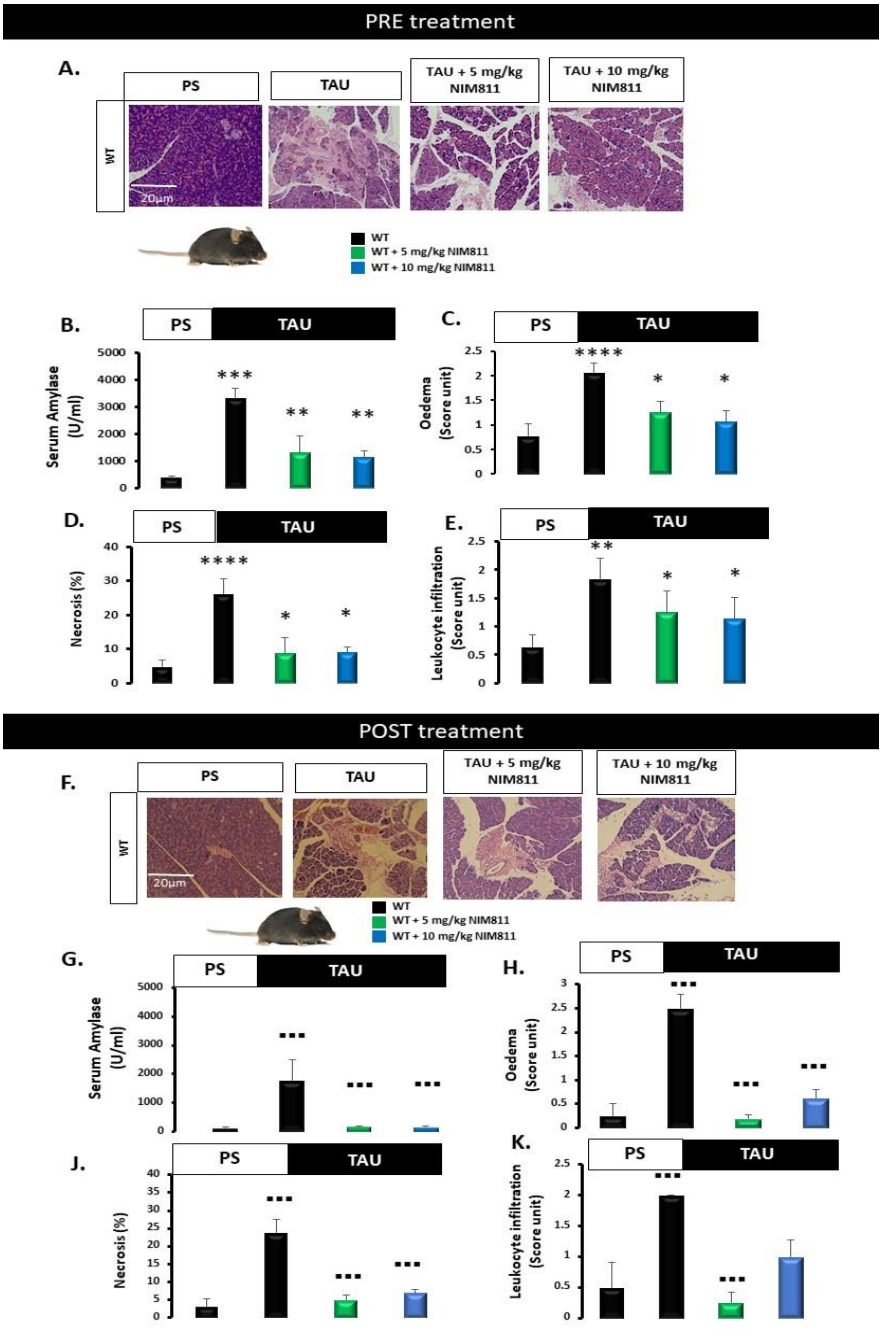

Figure8. NIM811 reduces the severity of TAU induced AP in mice.

We performed TAU induced pancreatitis(Fig.8A-K), serum amylase measurements revealed that due to retrograde infusion of TAU elevated serum amylase levels occured (***p<0.01 WT PS vs WT TAU Fig.6.B, ▪▪▪p<0.001 WT PS vs WT TAU Fig.8G) however 5 mg/bwkg or 10 mg/bwkg NIM811 treatment significantly reduced the enzyme levels both in the pre and post treatment (Fig. 8B **p<0.02 WT TAU vs pre 5mg/kg NIM811+TAU , ** p<0.02 WT TAU vs pre 10mg/kg NIM811+TAU, , ▪▪▪p<0.001 WT TAU vs. post 5mg/kg NIM811 TAU, ▪▪▪p<0.001 WT TAU vs post 10mg/kg NIM811 +TAU) the serum amylase levels were reduced compared to WT TAU treated groups (Fig.8B. and 7G *p<0.01 WT TAU vs. WT 5mg/bwkg NIM811 TAU and *p<0.01 WT TAU vs WT 10 mg/bwkg NIM811 TAU) . During pre NIM811 treatment oedema, necrosis and leukocyte infiltration scores were significantly decreased compared to the only TAU treated groups (Fig.8A,C,D,E p<0.05 WT TAU vs pre 5mg/bwkg NIM811 TAU/10mg/bwkg NIM811 TAU). Post insult administration of NIM811 decreased oedema, leukocyte infiltration and necrosis levels in the TAU group (▪▪▪p<0.001 Fig.8G-K) n=4-6 animals per group, data means ±SEM).

VI.2. Results from publication No.2.

VI.2.1. Aquaporin1 and CFTR expression in mouse pancreas

We performed immunostaining on the pancreas samples from AQP1 and CFTR KO mice in order to characterize the possible relation between the two channels. In WT mice, AQP1 expression was detected throughout the whole plasma membrane, whereas expression of CFTR exclusively localized to the apical membrane of the ducts (Fig.9A). The absence of AQP1 caused a slight but not significant decrease in the expression of CFTR, indicating that the impaired HCO_3^- secretion in the AQP1 KO mice is not due to the decreased expression of CFTR (Fig.9A). Interestingly, we have found that expression of AQP1 significantly decreased in the ducts of CFTR KO mice, especially at the apical membrane (Fig. 9A and Fig. 9B). During this study, we have also found that the absence of CFTR did not affect the expression of AQP1 in the blood vessels indicating that some kind of interaction may exist between these two channels in the pancreatic ductal cells (data is not part of the dissertation). Although further investigations are needed to clarify whether the two channels are able to regulate each others function, expression or trafficking.

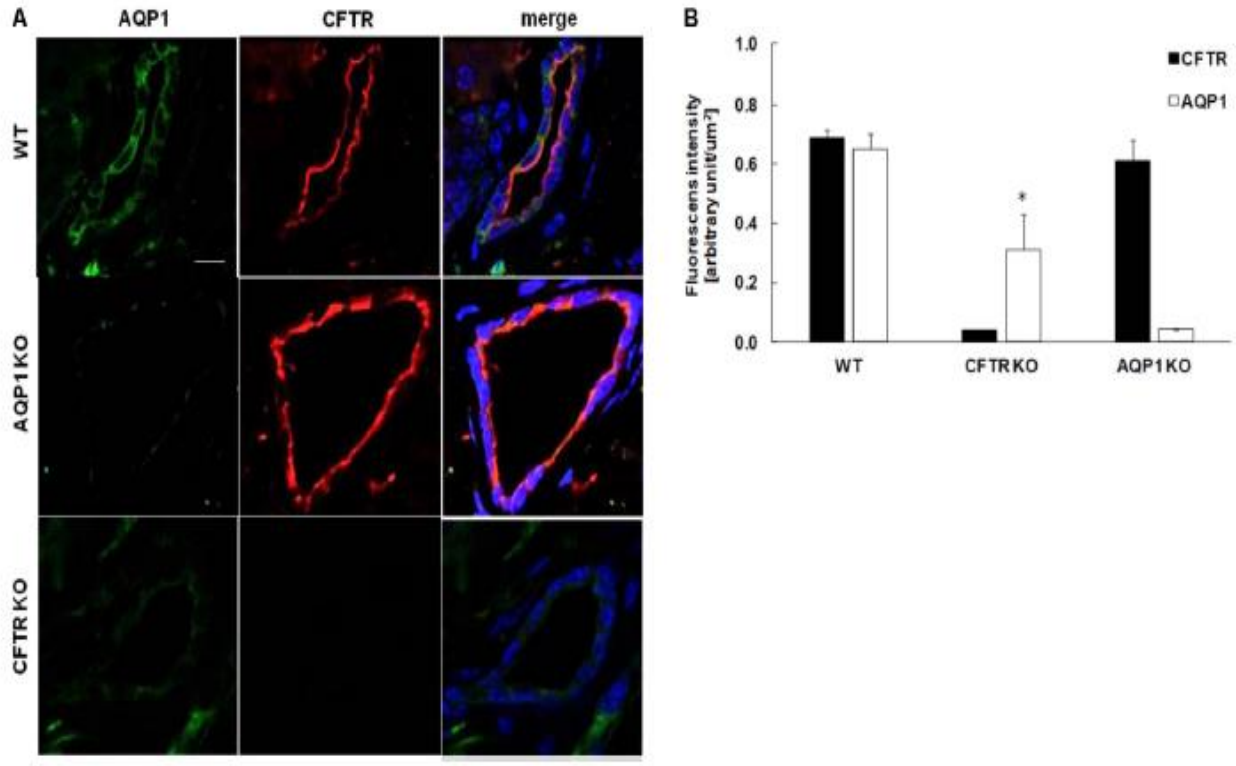

Figure 9. Expression of Aquaporin1 and CFTR in mouse pancreas. Expression of AQP1 and CFTR in the pancreas of KO. (A) Representative immunofluorescence staining of AQP1 and CFTR in wild type , AQP1 knock out and CFTR KO mice. (B) Summary bar chart shows the mean fluorescence intensity in the ductal cells normalized to the ductal area and expressed in arbitrary. Scale bar represents 10 mm. Data are presented as means ±SEM. *p <0.05 vs. WT (AQP1), n = 5.

VI.3. Results from publication No.3.

VI.3.1. Experimental modeling of MA in mice

As an induction of metabolic acidosis, we performed different types (oral, i.p. or both) of NH_4Cl^- administration in mice.

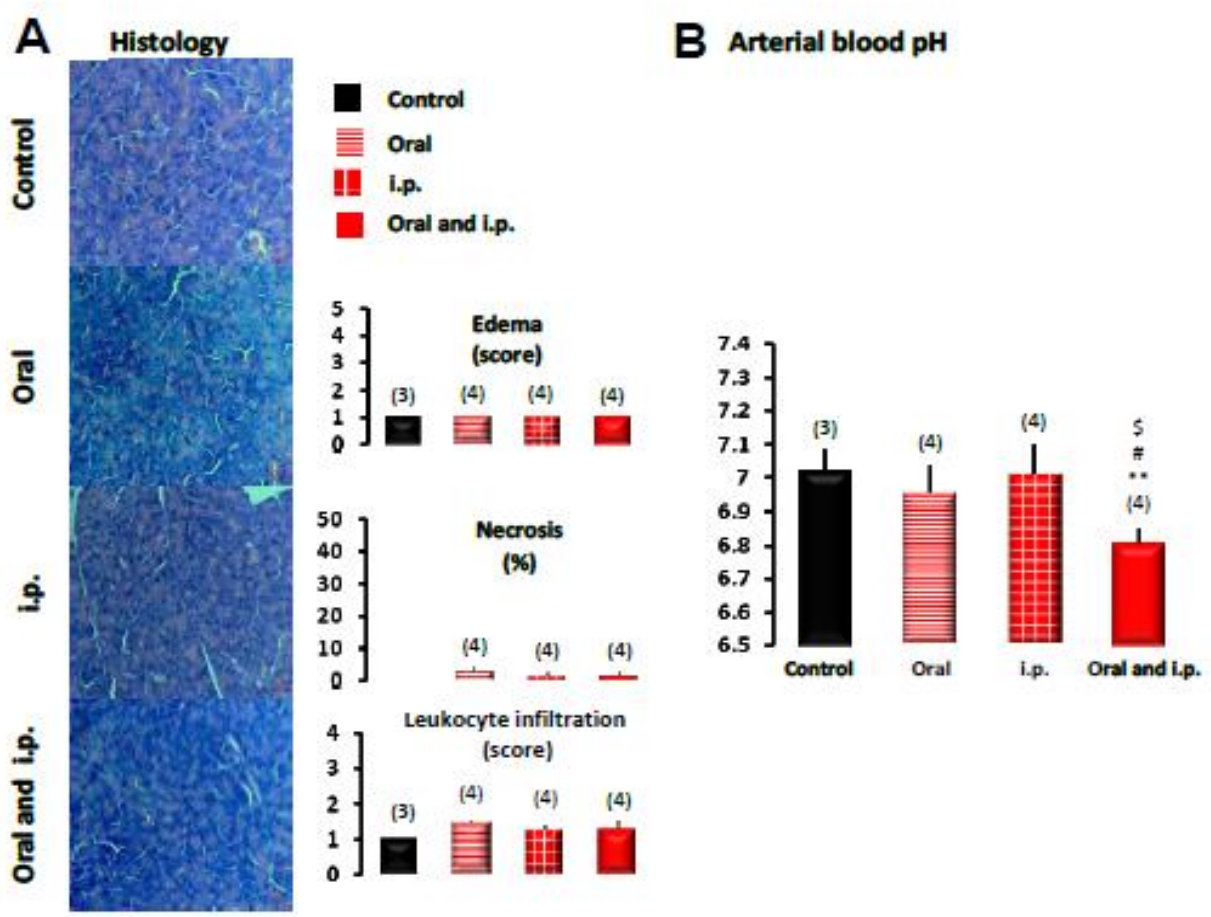

Figure 10: Development of a new method for metabolic acidosis

Induction of metabolic acidosis were performed by different administration of (oral, i.p. or both) of NH4Cl in animals. None of the treatments caused pancreatic edema or necrosis, and only minimal (not significant) increase of leukocyte infiltration was observed in all three NH4Cl treatment groups compared to the controls. (**B**) Arterial blood pH decreased minimally in the oral or i.p. treatment groups, while the combined (oral and i.p.) treatment significantly decreased arterial pH. **,$p < 0.01$ for the control group versus the oral and i.p. group; #, $p < 0.05$ for the oral group versus the oral and i.p. group; and $, $p < 0.05$ for the i.p. group versus the oral and i.p. group. Scale bar represents 20 μm. In figures 10-12. numbers in parentheses mean the number of animals in the corresponding groups.

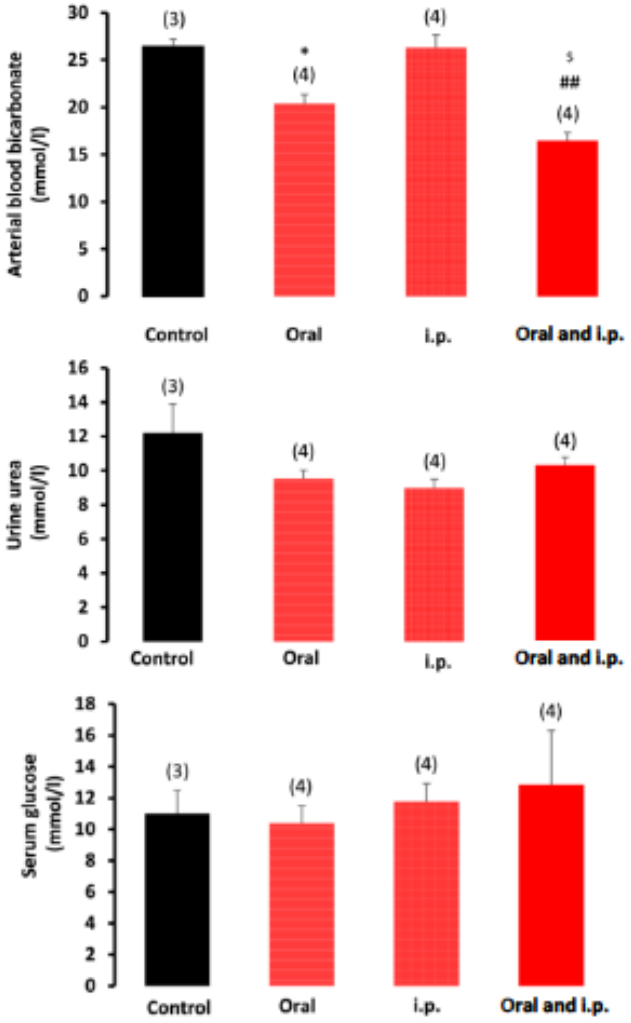

Figure 11. Laboratory parameters during the development MA groups

Scale bars represents arterial blood bicarbonate, urine urea, and serum glucose levels after different types (oral, i.p. or both) of NH_4Cl administration in mice. Minimal decrease of arterial blood bicarbonate level were detected in the i.p. treatment group, while the oral and the combined (oral and i.p.) treatment significantly decreased arterial bicarbonate level were observed. Urine urea and serum glucose and levels were not changed significantly in the treatment groups.(*p < 0.05 control vs. the oral group; ##, *P* < 0.001 e control vs. the oral and i.p. group; and $, *P* < 0.05 oral group vs. oral and i.p. group.

VI.3.2. Pre-existing Acidosis Deteriorates Both Mild and Severe Forms of AP in Mice

To determine whether the presence of pre-existing MA has any effects on the outcome of AP , pancreatic oedema, necrosis, and leukocyte infiltration scores were quantified in pancreatic sections of mice without AP, or with MAP or SAP in the presence and the absence of pre-existing MA. MA caused no pancreatic damage in the control group (no AP) (Fig. 12.), which

is line with our previous results (Fig.10-11.). On the other hand, in mice with preexisting MA, MAP resulted in significantly larger edema ($p < 0.05$), increased necrosis ($p < 0.05$), and elevated leukocyte infiltration $p < 0.05$) compared to MAP in mice with normal blood pH (Fig.12). Pancreatic damage was also more severe in SAP with pre-existing MA compared to SAP in mice with normal blood pH as indicated by increased edema $p < 0.05$), necrosis $p < 0.01$), leukocyte infiltration $p< 0.05$), and serum amylase activity $P < 0.05$) (**Fig.12.**).

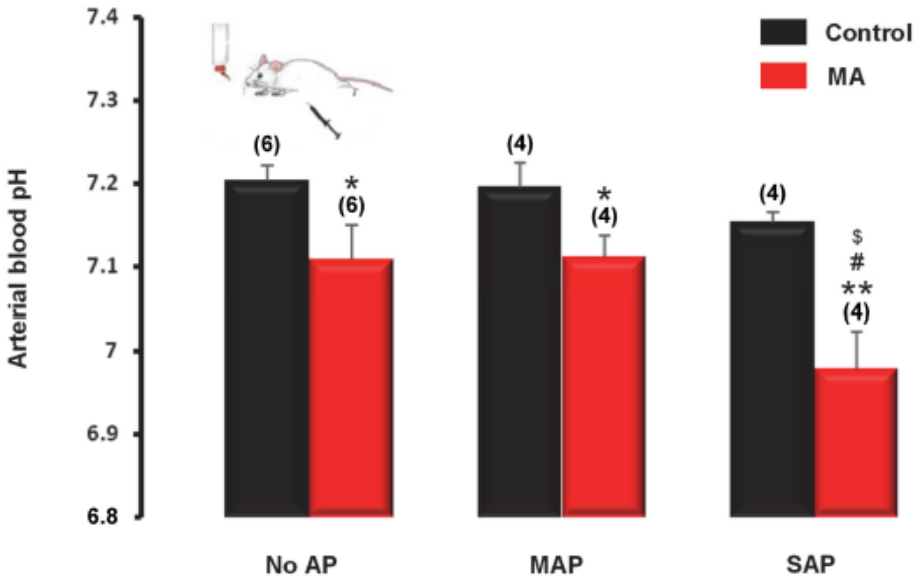

Figure 12. Arterial pH with MA induced by combination of oral and i.p. NH_4Cl^- treatment and without acidifying treatment (control groups). Mild acute pancreatitis (MAP) or severe acute pancreatitis (SAP) was induced by alcohol and fatty acid or cerulein, respectively. Mice in the control group (no AP) were injected i.p. with saline. Statistically significant differences are marked with *between MA and control (non-acidotic)groups, with #between no AP and MAP groups in MA, and with $between no AP and SAP groups in MA, as follows: $*p < 0.05$ and $**p< 0.01$ for MA vs. control in no AP, MAP, and SAP; $p< 0.05$ for no AP in MA vs. SAP in MA; $\$p < 0.05$ for MAP in MA vs. SAP in MA.

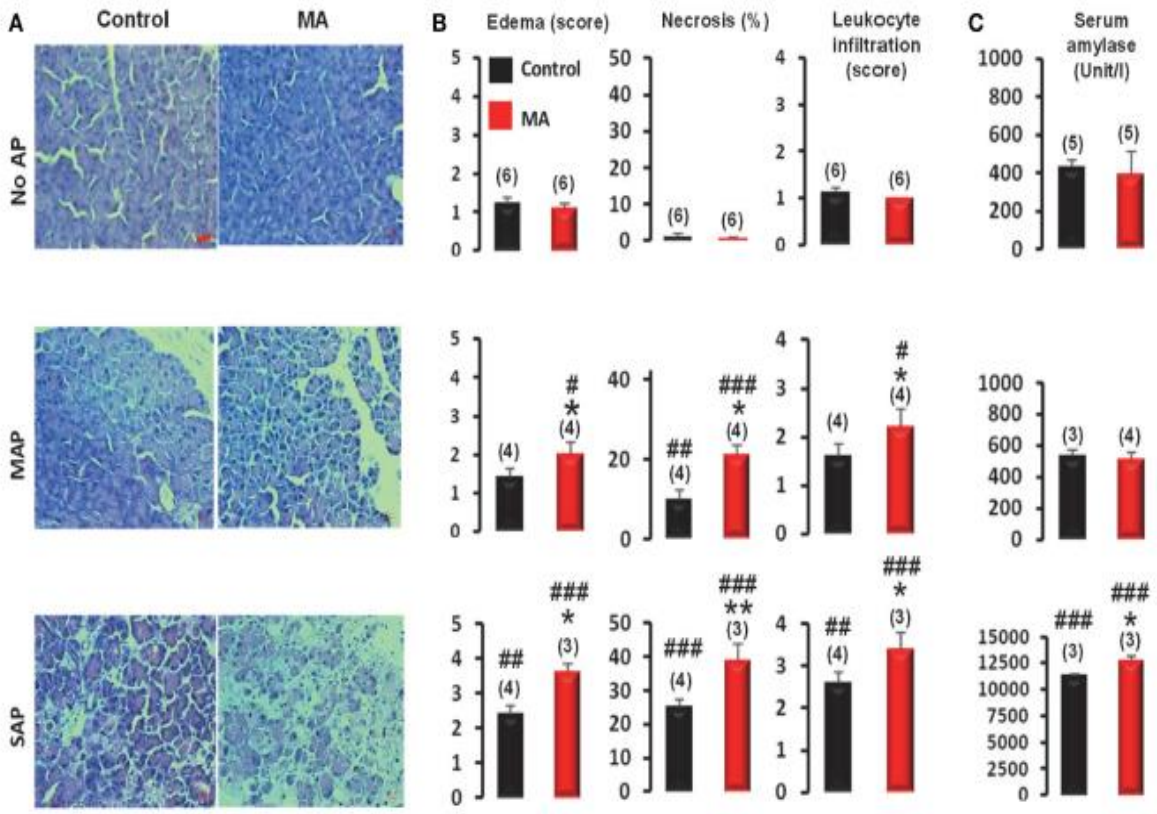

Figure 13. MA deteriorates AP in mice Representative images of pancreatic slices from mice (A), histological evaluation of oedema scores, necrosis, and leukocyte infiltration scores (B), and levels of serum amylase in MA and control mice with mild acute pancreatitis (MAP), severe acute pancreatitis (SAP) or without acute pancreatitis (no AP) (C). Scale bar represents 20μm. *p < 0.05; **p< 0.01 for MA vs. control in MAP and SAP; #p < 0.05, ##p < 0.01, and ###p < 0.001 for no AP vs. MAP and SAP.

VII. DISCUSSION

VII.1. Protecting the mitochondrial homeostasis as a novel therapeutic option in AP- Publication No.1.

Dysfunction of mitochondria is one of the main pathophysiological events in the early phase of AP in pancreatic ducts and acinar cells as well [10, 28, 85] . It decreases ATP production, causing 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[28, 30, 32, 50, 85-87] . Henceforth, mitochondrial damage is the main factor in determining cell death pathways necrosis and apoptosis. Release of mitochondrial cytochrome c into the cytosol causes apoptosis, whereas mitochondrial depolarization leads to necrosis[88] . Inhibition of mPTP could prevent both cell death mechanisms in DEC, which is different from that seen in acinar cells, where only necrosis could have been prevented. Taking it together, inhibition of mPTP seems to be beneficial in both cell types. In the last decade, it has been proved that genetic or pharmacological inhibition of mPTP reduces BA- or EtOH+FA-induced AC damage as well as augmenting the severity of AP [27, 30, 32, 89]. In the last few years our research group revealed that both BA and EtOH+FA induce inhibition of HCO_3^- secretion via severe mitochondrial damage in PDEC [50, 86][50, 85,36, 70] . During our studies we have continued the experiments investigating the role of mPTP and its inhibition in pancreatic ductal epithelial cells. In the first step, we characterized the role of mPTP (both genetic and pharmacological CyA) inhibition in PDEC 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 have 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 was also stopped before reaching the "proof of concept" phase 2 clinical trials in AP because of its inconsistent behavior in other trials due to the facts noted in the introduction. Recently, other new mPTP inhibitors have been introduced in experimental studies, until now with not much success. For example, isoxazoles had inconsistent effects in myocardial infarction[39] benzamides resulted in impaired ATP generation[33, 39] cinnamic anilides were shown to be effective in myocardial infarction [90].However, lately it has turned out that it has an age-related toxicity [91]. As mention in the introduction section, Nim811 has been shown to be protective in several experimental studies in different diseases, and until now no toxic effects have been demonstrated. We continued our

study by testing the effects of NIM811 on ductal and acinar cells in vitro (results from acinar cells are not part of this dissertation). We revealed 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. Surprisingly, inhibition of mPTP protected pancreatic ductal bicarbonate but fluid secretion during BA or EtOH+FA treatment. Considering these results, it is assumed that rescuing intracellular ATP level 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 [77]. *Per os* administration of 5 or 10 mg/kg NIM811 treatment alone had no toxic effect, but significantly reduced the severity of AP.

VII.2. The role of AQP1 in pancreatic ductal fluid secretion- Publication No.2.

Concerning, the AQPs role in the pancreatic ductal fluid secretion, by using double immunostaining of AQP1 and CFTR we have shown for the first time that AQP1 and CFTR are co-localized at the apical membrane of pancreatic ductal cells. Lack of CFTR significantly reduced the expression of AQP1, these data indicate that CFTR may control the water permeability of ductal cells. Our results also indicate that AQP1 interacts with the CFTR Cl^- channel and takes part in the formation of pancreatic fluid. Moreover, we have found that AQP1 plays role in the pathology of pancreatitis. Earlier, similar results have been found in respiratory epithelial cells, where the CFTR channel was mutant or inhibited the water permeability of the epithelial cells significantly decreased [51, 92]. This could highlight the significance of this water channel in disease of pancreatitis moreover in cystic fibrosis as well.

VII.3. The vicious cycle between reduced blood pH and AP-Publication No.3.

Since, in the literature there were no mouse model of MA, first we performed several methods of experiments to find the most beneficial MA model to use. Dual administration (oral and i.p.) of acidic fluid induced a marked pH drop in the blood without damaging the pancreas. By the oral treatment with i.p. acidification, our model is similar to such conditions, when primarily the pH of the peritoneal fluid is reduced such as bacterial peritonitis, carbon dioxide insufflation during laparoscopy [93], or peritoneal dialysis [94] In our model of MA , the MA manifested slowly and occured for several days in the mice which is very similar to what is happening in patients with MA. Furthermore, in human patients AP can manifest in pre-existing MA, for instance during hyperlipidemia or diabetic ketoacidosis [95, 96] However, in clinical settings MA typically occurs as a consequence of AP and in most cases it does not pre-exist.

In the future, it should be a great goal of clinical trials to find the beneficial effects of controlled pH management and to search for the optimal fluid resuscitation forms in patients with AP and

pre-existing MA. During our experiments we have shown experimental evidence to a bilateral link between pH and AP. We showed that pre-existing MA worsens the outcome of AP, whereas AP reduces pH in the blood which vicious cycle could be one of the main reasons for the high mortality rate in severe cases of AP. Future evaluations are needed to reveal the exact mechanism of how MA can deteriorate AP, but assumably a complex regulatory mechanisms is involved.

VIII. SUMMARY

VIII.1. Conclusions, new therapeutic options in the treatment of AP

1. **NIM811 is a suitable compound to be tested in clinical trials of AP.** 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, companies should organize phase 2 clinical trials with the use of this novel and promising drug candidate. (Publication No.1.)
2. **Protecting fluid secretion could be a new therapeutic option in AP.** AQP1 and CFTR channels are co-localized in the pancreatic ducts, we hypothesize that absence of the channel makes the pancreas more sensitive to pancreatitis, probably due to the decreased pancreatic fluid and HCO_3^- secretion. (Publication No.2.)
3. **Restoring the normal pH in patients with AP could be a beneficial therapeutic application in the treatment of the disease.** (Publication No.3.)

IX. MY OWN WORK

Publication No.1.: I was involved in: i) the study design, ii) article search iii) data extraction and statistical analysis, iv) experimental design v) performing of all types of the used experiments – in vivo and in vitro vi) developing the data interpretation. Also, I wrote the first version of the article and took part in developing the final version. I also prepared the version 1 of the "answers to the reviewers" and the revision of the article.

Publication No.2.: I was involved in: i) performing immunofluorescent stainings of mouse pancreas samples ii) quantification of the fluorescent data (from immunofluorescent stainings) iii, statistical analysis vi, data interpretation

Publication No.3.: I was involved in: i) the experimental study design, ii) experimental article search, iii) experimental data extraction and statistical analysis, iv) performing of all types of the used experiments – in vivo and in vitro vi) developing the experimental data interpretation. I also wrote the experimental part of the article.

X. TABLES

Table1. Solutions used during our studies

	HEPES (Standard) mM	**HCO_3-** (Standard) mM	**NH_4Cl-HCO_3- mM**	**1xTBS 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**

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"Success is walking from failure to failure with no loss of enthusiasm." —Winston Churchill

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

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KEYPOINTS

Bile acids, ethanol and fatty acids deteriorate pancreatic ductal fluid and bicarbonate secretion via mitochondrial damage, ATP depletion and calcium overload.

It is known that pancreatitis inducing factors open the membrane transition pore (mPTP) channel via cyclophilin D activation in acinar cells causing calcium overload and cell death and genetic or pharmacological inhibition of mPTP improves the outcome of acute pancreatitis in animal models.

In our study we show that genetic and pharmacological inhibition of mPTP protects mitochondrial homeostasis and cell function evoked by pancreatitis-inducing factors in pancreatic ductal cells.

Our results also reveal that the novel Cyclosporin A derivative NIM811 protects mitochondrial function in acinar and ductal cells, moreover it preserves bicarbonate transport mechanisms in pancreatic ductal cells.

We found that NIM811 is highly effective in different experimental pancreatitis models and that NIM811 has no side-effects. NIM811 is a highly suitable compound to be tested in clinical trials .

ABSTRACT

Background and aims

Mitochondrial dysfunction plays a crucial role in the development of acute pancreatitis (AP); however, no compound is currently available with clinically acceptable effectiveness and safety. In this study, we investigated the effects of a novel mitochondrial transition pore inhibitor, N-methyl-4-isoleucine cyclosporin (NIM811), in AP.

Methods

Pancreatic ductal and acinar cells were isolated by enzymatic digestion from Bl/6 mice. In vitro measurements were performed by confocal microscopy and microfluorometry. Preventive effects of pharmacological (cylosporin A (2μM), NIM811 (2μM)) 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 *per os*), were checked in

three different AP models induced by either caerulein (10x50µg/kg), ethanol+ fatty acid (1.75 g/kg ethanol and 750 mg/kg palmitic acid) or 4% taurocholic acid (2ml/kg).

Results

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.

Conclusion

The novel mitochondrial transition pore inhibitor NIM811 seems to be an exceptionally good candidate compound for clinical trials in AP.

KEYWORDS

Acute pancreatitis, mitochondrial transition pore, cyclophilin D, NIM811

INTRODUCTION

Acute pancreatitis (AP) is among the most common gastrointestinal disorders requiring hospitalization in the United States (Fangenholz *et al*,2007 ; Fagenholz *et al*, 2007 ; 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 cystic fibrosis transmembrane conductance regulator (CFTR) inhibition were shown to have a great 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 and Saluja,2011; Maleth *et al*, 2013; Abu-El-Haija *et al*, 2018 ; Biczo and Vegh *et al* ,2018 ;) . It has been shown in acinar cells that bile acids (BA) 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 is still unknown how the pancreatitis-inducing factors noted above modify mPTP channel activity in pancreatic ductal epithelial cells (PDEC), 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 licenced 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, CyA derivative Debio025 (Alispovirir, Debiopharm) has been found 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), 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) . Lately, it has turned out that TRO40303 does not even bind to Cyp D directly (Sileikyte,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 have reached "proof of concept" clinical trials in AP, most probably due to the clinical failures noted above. All in all, new compounds are crucially needed.

A novel cyclosporin A 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 central nervous system 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) , 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 NIM811 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 NIM811 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 euthanized by 200 mg/kg pentobarbital i.p. (Bimeda MTC, Cambridge, Canada).

Animals

A total of 70 wild type (WT) and cyclophilin D knockout (Cyp D KO, (B6;129-Ppiftm1Maf/J) mice were sacrificed. *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 us by the Department of Medical Biochemistry, Semmelweis University, Budapest, Hungary. Wild type and Cyp D-deficient littermate mice (of C57Bl/6 J 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 Termofischer Scientific . NIM811 were purchased from MedChem Express Europe (Sweden). Cyclosporin A (CYA) , caerulein (CER) , NIM811, CCCP and fluorescence dies were diluted in dimethyl sulfoxide (DMSO) . Table 1 describes the constitution of solutions that we used during the study. In this study 500µM Chenodeoxycholic acid (bile acid,BA) or 100mM ethanol (EtOH) + 200µM palmitoleic acid (fatty acid, FA) was 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 pharmachological inhibition of the mPTP in pancreatic ducts or acinar cells. 100 µM of Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) were used in the mitochondrional measurements as a positive control for mitochondrial damage.

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

Abbrevations used in this study:

AP- acute pancreatitis , NIM811- N-metil-izoleucine cyclosporine , mPTP- mitochondrial transition pore , mitochondrial membrane potencial- ψ, CFTR- cystic fibrosis transmembrane conductance regulator, PDEC-pancreatic ductal epithelial cells , Cyclophylin D- Cyp D , CYA- cylosporin A , Hepatitis C virus-HCV, Debio025- Alispovirir, Tro40303-3,5-seco-4-nor-cholestan-5-one-oxime-3-o, PCR-polymerase chain reaction, TMRM-Tetramethylrhodamine Methyl Ester Perchlorate, TOM20- Mitochondrial import receptor subunit, FA- fatty acid (palmitoliec acid), FAEE- Fatty acid ethyl ester , ETOH-ethanol , BA- CDC- chenodeoxycholic acid , BCECF-AM (2',7'-Bis-(2-Carboxyethyl)-5-(and-6)-Carboxyfluorescein, Acetoxymethyl Ester), CER-caerulein , TAU- sodium taurocholate , TBS- Tris Buffered Solution, BSA- Bovine Serum Albumin, HBSS- Hank1s Stock Solution, CBD- Common Pancreatic Biliary Duct, CCCP- Carbonyl cyanide 3-chlorophenylhydrazone

Methods

Mouse genotyping

Genotypes of cyclophilin D deficient mice were identified by PCR (typical polymerase chain reaction , analyses from tail genomic DNA) . PCR-mix contained: Taq DNA pol 5 U and 10xTaq Buffer (Abgene, Portmouth, USA), $MgCl_2$ 1,5 mM, dNTP 2.5mM, F-null2/LoxP1f /CyPuP2 primers (20-20µM), dH_2O and template DNA sample. Total reactions mix volume was 25 µl.
The wild type allele was detected using LoxP1f, 5'-AAA CTT CTC AGT CAG CTG TTG CCT CTG-3' as a forward primer and F-null2, 5'- GCT TTG TTA TCC CAG CTG GCG C-3' as a reverse primer. For genotyping of the mutant cyclophilin D deficient allele, F-null2, 5'-TTC TCA CCA GTG CAT AGG GCT CTG –3' was used as a forward primer with the reverse primer for WT (Table 2.) . DNA was denatured at 95°C for 2 mins, followed by 30 cycles of amplification: 94°C for 30 secs, 60°C for 30 secs, 72°C for 45 secs and a final primer extension step at 72°C for 7 mins. Bands of 270 and 470 base pairs were amplified for WT and CypDKO mice, respectively.

Pancreatic ducts and acinar cells were isolated by microdissection and enzymatic digestion as described earlier (Argent *et al*,1986 ; Gout *et al*, 2013) (Argent, Arkle et al. 1986, Gout, Pommier et al. 2013).

Mitochondrial membrane potential (Ψ) were determined by 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 (Tetramethylrhodamine Methyl Ester Perchlorate ,100 nmol/L).

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

Microfluorometry was used to measure pancreatic ductal HCO_3^- secretion as described earlier (Hegyi *et al*, 2003, Hegyi *et al*, 2004) by using BCECF-AM (2',7'-Bis-(2-Carboxyethyl)-5-(and-6)-Carboxyfluorescein, Acetoxymethyl Ester, 1.5 mmol/L).

Functionally active mitochondria were detected with immunofluorescent staining (TOM20 mitochondrial marker, (EPR15581-39, Abcam)). In order to determine mitochondrial localisation in isolated pancreatic ductal or acinar cells we labeled the mitochondria by the using of TOM20 primary antibody (Abcam, EPR15581-39). TOM20 is the central unit of the receptor TOM complex in the mitochondrial outer membrane and the role of it is to recognise and translocate cytosolically synthetized mitochondrial preproteins (Shatz *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 by Leica Cryostat. Sections were fixed in 4% paraformaldehyde . Washing periods were administered with 1xTBS solution. Antigen retrieval was performed with 10 mM Sodium –Citrate solution at the pH of 6 at 95 °C for 15 minutes. Blocking was obtained for 1h with 1% goat serum in 5% BSA-TBS solution. After these sections were incubated with TOM20 rabbit monoclonal antibody (dilution 1:400,Abcam) overnight incubation at 4°C. The following day the samples were incubated with goat anti rabbit secondary antibody (Alexa fluor 488, Thermo Fisher, Rockford, IL, United States) for 2 hours at dark in room temperature. The nuclei were counterstained with Hoechst 33342 (Termofischer, Rockford,IL,United States) . Immunofluorescence staining of the isolated pancreatic acinar cells were performed freshly after the isolation procedure with the same conditions as stated above, (except two parameters ; cells were fixed in 2% paraformaldehyde and dilution fo the primary antibody was 1:200) as stated above. Both ductal and acinar cell samples were mounted with Fluoromount and then analyzed using a Zeiss LSM 880 confocal laser scanning microscope (Carl Zeiss Technika Kft., Budaörs, Hungary). To quantify TOM20 positively stained area, 5-6 representative images from each group were taken by Zeiss LSM 880 Confocal Scannig Microscope (Carl Zeiss Technika Kft., Budaörs, Hungary). Image J software was used to convert images to gray scale (16 bit), threshold function was used to select the positively stained area. The fluorescence signal were calculated by the software (arbitary scale from 0-negative (white) to 255-maximal staining (black)) (Venglovecz *et al*,2018). Fluorescence intensity of the images were then normalized to the own total ductal or acinar area of the samples, which were measured in arbitary units. Fluorescence intensity was given in %, normalized to the total ductal or acinar total area.

AP was induced by caerulein (CER,10x50µg/kg); 4% sodium taurocholate (TAU, 2ml/kg,4%) (Niederau *et al*, 1985 ; Ding *et al*,2003 ; Perides *et al*,2010; Pallagi and Balla *et al*;2014 ;) or alcohol and fatty acid (intraperitonal injection of 1.75 g/kg ethanol and 750 mg/kg palmitic acid , EtOH+FA) as described earlier (Huang *et al*.,2014;Maleth *et al*, 2015). 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 AP, concentrations of NIM811 were 10 mg/kg or 5mg/kg. Dosage 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 (20ga x 38mm, Instech Laboratories, 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 hour 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 3rd injection of CER. The method for retrograde intraductal infusion of TAU has been described by Perides et al (Perides *et al*, 2010) . The surgery was performed on anesthetized mice (with ketamine-xylazine, dosage: 87.5 mg/kg ketamine-12.5 mg/kg xylazine). At the end of the procedure the mice were placed on a heating pad for 40 minutes and received buprenorphine i.p. injection (0.075 mg/kg) at once to reduce their occurrent pain. Following these mice were replaced into their cages for 24hours. They had free access to food and water. 24 hours after the TAU or EtOH +FA induced AP the mice were euthanized by 200 mg/kg pentobarbital i.p. (Bimeda MTC, Cambridge, Canada), . During the CER induced AP mice were euthanized with 200 mg/ kg pentobarbital i.p. (Bimeda MTC, Cambridge, and Canada) 2 hours after the last injections of CER. Mice were exsanguinated through cardiac puncture and the pancreas were removed. Blood from the cardiac puncture was placed on ice, then centrifuged with 2500 RCF for 15 mins 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 hematoxylin –eosin staining was performed. A colorimetric kit was used to measure serum amylase activity (Diagnosticum, Budapest, Hungary). Absorbance of the samples were detected at 405 nm with the use of 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 hematoxylin-

eosin by using a standard laboratory method. To quantify oedema, necrosis and leukocyte infiltration grades a semiquantitative scoring system was used as Kui et al described previously (Kui and Balla *et al*, 2015) .

In vitro pancreatic ductal fluid secretion (luminal swelling) assays were developed by Fernández-Salazar et al, (Fernández-Salazar et al,2004) performed by videomicroscopy as described earlier (Balázs *et al*,2018). Briefly, stimulaton of pancreatic ductal fluid secretion was induced by 5 μM forskolin and 100 μM 3-isobutyl-1-methylxanthine (IBMX) , quantification were performed by Image J Software (Balázs *et al*,2018). In vivo fluid secretion measurements were performed on anesthetized (by i.p. 87.5 mg/kg ketamine-12.5 mg/kg xylazine) mice after CER or EtOH+FA induced AP prior to euthanasia. Animals were placed on warm pads (37◦ C) to maintain the body temperature. Briefly, the abdomen of the mice were opened and cannucaltion of the lumen of the common biliopancreatic duct was performed by a 30-gauge needle (Maléth *et al*, 2015). Then 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 PE-10 tube for 15 min. In vivo secretion was induced by i.p. administration of 0.75CU/kg secretin (Maléth *et al*, 2015).

Statistical Analysis

All data are expressed as means ± SEM. Data were compared by either one- or two-way analysis of variance (ANOVA) or Kruskal–Wallis tests followed by the Holm–Sidak Method as appropriate (Sigma Plot). The effects were considered significant when $p < 0.05$.

RESULTS

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

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 performed with TMRM and TOM20 revealed that genetic inhibition of mPTP decreased both the loss of $\Delta\psi$ (Fig. 1A) and mitochondrial mass (Fig. 1B) caused by 500 μM CDC (BA) or co-administration of 100mM ethanol and 200μM 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. 1C), suggesting that genetic inhibition of Cyp D has a protective effect on PDEC. Next, we investigated how the genetically preserved mitochondrial function affects the cellular function of PDEC (Fig. 1D). 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) are totally blocked in $Ppif^{-/-}$ vs WT mice, suggesting that inhibition of mPTP can preserve ductal function and thus has therapeutic benefits (Fig. 1D–F).

Pharmacological inhibition of mPTP by CyA effectively prevents mitochondrial damage evoked by pancreatitis-inducing factors in PDEC

Both BA and EtOH+FA significantly decreased the ψ of PDEC (Fig. 2A). Importantly, 2μM 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 we could see during the genetic inhibition of mPTP (Fig. 2B). 2μM CYA decreased the extent of necrosis and apoptosis during the administration of BA or EtOH+FA in PDEC (Fig. 2C). 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. 2D–F).

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

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. 3A–B). Experiments using CytoCalcein Violet, Apopxin Deep Red and Nuclear Green showed that NIM811 alone has no toxic effects on

PDEC. Furthermore, it can strongly decrease BA- or EtOH-FA-evoked necrosis and apoptosis (Fig. 3C). 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 PDEC (Fig. 3D).

NIM811 and CYA have no effects on pancreatic ductal fluid secretion

Both in vivo and in vitro measurements revealed that NIM811 or CyA treatment can not prevent BA or EtOH+FA induced fluid secretiory damage in isolated ducts (Fig.4 A-D, 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 we have seen in PDEC (Fig. 4A). However, results obtained from TOM20 staining suggest that NIM811 has no effect on mitochondrial mass in acinar cells (Fig. 5B). 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. 5C).

NIM811 has therapeutic benefits in caerulein, taurolithocholic acid sulfate and ethanol and fatty acid induced AP

Firstly, we confirmed that per os administration of either 5 or 10mg/kg NIM811 alone has no toxic effect on the pancreas (Fig 9.) . Secondly, we tested the compound in three different experimental AP models, the caerulein (CER) , alcohol and fatty acid (EtOH+FA) and the taurocholic (TAU)-induced ones (Niederau et al,1985; Huang et al, 2014; Perides et al,2010) . Importantly, both pretreatment 5 or 10mg/kg 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). In our study we also confirmed, that post treatment of 5mg/kg or 10 mg/kg NIM811 has protective effects against pancreatic damage (Figs. 6-8.).

DISCUSSION

Acute pancreatitis is a multifactorial disease (Hegyi and Petersen ,2013; Sahin-Toth and Hegyi, 2017) involving several types of cell, including acinar and ductal cells. None of the therapeutic efforts targeting only one of them have been successful. Intravenous administration of secretin, which targeted ductal cells only, was found either to be slightly beneficial or natural in AP (Renner *et al*,1983; Lankisch *et al*, 1983; Keim *et al*, 1985). On the other hand, neither gabexate mesilate nor trasylol, which effectively inhibit trypsin activity, had beneficial effects in AP (Imrie *et al*,1978; Buchler *et al*, 1993) (Imrie, Benjamin et al. 1978, Buchler, Malfertheiner et al. 1993). Therefore, we need to find common targets which can restore both acinar and ductal cell functions in AP.

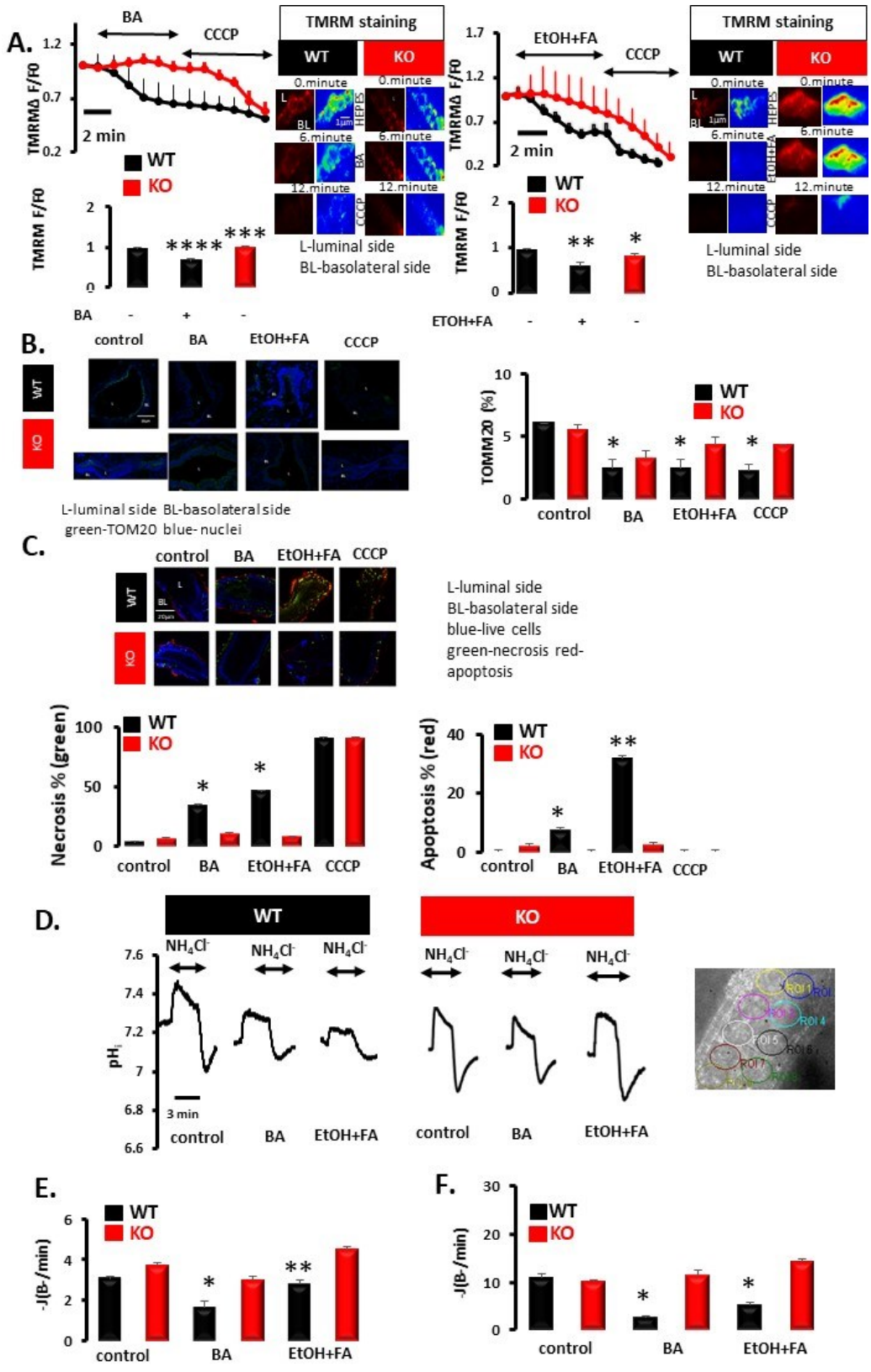
Mitochondrial damage is one of the key pathophysiological events in the early phase of AP in both types of cell (Maleth *et al*, 2013; Hegyi and Petersen, 2013 ; Maleth and Hegyi,2016) It decreases ATP production, causing elevation of intracellular calcium concentration; moreover, it negatively influences ATP-dependent $Cl^-HCO_3^-$ exchangers, CFTR Cl^- channels in ductal cells and enzyme secretory processes in acinar cells (Maleth *et al*,2011; Maleth *et al*, 2013 ; Judak *et al*, 2014; Maleth *et al*,2015 ; Mukherjee *et al*,2016; Maleth and Hegyi,2016, Biczo and Vegh *et al*, 2018). In addition, mitochondrial damage is the main factor in determining cell death pathways 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 like cytochrome c to be released from the inner membrane to the cytosol (Tait *et al*, 2010; Maleth *et al*, 2016). On the other hand, the opening of the mPTP leads to loss of ψ , ATP depletion, increased inner membrane permeability, mitochondrial swelling and necrotic cell death (Golstein *et al*, 2007; Halestrap *et al*, 2009; Maleth *et al*, 2016). Very uniquely, inhibition of mPTP could prevent both cell death mechanisms in PDEC, which is different from that seen in acinar cells, where only necrosis could have been prevented. All in all, inhibition of mPTP seems to be highly beneficial in both cell types. In the last decade, it has been proved that genetic or pharmacological inhibition of mPTP reduces BA- or EtOH+FA-induced AC damage as well as augmenting the severity of AP (Sah et al,2011; Mukherjee et al, 2016 ; Gukovskaya et al, 2016; Biczo and Vegh 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 PDEC) (Maleth *et al*,. 2011, Maleth *et al*. 2015). 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 PDEC 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 have 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 the "proof of concept" phase 2 clinical trials in AP because of its inconsistent behavior in other trials due to the facts noted in the introduction. Recently, other new mPTP inhibitors have been introduced in experimental studies. Isoxazoles had inconsistent effects in myocardial infarction (Sileikyte *et al*, 2016). Benzamides resulted in impaired ATP generation (Sileikyte *et al*, 2016; Javed et al, 2018) . Cinnamic anilides were shown to be effective in myocardial infarction (Fancelli *et al*, 2010) ; however, lately it has turned out that it has an age-related toxicity (Fang *et al*, 2019). Besides unsuccessful attempts, NIM811 seemed 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 fluid secretion during BA or EtOH+FA treatment. These data suggest that rescuing intracellular ATP level 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 NIM811 treatment alone had no toxic effect, but significantly reduced the severity of AP. We found that NIM811 treatment was more beneficial in the TAU than the EtOH+FA induced AP model. One of the explanations could be that besides the direct toxic effect of EtOH and FA, the non-oxidative metabolites of FA

namely FAEE has even higher toxicity on the mitochondria both in acinar and ductal cells (Criddle *et al*, 2006; Petersen *et al*, 2009).

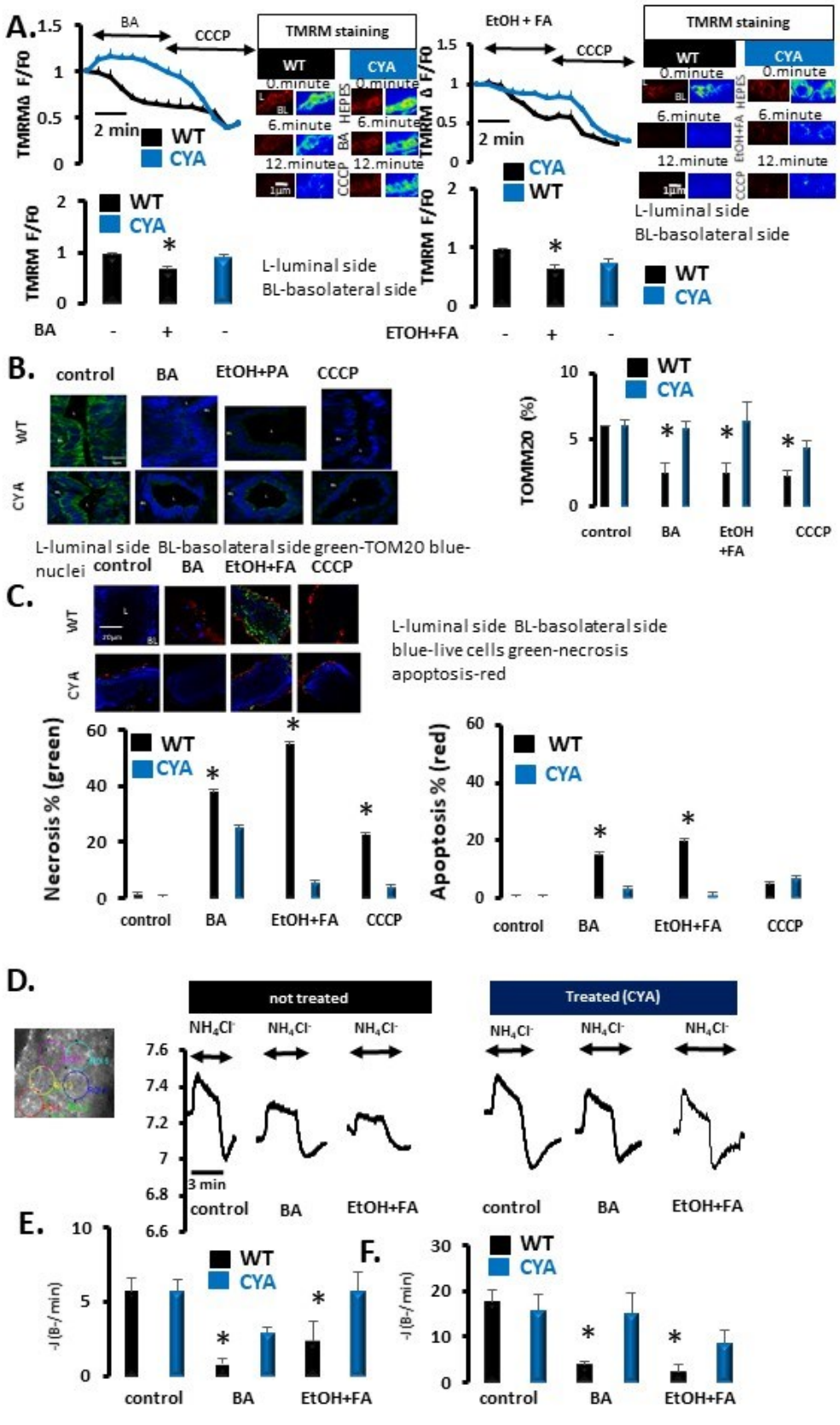
Taken together, mitochondrial function and bioenergetics play a crucial role in the development of AP; however, translation of the results to patient benefit is still missing (Maleth *et al*,2013 ;Mukherjee *et al*,2016 ;Maleth and Hegyi,2016 ; Gukovskaya *et al*, 2016; Biczo and Vegh *et al*,2018). In this study, we were the first to confirm that the mPTP inhibitor NIM811 is a highly suitable compound to be tested in clinical trials. As a next step, the companies should organize phase 2 clinical trials with the use of this novel and promising drug candidate.

Figure1. Genetic inhibition of Cyp D reduces the severity of bile acid or ethanol and fatty acid induced damage in PDEC



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 (100mM) and fatty acid (200μM FA) treatment (Fig1. A) (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=07.12,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/group, data means ±SEM. Results from the immunostainings revealed a significant decrease of the TOM20 stainings in BA; EtOH+PA or CCCP treated WT ducts , results were compared to Cyp D KO stainings . (Fig1.B) (*p<0.05). Genetic inhibition of mPTP also decreased the necrosis and apoptosis levels during bile acid; ethanol or fatty acid or CCCP treatment (Fig1.C). (*p<0.05) Representative traces from the pancreatic ductal HCO_3- secretion measurements (Fig.1.D) Our data revealed that recovery from the alkalosis grades were significantly lower due to BA or ETOH+FA administration (*p<0.05) compared to the results from Cyp D KO ducts (Fig1.E). Recovery from the acidosis grades were significantly lower in the WT ducts due to the treatment with BA or EtOH and FA (*p<0.05), while in Cyp D KO ducts these grades were significantly higher (*p<0.05) . n=5-7 experiments/group, data means ±SEM.

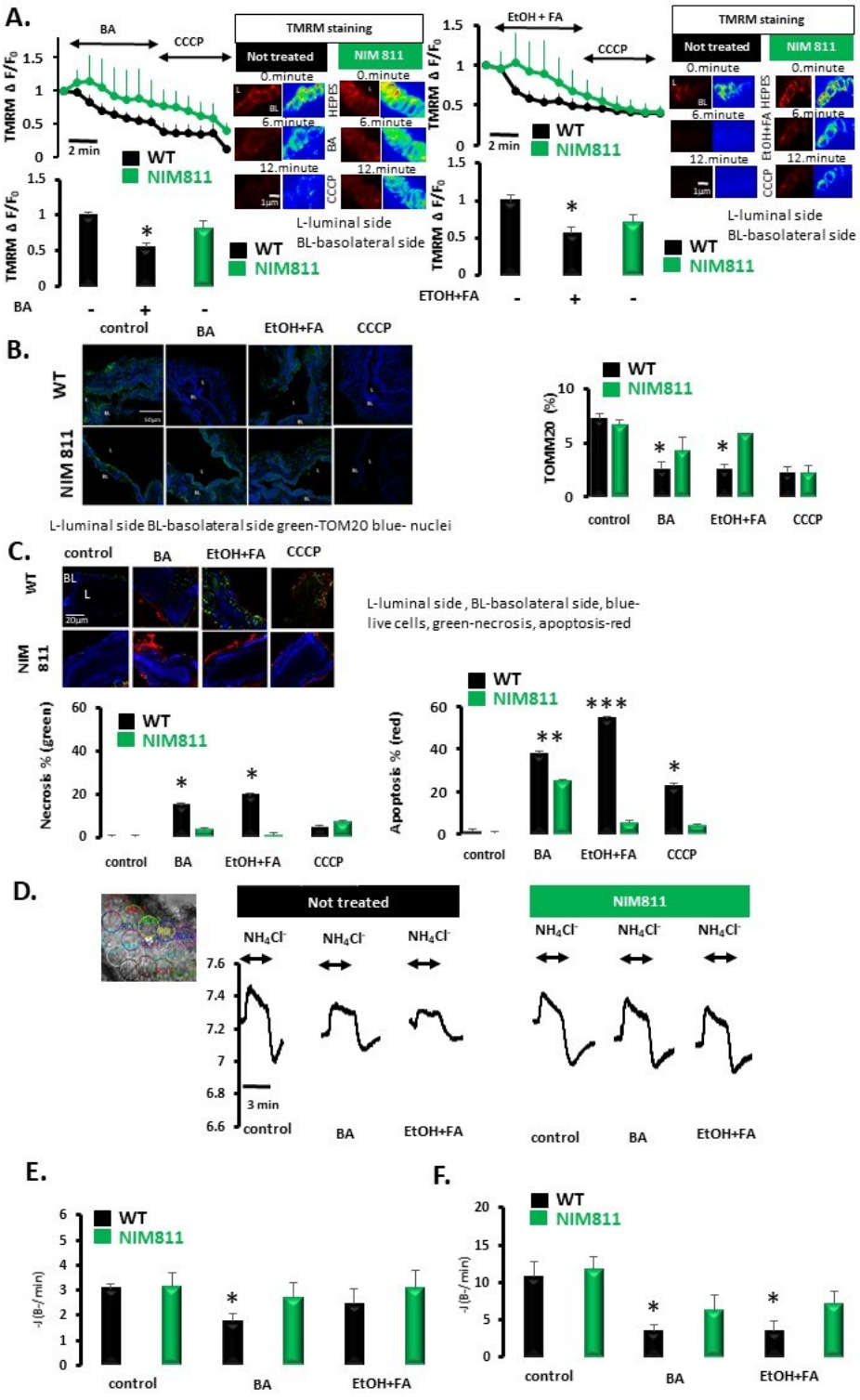
Figure2. CYA reduces the severity of bile acid or ethanol and fatty acid induced pancreatic ductal damage



2 μM CYA treatment reduced the drop of mitochondrial membrane potencial loss which accured due to the BA or ETOH+FA treatment. (WT vs. CYA) (Fig.2.A). 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 were no significant decrease. 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 were significantly higher (Fig2.B) *$p<0.05$. Between the control groups (WT control or only CYA treated samples) we found no significant alterations in the stainings. Necrosis levels were intensively elevated in BA or EtOH treated groups in WT ducts but not in CYA treated groups (Fig.2.C). Apoptosis levels were significantly higher as well in the not CYA treated groups compared to the CYA treated groups (Fig2. C).

Measurements of HCO_3^- secretion levels revealed a significant difference in WT and CYA treated ducts during the administration of BA ($p<0.05$ WT BA vs CYA BA) or EtOH+FA (*$p<0.05$). In WT ducts the levels of base flux (-J(B-/min) grades were significantly decreased (Fig2.E,F) due to BA (WT vs WT BA $p<0.05$) or ETOH+FA (WT vs WT EtOH+PA $p<0.05$) treatment (Fig2 E,F). Recovery from alkalosis (Figure 2. E) and recovery from acidosis values are presented in base flux ((-J(B-/min) grades respectively, with ±SEM. Comparison within CYA treated groups revealed no significant difference (CYA control vs CYA BA $p=0.644$).

Figure3. NIM811 protects mitochondrial and cell function in PDEC

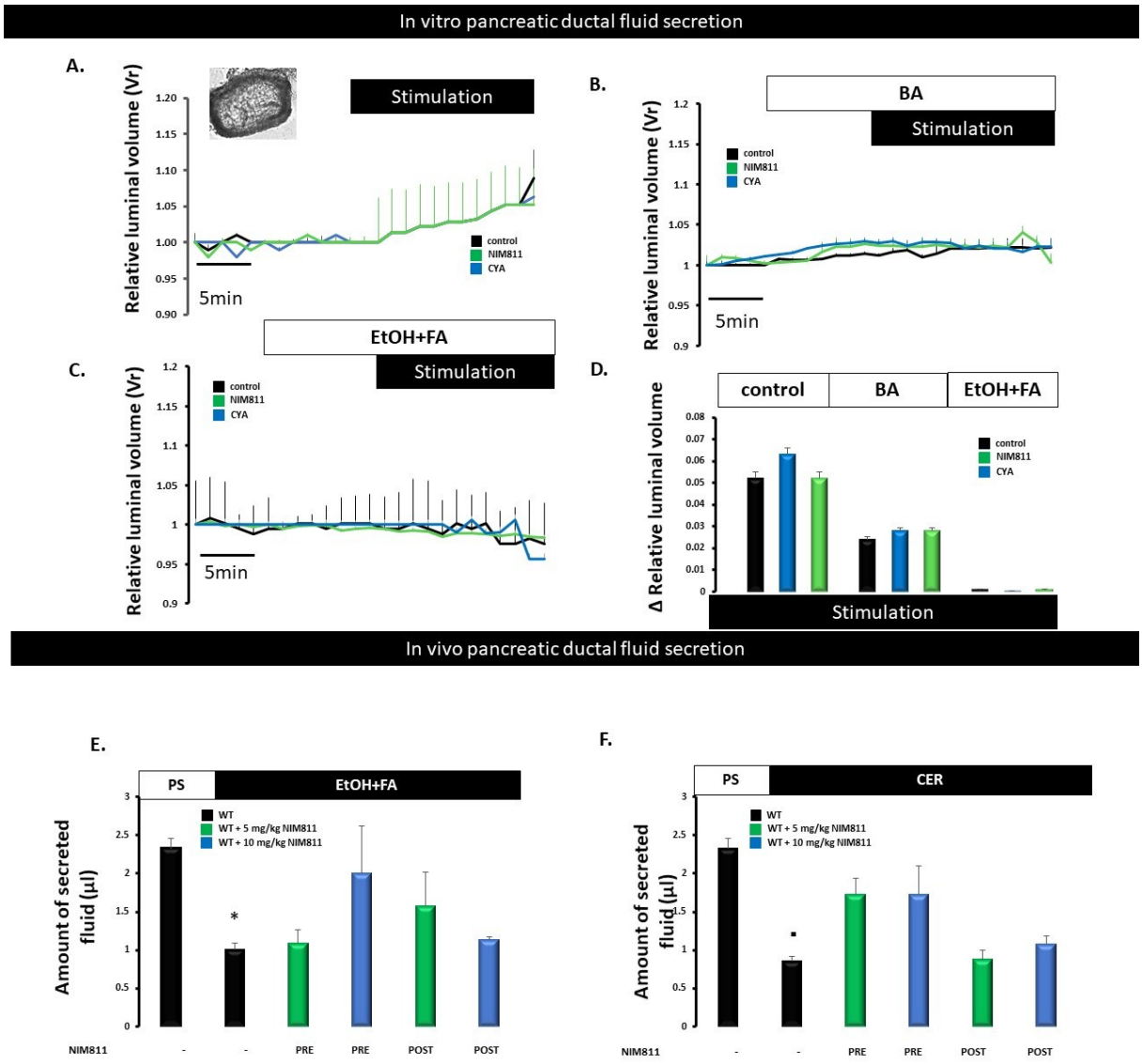


NIM811 treated ducts revealed a significantly consolidated loss of mitochondrial membrane potencial during the BA (WT BA vs NIM811 BA *p<0.05) or ETOH+FA (WT ETOH+FA vs NIM811 ETOH+FA *p<0.05) treatment (Fig.3A) . In NIM811 treated ducts the percentage of fluorescence intensity were significantly higher compared to not NIM811 treated ducts during BA or ETOH+FA administration. In CCCP treated ducts we found no significant difference in the amount of TOM20 stainings in the aspect of NIM811 treated or not treated groups. NIM811 itself did not alter the value of TOM20 stainings compared to the WT control samples (Fig.3B).

NIM811 decreased the numbers of apoptotic and necrotic cells during bile acid or ethanol and fatty acid treatment (Fig.3C) (WT BA vs NIM811 BA *p<0.05, WT EtOH+FA vs NIM811 *p<0.05). While during the administration of CCCP the apoptosis and necrosis grades were not significantly different in the comparative groups (Fig.3.C).

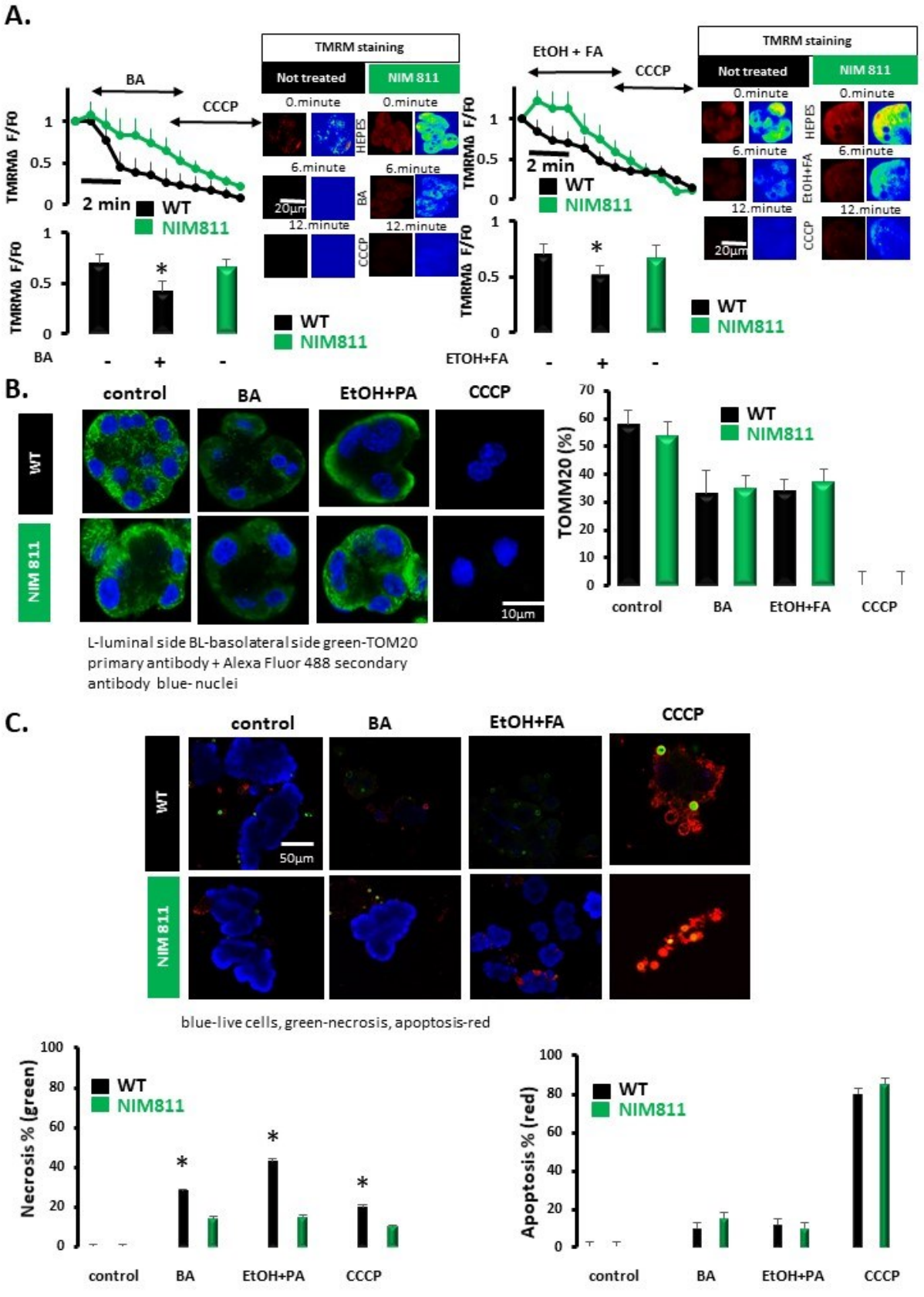
NIM811 treatment did not decreased the HCO_3^- secretion grades (control, Fig.3 D,E,F) , while during the adminsitration of BA or ETOH+FA treatment it had a protective effect against the reduction of HCO_3^- secretory levels (Fig.3E/F) (WT BA vs NIM811 BA *p<0.05, WT EtOH+FA vs NIM811 EtOH+FA *p<0.05). In the aspect of recovery levels from alkali load during EtOH and FA treatment , the difference were not sigfnificant in WT EtOH+FA compared to the NIM811 and EtOH+FA treated groups (Fig.3E).

Figure 4. Pancreatic ductal fluid secretion is not altered by NIM811 or CYA treatment



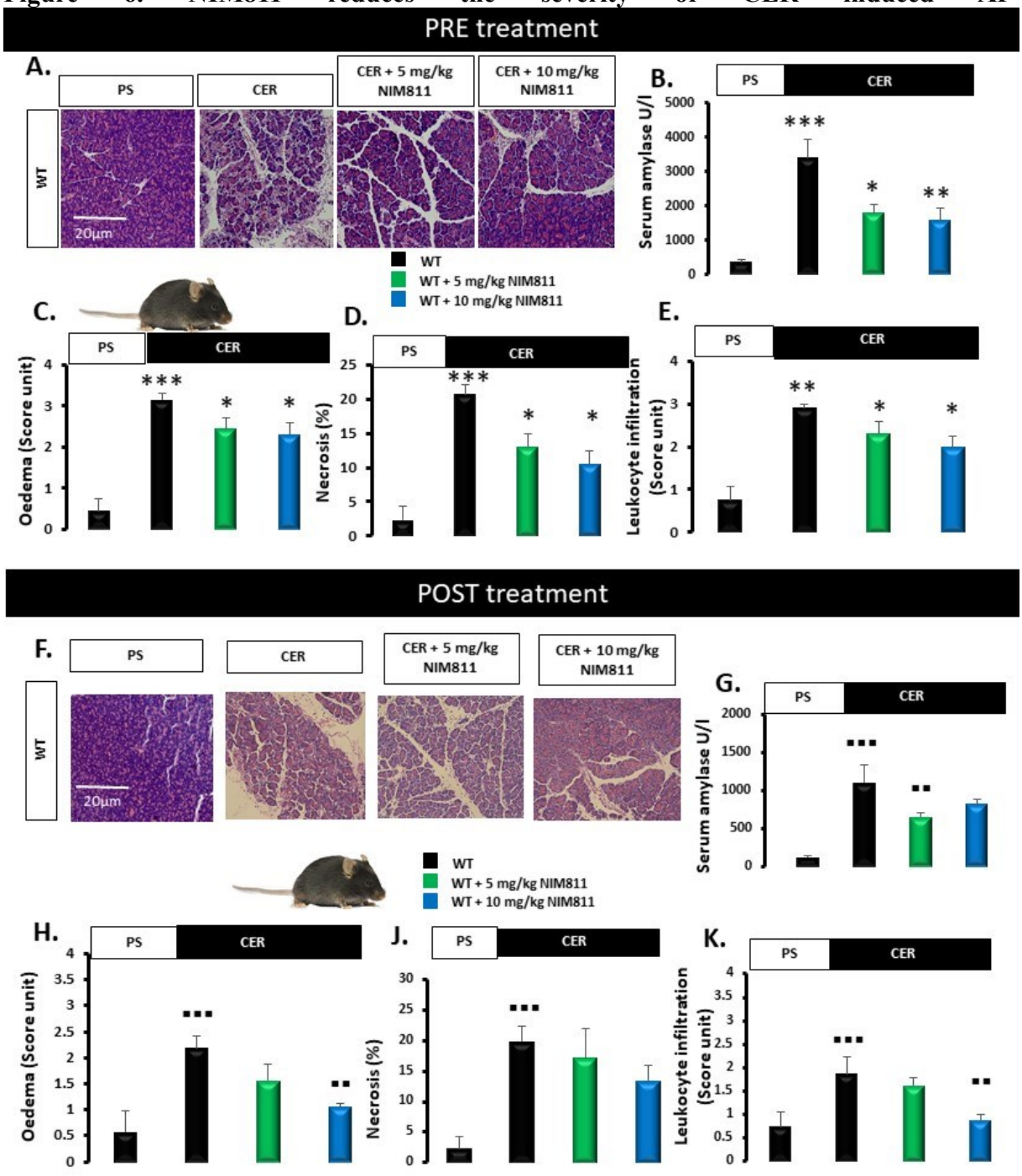
In vitro fluid secretion was stimulated by 5µM forskolin and 100µM IBMX (stimulation). BA or EtOH+PA treatment inhibited the luminal swelling (Fig.4.B-C). Figure 4D represents the relative luminal volume changes during forskolin and IBMX stimulation (Figure4.D). Means ±SEM. n= 5-10 ducts/group. In vivo fluid secretion measurements were performed after the induction of CER or EtOH+FA induced AP (Fig.4.E-F.). These experiments confirmed that pancreatic ductal fluid secretion is not affected by NIM811 or CyA. (Fig.4.E-F). *p<0.05 WT PS vs. WT EtOH+FA, ▪p<0.05 WT PS vs. WT CER n=4-7 animal/group

Figure5. NIM811 treatment protects mitochondrial function in pancreatic acinar cells



Mitochondrial membrane potencial measurements revealed a significant difference between WT not NIM811 treated and the NIM811 treated acinar cell response due to bile acid or ethanol and fatty acid treatment (Fig.5A) (WT BA vs NIM811 BA *$p<0.05$; WT EtOH+FA vs NIm811 ETOH+FA * $p<0.05$). Significant difference was detected between the NIM811 treated acinar cells and the groups which were not treated with NIM811 (Fig.5A) during BA or ETOH+FA treatment. Mitochondrial protein TOM20 levels did not show difference in the NIM811 treated or not treated groups after BA, ETOH+FA or CCCP treatment (Fig.5B) ($p>0.05$). In necrosis levels we found significant difference between NIM811 treated and not treated groups in BA or ETOH+FA (Fig.5C) (*$p<0.05$). However, in CCCP treated groups we found no difference (Fig.5C). Apoptosis levels were not altered significantly by NIM811 during BA or ETOH+FA treatment.

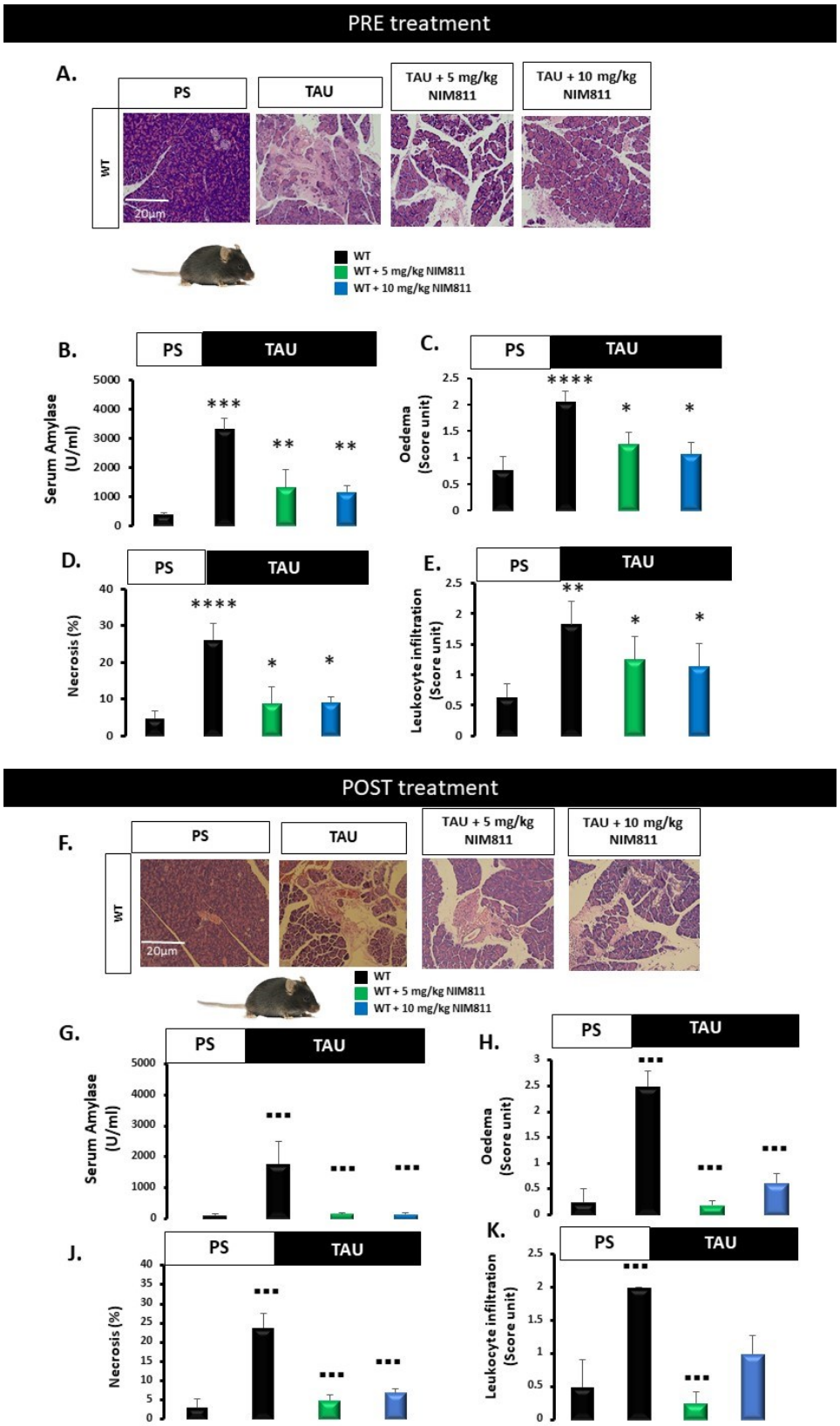
Figure 6. NIM811 reduces the severity of CER induced AP



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 WT CER group (Fig. 6B ***p<0.01 WT PS vs WT CER, **p<0.02 WT CER vs pre10mg/kg NIM811 CER, *p<0.05 WT CER vs pre 5mg/kg NIM811 CER, p=0.717 CER+ pre 5mg/kg NIM811

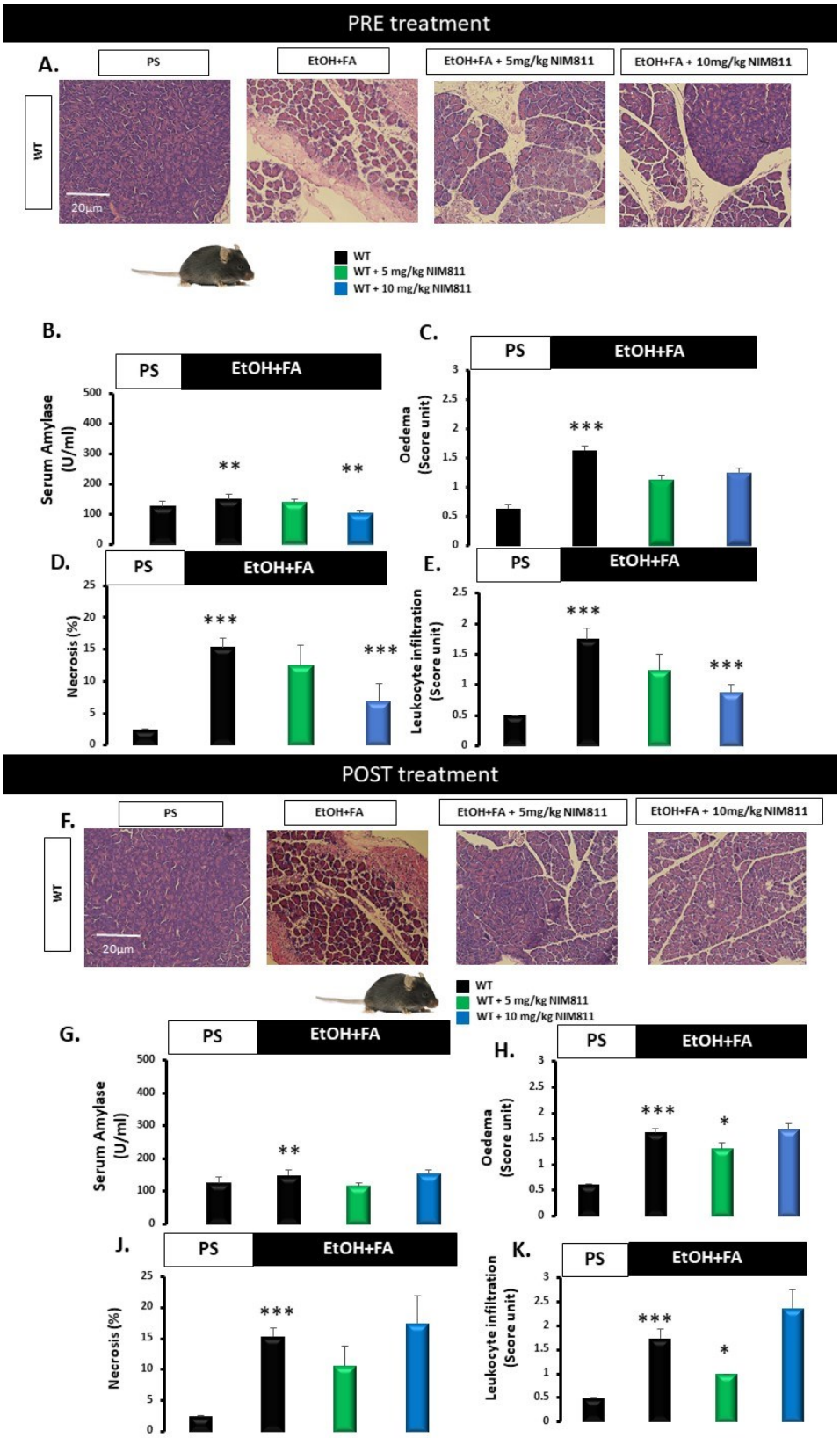
vs CER + pre 10mg/kg NIM811). In the aspect of CER induced pancreatitis both 5 mg/bwkg NIM811 (Fig.6 A-F, $p<0.05$ WT CER vs. pre 5mg/bwkg NIM811 CER) and pre 10 mg/bwkg NIM811 (Fig.6 A-F, $p<0.05$ WT CER vs. Pre 10mg/bwkg NIM811 CER)) treatment reduced the CER-induced damage. Post 5mg/kg NIM811 treatment significantly reduced serum amylase levels compared to WT CER ▪$p<0.05$, ▪▪▪$p<0.001$ WT PS vs WT CER (Fig.6G-E). Post insult administration of 10mg/kg NIM811 significantly reduced oedema and leukocyte infiltration levels compared to WT CER treated groups ▪$p<0.05$ (Fig.6H), n=8-10 animals per group, data means ±SEM).

Figure7. NIM811 reduces the severity of TAU induced AP in mice



We performed TAU induced pancreatitis(Fig.7A-K), serum amylase measurements revealed that due to retrogrode infusion of TAU elevated serum amylase levels occured (***$p<0.01$ WT PS vs WT TAU Fig.6.B, ▪▪▪$p<0.001$ WT PS vs WT TAU Fig.7G) howewer 5 mg/bwkg or 10 mg/bwkg NIM811 treatment significantly reduced the enzyme levels both in the pre and post treatment (Fig. 7B **$p<0.02$ WT TAU vs pre 5mg/kg NIM811+TAU , ** $p<0.02$ WT TAU vs pre 10mg/kg NIM811+TAU, , ▪▪▪$p<0.001$ WT TAU vs. post 5mg/kg NIM811 TAU, ▪▪▪$p<0.001$ WT TAU vs post 10mg/kg NIM811 +TAU) the serum amylase levels were reduced compared to WT TAU treated groups (Fig.7B. and 7G *$p<0.01$ WT TAU vs. WT 5mg/bwkg NIM811 TAU and *$p<0.01$ WT TAU vs WT 10 mg/bwkg NIM811 TAU) . During pre NIM811 treatment oedema, necrosis and leukocyte infiltration scores were significantly decreased compared to the only TAU treated groups (Fig.7A,C,D,E $p<0.05$ WT TAU vs pre 5mg/bwkg NIM811 TAU/10mg/bwkg NIM811 TAU). Post insult administration of NIM811 decreased oedema, leukocyte infiltration and necrosis levels in the TAU group (▪▪▪$p<0.001$ Fig.7G-K) n=4-6 animals per group, data means ±SEM).

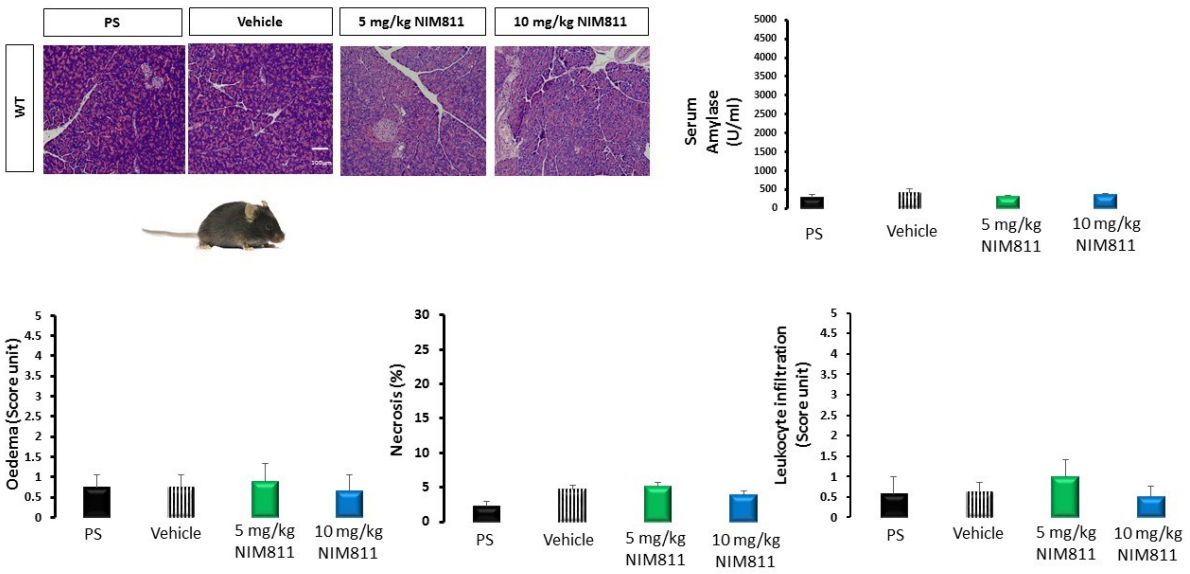
Figure8. NIM811 has protective effect against EtOH+FA induced pancreatic damage



We performed EtOH+FA induced pancreatitis (Fig.8A-K). Serum amylase measurements revealed that in pre treatment of 10 mg/kg NIM811 significantly reduced serum amylase

levels **p<0.002 WT EtOH+FA vs pre 10mg/kg NIM811 +EtOH+FA (Fig.8B), **p<0.002 WT PS vs Wt EtOH+FA (Fig.8B and G), in post NIM811 treatment serum amylase levels did not differ significantly compared to its ETOH+FA control (Fig.8G). In pre 10mg/kg NIM811 treatment leukocyte infiltration (***p<0.001 WT EtOH+FA vs 10mg/kg NIM811) and necrosis levels (***p<0.001 Wt EtOH+FA vs 10 mg/kg NIM811) were significantly reduced compared to EtOH+FA AP group (Fig.8D-E). ***p<0.001 WT PS vs Wt EtOH+FA in Fig.8C-E. Oedema and leukocyte infiltration levels were significantly reduced in post 5mg/kg NIM811 treated groups compared to WT EtOH+FA groups (*p<0.05 WT EtOH+FA vs post 5 mg/kg NIM811) (Fig.8H and K) n=4-7 animals per group, data means ±SEM).

Figure9. 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 animal/group

Table1. Solutions used in our study

	HEPES (Standard) mM	**HCO_3-** (Standard) mM	**NH_4Cl-HCO_3- mM**	**1xTBS 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	**-**	**-**	**-**	**-**	**-**
$NaHCO_3$-	**-**	**25**	**25**	**-**	**4.2**
NH_4Cl-	**-**	**-**	**20**	**-**	**-**
Trisma Base	**-**	**-**	**-**	**50**	**-**
Na_2HPO_4	**-**	**-**	**-**	**-**	**0.25**
KH_2PO_4	**-**	**-**	**-**	**-**	**0.44**
$MgSO_4$	**-**	**-**	**-**	**-**	**1.03**

Table 2. Oligonucleotide primers used in genotyping

Primers	
F-null2	TTCTCACCAGTGCATAGGGCTCTG
LoxP1f	AAACTTCTCAGTCAGCTGTTGCCTCTG
CyPuP2	GCTTTGTTATCCCAGCTGGCG

ADDITIONAL INFORMATION SECTION

COMPETING INTEREST

The authors have no conflicts of interest to disclose.

AUTHOR CONTRIBUTION

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 to us and were involved in the 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 the authors approved the final manuscript.

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AUTHORS' 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 the disease initiation is the mitochondrial dysfunction and ATP depletion. It has been shown that the pancreatitis-

inducing factors namely ethanol, fatty acids and bile acids open the membrane transition pore (mPTP) channel, keeping the channel continuously opened 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, companies should organize phase 2 clinical trials with the use of this novel and promising drug candidate.

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The Importance of Aquaporin 1 in Pancreatitis and Its Relation to the CFTR Cl^- Channel

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Aquaporins (AQPs) facilitate the transepithelial water flow involved in epithelial fluid secretion in numerous tissues; however, their function in the pancreas is less characterized. Acute pancreatitis (AP) is a serious disorder in which specific treatment is still not possible. Accumulating evidence indicate that decreased pancreatic ductal fluid secretion plays an essential role in AP; therefore, the aim of this study was to investigate the physiological and pathophysiological role of AQPs in the pancreas. Expression and localization of AQPs were investigated by real-time PCR and immunocytochemistry, whereas osmotic transmembrane water permeability was estimated by the dye dilution technique, in Capan-1 cells. The presence of AQP1 and CFTR in the mice and human pancreas were investigated by immunohistochemistry. Pancreatic ductal HCO_3^- and fluid secretion were studied on pancreatic ducts isolated from wild-type (WT) and AQP1 knock out (KO) mice using microfluorometry and videomicroscopy, respectively. *In vivo* pancreatic fluid secretion was estimated by magnetic resonance imaging. AP was induced by intraperitoneal injection of cerulein and disease severity was assessed by measuring biochemical and histological parameters. In the mice, the presence of AQP1 was detected throughout the whole plasma membrane of the ductal cells and its expression highly depends on the presence of CFTR Cl^- channel. In contrast, the expression of AQP1 is mainly localized to the apical membrane of ductal cells in the human pancreas. Bile acid treatment dose- and time-dependently decreased mRNA and protein expression of AQP1 and reduced expression of this channel was also demonstrated in patients suffering from acute and chronic pancreatitis. HCO_3^- and fluid secretion significantly decreased in AQP1 KO versus WT mice and the absence of

AQP1 also worsened the severity of pancreatitis. Our results suggest that AQP1 plays an essential role in pancreatic ductal fluid and HCO_3^- secretion and decreased expression of the channel alters fluid secretion which probably contribute to increased susceptibility of the pancreas to inflammation.

Keywords: pancreas, aquaporins, bile acids, HCO_3^- secretion, CFTR Cl^- channel

INTRODUCTION

The pancreas secretes 1.5–2 l of fluid per day (Hegyi et al., 2011a; Pallagi et al., 2015). This fluid contains the inactive form of digestive enzymes and is prominently rich in HCO_3^- produced by the ductal cells (Pallagi et al., 2015). The main functions of this HCO_3^--rich fluid are (i) to prevent the premature activation of zymogens (Pallagi et al., 2011), (ii) to wash out the toxic factors (such as bile acids) from the ductal tree (Venglovecz et al., 2008), and (iii) to provide an alkaline environment in the duodenum for the optimal function of digestive enzymes (Steward et al., 2005). In the last few years, the importance of ion transport proteins has been highlighted in the course of pancreatitis; therefore intensive research has been conducted in order to characterize their pathological roles (Hegyi and Rakonczay, 2015; Pallagi et al., 2015). These clinical and experimental studies indicate that impaired ductal HCO_3^- secretion makes the pancreas more susceptible to inflammatory diseases such as acute or chronic pancreatitis (CP) (Hegyi and Rakonczay, 2010; Hegyi et al., 2011b; Takacs et al., 2013; Pallagi et al., 2014; Maleth et al., 2015). In contrast, much less is known about the water transport processes, despite the fact that movement of electrolytes is osmotically coupled to water flow.

Aquaporins (AQPs) are small membrane proteins that primarily mediate the transport of water molecules and recent research also emphasize their importance in certain regulatory processes (Rodriguez et al., 2011; Ribatti et al., 2014; Madeira et al., 2015). In mammals, 13 AQP isoforms have been identified so far and a few of them show a species-specific expression pattern in the pancreas. In humans, AQP1, -5, -8, and -12 are present in the pancreas (Hurley et al., 2001; Tani et al., 2001; Furuya et al., 2002; Burghardt et al., 2003; Itoh et al., 2005). AQP1 is the first AQP which has been described and is exclusively permeable to water (Preston et al., 1992). The presence of this channel has been shown in the apical and lateral plasma membrane of centroacinar cells, and in the apical and basolateral membranes of intercalated and intralobular ducts (Furuya et al., 2002; Burghardt et al., 2003). AQP5 is an aquaglyceroporin which mediates the transport of glycerol, urea and other small solutes beside water. Expression of AQP5 has only been detected in the apical plasma membrane of intercalated ducts but not in the centroacinar cells (Burghardt et al., 2003). AQP1 and -5 are colocalized with the cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channel at the apical membrane of the ductal cells which indicates that these channels influence each other's function (Burghardt et al., 2003). AQP8 and -12 are exclusively localized to centroacinar cells. AQP8 is expressed on the apical plasma membrane (Hurley et al., 2001; Tani et al., 2001), whereas AQP12, a relatively new member of the AQP family, is an intracellular water channel (Itoh et al., 2005). Several studies suggest that altered expression or functions of AQPs are often associated with different diseases affecting the kidney, colon, lacrimal, or salivary glands (Steinfeld et al., 2001; Tsubota et al., 2001; Bedford et al., 2003; Zhu et al., 2016). However, only scarce information is available regarding the role of AQPs in pancreatitis. Ohta et al. (2009) have shown that in the absence of AQP12 the course of cerulein-induced pancreatitis is much worse, probably due to the defect in the secretion of zymogens. In addition, decreased expression of AQP1 and -8 has been found in this pancreatitis model that may also affects the outcome of pancreatitis (Kitami et al., 2007).

The aim of this study is to provide the first detailed characterization regarding the role of AQPs in the pancreas, both under physiological and pathophysiological conditions and to study its relationship to the CFTR Cl^- channel. We have chosen to focus on AQP1, since this isoform is abundantly expressed in both acinar and ductal cells of mouse and human pancreas and the role of this AQP has not been evaluated in pancreatitis yet. Using *in vitro* models, human tissues and transgenic mice, we have shown that AQP1 plays essential role in ductal fluid and HCO_3^- secretion, lack of CFTR decreases its expression and deletion of AQP1 is strongly associated with increased susceptibility of the gland to pancreatitis.

MATERIALS AND METHODS

Ethical Approval

Animal experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (United States, Department of Health and Human Services). In addition, the experimental protocol was approved by the local Ethical Board of the University of Szeged, Hungary and by the National Scientific Ethical Committee on Animal Experimentation (Budapest, Hungary). The use of human tissue was approved by the local ethical committee (University of Szeged, Hungary) and written informed consent was obtained from the patients.

Transgenic Mice

AQP1 knock out (KO) mice were a kind gift from Dr. Alan Verkman (University of California, CA, United States) and Dr. Alastair Poole (University of Bristol, United Kingdom). CFTR KO mice were kindly supplied by Dr. Ursula Seidler (Hannover Medical School, Hannover, Germany). Animals were kept in standard plastic cages on 12:12 h light-dark cycle at room temperature ($23 \pm 1°C$) and had free access to standard or CFTR

specific laboratory chow and drinking solutions. Functional experiments were performed on litter-matched (age 12–16 weeks, both sexes) wild-type (WT) and AQP1 KO mice. All mice were genotyped prior to the experiments. For genotyping, genomic DNA from the tail was isolated and amplified by traditional PCR.

Human Pancreatic Tissue Samples

Human pancreatic tissue samples were obtained from autopsy and from surgical resections. Control tissue ($n = 5$) were collected from the tumor-free region of the pancreas of patients with neuroendocrine tumors. Tissue samples from patients with acute necrotizing pancreatitis (ANP; $n = 5$) or CP ($n = 5$) were from autopsy and from surgical resections. The average age of ANP patients was 56 ± 2.8 years, and the male/female ratio was 1.5:1. The average age of CP patients was 56.8 ± 2.8 years, and the male/female ratio was 4:1.

Cell Cultures and Treatments

Capan-1, Panc-1, and Miapaca-2 cells were obtained from the American Type Culture Collection (Manassas, VA, United States). Capan-1 cells were maintained in Roswell Park Memorial Institute (RPMI)-1640 Medium supplemented with 15% (v/v) fetal bovine serum (FBS), 1% (v/v) L-glutamine and 1% (v/v) Penicillin–Streptomycin (PS). Panc-1 and Miapaca-2 were maintained in Dulbecco's Modified Eagle's Medium (DMEM) high glucose Medium supplemented with 10% (v/v) FBS, 1% (v/v) L-glutamine, 2.5% (v/v) horse serum and 1% (v/v) PS. All three cell lines were kept in a humidified incubator at 37°C. Cells from passage numbers 20–60 were used in this study. In case of PCR experiments 10^6, whereas in the case of immunostaining 10^4 cells were seeded into 75 cm^2 tissue culture flasks or glass bottom petri dishes, respectively, and incubated for 24 h at 37°C. After the incubation, cells were treated with chenodeoxycholic acid (CDCA; 100, 300, and 500 μM), glycochenodeoxycholic acid (GCDCA; 100, 300, and 500 μM), ethanol (EtOH; 1, 10, and 100 mM), palmitoleic acid (POA; 10, 100, and 200 μM) and palmitoleic acid ethyl ester (POAEE; 10, 100 and 200 μM) for 6, 12, 24, and 48 h and the mRNA and protein expression were investigated by real-time PCR and immunohistochemistry.

Chemicals and Solutions

2,7-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM) was from Invitrogen (Eugene, OR, United States). BCECF-AM (2 mmol/l) were prepared in dimethyl sulfoxide (DMSO) and stored at −20°C. POAEE was purchased from Cayman Chemical (Tallinn, Estonia). POA and POAEE were made up as a 10 mM stock solution in DMSO and stored at −20°C. Chromatographically pure collagenase was purchased from Worthington (Lakewood, NJ, United States). All other chemicals were obtained from Sigma-Aldrich (Budapest, Hungary).

For microfluorimetry studies, the standard HEPES-buffered solution contained (in mM): 130 NaCl, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 D-glucose and 10 Na-HEPES. HEPES-buffered solutions were gassed with 100% O_2 and their pH was set to 7.4 with HCl. The standard HCO_3^-/CO_2-buffered solution contained (in mM): 115 NaCl, 25 $NaHCO_3$, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, and 10 D-glucose. The Cl^--free HCO_3^-/CO_2-buffered solution contained (in mM): 115 Na-gluconate, 25 $NaHCO_3$, 6 Ca-gluconate, 1 Mg-gluconate, 2.5 K_2H-sulfate, and 10 D-glucose. HCO_3^-/CO_2-buffered solutions were gassed with 95% O_2/5% CO_2 to set the pH to 7.4.

Isolation of Pancreatic Ducts and Measurement of Intracellular pH

Intra/interlobular ducts were isolated from the pancreas of WT and AQP KO mice using the microdissection technique as described previously (Argent et al., 1986). Changes in intracellular pH (pH_i) were detected using the pH-sensitive fluorescence dye, BCECF. Pancreatic ducts were incubated with BCECF-AM (2 μM) for 30–60 min, at room temperature. After the incubation, ducts were attached to a cover glass, which formed the base of a perfusion chamber, mounted on the stage of an IX71 live cell imaging fluorescence microscope (Olympus, Budapest, Hungary) and excited at 440 and 490 nm. Emissions were monitored at 530 nm. Five to seven region of interests (ROIs) were examined in each experiment, and one measurement per second was obtained. The 490/440 fluorescence ratio was calibrated to pH_i using the high K^+-nigericin technique, as previously described (Thomas et al., 1979; Hegyi et al., 2004).

Measurement of HCO_3^- Secretion

In order to estimate HCO_3^- efflux, the activity of the Cl^-/HCO_3^- exchanger was measured by the Cl^- withdrawal technique. Removal of luminal Cl^- from the standard HCO_3^-/CO_2-buffered solution induced an alkalization in the cells due to the reverse mode of the exchanger. Re-addition of Cl^- induces HCO_3^- secretion via the Cl^-/HCO_3^- exchanger. Under these conditions the initial rate of acidification reflects the activity of the Cl^-/HCO_3^- exchanger. In order to evaluate base efflux [$-J(B^-/min)$] the following equation was used: $-J(B^-/min) = \Delta pH/\Delta t \times \beta_{total}$, where $\Delta pH/\Delta t$ is the rate of acidification measured over the first 60 s and β_{total} is the total buffering capacity of the cell.

Measurement of *in Vitro* Ductal Fluid Secretion

Fluid secretion of intra/interlobular pancreatic ducts was measured using a swelling method, as described previously (Fernandez-Salazar et al., 2004). Briefly, isolated pancreatic ducts were attached to a cover glass which formed the base of a perfusion chamber and mounted on the stage of an IX71 live cell imaging fluorescence microscope. Low magnification, bright-field images were acquired at 1-min intervals using a CCD camera (Hamamatsu ORCA-ER, Olympus, Budapest, Hungary). At the end of each experiment, ducts were perfused with hypotonic solution in order to check the integrity of the duct wall. Ducts that not respond to the hypotonic challenge were excluded from the analysis. Changes in relative luminal volume was analyzed by Scion Image software (Scion Corporation, Frederick, MD, United States) (Fernandez-Salazar et al., 2004; Pascua et al., 2009).

Measurement of *in Vivo* Pancreatic Fluid Secretion

In order to measure pancreatic fluid secretion *in vivo*, magnetic resonance imaging (MRI) was performed on WT and AQP1 KO mice (Maleth et al., 2015). Animals were allowed free access to pineapple juice 12 h before the MRI examination. MRI was performed in a 7.1 Tesla animal scanner (Bruker, Ettlingen, Germany). Strong T2-weighted series of the complete abdomen were acquired before and after retroorbital injection of 10 IU units/kg body weight (b.w.) secretin (ChiroStim, ChiRhoClin, Burtonville, MD, United States). The time between injection and MRI was 6 min. The sequences were acquired using the image parameters: TR/TE 4400/83 ms; flip angle: 180°; matrix 256 × 256; field of view 40 × 40 mm; bandwidth 315 Hz/pixel; slice thickness 1 mm; 20 slices. All image analyses were performed using Osirix (version 5; Pixameo, Bernex, Switzerland). In order to exclude effects of the basal secretion, MRI datasets after and before secretion were subtracted. The created images show the total excretion after secretin stimulation. Excreted fluid is defined as high signal intensity in created images. The fluid excretion into the small intestine was segmented in each slice. The software calculated the volume of the segmented areas. This volume represents the total excreted volume (TEV). In order to minimize artifacts, image noise was reduced.

Measurement of Osmotic Transepithelial Water Permeability

The osmotic transcellular water movement (P_f) was estimated using the cell-impermeant dye, Texas Red^{TM} Dextran as previously described (Levin et al., 2006). Briefly, cells (5×10^5) were grown on a polyester permeable support (Transwell, 12 mm diameter and 0.4 μm pore size). Monolayer confluence was checked by measuring the transepithelial electrical resistance (R_T) using an EVOM-G Volt/Ohm Meter (World Precision Instruments, Sarasota, FL, United States). Cells were washed with isoosmolar phosphate-buffered saline (PBS) from the basolateral surface and hyperosmolar PBS (complemented with 300 mM D-mannitol and 0.25 μg/mL Texas Red Dextran) from the luminal surface. In some experiments, different concentrations of bile acids were added to the apical solution. Transwells were than placed into a CO_2 incubator and 5 μl samples were collected from the apical solution at specified time points. Water moves along the osmotic gradient that causes the dilution of the fluorescent dye, in the apical solution. Fluorescence was measured at 595 nm excitation and 615 nm emission, using a Fluoro Max-4 spectrofluorometer (Horiba Scientific, Tokyo, Japan). For the calculation of P_f, the following formula was used: $dV(0)/dt = P_f{}^*S^*v_w{}^*(\Phi_1 - \Phi_2)$, where S is the tissue surface area, v_w is the partial molar volume of water and $\Phi_1 - \Phi_2$ is the transepithelial osmotic gradient.

Real-Time PCR

Total RNA was purified from individual cell culture samples using the RNA isolation kit of Macherey-Nagel (Macherey-Nagel, Düren, Germany). All the preparation steps were carried out according the manufacturer's instructions. RNA samples were stored at –80°C in the presence of 30 U of Prime RNAse inhibitor (Fermentas, Lithuania) for further analysis. The quantity of isolated RNA samples was checked by spectrophotometry (NanoDrop 3.1.0, Rockland, DE, United States). In order to monitor gene expression, QRT-PCR was performed on a RotorGene 3000 instrument (Corbett Research, Sydney, NSW, Australia) using the TaqMan probe sets of specific Aquaporin genes (Applied Biosystems, Foster City, CA, United States). Information about the genes and the TaqMan assays is collected in **Table 1**. 3 μg of total RNA was reverse transcribed using the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, United States) according to the manufacturer's instructions in final volume of 30 μL. The temperature profile of the reverse transcription was the following: 10 min at room temperature, 2 h at 37°C, 5 min on ice and finally 10 min at 75°C for enzyme inactivation. These steps were carried out in a Thermal Cycler machine (MJ Research, Waltham, MA, United States). After dilution with 30 μL of water, 1 μL of the diluted reaction mix was used as template in the QRT-PCR. For all the reactions TaqMan Universal Master Mix (Applied Biosystems, Foster City, CA, United States) were used according to the manufacturer's instructions. Each reaction mixture (final volume 20 μL) contained 1 μL of primer-TaqMan probe mix. The QRT-PCR reactions were carried out under the following conditions: 15 min at 95°C and 45 cycles of 95°C for 15 s, 60°C for 1 min. Fluorescein dye (FAM) intensity was detected after each cycle. Non-template control sample was used for each PCR run to check the primer-dimer formation. Relative gene expression ratios were calculated as ΔCt values (Ct values of gene of interest versus Ct values of human hypoxanthine phosphoribosyltransferase gene). In the case of treatments, relative changes in gene expression were determined using the $\Delta\Delta C_T$ method as described in *Applied Biosystems User Bulletin* No. 2 (P/N 4303859). $\Delta\Delta C_T$ was calculated using the following formula: $\Delta\Delta C_T = \Delta C_T$ of treated cells – ΔC_T of control, non-treated cells. The N-fold differential expression in the target gene was expressed as $2^{-\Delta\Delta C_T}$. Genes with expression values less than or equal to 0.5 were considered to be down-regulated, whereas values higher than or equal to 2 were considered to be up-regulated. Values ranging from 0.51 to 1.99 were not considered to be significant.

Immunocytochemistry

CAPAN-1 cells were washed with PBS twice and fixed in paraformaldehyde (4% in PBS) for 30 min at room temperature (RT). In order to avoid non-specific antibody binding cells were incubated with 10% donkey serum and 1% BSA for further 30 min. After blocking, cells were incubated with primary AQPs human, polyclonal antibodies (1:100 dilutions; Abcam, Cambridge, United Kingdom) at 4°C overnight. Petri dishes were then washed with PBS and incubated with FITC-conjugated AffiniPure Donkey Anti-Rabbit IgG secondary antibody (1:400 dilutions; DAKO, Milan, Italy) for 60 min at RT. Nuclei were counterstained with Dapi. Dishes were then mounted and observed by a

TABLE 1 | TaqMan assays used for the investigation of AQP expression.

Definition	Gene symbol	Ref Seq Acc. number	ABI TaqMan assay ID
Homo sapiens aquaporin 1 (Colton blood group)	AQP1	NM_198098	Hs00166067_m1
Homo sapiens aquaporin 2 (collecting duct)	AQP2	NM_000486	Hs00166640_m1
Homo sapiens aquaporin 3 (Gill blood group)	AQP3	NM_004925	Hs00185020_m1
Homo sapiens aquaporin 4	AQP4	NM_001650	Hs00242341_m1
Homo sapiens aquaporin 5	AQP5	NM_001651	Hs00387048_m1
Homo sapiens aquaporin 6, kidney specific	AQP6	NM_001652	Hs01546883_m1
Homo sapiens aquaporin 7	AQP7	NM_001170	Hs00357359_m1
Homo sapiens aquaporin 8	AQP8	NM_001169	Hs00154124_m1
Homo sapiens aquaporin 9	AQP9	NM_020980	Hs00175573_m1
Homo sapiens aquaporin 10	AQP10	NM_080429	Hs00369738_m1
Homo sapiens aquaporin 11	AQP11	NM_173039	Hs00542681_m1
Homo sapiens aquaporin 12A	AQP12A	NM_198998	Hs01651303_m1
Homo sapiens aquaporin 12B	AQP12B	NM_001102467.1	

Fluowiew 10i-W confocal microscopy (Olympus, Budapest, Hungary).

Immunohistochemistry

Paraffin-embedded, 3- to 4-μm-thick sections of surgically removed resection specimens and autopsy tissue samples were used for immunohistochemistry. After deparaffinization of tissue samples with EZ Prep Concentrate 10X (Ventana Medical Systems, Tucson, AZ, United States), endogenous peroxidase blocking and antigen retrieval (CC1; Ventana Medical Systems), pancreas sections were incubated with polyclonal AQP1 antibody (1:100 dilution; Alomone Labs, Jerusalem, Israel) overnight at 4°C. Immunohistochemical staining was performed with horseradish peroxidase multimer-based, biotin-free detection technique according to the protocol of the automated Ventana system (Ventana Benchmark XT; Ventana Medical Systems). For visualization, the UltraView Universal diaminobenzidine (DAB) Detection Kit (Ventana Medical Systems) was applied. Sections from human pancreas were used as positive controls. For negative control, primary antibodies were substituted with antibody diluent (Ventana Medical Systems). The stained slides were digitized with Mirax Pannoramic MIDI and Mirax Pannoramic SCAN digital slide scanners (3DHistech Ltd., Budapest, Hungary).

Cryosections from WT, AQP1, and CFTR KO mice pancreas were fixed in 2% paraformaldehyde, permeabilized in 10% Tween 20-sodium citrate, blocked with 5% goat serum followed by immunofluorescent double staining for AQP1 mouse monoclonal antibody (1:500 dilutions; Thermo Fisher, Rockford, IL, United States) and CFTR rabbit polyclonal antibody (1:100 dilutions; Alomone Labs, Jerusalem, Israel) at 4°C, overnight. Following washing, sections were incubated with secondary antibodies goat-anti-mouse (Alexa fluor 488, Thermo Fisher, Rockford, IL, United States) and goat-anti-rabbit (Alexa fluor 568, Thermo Fisher, Rockford, IL, United States) for 2 h at room temperature in the dark. Nuclei were counterstained with Dapi. Sections were then mounted and analyzed using a Zeiss LSM 880 confocal laser scanning microscope (Carl Zeiss Technika Kft., Budaörs, Hungary).

Quantification of the Immunostainings

In order to quantify AQP1 or CFTR positively stained area, 10–12 representative, digital images were taken from the human (normal pancreas, AP and CP) and mice (WT, AQP1 KO and CFTR KO) pancreas sections and from Capan-1 cells. Pictures were than converted to gray scale (16-bit) and thresholded in order to select the positively stained area, using the ImageJ software. The intensity of DAB (human pancreas) or fluorescence signal (mice pancreas and Capan-1 cells) varied on an arbitrary scale from 0 to 255, where 0 is the negative staining (white pixels) and 255 is the maximal staining (black pixels). In the human samples, the mean integrated density of the positively stained area was normalized to the mean integrated density of the total image and converted into percentage. In the cell line, the mean integrated density of the positively stained area was normalized to the total cell number and expressed in arbitrary units. In the mice samples, the ductal area was selected and the total fluorescence intensity was calculated and summarized which was than normalized to the ductal area (μm^2) and expressed in arbitrary units/μm^2. Comparison of each groups were calculated by one-way ANOVA, followed by the Holm-Sidak method. Statistical significance was defined as $p \leq 0.05$. Data are expressed as means ± SEM.

Induction of Acute Pancreatitis in Mice

Acute pancreatitis (AP) was induced in mice by hourly (10 times) intraperitoneal (i.p.) injections of cerulein (50 μg/kg) (Niederau et al., 1985; Ding et al., 2003; Pallagi et al., 2014) following anesthesia with i.p. 85 mg/bwkg pentobarbital (Bimeda MTC, Cambridge, ON, Canada). The control animals received the same amount of saline. Two hours after the final injection, mice were euthanized by pentobarbital overdose (200 mg/bwkg i.p.). Animals were exsanguinated through the cardiac puncture and the pancreata were immediately removed. The collected blood was centrifuged at 4°C with 2500 RCF for 15 min and the sera were stored at −20°C until use. The pancreas was trimmed from fat and lymphatic tissue, put into 6% neutral formaldehyde solution and stored at −80°C until use. Serum amylase activity was measured with a commercial colorimetric kit

(Diagnosticum, Budapest, Hungary) with a FLUOstar OPTIMA (BMG Labtech, Budapest, Hungary) microplate reader at 405 nm. The formaldehyde-fixed pancreatic tissue was embedded in paraffin blocks, cut into 3 μm thick sections and stained for hematoxylin-eosin using standard techniques and viewed by light microscopy. A semiquantitative scoring system was used to evaluate the presence of edema, the rate of necrosis and infiltration of inflammatory cells according to the following scoring system (Kui et al., 2015): Edema (0: none, 1: patchy interlobular; 2: diffuse interlobular; 3: diffuse interlobular, and intraacinar), necrosis (0: none, 1: patchy interlobular; 2: diffuse interlobular; 3: diffuse interlobular and patchy intraacinar; 4: diffuse interlobular and intraacinar) and leukocytic infiltration (0: none; 1: patchy interlobular; 2: diffuse interlobular; 3: diffuse interlobular and intraacinar). The rate of necrosis was expressed as percentage of the total analyzed pancreatic area.

Statistical Analysis

Data are expressed as means ± SEM. In the case of pancreatic fluid and HCO_3^- secretion measurements, significant difference between groups was determined by ANOVA. Statistical analysis of the immunohistochemical data was performed using the Student's *t*-test. Probability values of $p \leq 0.05$ were accepted as being significant.

RESULTS

Capan-1 Cells Express Functionally Active AQPs

The relative gene expressions of AQP isoforms were studied in different pancreatic ductal cell lines (Capan-1, Panc-1, and Miapaca-2) using RT-PCR and TaqMan primer-probe sets, specific for AQP1-AQP12 isoforms (**Table 1**). Expressions of AQPs were investigated at different time points (6, 12, 24, and 48 h) 24 h after plating the cells, and as an internal gene, human hypoxanthine phosphoribosyltransferase (HPRT) was used. RT-PCR analysis revealed that among the three cell lines, AQPs were expressed at the highest level in Capan-1 cells, whereas in Panc-1 and Miapaca-2 the expression of AQPs was hardly detectable (**Figures 1A–C**). In Capan-1 cells high levels of AQP1, -3, and -5 was detected, which is in agreement with previous observations (Burghardt et al., 2003). The presence of these isoforms was also confirmed at protein level in the Capan-1 cells (**Figure 1D**). In order to test that the AQPs expressed on Capan-1 cells are functionally active, we investigated the transepithelial water flow in the presence and absence of the non-specific AQP inhibitor $HgCl_2$. As shown in **Figures 1E,F**, administration of 0.3 mM $HgCl_2$ to the apical solution inhibited the osmotic water movement by 52.6 ± 2.4%, indicating that significant proportion of water is transported through AQPs.

AQP1 Is Essential for Pancreatic Ductal Fluid and HCO_3^- Secretion

Aquaporins are involved in transepithelial water transport in numerous tissues (Tradtrantip et al., 2009). In the next step, we investigated the role of this protein in pancreatic ductal fluid secretion using gene modified mice. Using traditional PCR we have shown the presence of AQP1 both in the isolated, intra/interlobular pancreatic ducts and the total pancreas (**Figure 2A**). Slight expression of AQP5 was also detected in the pancreatic ducts, whereas the mRNA presence of AQP8 and -12 was only observed in the total pancreas (data not shown). We decided to characterize the role of AQP1, since this isoform is constitutively expressed in both the ductal and acinar cells and also can be found in the mouse and human pancreas (**Table 2**). In contrast to AQP1, AQP3 is not expressed in the mouse pancreas and this isoform is either involved in tumor progression than pancreatitis (Direito et al., 2017; Huang et al., 2017). Although AQP5 is present in the intra/interlobular pancreatic ducts of mice and human, and probably plays role in ductal fluid secretion (Burghardt et al., 2003), KO animal for this isoform was not available to us. **Table 2** summarizes the presence of AQP isoforms in mice and human.

The rate of fluid secretion was measured over 30–40 min using sealed ducts and the swelling technique. Initially, ducts were perfused with the standard HEPES-buffered solution and then perfusion was switched to HCO_3^-/CO_2-buffered solution to initiate HCO_3^--dependent secretion. As shown in **Figure 2B**, the presence of HCO_3^- induced a dynamic swelling of the WT ducts as a result of fluid secretion into the closed luminal space. In contrast, ducts from AQP1 KO mice showed no or only a slight response to HCO_3^-. Under stimulated conditions (forskolin; 10 μM), the rate of fluid secretion further increased in the WT ducts, whereas in the case of KO ducts, no response was detected. **Figure 2C** summarizes the relative luminal volume changes in WT and KO ducts after forskolin stimulation. As shown in **Figure 2C**, the relative luminal volume increase reduced by 89.9 ± 6.2% in the absence of AQP1, indicating that this AQP isoform plays an essential role in fluid secretion.

We also investigated the rate of pancreatic fluid secretion *in vivo* by MRI cholangiopancreatography in anesthetized mice. Fluid secretion was stimulated by retroorbital administration of 10 U/kg b.w. secretin, and then TEV was measured in WT and KO animals. Similarly, to the *in vitro* measurements, the pancreatic fluid secretion significantly reduced in AQP1 KO (0.0041 TEV/cm^3) vs. WT (0.023 TEV/cm^3) mice (**Figures 2D,E**).

Next, we were curious whether the decreased ductal fluid secretion is also associated with impaired HCO_3^- efflux in AQP1 KO animals. For the measurement of ductal HCO_3^- secretion, intra/interlobular pancreatic ducts were isolated from the pancreas of WT and AQP1 KO mice. Ducts were perfused both from the luminal and basolateral membrane with HCO_3^-/CO_2-buffered solution. Removal of Cl^- from the apical solution induced an alkalisation inside the cells, due to the reverse mode of the Cl^-/HCO_3^- exchanger. Addition of Cl^- back to the external solution decreased the pH_i indicating HCO_3^- efflux through the exchanger. Under these conditions, the initial rate of recovery from alkalosis reflects the activity of the Cl^-/HCO_3^- exchanger [$(-J(B^-/min)$]. Using this technique, we showed that the rate of acidification significantly reduced in AQP1 KO (42 ± 3.2%) vs. WT ducts indicating that pancreatic ductal HCO_3^- secretion is impaired in the absence of AQP1 (**Figures 2F,G**).

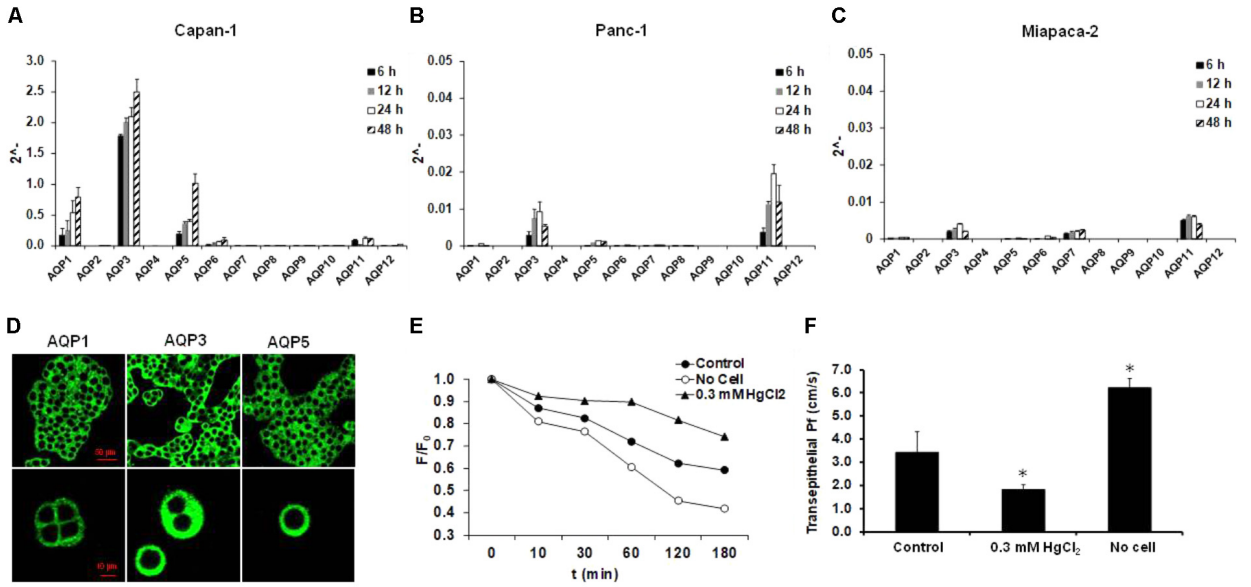

FIGURE 1 | Expression and activity of AQPs in pancreatic ductal cells. Expression of different AQP isoforms was investigated by real-time PCR in **(A)** Capan-1, **(B)** Panc-1, and **(C)** Miapaca-2 pancreatic ductal cell lines, 6, 12, 24, and 48 h after the plating the cells. Data represent mean ± SEM of three, independent experiments. **(D)** Immunofluorescence staining of Capan-1 cells using FITC-conjugated anti-AQP1, -3, and -5 antibodies. **(E)** Osmotic water movement was investigated in Capan-1 cells. Representative graph shows changes in the fluorescence intensity of the apical solution, at different time points, in the present (triangle) and absence (black circle) of luminal $HgCl_2$ (0.3 mM). To estimate the changes, the fluorescence intensity **(F)** was normalized to the initial value (F_0). Transwell without cells was used for absolute positive control (open circle). **(F)** Summary of the P_f values obtained from the experiments in **(E)**. Data represent mean ± SEM of three, independent experiments. $^*p \leq 0.05$ vs. Control.

AQP1 Expression Decreased in CFTR KO Mice

The decreased HCO_3^- secretion in AQP1 KO mice indicates that beside the transport of water, AQP1 interacts with one or more ion transporters which are involved in HCO_3^- secretion. The CFTR Cl^- channel plays essential role in ductal HCO_3^- secretion by maintaining a luminal [Cl^-] which is necessary for HCO_3^- efflux through the Cl^-/HCO_3^- exchanger. Several studies presume a physical interaction between the CFTR Cl^- channel and certain AQP isoforms (Schreiber et al., 1999; Cheung et al., 2003; Jesus et al., 2014a,b). Colocalization of this two channel has also been found in the human pancreas (Burghardt et al., 2003). In the following step, we performed immunostaining on the pancreas of AQP1 and CFTR KO mice in order to characterize the possible relation between the two channels. In WT mice, AQP1 expression was detected throughout the whole plasma membrane, whereas expression of CFTR exclusively localized to the apical membrane of the ducts (**Figure 3A**). The absence of AQP1 caused a slight but not significant decrease in the expression of CFTR, indicating that the impaired HCO_3^- secretion in the AQP1 KO mice is not due to the decreased expression of CFTR (**Figure 3A**, middle line). Interestingly we have found that expression of AQP1 dramatically decreased in the intra/interlobular ducts of CFTR KO mice, especially at the apical membrane (**Figure 3A**, bottom line and **Figure 3B**). We have also found that the absence of CFTR did not affect the expression of AQP1 in the blood vessels indicating that some kind of interaction may exist between these two channels in the pancreatic ductal cells. Although further investigations are needed to clarify whether the two channels are able to regulate each others function, expression or trafficking.

Expression and Function of AQPs Significantly Decreased After Bile Acid Treatment

In the next step, we studied the effect of pancreatitis-inducing factors on the expression of AQP1. Gallstone obstruction and heavy alcohol consumption are the two major causes of pancreatitis. Capan-1 cells were treated with CDCA and GCDCA (100, 300, and 500 μM) (Venglovecz et al., 2008, 2011; Muili et al., 2013), EtOH (1, 10, and 100 mM), POA and POAEE (10, 100, and 200 μM) (Criddle et al., 2006; Judak et al., 2014; Maleth et al., 2015) for 6, 12, 24, and 48 h and the mRNA expressions of AQP1 were analyzed by RT-PCR. Among the investigated agents, CDCA had the most marked effect, it dose- and time-dependently decreased the expression of AQP1 (**Figure 4A**). GCDCA, POA, and POAEE caused a significant decrease at 12 and 24 h, primarily at higher doses, which partially regenerated after 48 h in the continuous presence of the agents (**Supplementary Figures 1A–C**). In contrast to bile acids, EtOH initially (24 h) increased the expression of AQP1 that was followed by a decrease (**Supplementary Figure 1D**). In order to decide whether the downregulating effect of CDCA can also be observed at protein level, we performed immunostaining on the CDCA-treated cells, using specific antibodies against AQP1. CDCA dose- and time-dependently

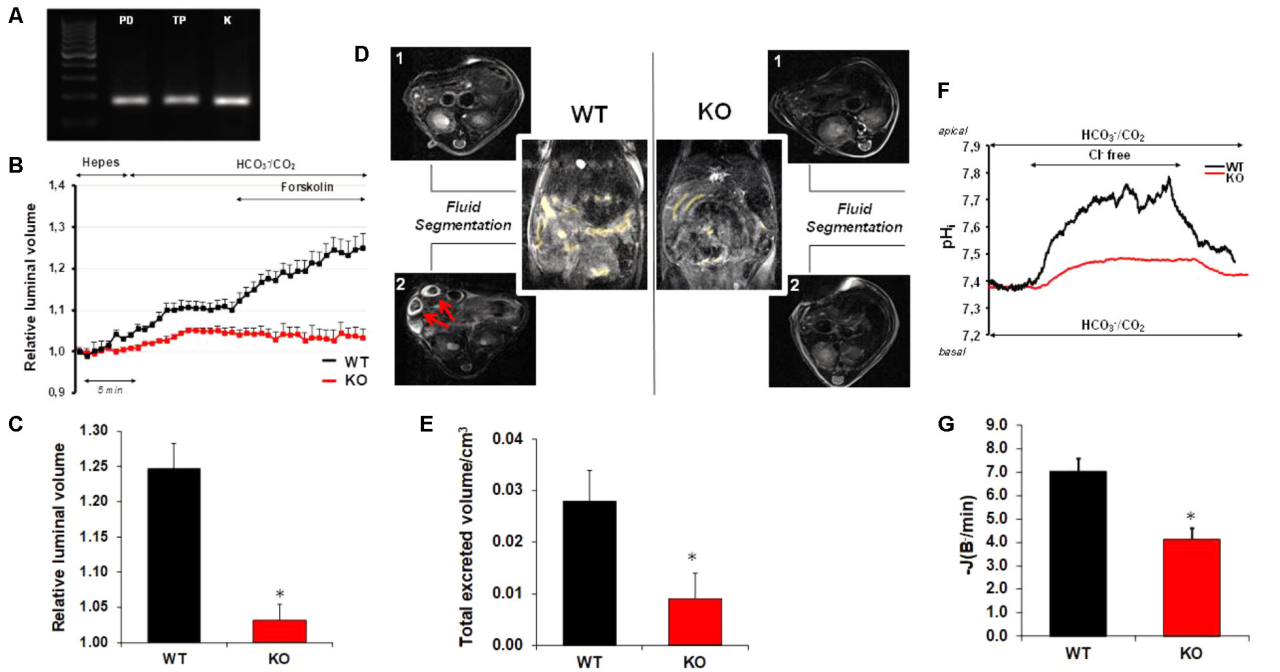

FIGURE 2 | Pancreatic ductal HCO_3^- and fluid secretion in AQP1 knock out mice. **(A)** Traditional PCR shows the presence of AQP1 mRNA both in the isolated pancreatic ducts (PD) and in the total pancreas (TP). As positive control, kidney was used (K). **(B)** Pancreatic ductal fluid secretion was measured on isolated pancreatic ducts from wild type (WT, black line) and AQP1 knock out (KO, red line) mice. Changes in the relative luminal volume of the ducts was measured by videomicroscopy and analyzed by Scion Image software. **(C)** Changes in the relative luminal volume of the pancreatic ducts of AQP1 WT and KO mice measured at the final time point (40 min). Data are shown as means ± SEM. n = 4–8 ducts/groups. *$p \leq 0.05$ vs. WT. **(D)** Reconstructed images of the duodenal filling before (1) and after (2) secretin stimulation. Yellow color shows the presence of fluid. Red arrows indicate excreted volume in the small bowel after stimulation with secretin. In the case of KO mice, the secretin-stimulated fluid secretion significantly decreased. **(E)** *In vivo* pancreatic fluid secretion was measured as total excreted volume (TEV) using small animal magnetic resonance imaging. Raw data were acquired in axial and coronar view. Data are shown as means ± SEM. n = 4 animals/groups. *$p \leq 0.05$ vs. WT. **(F)** Representative experimental traces show pancreatic ductal HCO_3^- secretion in AQP1 WT (black line) and AQP1 KO (red line) mice. **(G)** Base flux [$-J(B^-/min)$] was calculated from the $\Delta pH/\Delta t$ obtained by linear regression analysis of pH_i measurements made over the first 60 s after readdition of extracellular Cl^-. Means ± SEM are from 20 ROIs of four ducts. *$p \leq 0.05$ vs. WT.

TABLE 2 | mRNA expression of AQP isoforms in mice and human.

Known isoforms	Isoforms in mice pancreas	Isoforms in mice pancreatic ducts	Isoforms in human pancreas	Isoforms in human pancreatic ducts	Available KO mice
AQP0	–	–	–	–	–
AQP1	√	√	√	√	√
AQP2	–	–	–	–	–
AQP3	–	–	–	√	–
AQP4	–	–	–	–	√
AQP5	√	√	√	√	–
AQP6	–	–	–	–	–
AQP7	–	–	–	–	–
AQP8	√	–	√	–	–
AQP9	–	–	–	–	–
AQP10	–	–	–	–	–
AQP11	–	–	–	–	–
AQP12	√	–	√	–	–

decreased the protein expression of AQP1 was consistent with the PCR data (**Figure 4B**). Representative ICC pictures show that incubation with 500 μM CDCA time-dependently decreased the AQP expression in the cells (**Figure 4C**). The effect of CDCA on the activity of AQPs was also investigated. As shown in **Figure 4D**, 100 and 300 μM CDCA had no effect on the transepithelial water movement, however, at 500 μM P_f was significantly impaired.

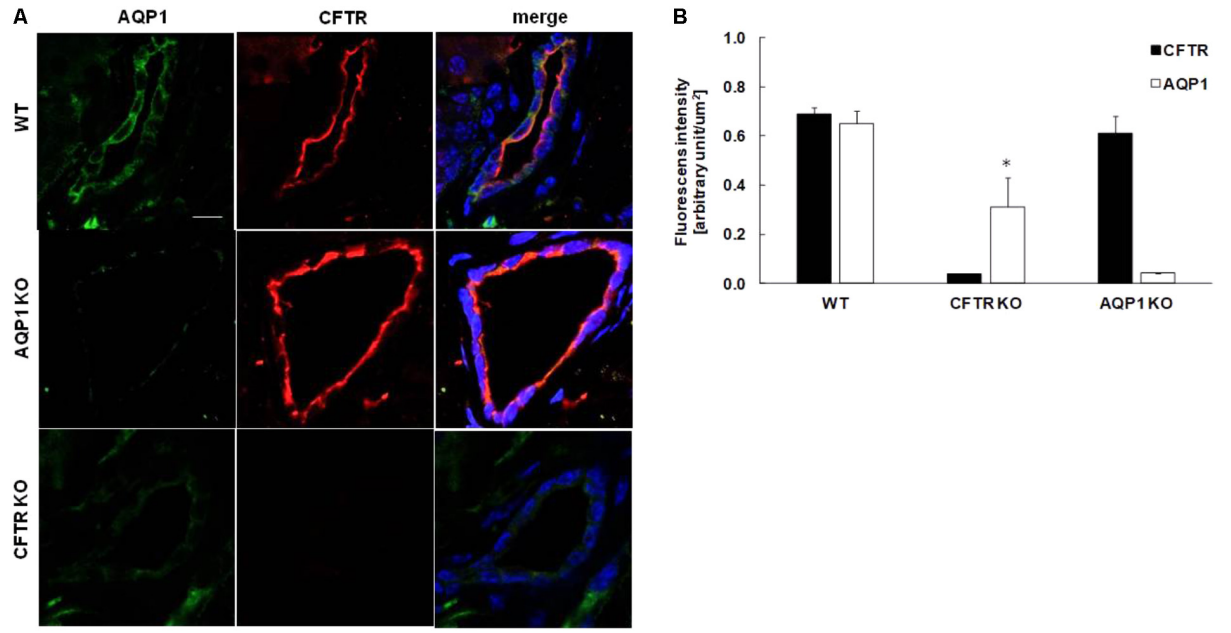

FIGURE 3 | Expression of AQP1 and CFTR in the pancreas of knock out mice. **(A)** Representative immunofluorescence staining of AQP1 and CFTR in wild type (WT, upper line), AQP1 knock out (AQP1 KO, middle line) and CFTR KO (bottom line) mice. Pictures were taken at 40× magnification. Pancreas slices were excited at 405 (Dapi), 488 (Alexa fluor 488) and 568 (Alexa fluor 568) nm and emissions were collected at 453, 516, and 603 nm, respectively. Scale bar represents 10 μm. **(B)** Summary bar chart shows the mean fluorescence intensity in the ductal cells normalized to the ductal area and expressed in arbitrary units/μm^2. Data are presented as means ± SEM. *$p \leq 0.05$ vs. WT (AQP1), $n = 5$.

Acute Pancreatitis Is Aggravated in AQP1 KO Mice

In order to test the hypothesis that AQP1 may be involved in the pathomechanism of pancreatitis, we investigated the severity of cerulein-induced AP in WT and AQP1 KO mice. WT and AQP1 KO mice were given 10 hourly injections of either physiological saline (control) or supramaximal doses of cerulein (50 μg/kg per injection) i.p. to induce AP. After saline injection, the pancreas had normal histology in both WT and KO animals. In contrast, i.p. injections of cerulein caused extensive cell damage both in the WT and KO animals. The extension of pancreatic necrosis were markedly higher in the AQP1 KO (25 ± 2.8%) vs. WT mice (12.1 ± 3.2%), whereas no differences were observed in the extent of edema and in the infiltration of inflammatory cells (**Figures 5A–C**). Serum amylase activities were significantly higher in KO (1605 ± 6 U/l) vs. WT mice (1285 ± 51 U/l) after the induction of AP (**Figure 5D**). As shown on the representative histological images (**Figure 5E**), the rate of pancreatic necrosis was more extensive in AQP1 KO mice. Overall, these results indicate that in the absence of AQP1 the course of pancreatitis is more severe.

Expression of AQP1 Is Decreased in Acute and Chronic Pancreatitis

Since we found that the lack of AQP1 exacerbates the course of cerulein-induced pancreatitis in mice, we tested whether this water channel is also involved in the pathomechanism of pancreatitis in humans. Therefore, in the next step we investigated the expression of AQP1 in pancreatic tissues samples obtained from five patients with ANP and five patients with CP. Control pancreatic tissue were obtained from tumor-free tissue surrounding neuroendocrine pancreatic tumors. In order to localize AQP1, IHC was performed. In the normal pancreas, strong AQP1 immunoreactivity was detected in the intra/interlobular and intercalated ducts, the acinar and centroacinar cells (**Figures 6A–C**). The staining in acinar cells and smaller ducts is mainly localized to the lateral and apical surface of the cells (**Figures 6B,C**). In the interlobular ducts, the apical plasma membrane was positive to AQP1 with some cytoplasmic staining, whereas Langerhans islets were completely negative for AQP1. These results are consistent with previous observations (Burghardt et al., 2003). In the ANP and CP pancreatic tissue sections the expression of AQP1 strongly reduced in the interlobular ducts, whereas intralobular and intercalated ducts still exhibited weak to moderate AQP1 immunoreactivity (**Figures 6D–G**). In case of acinar cells, the expression of AQP1 slightly decreased in the inflamed pancreas, especially in ANP. Quantification of DAB intensity showed that AQP1 staining was significantly higher in normal pancreas vs. the ANP or CP groups (**Figure 6H**).

DISCUSSION

Acute and chronic pancreatitis are serious disorders characterized by inflammation and injury of the gland.

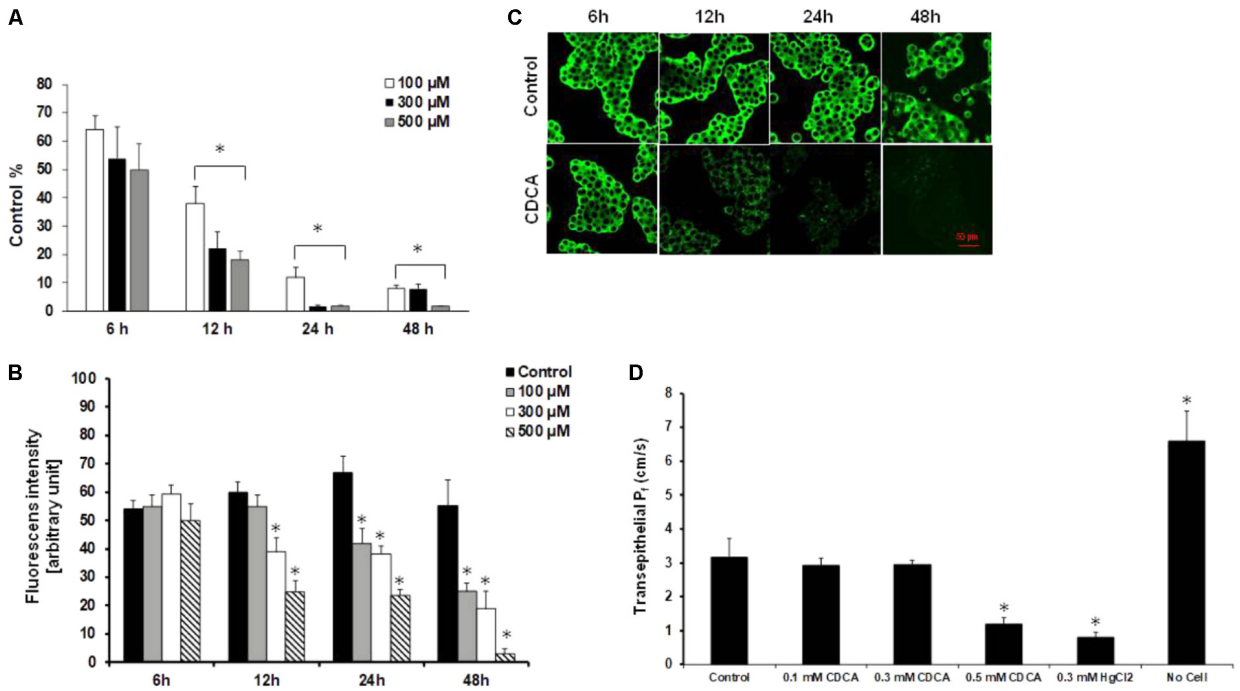

FIGURE 4 | Effect of chenodeoxycholic acid on the expression and activity of AQP1. **(A)** Capan-1 cells were treated with chenodeoxycholic acid (CDCA) for 6, 12, 24, and 48 h and the relative gene expression of AQP1 was investigated by real-time PCR. Data represent mean ± SEM of three, independent experiments. **(B)** Protein expression of AQP1 was investigated by immunocytochemistry in Capan-1 cells. Protein expression was measured from the mean fluorescence intensity, normalized to the total cell number and expressed in arbitrary units. Data represent mean ± SEM of three, independent experiments. **(C)** Representative immunofluorescence staining of Capan-1 cells show the expression of AQP1 after the treatment with CDCA (500 µM) for 6, 12, 24, and 48 h. **(D)** Summary bar chart shows the changes in osmotic transcellular water movement (P_f) of Capan-1 cells in the presence of various concentrations of CDCA. Transwell without cells was used for absolute positive control. Data represent mean ± SEM of three, independent experiments. *$p \leq 0.05$ vs. Control.

Gallstones and heavy alcohol consumption are responsible for approximately 80% of all AP patients (Parniczky et al., 2016), whereas alcohol abuse is the primary cause of CP (Szucs et al., 2017). Although the therapy improved a lot in the last few years the morbidity and mortality of pancreatitis is still excessively high (Parniczky et al., 2016). Defects in ductal fluid secretion have been proposed as an important factor in the pathomechanism of pancreatitis (Hegyi et al., 2011b). This fluid is high in HCO_3^- which prevents the premature activation of zymogens and provides an optimal environment in the duodenum for the action of digestive enzymes. The secreted fluid is also beneficial from the point of view that washing out the potential toxic factors such as activated digestive enzymes or bile acids from the ductal tree and therefore prevents the pancreas from their damaging effects (Venglovecz et al., 2008). AQP1 is a water channel that extensively expressed in the acinar and ductal cells where mediates pancreatic fluid production. Several studies indicate that altered expression or localization of AQPs associates with different gastrointestinal disorders, such as gastritis or diarrhea; therefore, many studies have been conducted to identify the specific role of particular AQP isoforms. Although, AQP1 plays an essential role in pancreatic physiology its function under pathological conditions is not known.

In the present study, we showed that (i) bile acids strongly decrease the expression of AQPs in pancreatic ductal cells, (ii) chronic or acute inflammation of the pancreas is associated with decreased expression of AQP1, (iii) the absence of AQP1 reduces ductal fluid and HCO_3^- secretion, (iv) and induces a more severe pancreatitis in a cerulein-induced pancreatitis model.

Basically there are two ways for water transport. One is the paracellular way along the osmotic gradient generated by the ion transporters and the other one is the transcellular pathway through the AQPs. In case of the pancreatic ducts, it is not clear which pathway is the dominant. The pancreatic ductal epithelium is a moderately leaky epithelium which favors the paracellular movement of water (Novak and Greger, 1988). Nevertheless, administration of luminal Hg^{2+}, a non-specific inhibitor of AQPs, decreased ductal fluid secretion by more than 90% in rat pancreatic ducts, which strengthen the importance of transcellular pathway (Ko et al., 2002). In the present study, we demonstrated that pancreatic ductal cells express functionally active AQPs. We have identified the presence of AQP1, -3, and -5 on the ductal cells that are consistent with previous observations (Burghardt et al., 2003). Treatment of the cells with pancreatitis-inducing agents, mostly decreased the expression of AQPs both at mRNA and protein levels. Among the investigated agents, CDCA had the biggest effect, which dose- and time-dependently decreased the expression of AQP1. These data are partly consistent with previous findings in the colon, where bile acid treatment reduced the protein expression of AQP3,

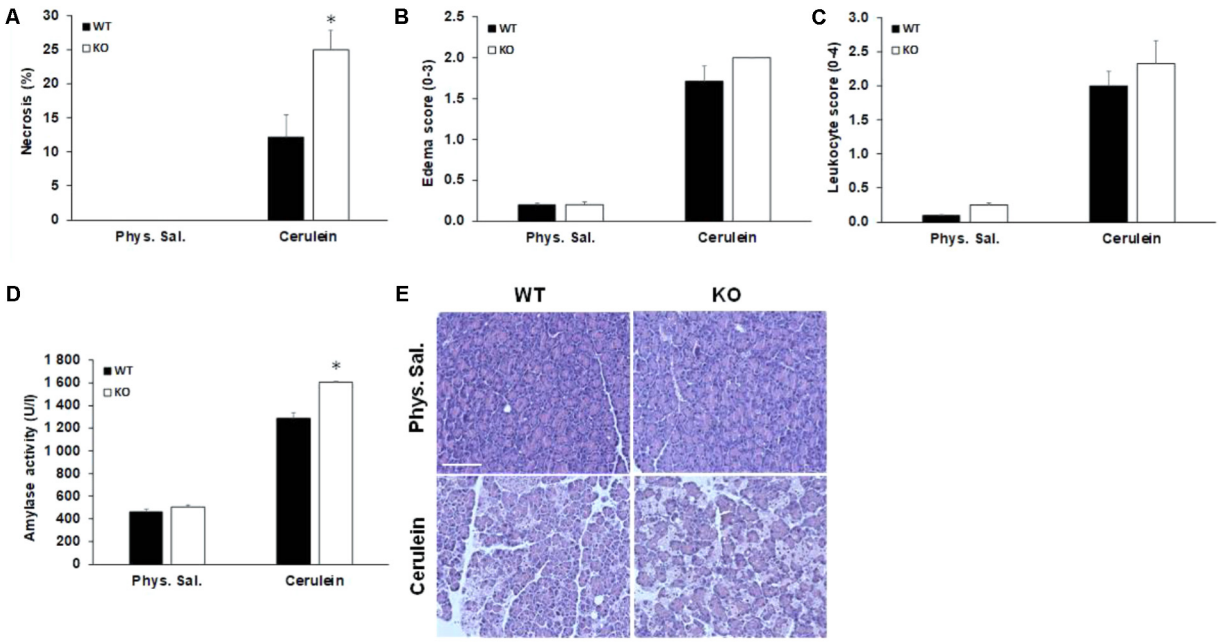

FIGURE 5 | Characterization of acute pancreatitis in AQP1 knock out mice. **(A)** Pancreatic cell necrosis, **(B)** edema, **(C)** leukocyte infiltration, and **(D)** serum amylase activity (U/l) were measured in wild-type (WT) and AQP1 knock out (KO) mice after induction of pancreatitis. Control animals received the same amount of saline. Data are presented as means ± SEM. $^*p \leq 0.05$ vs. WT. **(E)** Representative histological images show pancreatic sections from control (physiological saline) and cerulein-treated animals. Phys sal., physiological saline. Scale bar represents 50 μm.

whereas increased the levels of AQP7 and -8 (Yde et al., 2016). In case of EtOH, the expression of AQPs initially increased (presumably due to a compensatory mechanism), then a decrease was observed, similarly to the rat stomach, where intragastrical administration of 1 mM EtOH caused analogous changes in the expression of AQPs (Bodis et al., 2001). High concentration of CDCA also decreased the activity of the water channels, which is somewhat related to our previous findings on guinea pig pancreatic ducts, where 1 mM CDCA strongly inhibited ductal HCO_3^- and thus fluid secretion (Venglovecz et al., 2008). The involvement of AQP1 in epithelial water movements has been described in various tissues, although there is still no consensus among researchers regarding the functional importance of this water channel (Marples, 2000). In cholangiocytes, the absence of AQP1 does not affect the fluid secretion (Mennone et al., 2002), although inhibition of the secretin-induced translocation of AQP1 reduces bile flow more than half (Marinelli et al., 1997, 1999). It has been also demonstrated that inhibition of AQPs by $HgCl_2$ dose-dependently reduced the osmotically induced volume increase in these cells (Roberts et al., 1994). In the kidney, the absence of AQP1 dramatically decreased the urine concentrating ability of mice due to the impaired water permeability of the proximal tubule, limb of Henle and vasa recta (Ma et al., 1998; Schnermann et al., 1998; Pallone et al., 2000). The importance of AQP1 has been also highlighted in the brain where cerebrospinal fluid production decreased by fivefold in mice lacking AQP1 (Oshio et al., 2004).

In order to determine the role of AQP1 in pancreatic fluid secretion, we used AQP1 KO mice. No differences were observed in body weight and physical appearance between WT and KO mice, although the lifespan of AQP1 deficient mice was slightly lower. Using both *in vitro* and *in vivo* approaches, we found that fluid and HCO_3^- secretion significantly reduced in the absence of AQP1. These data are partly in contrast with previous observations demonstrating that defect in AQP1 expression cause only a small, but not significant decrease in the rate of stimulated pancreatic fluid secretion (Ma et al., 2001). Although this discrepancy can be explained by the different methods used for the measurement of pancreatic fluid. Nevertheless, other studies have found that decreased expression or function of AQP1 dramatically reduce ductal fluid secretion (Ko et al., 2002; Gabbi et al., 2008). Ko et al. (2002) have shown that luminal or basolateral administration of $HgCl_2$ dose-dependently decrease the fluid secretory rate and osmotic water permeability of isolated pancreatic ducts. It has been also described that the defect in pancreatic fluid secretion in liver X receptor β-deficient mice is related to the decreased expression of AQP1 in the pancreatic ducts of these mice (Gabbi et al., 2008).

In order to identify the mechanism by which AQP1 influences ductal HCO_3^- secretion, we investigated the relation of this channel with the CFTR Cl^- channel. CFTR is a cAMP-activated Cl^- channel, which primarily located at the apical membrane of epithelial cells and plays a crucial role in the maintenance of fluid homeostasis. Growing number of studies indicate that a molecular interaction exists between CFTR and certain AQP isoforms. It has been demonstrated that activation of CFTR by cAMP, increases the water permeability of the cells through the activation of AQP3 (Schreiber et al., 1999; Jourdain et al., 2014).

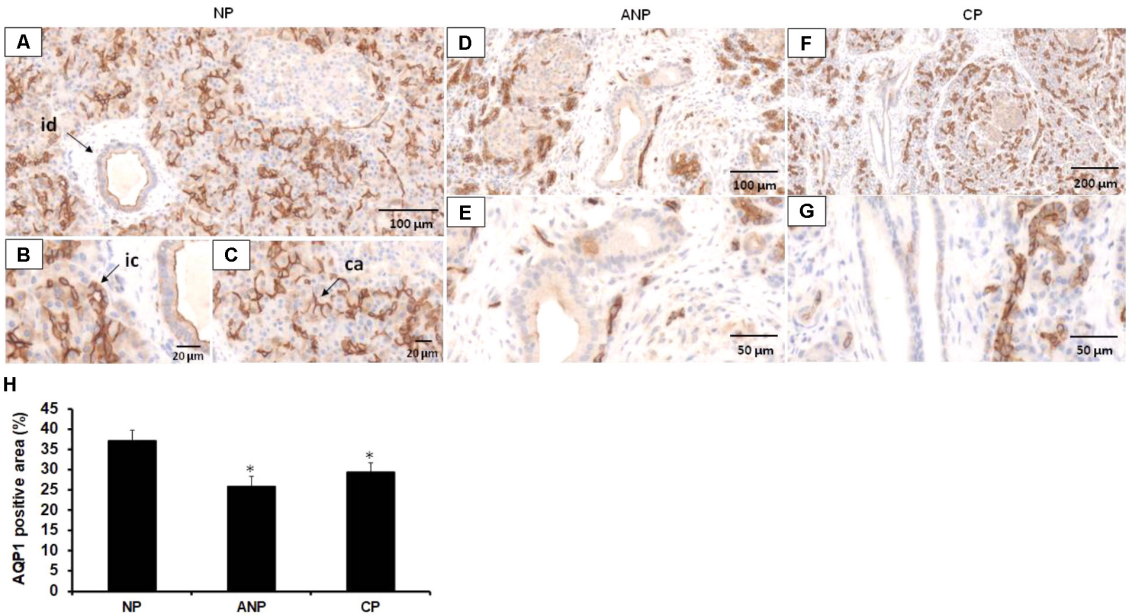

FIGURE 6 | Expression of AQP1 in patients with acute and chronic pancreatitis. Representative histological images show expression and localization of AQP1 in normal human pancreas (NP, **A–C**) acute necrotizing pancreatitis (ANP, **D,E**) and chronic pancreatitis (CP, **F,G**) samples. AQP1 immunoreactivity was detected in inter/intralobular and intercalated ducts, in the acinar and centroacinar cells of the normal pancreas. In contrast, expression of AQP1 strongly decreased in ANP and CP. id, interlobular duct; ic, intercalated duct; ca, centroacinar cells. **(H)** Intensity of diaminobenzidine staining was measured in pancreas samples by ImageJ software and expressed as percentage of total pancreatic area. $^*p \leq 0.05$ vs. normal pancreas, $n = 10–12$.

The relation between AQPs and CFTR has been also confirmed in rat epididymis, where CFTR potentiates AQP9-mediated water permeability (Cheung et al., 2003). Moreover, a direct interaction between AQPs and CFTR has been also observed in Sertoli cells (Jesus et al., 2014a,b). Using double immunostaining we showed for the first time that AQP1 and CFTR are co-localized at the apical membrane of pancreatic ductal cells. Lack of CFTR significantly decreased the expression of AQP1, indicating that CFTR somehow controls the water permeability of ductal cells. Similar results have been found in respiratory epithelial cells, where in the presence of CFTR inhibitor or mutant CFTR the water permeability significantly decreased (Schreiber et al., 1999, 2000; Jourdain et al., 2014) which highlights the significance of this water channel in cystic fibrosis. In contrast, expression of CFTR does not depend on the presence of AQP1, since in the absence of this water channel, strong CFTR staining was detected. This result indicates that the decreased HCO_3^- secretion in the AQP1 KO mice is not due to the impaired expression of CFTR. Nevertheless, we cannot exclude that although the expression of CFTR did not change but the channel does not work correctly, however, further functional experiments are needed to confirm this hypothesis.

There is more and more evidence that impaired pancreatic fluid secretion plays role in the pathomechanism of pancreatitis (Hegyi and Rakonczay, 2010; Pallagi et al., 2014; Maleth et al., 2015). So in the next step, we investigated whether the lack of AQP1 has any effects on the progression of pancreatitis. The loss of AQP1 itself does not damage the pancreas and does not cause pancreatitis in mice, which assuming the compensating effect of AQP5; however, induces a more severe disease progression. The involvement of AQP1 in the pathophysiology of pancreatitis has been already raised previously (Kitami et al., 2007). Kitami et al. (2007) have found that AQP1 expression decreased both in the ductal and acinar cells in a cerulein-induced pancreatitis model, which indicate that reduced levels of AQP1 may contribute to exocrine insufficiency. These findings are in accordance with our observation that expression of AQP1 decreased in the ductal and acinar cells of patients with AP or CP. The importance of AQP1 in the exocrine pancreas has been also confirmed by the fact that this water channel is abundantly expressed in the zymogen granules of acinar cells and plays an essential role in zymogen swelling and probably secretion (Cho et al., 2002). AQP12 is also expressed in the zymogen granule of acinar cells and huge amount of this isoform is present in the rough endoplasmic reticulum (Ohta et al., 2009). In the absence of AQP12, high concentration of cholecystokinin octapeptide makes the pancreas more susceptible to pancreatitis, presumably by the aberrant exocytosis of zymogen granules in these mice (Ohta et al., 2009). All of these previous observations indicate that AQP1 plays essential role both in ductal and acinar functions and we speculate that the absence of AQP1 makes the pancreas more sensitive in two ways: (1) ductal fluid secretion is not sufficient and (2) exocytosis of zymogen granules is impaired.

In this study, we provided an overview regarding the expression and role of AQP1 in the physiology and pathophysiology of the pancreas. Our data indicate that AQP1 interacts with the CFTR Cl^- channel and takes part in the formation of pancreatic fluid. Moreover, we have found that

AQP1 plays role in the pathology of pancreatitis. We hypothesize that absence of the channel makes the pancreas more sensitive to pancreatitis, probably due to the decreased pancreatic fluid and HCO_3^- secretion. Our novel findings not only help to understand the pathomechanism of pancreatitis better, but open up new therapeutic opportunities in the treatment of the disease.

AUTHOR CONTRIBUTIONS

PP performed microfluorimetric and videomicroscopy experiments. LK, ÁZ, and LP were involved in molecular biology experiments. AB, MS, and J-PK performed MRI and JM and ML interpreted the MRI pictures. Genotyping and breeding of AQP mice were done by EB and EG. Pancreatitis was induced by ZB. KB and ET did the immunostainings on the human and mice pancreatic samples, respectively, and quantified fluorescence intensity. ZR was involved in data interpretation and edited the manuscript. VV was involved in all of the above mentioned experiments, analyzed the data, and drafted the manuscript. PH supervised the project and edited the manuscript. All authors approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2018.00854/full#supplementary-material

FIGURE S1 | Expression of AQP after various treatments. Capan-1 cells were treated with **(A)** glycochenodeoxycholic acid (GCDCA), **(B)** palmitoleic acid (POA), **(C)** palmitoleic acid ethyl ester and **(D)** ethanol (EtOH) for 6, 12, 24, and 48 h and the relative gene expression of AQP1 was investigated by real-time PCR. Data represent mean ± SEM of three, independent experiments. $^*p \leq 0.05$ vs. Control.

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Bidirectional Relationship Between Reduced Blood pH and Acute Pancreatitis: A Translational Study of Their Noxious Combination

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Acute pancreatitis (AP) is often accompanied by alterations in the acid-base balance, but how blood pH influences the outcome of AP is largely unknown. We studied the association between blood pH and the outcome of AP with meta-analysis of clinical trials, and aimed to discover the causative relationship between blood pH and AP in animal models. PubMed, EMBASE, and Cochrane Controlled Trials Registry databases were searched from inception to January 2017. Human studies reporting systemic pH status and outcomes (mortality rate, severity scores, and length of hospital stay) of patient groups with AP were included in the analyses. We developed a new mouse model of chronic metabolic acidosis (MA) and induced mild or severe AP in the mice. Besides laboratory blood testing, the extent of pancreatic edema, necrosis, and leukocyte infiltration were assessed in tissue sections of the mice. Thirteen studies reported sufficient data in patient groups with AP (n = 2,311). Meta-analysis revealed markedly higher mortality, elevated severity scores, and longer hospital stay in AP patients with lower blood pH or base excess ($P < 0.001$ for all studied outcomes). Meta-regression analysis showed significant negative correlation between blood pH and mortality in severe AP. In our mouse model, pre-existing MA deteriorated the pancreatic damage in mild and severe AP and, vice versa, severe AP further decreased the blood pH of mice with MA. In conclusion, MA worsens the outcome of AP, while severe AP augments the decrease of blood pH. The discovery of this vicious metabolic cycle opens up new therapeutic possibilities in AP.

Keywords: experimental pancreatitis, acidosis, acid-base balance, meta-analysis, mortality

INTRODUCTION

Acute pancreatitis (AP) is one of the most frequent gastrointestinal causes of hospitalization with significant morbidity and mortality in the US (Yadav and Lowenfels, 2013; Parniczky et al., 2016). Although the mortality rate in mild and moderate AP is low, this value is still unacceptably high (30%) in its severe form (Parniczky et al., 2016). Since no specific therapy is available, only prompt and accurate interventions, such as aggressive fluid therapy can be beneficial (Vinish et al., 2017).

An important function of the pancreas is bicarbonate production, which is required to maintain its constant "milieu intérieur," thereby to prevent premature activation of pancreatic proteases (Pallagi et al., 2011, 2015; Hegyi and Petersen, 2013). When pancreatic bicarbonate production is challenged by local or systemic acid load (i.e., metabolic acidosis, MA), the resulting lower pH can facilitate pancreatic enzyme activation and deteriorate cell damage (Reed et al., 2011). Furthermore, injection of acidic contrast solution either into the pancreatic duct or into the vein significantly increased the severity of AP in rats (Noble et al., 2008; Bhoomagoud et al., 2009). Beside an external acid load, the pancreatic pH balance can also be compromised by tissue injury such as AP, which can lead to acidification of local tissues, thus deteriorate cell damage (Behrendorff et al., 2010). The luminal pH of the main pancreatic duct was also lower in human patients with AP compared to controls (Takacs et al., 2013), suggesting that the development of AP is accompanied by a reduction of local pH. Multiple mechanisms have been implicated in AP which can lead to MA, including direct mechanisms such as the loss of bicarbonate-rich pancreatic juice via pancreatic fistula or drainage (Rice et al., 2014), as well as indirect ones through lactic acidosis which can sequentially occur in AP due to shock, sepsis, cardiovascular failure, or upper gastrointestinal bleeding (Zhan et al., 2015). However, the interaction between AP and systemic pH is still not fully clarified.

Acidosis is often considered as a marker of disease severity, viz., a by-product of systemic dysregulation, and as such it is a proven prognostic factor in the assessment of critically ill patients (Vincent and Moreno, 2010). Despite the fact that scoring systems, which are used to help the diagnosis and the assessment of the progression of AP, include the changes in systemic pH balance of the patients (e.g., Acute Physiology and Chronic Health Evaluation, APACHE II and Ranson scores), clinical trials aiming to reveal a correlation between the acid-base status and the outcome of AP are scarce. To our knowledge, the sole published human study, which aimed to directly answer this question showed that changes in the parameters of systemic acid-base status can predict mortality in AP (Sharma et al., 2014). On the contrary, the necessity of arterial blood gas sampling was questioned in patients with AP in another human study (Ward et al., 2008). With regards to the results obtained in experimental animals, a detailed analysis of the correlation between systemic pH and the outcome of AP would be of utmost importance, because it could establish blood pH as a predictor of the severity and the outcome of the disease and, arguably, identify acidosis as a therapeutic target in AP.

In the present study, by using a dual, translational approach, we have discovered a vicious, bidirectional interaction between blood pH and the outcome of AP. Based on our discovery, the possibility of new therapeutic approaches in AP can be suggested.

Abbreviations: AP, acute pancreatitis; APACHE, acute physiology and chronic health evaluation; CI, confidence interval; CRP, C-reactive protein; ES, estimated logit mortality rate; IL, interleukin; i.p., intraperitoneal(ly); LOS, length of hospital stay; MA, metabolic acidosis; MAP and SAP, mild and severe acute pancreatitis, respectively; SEM, standard error of mean; SMD, standardized mean difference; TNF, tumor necrosis factor.

MATERIALS AND METHODS

Study Design 1: Meta-Analysis of Clinical Trials

Search Strategy

Our meta-analysis was conducted in accordance with the guidelines of the Preferred Reporting Items for Systematic Reviews and Meta-Analysis Protocols (Moher et al., 2009) (**Supplementary Table 1**), similarly as in our recent study (Olah et al., 2018). The analysis was based on the Patients, Intervention (or indicator), Comparison, Outcome (PICO) model: in patients with AP, we aimed to assess the predictive role of the change in pH status (as assessed by blood pH, bicarbonate concentration, base excess, or base deficit) on disease severity (indicated by clinical scores), length of hospital stay (LOS), and mortality ratio. This meta-analysis has been registered with PROSPERO (CRD42017055396).

A search in the PubMed, EMBASE, and Cochrane Controlled Trials Registry databases was performed from inception to January 2017 using the following terms: "pancreatitis AND (mortality OR survival OR severity) AND ("arterial pH" OR "blood pH" OR "systemic pH" OR "base deficit" OR "base excess" OR bicarbonate OR HCO3- OR "anion gap" OR acidosis OR alkalosis OR acid-base)." We restricted our search to original human studies published in English without time period limitations. A manual search of the reference lists of relevant full-text articles was conducted to identify further potentially eligible articles. The search was conducted separately by two authors (ZRu, AG), who also assessed study eligibility and extracted data from the selected studies independently. Disagreements were resolved by consensus with the help of a third party (PH).

Study Selection and Data Extraction

The titles and abstracts of the publications from the literature search were screened and the full text of potentially eligible articles was obtained. We included studies in which blood pH or a related parameter (e.g., base excess, base deficit, or bicarbonate) and severity scores or LOS or mortality ratios were reported for the same group(s) of patients with AP. From all included articles we extracted the sample size, the reported mean pH or its related parameter for the studied patient groups with the corresponding standard error (SEM) or deviation, as well as the severity score, LOS, and mortality ratio within the group. To analyze the influence of the change in acid-base status on the severity and the outcome of AP, in each study we assigned the patient groups as a lower pH group and as a higher pH group,

irrespective from the original basis for grouping used by the authors of the study.

Outcomes of Interest

We used mortality ratio of the AP patients groups as the primary outcome. Regarding secondary outcomes, we used two commonly applied severity indices (i.e., APACHE II and Ranson scores) and the LOS.

Quality Assessment

We assessed the quality of each study included in the meta-analysis by using the Newcastle-Ottawa Scale (Wells et al., 2000; **Supplementary Table 2**).

Statistical Analysis

We used logit transformation of event rates for mortality ratios and standardized mean difference (SMD) for LOS and severity scores as the effect size data. The secondary outcomes were compared between the lower and higher pH groups (see above) within each study, and then the estimated pooled mean values were calculated. The relevant studies were compared with standard meta-analysis tools (e.g., forest plot) in case of each outcome.

Between-study heterogeneity was assessed by I^2 statistical test, where I^2 is the proportion of total variation attributable to between-study variability (an I^2 value of more than 50 was considered as indication of considerable statistical heterogeneity). The selection of patients, study design, and the used methods showed variability among the studies included in our analyses, which also resulted in statistical heterogeneity. Since the lack of statistical significant results on these heterogeneity tests could be also due to the lack of power because of the small number of studies eligible for the analyses, we used the random effect model in case of each forest plot, similarly to our earlier meta-analysis (Rumbus et al., 2017). Publication bias was assessed by funnel-plot analysis, Egger's test and Duval and Tweedie trim and fill method (**Supplementary Figures 1–5**). Publication bias plots were used to assess whether studies with small sizes could have been missed in our analyses, however, due to the design of these tests they do not allow to firmly rule out the possibility that some papers missed the inclusion criteria of our search.

As a different statistical approach to reveal a correlation between systemic pH and mortality in moderate and severe forms of AP, we performed meta-regression analysis of those studies in which both blood pH and mortality rate were reported within the same patient group. The meta-analyses were performed with Comprehensive Meta-Analysis (version 3.3; Biostat, Inc., Engelwood, MJ, USA) and Stata (version 11.1; StataCorp, College Station, TX, USA) software.

Study Design 2: Experimental Procedures

Animals

The experiments were performed in 40 female FVB/N mice (Charles Rivers Laboratories, Wilmington, MA, USA). This commercially available, multipurpose mouse strain is characterized by excellent reproductive performance and it was repeatedly used by our group to study the mechanisms of AP (Kui et al., 2015; Maleth et al., 2015). The mice were housed in standard plastic cages kept in a room with an ambient temperature of 24°C on a 12-h light-dark cycle in the animal facility of the First Department of Medicine at the University of Szeged. The mice were allowed free access to water and standard laboratory chow for rodents (Biofarm, Zagyvaszanto, Hungary).

Ethics

All experiments were approved by the Institutional Animal Care and Use Committee of the University of Szeged and also by an independent committee assembled by national authorities (XII/3773/2012.). All experiments were conducted in compliance with the European Union Directive (2010/63/EU) and the Hungarian government decree (40/2013, II.14.).

Experimental Modeling of Chronic MA in Mice

To develop a mouse model of chronic MA, the mice were randomly divided into the following 4 groups for a 12-day treatment: (i) ammonium chloride (NH_4Cl) administration with drinking water (8.2 ± 0.5 ml/day/mouse) as reported in earlier studies (Galicek et al., 1981; Nowik et al., 2010); (ii) intraperitoneal (i.p.) injections of NH_4Cl (0.5 ml, 0.28 M) on days 1 and 6; (iii) administration of NH_4Cl with drinking water (as in group 1) and i.p. injections (as in group 2); and (iv) controls, receiving NH_4Cl-free tap water and 2 i.p. injections of saline on days 1 and 6.

Experimental Modeling of AP

Two different types of AP were used in this study. Mild acute pancreatitis (MAP) was induced by alcohol and fatty acid as described earlier (Huang et al., 2014; Maleth et al., 2015). Severe acute pancreatitis (SAP) was induced by the injections of cerulein (50 μg/kg, i.p.) at start time, and then at every hour for 9 h (Mareninova et al., 2006). In the chronic MA model, MAP and SAP were induced on day 12 of the acidifying treatment.

Laboratory Measurements

Animals were euthanized by i.p. injection of sodium pentobarbital (50 mg/kg). Blood samples were collected by cardiac puncture. Serum amylase activity was measured by using a colorimetric kinetic method (Diagnosticum, Budapest, Hungary). Serum concentrations of creatinine and glucose as well as urea concentration in urine were measured with commercially available laboratory kits (Institute of Laboratory Medicine, University of Szeged). Arterial blood samples were collected in sealed plastic capillaries (170 μl), which were previously treated with lithium and heparin. Analysis of the arterial blood samples was performed by a blood gas analyzer (Cobas b221 system; Roche Ltd., Basel, Switzerland) within 1 min after blood collection at room temperature (22°C).

Histology

Histological evaluations were performed as described earlier (Kui et al., 2015). In brief, the extent of pancreatic edema (0: none; 1: patchy interlobular; 2: diffuse interlobular; 3: diffuse interlobular and intraacinar), necrosis (%), and leukocyte infiltration (0: none; 1: rare patchy interlobular; 2: patchy interlobular; 3: diffuse

interlobular; 4: diffuse interlobular and intraacinar) were assessed in pancreatic tissue sections stained with haematoxylin and eosin under a light microscope (Zeiss Axio scope A1 microscope) at 40x magnification by an investigator who was expert in pancreas histology, however blinded to the animal's treatment group. The percentage of acinar cell necrosis was evaluated by ImageJ software (NIH, Bethesda, MD, USA).

Statistical Analysis

Data were compared by one-way ANOVA followed by Holm-Sidak test, two-way ANOVA followed by Fischer Least Significant Difference test, or two-tailed Student's *t* test, as appropriate. SPSS 23.0 (IBM, Armonk, NY, USA) and Microsoft Excel (Microsoft Corporation, Redmond, WA, USA) software was used for statistical analysis. The effects were considered significant when $P < 0.05$. In the experimental part of the study, data are reported in the Mean ± SEM format.

RESULTS

Meta-Analysis

Study Selection

The flow chart of the study selection is presented in **Figure 1**. Until January 2017 the electronic literature search identified altogether 1,076 studies from the PubMed, EMBASE, and Cochrane databases. After enabling filters for human studies and English language and removal of duplicates, 793 articles remained, which were screened on title and abstract for inclusion criteria. Full texts of the remaining 122 articles were reviewed in detail. In 109 studies pH parameters or outcomes were not suitably reported in the patients with AP, therefore these were also excluded. As a result, 13 full-text publications were found eligible for statistical analysis which included data from a total of 2,311 patients (Ranson et al., 1976; Nair et al., 2000; Eachempati et al., 2002; Zhu et al., 2003; Kaya et al., 2007; Keskinen et al., 2007; Pupelis et al., 2007; De Campos et al., 2008; Shinzeki et al., 2008; Lei et al., 2013; Sharma et al., 2014; Zhan et al., 2015; Shen et al., 2016). The characteristics of these studies are summarized in **Supplementary Table 3**.

Reduction of Blood pH Is Associated With Higher Mortality Rate in AP

First, we investigated the association between systemic (blood) pH status and our strongest endpoint, viz., the mortality. Our meta-analysis revealed a logit event rate of −0.09 (95% CI, −0.79, 0.61), corresponding to an average mortality rate of 51.0% (95% CI, 31.5, 70.1) in the more acidotic patient groups, while in the patient groups with higher pH or bicarbonate level the logit event rate was −3.68 (95% CI, −4.81, −2.55), which corresponds to an average mortality rate of 3.0% (95% CI, 1.2, 7.1) (**Figure 2**). The mortality ratios were significantly different between the two groups ($P < 0.001$).

Lower pH or Bicarbonate Concentration Worsens the Severity of AP

We wanted to know whether the change in acid-base status can also predict the severity of AP as assessed by clinical scores. Thus, we studied the association between blood pH and clinical severity scores. We found two scores, the Ranson and the APACHE II scores, which were reported in sufficient number of studies for statistical analysis (Ranson et al., 1976; Nair et al., 2000; Eachempati et al., 2002; Zhu et al., 2003; Kaya et al., 2007; Keskinen et al., 2007; Pupelis et al., 2007; De Campos et al., 2008; Shinzeki et al., 2008; Lei et al., 2013; Sharma et al., 2014; Zhan et al., 2015; Shen et al., 2016). Meta-analysis revealed that the pooled SMDs of the Ranson score (0.92, 95% CI, 0.58, 1.26) and the APACHE II score (1.38, 95% CI, 0.95, 1.81) were significantly positive between the patient groups with lower pH or bicarbonate levels and the less acidotic groups of patients ($P < 0.001$) (**Figures 3A,B**). These standardized values correspond to 1.60 (95% CI, 0.77, 2.42) higher Ranson score and 7.40 (95% CI, 5.05, 9.75) higher APACHE II score in the more acidotic patients with AP. The correlation found between lower blood pH and higher clinical scores could be expected as these scores also include blood pH in their calculation (Vincent and Moreno, 2010), nevertheless, these results confirm the feasibility of our meta-analysis approach to reveal an interaction between systemic pH and the outcome of AP.

Acidosis Is Associated With Longer Hospitalization in AP

Next, we analyzed the LOS in patients with AP by using the same grouping of acid-base status as used for mortality and severity scores. For the meta-analysis, LOS was expressed as SMD between the patient groups. We found that the pooled difference was significantly positive between the more acidotic patient groups and the groups with higher pH or bicarbonate concentrations (0.89, 95% CI, 0.73, 1.04; $P < 0.001$) (**Figure 4**), which difference corresponds to 15.05 days (95% CI, 10.84, 19.19) longer LOS in the more acidotic AP patient group.

Meta-Regression Analysis

As a further statistical approach to determine a correlation between blood pH and mortality in the more progressed forms of AP, we also performed a meta-regression analysis on the collected data. For that, we used those study groups, in which pH and mortality rate was reported in moderately severe or severe manifestations of AP for the same patient groups (Zhu et al., 2003; Lei et al., 2013; Shen et al., 2016). We found a significant correlation between pH and mortality rate with a regression slope of −55.4 (95% CI, −97.9, −12.9; $P = 0.011$) (**Figure 5**). The potential reason for statistical heterogeneity, as revealed in the forest plots, could not be evaluated in the meta-regression analysis because of the small number of eligible studies.

Experimental Animal Model

Acidosis Is Augmented in Severe Form of AP

Analyses of data from 2,311 patients showed strong association between acidosis and the outcome of AP. Therefore, we moved from the "bedside to the bench" to clarify their causative relationship. First, we developed a new experimental model to mimic chronic MA by comparing different types (oral or i.p. or both) of acidifying treatments in mice. We

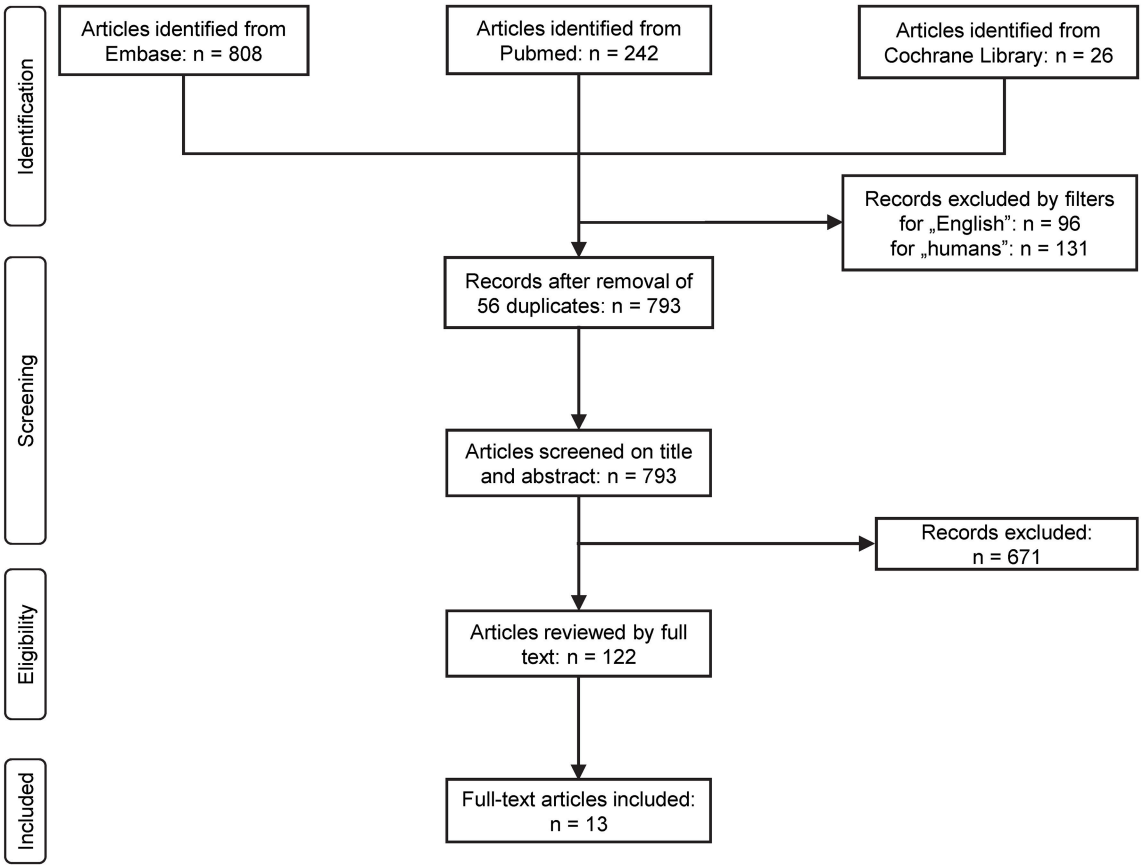

FIGURE 1 | Flow chart of study selection and inclusion.

found that MA can be induced in mice by the combined administration of oral and i.p. NH_4Cl, which decreased blood pH to 6.80 ± 0.04, but it did not cause any pancreatic damage (**Supplementary Figure 6**), nor did it change serum glucose and urine urea levels (**Supplementary Figure 7**). Similarly to pH, arterial blood bicarbonate level decreased most significantly ($P < 0.001$) in the combination (oral and i.p.) treatment group as compared to controls (16.5 ± 0.9 vs. 26.4 ± 0.8 mmol/l) (**Supplementary Figure 7**). We detected no significant differences in the serum concentrations of creatinine, sodium, and potassium among the different treatment groups (data not shown). We used this MA model to study the interaction between acidosis and AP. For that, mice with or without pre-existing MA were assigned to MAP, SAP, and control (no AP) groups and their arterial blood pH were compared. As expected, pre-existing MA induced by dual (oral and i.p.) acidifying treatment resulted in significantly decreased blood pH in the mice without AP, as well as in mice with either MAP or SAP (**Figure 6**). In the mice with pre-existing MA, the extent of the pH reduction was similar in the sham AP and MAP groups (7.08 ± 0.04 and 7.11 ± 0.03, respectively), while in the mice with SAP the arterial pH decreased to 6.97 ± 0.05, which was significantly lower than in the sham AP and MAP groups ($P < 0.05$ compared to both), suggesting that SAP further deteriorates MA (**Figure 6**). As expected, MA also resulted in decreased arterial blood bicarbonate levels, which reached the level of significance in the MAP group (16.7 ± 1.4 mmol/l; $P < 0.01$) (**Supplementary Figure 8**). The levels of urea in the urine were markedly decreased in mice with SAP (3.6 ± 0.2 mmol/l) regardless of their pH status as compared to the urine urea levels in the sham AP groups without and with pre-existing MA (7.4 ± 0.2 and 7.5 ± 0.1 mmol/l, respectively; $P < 0.001$ for both). Importantly, MA significantly lowered urine urea levels in mice with MAP compared to mice with MAP without pre-existing MA (5.9 ± 0.7 vs. 8.9 ± 1.0 mmol/l; $P < 0.05$) (**Supplementary Figure 8**). We did not detect significant difference in the serum concentrations of glucose (**Supplementary Figure 8**), and in the levels of creatinine, sodium, and potassium among the different treatment groups (data not shown).

Pre-existing Acidosis Deteriorates Both Mild and Severe Forms of AP in Mice

To determine whether the presence of pre-existing MA has any effects on the pancreatic damage during AP, pancreatic edema, necrosis, and leukocyte infiltration scores were assessed in pancreatic sections of mice without AP, or with MAP or SAP in the presence and the absence of pre-existing MA. The

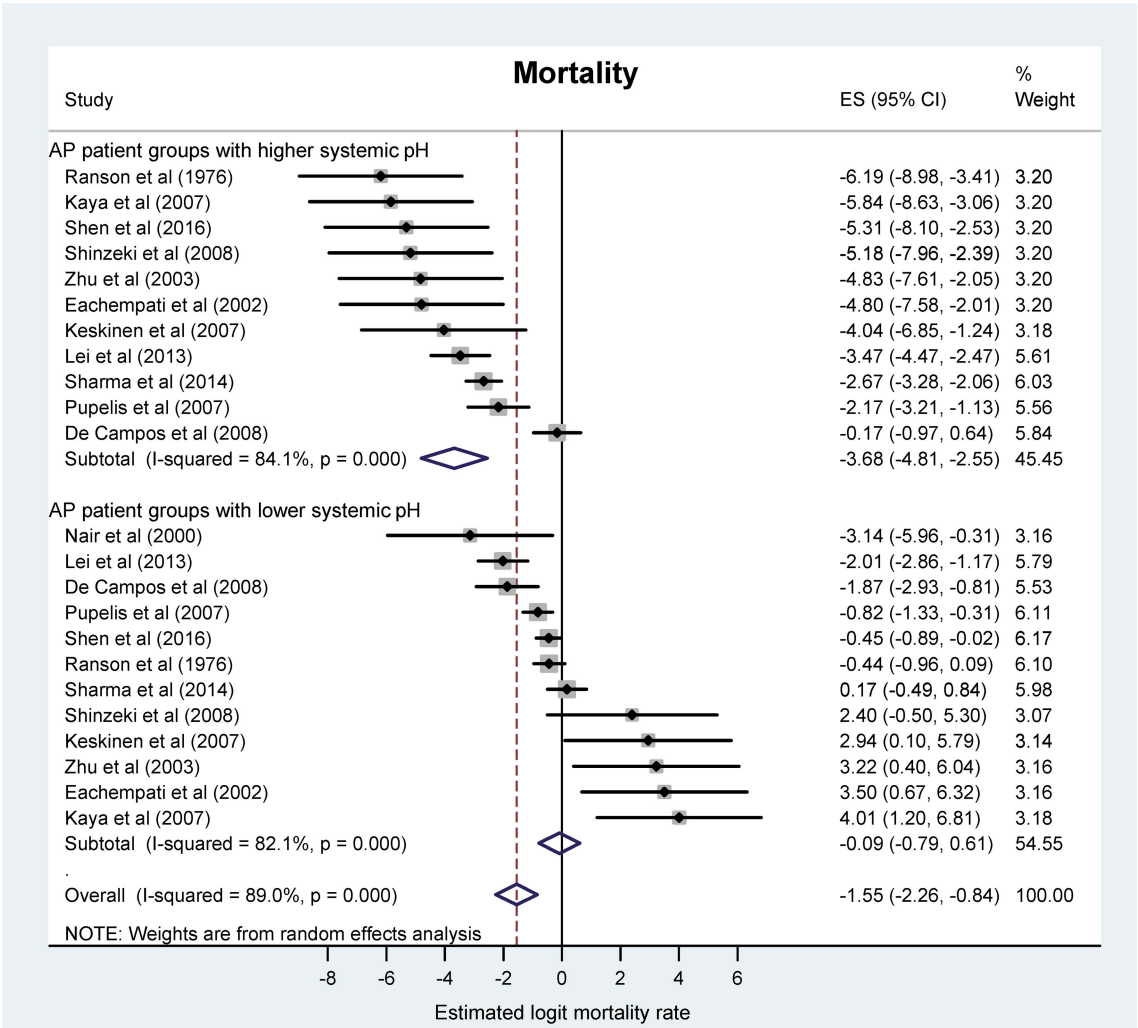

FIGURE 2 | Forest plot of mortality rate using random-effects model in different systemic pH groups of patients with acute pancreatitis (AP). For each patient group, black circles and horizontal lines represent the estimated logit mortality rate (ES) and the corresponding confidence interval (CI), respectively. Lower ES corresponds with lower mortality rate and vice versa. Gray squares indicate the relative statistical weight of a given patient group. Open diamonds show the average ES and CI of patient groups with higher systemic pH (top), lower systemic pH (middle), and all patient groups (bottom).

acidifying treatment caused no pancreatic damage in the control (no AP) mice (**Figure 7**), which is line with our previous results (**Supplementary Figure 6**). On the contrary, in mice with pre-existing MA, MAP resulted in significantly larger edema (2.0 ± 0.3 vs. 1.4 ± 0.2; $P < 0.05$), increased necrosis (21.0 ± 2.4 vs. 10.0 ± 2.2%; $P < 0.05$), and elevated leukocyte infiltration (2.5 ± 0.4 vs. 1.6 ± 0.2; $P < 0.05$) compared to MAP in mice with normal blood pH (**Figure 7**). Pancreatic damage was also markedly more pronounced in SAP in mice with pre-existing MA compared to SAP in mice with normal blood pH as indicated by increased edema (3.6 ± 0.2 vs. 2.4 ± 0.2; $P < 0.05$), necrosis (38.6 ± 5.0 vs. 25 ± 2.2%; $P < 0.01$), leukocyte infiltration (3.6 ± 0.4 vs. 2.6 ± 0.2; $P < 0.05$), and serum amylase activity (12,730 ± 384 vs. 11,362 ± 106 Unit/l; $P < 0.05$) (**Figure 7**). These results suggest that MA further deteriorates pancreatic damage in both MAP and SAP.

DISCUSSION

In the present study, we revealed a strong association between blood pH and the outcome of AP with meta-analysis of human studies. Our analyses showed that lower blood pH predicts higher mortality rate, longer LOS, and worsens the severity of AP. A significant negative correlation between blood pH and mortality rate in severe forms of AP was found with meta-regression analysis of the human studies. To better clarify how MA can interact with AP, we developed a mouse model of chronic MA and showed that SAP worsens the MA in the mice. In the same model we also demonstrated that pre-existing MA further deteriorates the tissue damage in both mild and severe forms of AP.

Although previous human studies indicated a link between MA and AP (Nair et al., 2000; Zhu et al., 2003; Shinzeki et al., 2008; Sharma et al., 2014; Shen et al., 2016), we found only one,

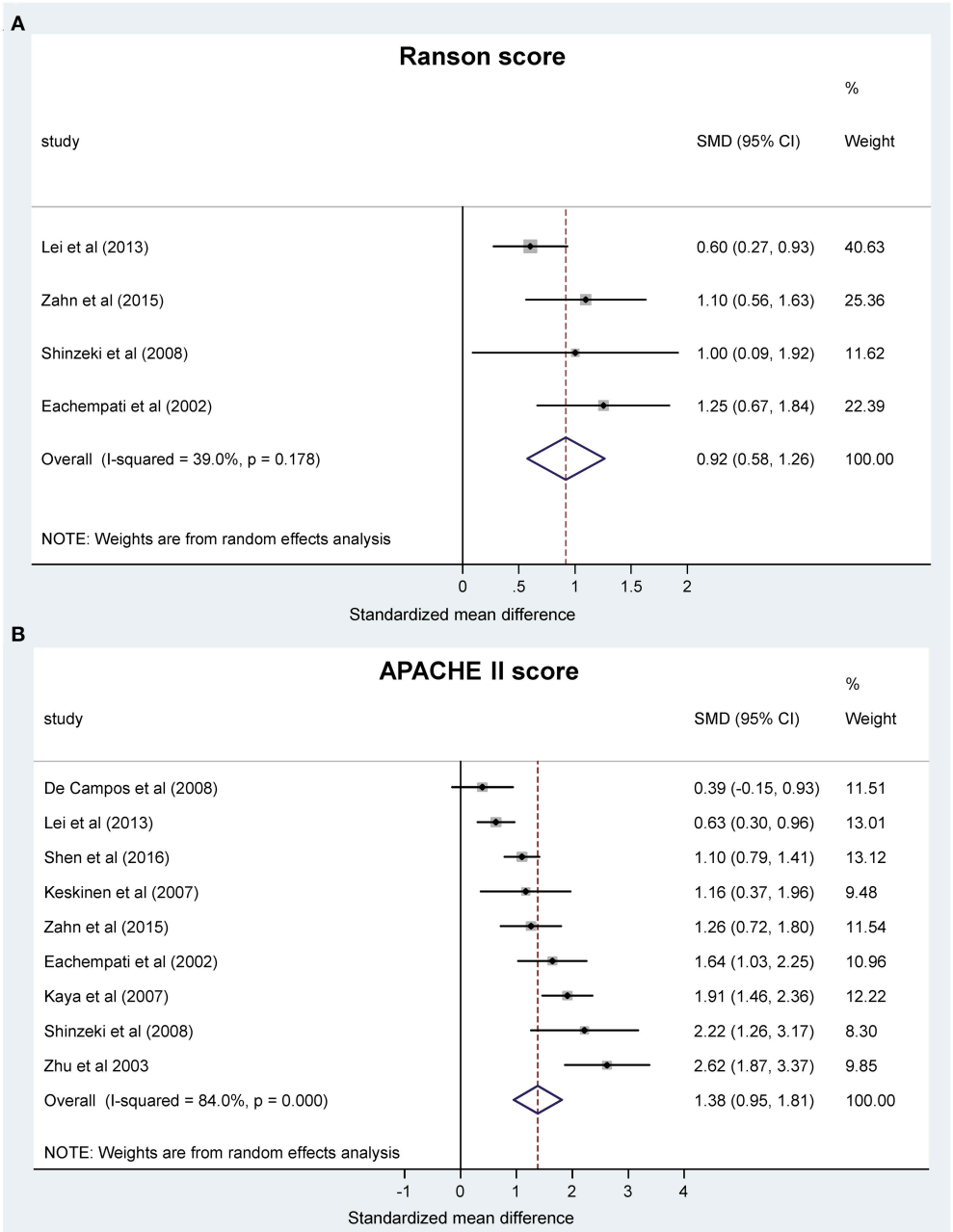

FIGURE 3 | Forest plot of **(A)** Ranson scores and **(B)** Acute Physiology and Chronic Health Evaluation (APACHE II) scores using random-effects model in different systemic pH groups of patients with acute pancreatitis. Here and in **Figure 4**, in each study the standardized mean difference (SMD) of the outcome was calculated between the patient group with lower and higher pH. Black circles represent the SMD for each study, while the left and right horizontal arms of the circles indicate the corresponding 95% confidence intervals (CI) for the SMD for each study. The size of the gray box is proportional to the sample size of the study; bigger box represents larger sample size, thus bigger relative weight of the study, and vice versa. Circles close to zero represent smaller SMD between the lower and higher pH groups in the given study. A positive SMD means higher score (**Figure 3**) or longer hospital stay (**Figure 4**) in the patient group with lower pH compared to the patient group with higher pH. The diamond on the bottom represents the averaged SMD calculated from the SMDs of all the individual studies. The vertical dashed line is determined by the two vertical points of the diamond and indicates the value of the averaged SMD of all studies. The horizontal points of the diamond represent the 95% CI of the averaged SMD.

single-center prospective study which directly aimed to explore this correlation (Sharma et al., 2014). Because of the scarcity of data available from targeted clinical trials, we aimed to clarify the association between MA and AP by systematic review of the current literature and by meta-analysis of the available data. By identifying 13 eligible studies for the analysis (Ranson et al.,

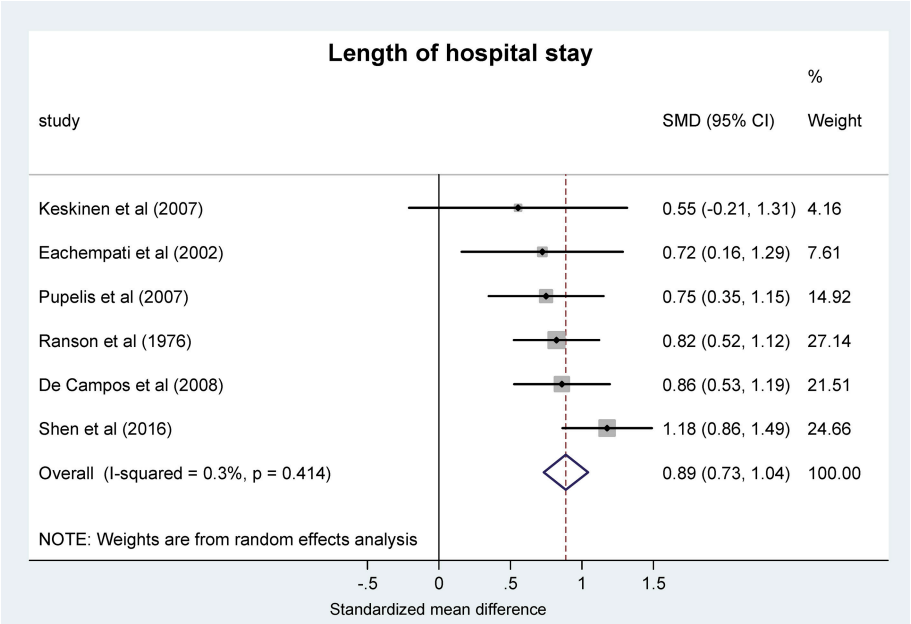

FIGURE 4 | Forest plot analysis of the length of hospital stay using random-effects model in different systemic pH groups of patients with acute pancreatitis. For explanation, see the legend of **Figure 3**. SMD, standardized mean difference; CI, confidence interval.

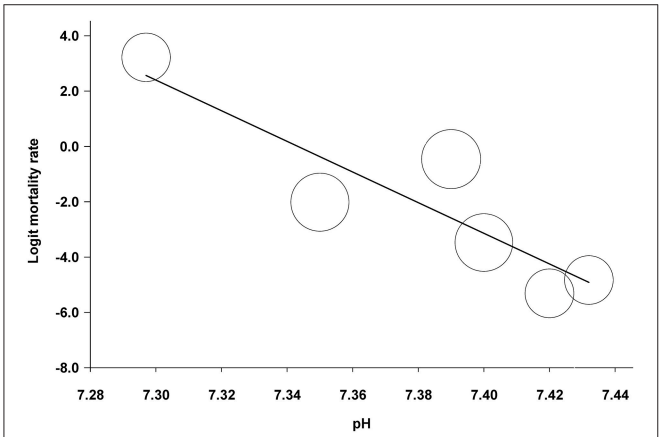

FIGURE 5 | Meta-regression analysis of the association between blood pH and mortality rate in patients with moderately severe and severe forms of acute pancreatitis. The circles indicate estimated logit mortality rate calculated for each patient group. A lower calculated value corresponds with lower mortality rate and vice versa. The circle size is proportional to the precision of the estimated logit mortality rate. The solid black line represents the weighted regression line based on variance-weighted least squares.

1976; Nair et al., 2000; Eachempati et al., 2002; Zhu et al., 2003; Kaya et al., 2007; Keskinen et al., 2007; Pupelis et al., 2007; De Campos et al., 2008; Shinzeki et al., 2008; Lei et al., 2013; Sharma et al., 2014; Zhan et al., 2015; Shen et al., 2016), we included 2,311 patients with AP in the analyses. In all of these studies, blood sample analysis was performed at admission or within 24 h thereafter, hence the blood pH parameters were determined with practically the same latency compared to the time when AP was diagnosed. Unavoidably however, the disease could progress to different stages in the different patients before the diagnosis has been reached. There were huge differences between the protocols of the individual studies, but it is remarkable that no matter how the patients were grouped by the authors originally, the patient group with lower pH had always (with no exceptions) worse outcomes (mortality rate, LOS, severity scores) than the group with higher pH in AP, which suggests that in the early stages (viz., until the time of diagnosis) of AP acidosis is an important influencing factor of the outcome regardless from the actual progression of the disease. Unfortunately, the design of the studies did not allow to analyze the causative relationship between MA and AP. In most of the studies, the systemic pH status of the patients prior to or repeatedly after the diagnosis of AP was not reported, thus the dynamics in the changes of pH during the time course of AP could not be assessed in the current analysis, but it is notable that the average base deficit was markedly (4-8 fold) higher in populations of patients, who did not survive SAP (Kaya et al., 2007; Keskinen et al., 2007). In the prospective trial by Sharma et al. (2014), in those SAP patients, who had a blood pH of less than 7.35, the mortality rate was nearly 10 times higher than in those patients whose pH was above this level (54 vs. 6.5%).

As limitations of our study, it should be mentioned that even though our meta-analysis showed a clear association between blood pH and the outcome of AP, since originally the patients were not divided into subgroups based on their blood pH by the authors, the independent effect of lower blood pH on the outcome and the cause-effect relationship between MA and AP could not be assessed. Because of the same reason and also to reduce the inter-study heterogeneity, in each of the analyzed

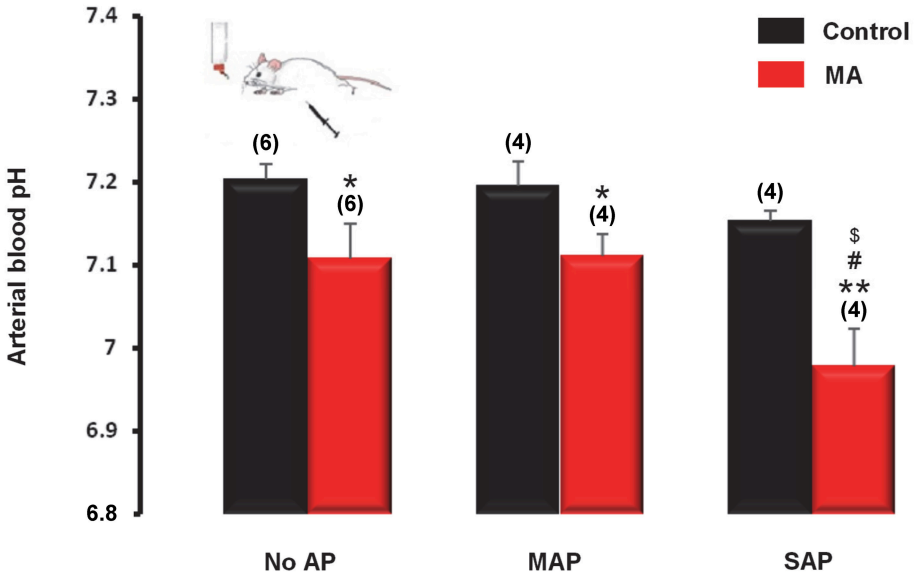

FIGURE 6 | Arterial pH of mice with metabolic acidosis (MA) induced by combination of oral and i.p. NH_4Cl administration and without acidifying treatment (control). On day 12 of the acidifying treatment, mild acute pancreatitis (MAP) or severe acute pancreatitis (SAP) was induced by alcohol and fatty acid or cerulein, respectively. Mice in the sham pancreatitis group (no AP) were injected i.p. with saline. Statistically significant differences are marked with *between MA and control (non-acidotic) groups, with $^{\#}$between no AP and MAP groups in MA, and with $^{\$}$between no AP and SAP groups in MA, as follows: $^{*}P < 0.05$ and $^{**}P < 0.01$ for MA vs. control in no AP, MAP, and SAP; $^{\#}P < 0.05$ for no AP in MA vs. SAP in MA; $^{\$}P < 0.05$ for MAP in MA vs. SAP in MA.

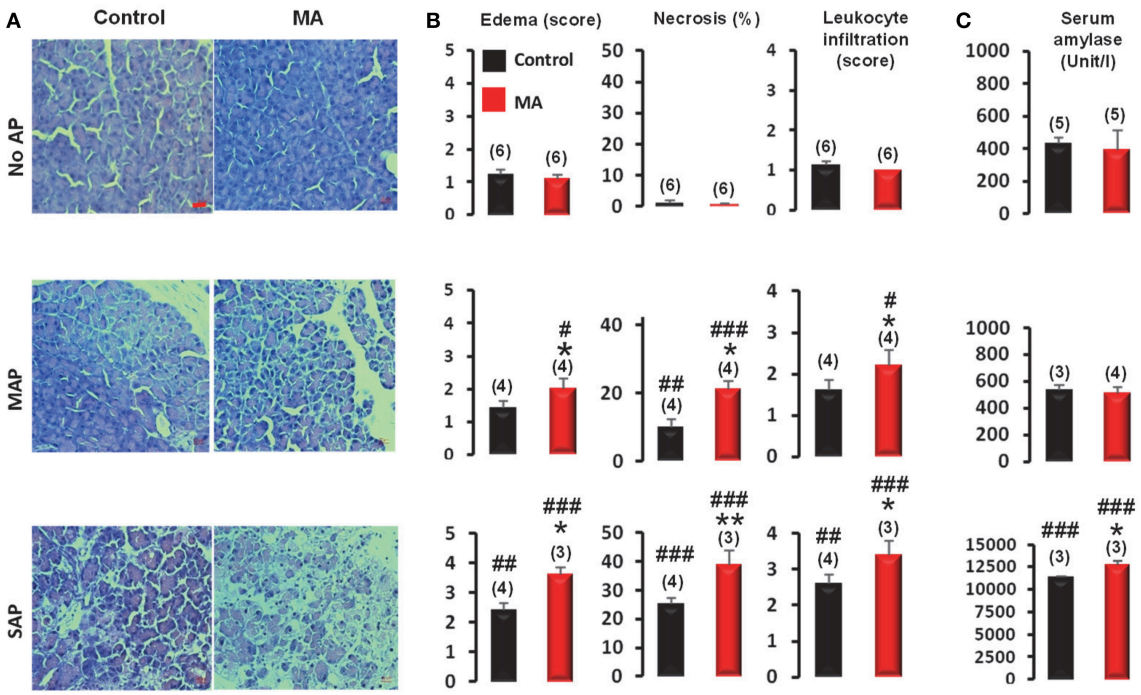

FIGURE 7 | Assessment of the severity of acute pancreatitis (AP) in mice with and without metabolic acidosis (MA and control, respectively). **(A)** Representative microphotographs of pancreatic sections, **(B)** histological evaluation of edema scores, necrosis, and leukocyte infiltration scores, and **(C)** serum amylase levels of MA and control mice with mild acute pancreatitis (MAP), severe acute pancreatitis (SAP) or without acute pancreatitis (no AP). Scale bar represents 20 μm. Statistically significant differences are marked with *between MA and control (non-acidotic) groups and with $^{\#}$between no AP and either MAP or SAP groups, as follows: $^{*}P <$ 0.05; $^{**}P < 0.01$ for MA vs. control in MAP and SAP; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ for no AP vs. MAP and SAP.

studies we assigned one patient group as the lower pH group and the other one as the higher pH group. Since the reported pH values differed substantially among the analyzed studies, the cut-off value between the lower and the higher pH groups was individually determined for each study. Consequently, in the present analysis we could not determine a specific cut-off pH value which would be detrimental for the outcome of AP. The most convincing method to obtain direct evidence for the role of acidosis as an independent risk factor in AP, determine a detrimental cut-off pH value, and gain insight into the cause-effect relationship in humans would be to conduct targeted clinical trials in which patients with AP are grouped based on their blood pH at admission and their acid-base status as well as the severity and the outcome of AP is continuously monitored. By collecting data of individual patients in such clinical trials, it would be possible to statistically analyze the direct (independent) effect of acid-base disturbances on AP. Until such or similar trials are conducted, we are restricted to use different (not so direct) approaches such as meta-analyses and animal experiments.

To discover whether a pre-existing MA worsens the outcome of AP or MA is rather the result of the progression of AP, we moved from the "bedside to the bench." Gorelick's workgroup has discovered that low extracellular pH induces pathophysiological changes in acinar cells (Bhoomagoud et al., 2009; Reed et al., 2011). They described that reduced pH sensitizes the acinar cell to secretagogue-induced pancreatitis responses in rats, and enhances connexin32 degradation and ryanodine receptor-mediated calcium signaling in the basolateral region of the acinar cell which mechanisms are responsible for the injurious effects of low extracellular pH on the exocrine pancreas (Bhoomagoud et al., 2009; Reed et al., 2011, 2014). However, the authors have not investigated the causative relationship between low pH and pancreatitis. Since no mouse model of chronic MA was available in the literature, first we designed a set of experiments to develop the most suitable MA model. Dual administration (oral and i.p.) of acidic fluid induced a marked pH drop in the blood without damaging the pancreas. By supplementing the oral treatment with i.p. acidification, our model also accounted for such conditions, when primarily the pH of the peritoneal fluid is reduced such as bacterial peritonitis (Glinska-Suchocka et al., 2016), carbon dioxide insufflation during laparoscopy (Duerr et al., 2008), or peritoneal dialysis (Farhat et al., 2008). Notably, in our model, MA developed gradually and persisted for several days in the mice which is very similar to the development of MA in human patients. Indeed, there is evidence that in human patients AP can develop in pre-existing MA, for instance in diabetic ketoacidosis either via hyperlipidemia (Nair and Pitchumoni, 1997; Nair et al., 2000) or through distinct mechanisms (Gianfrate and Ferraris, 1998). It should be noted however that in clinical settings MA typically occurs as a consequence of AP and in many cases it does not pre-exist. The shown reverse relationship between MA and AP, namely that the presence of a pre-existing acidosis can influence the severity of AP, warrants for careful pH management in such clinical situations.

Sodium bicarbonate therapy is widely accepted for the treatment of MA in conditions associated with the loss of bicarbonate (e.g., renal tubular acidosis, diarrhea), however its use to increase pH in diseases associated with acidosis not due to bicarbonate loss is questionable because of its adverse effects, for example, intracellular acidosis, hypokalemia, and decreased serum ionized calcium concentration (for reviews, see Adeva-Andany et al., 2014; Hopper, 2017). In AP, bicarbonate production becomes impaired due to the damage of the pancreatic tissue, hence when systemic pH decreases bicarbonate administration can be beneficial to maintain normal pH, thereby to improve the outcome based on our results. It has to be noted that, to our knowledge, currently there is no evidence for the benefits of bicarbonate administration in AP. In contrast with sodium bicarbonate therapy, a growing body of evidence supports the beneficial effects of lactated Ringer's solution in the treatment of AP. Since lactate is metabolized to bicarbonate in the liver, lactated Ringer's solution was successfully used to lessen the metabolic acidosis by elevating blood bicarbonate levels and to attenuate the systemic inflammation response as assessed by lower C-reactive protein (CRP) levels in patients with AP (Wu et al., 2011; de-Madaria et al., 2018). Administration of lactated Ringer's solution resulted in lower mortality rate in critically ill patients with AP (Aboelsoud et al., 2016) and it lead to transiently reduced systemic inflammation in patients with MAP (Choosakul et al., 2018), although it had no therapeutic benefits in AP in a retrospective study (Lipinski et al., 2015). For the initial management of AP, the American College of Gastroenterology guideline recommends lactated Ringer's solution as the preferred isotonic crystalloid fluid replacement with moderate quality of evidence (Tenner et al., 2013). Future clinical trials are warranted to confirm the beneficial effects of tightly controlled pH management and to identify the optimal type of fluid resuscitation in patients with AP and pre-existing MA.

Our experiments clearly showed a strong bilateral link between pH and AP. We showed that pre-existing MA worsens the outcome of AP, whereas AP reduces pH in the blood which vicious cycle could be one of the main reasons for the high mortality rate in AP. The exact mechanism of how MA can deteriorate AP remains subject for future studies, but it can be assumed that complex regulatory mechanisms, such as the pancreatic damage and zymogen activation, neurogenic inflammation, and activation of inflammatory cells and mediators, are involved; for a comprehensive review, see Gorelick and Thrower (2009). Similarly, the question of whether the augmented acidosis is a direct or an indirect consequence (e.g., through impaired kidney and/or lung functions) or a combination of these in AP remains to be answered. Indeed, several complications of AP such as renal, pulmonary, and cardiovascular failure can cause disturbances in the acid-base balance. The development of acute renal dysfunction was reported in several of the analyzed studies (Keskinen et al., 2007; Pupelis et al., 2007; Lei et al., 2013; Sharma et al., 2014; Shen et al., 2016), and the impaired kidney function (determined by increased serum creatinine and blood urea nitrogen levels) was associated with significantly worse outcome, including higher mortality rates in AP (Talamini et al., 1999; Eachempati et al., 2002). Moreover, the frequency of renal failure increased by 5-10 times if acidosis (i.e., blood pH $<$ 7.35, base deficit $>$ 4 mEq/l, or bicarbonate $<$ 22 mEq/l) occurred in AP (Sharma et al., 2014). Since we found only one clinical trial which directly

investigated the relationship between renal failure and acidosis in AP (Sharma et al., 2014), the available data in humans were not sufficient for meta-analysis. In our experimental model, acute renal dysfunction occurred in mice with SAP regardless of their pH status, moreover the presence of pre-existing MA significantly impaired the kidney functions in mice with MAP, which is in harmony with the observations in humans and provides direct experimental support to the association between acidosis and renal failure in AP.

Cytokines are important mediators in the whole process of AP. A number of proinflammatory mediators, such as interleukin (IL)-1, 6, 8, and tumor necrosis factor (TNF)-α, were shown to play a role in AP in experimental animals (Norman et al., 1997; Liu et al., 2003; Meng et al., 2005) and in human patients (de Beaux et al., 1996; McKay et al., 1996; Brivet et al., 1999; Mayer et al., 2000). Cytokine production occurs in the pancreas first, and then with the progression of the disease in distant organs like the lungs, liver, and spleen (Norman et al., 1997). The levels of IL-6, 8, and TNF-α are even more increased in SAP than in MAP (McKay et al., 1996; Pooran et al., 2003). Active digestive enzymes which are released from injured pancreatic cells can potently stimulate proinflammatory cytokine production in macrophages (Desser et al., 1994; Lundberg et al., 2000), moreover the pancreatic acinar cells can also produce proinflammatory cytokines (Gukovskaya et al., 1997; Brady et al., 2002). For example, amylase can induce the production of IL-1, 6, and TNF-α in human peripheral blood mononuclear cells and in dermal fibroblasts (Desser et al., 1994; Malpass et al., 2013). Lipase markedly induced TNF-α production in rat macrophages (Jaffray et al., 2000), while CRP was shown to strongly correlate with IL-6 levels in patients with AP (Viedma et al., 1992). In our mouse model, we found that amylase level was elevated in SAP, and it was further increased in the presence of a pre-existing acidosis, therefore it can be expected that circulating cytokine levels are also higher in the co-existence of SAP and MA than in SAP without acidosis. In patients with SAP, the serum lipase and CRP levels were higher when their blood pH was lower (Pupelis et al., 2007), thus suggesting higher levels of circulating cytokines.

The production of pro- and anti-inflammatory cytokines was repeatedly shown to depend from the extracellular pH (for reviews, see Kellum et al., 2004b; Okajima, 2013; Casimir et al., 2018). Although the different forms and severities of acidosis can differently influence cytokine production (Kellum et al., 2004b), a proinflammatory effect, including enhanced TNF-α synthesis and augmented nuclear factor-κB activation, of hyperchloremic acidification was shown in activated macrophages by independent groups (Bellocq et al., 1998; Heming et al., 2001; Kellum et al., 2004a). Also, decreasing extracellular pH caused increasing IL-8 expression and nuclear factor-κB activation in human pancreatic tumor cells (Shi et al., 2000). In addition to the recruitment of immune cells by the low pH-induced cytokine release, extracellular acidosis *per se* promotes the activation of neutrophils (Martinez et al., 2006), which is in line with the increased leukocyte infiltration in MAP and SAP with pre-existing acidosis compared to MAP and SAP with initially normal blood pH, as observed in the present study (**Figure 7B**). Here, we revealed a bidirectional relationship constituting a vicious circle between AP and acidosis and developed a mouse model for studying the underlying mechanisms of the progression of AP in pre-existing MA. However, the experimental conformation of the dynamics of tissue and circulating cytokine concentrations and other potential processes (e.g., calcium signaling) in this model remains subject for future studies.

In summary, by the meta-analysis of literature data available from human studies we found a significant correlation between low systemic pH and the outcome of AP, indicating that lower pH level is associated with higher mortality rates, longer LOS, and more severe AP. With regards to the mechanism, in experimental animals we showed the existence of a bidirectional interaction between MA and AP, in which pre-existing MA deteriorates AP and, vice versa, AP further increases the severity of MA. Our findings suggest that systemic pH level should be closely monitored in patients with AP and that interventions to normalize the low pH of patients with AP should be considered in clinical settings. Well-designed, targeted clinical trials are warranted to evaluate the effects of the therapeutic interventions of acidosis in patients with AP.

AUTHOR CONTRIBUTIONS

ZRu and AG conducted the literature search of meta-analysis and the quality assessment of included studies, and extracted data from the articles. ET, JM, ZB, and PH performed the experiments. PH and AG conceived and supervised the meta-analysis and the experimental procedures and obtained funding. ZRu, ET, LP, JM, AG, and PH analyzed and interpreted the data. ZRu, ET, LP, PH, and AG wrote the paper. LP, EO, AV, GV, LC, KM, AM, ZRa, ZB, JK, IF, and JM reviewed and contributed to the manuscript. All authors approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2018.01360/full#supplementary-material

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