IMPORTANT THERAPEUTIC TARGETS IN ACUTE PANCREATITIS

Ph.D. Thesis

Emese Tóth

Supervisors:

Prof. Péter Hegyi M.D., Ph.D., D.Sc., MAE [1,2,3]

József Maléth M.D., Ph.D.[2,4]

[1]Momentum Translational Gastroenterology Research Group, Hungarian Academy of Sciences–University of Szeged, Szeged, Hungary

[2]First Department of Medicine, University of Szeged, Szeged, Hungary

[3]Institute for Translational Medicine and First Department of Medicine, University of Pécs, Pécs, Hungary,

[4]Momentum Epithelial Cell Signalling and Secretion Research Group, Hungarian Academy of Sciences–University of Szeged, Szeged, Hungary

Szeged

2019.

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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)* 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)* 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 Szentesi1, **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 Blasko´ 1 , Alexandra Demcsák , Andrea Párniczky , Gabriella Pár, Szilárd Gódi , Dóra Mosztbacher , Ákos Szűcs, Adrienn Halász1, Ferenc Izbéki, Nelli Farkas, Péter Hegyi, Hungarian Pancreatic Study Group 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)	**24**
Hirsch index	**2**
Number of total citations (MTMT2)	**19**
Hirsch index	**2**

II. INTRODUCTION

II.1. Targeting the mitochondrial transition pore as potential therapeutic target in AP

Mitochondrial dysfunction is one of the earliest events in the disease [1-4]. 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 [3, 5, 6]. 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 [7] . 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 [7-9]. 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 [10, 11]. 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 [7, 12, 13]. 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[14-19]. 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 [20].

II.2. Importance of pancreatic ductal fluid secretion

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) [21-25]. 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 [26-28] Henceforth, colocalization of this two channel has been revealed in the human pancreas [29]. 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 [30, 31]. In the peritoneum the lack of AQP1 ends up in significantly reduced osmotical water transport. [30, 32-34] 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.

II.3. 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[35]. 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 [36]. Moreover, injection of acidic contrast solution into the pancreatic duct increased the severity of experimental AP in rats [37, 38]. 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 [23]. These suggest that may the development of AP is coupled with the decrease of local pH. 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. The discovery of how the metabolic acidosis affect the outcome of AP in animals could open new therapeutic ways in the treatment of AP. [39]

III. 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.**

IV.MATERIALS AND METHODS

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

IV.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.

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

All data are expressed as means ± SEM. Analysis were performed by Sigma Plot Software.

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

IV.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 animals were provided for us by the Department of Medical Biochemistry, Semmelweis University, Budapest, Hungary.

IV.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).

IV.4.3. Isolation

Isolation of pancreatic ducts and acinar cells were performed by microdissection and enzymatic digestion as described earlier [40, 41]

IV.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 fluorescence dies (ab176750, Abcam) were used.

IV.4.5. Fluorescent microscopy

Microfluorometry was used to measure pancreatic ductal HCO_3^- secretion as described earlier [42, 43] by using BCECF-AM (2',7'-Bis-(2-Carboxyethyl)-5-(and-6)-Carboxyfluorescein, Acetoxymethyl Ester, 1.5 mmol/L).

IV.4.6. Videomicroscopy

In vitro pancreatic ductal fluid secretion (luminal swelling) assays were developed by Fernández-Salazar et al, [44] performed by videomicroscopy as described earlier [45].

IV.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 [46-48] 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 for 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).

IV.4.8. In vivo measurements

IV.4.8.1. Induction of acute pancreatitis

AP was induced by caerulein (CER,10x50µg/kg) and 4% sodium taurocholate (TAU, 2ml/kg,4%)[24, 49-51]. 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 [25, 52], 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.

IV.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 [17]. 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 [17]. 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.

IV.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.

IV.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 [53][55][80][81][[80][][79][78][77][76][75][74][75][74][73][72][71][70][69][68)[67][66].

IV.5. Materials and methods used in Publication No.2.

IV.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).

IV.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, as described earlier.[54]

IV.6. Methods used in publication No.3.

IV.6.1. Animals

We performed our experiments on female FVB/N mice (Charles Rivers Laboratories, Wilmington, MA, USA).

IV.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 [55, 56]
- 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.

IV.6.3. Induction of AP

Severe AP (SAP) was induced by caerulein (CER,10x50µg/kg), CER was administered i.p.[49]. 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 [25, 52]. During the experimental model of MA, MAP and SAP were induced on day 12 of the acidifying treatment.

IV.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).

V. RESULTS

V.1.Results of publication No.1.

Both genetic and pharmacological inhibition of Cyp D significantly prevented the toxic effects of BA and EtOH+FA by restoring mitochondrial membrane potential ($\Delta\psi$) and preventing the loss of mitochondrial mass. In vivo experiments revealed that per os administration of NIM811 has a protective effect in AP by reducing oedema, necrosis, leukocyte infiltration and serum amylase level in AP models. Administration of NIM811 had no toxic effects.

V.2. Results from publication No.2.

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.

V.3.Results from publication No.3.

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.

VI. DISCUSSION

VI.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 [2, 57, 58] . 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[2, 4, 6, 25, 58-60] . 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[61] . 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 [1, 4, 6, 62]. 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 [25, 59][25, 61][50, 85][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. 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 [54]. *Per os* administration of 5 or 10 mg/kg NIM811 treatment alone had no toxic effect, but significantly reduced the severity of AP.

VI.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 [26, 63]. This could highlight the significance of this water channel in disease of pancreatitis moreover in cystic fibrosis as well.

VI.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. 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 [64, 65] 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.

VII. SUMMARY

VII.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.)

VIII. FUNDING

My PhD work was funded by a Momentum Grant from the Hungarian Academy of Sciences (LP2014-10/2014 to PH) as well as Economic Development and Innovation Operational Programme Grants and Project Grants from the National Research, Development and Innovation Office (GINOP-2.3.2-15-2016-00015, EFOP-3.6.2-16-2017-00006, K116634 to PH, K109756 to VV and PD115974 to JM and K119938 to ZR).

IX. ACKNOWLEDGEMENTS

Firstly, I would like to express my gratitude and thanks to my mentor and supervisor **Prof. Dr. Péter Hegyi** (Momentum Translational Gastroenterology Research Group, Hungarian Academy of Sciences–University of Szeged, Institute for Translational Medicine, University of Pécs) who provided the scientific and financial support throughout my PhD. studies. Without his outstanding scientific work and supervision this dissertation would not have been possible. I am very grateful for my other supervisor **Dr. József Maléth** (First Department of Medicine University of Szeged, Momentum Epithelial Cell Signalling and Secretion Research Group, Hungarian Academy of Sciences–University of Szeged), for the scientific counselling, valuable comments and methodological advices. I would like to thank **Prof. Dr. Csaba Lengyel and Prof. Dr. György Ábrahám,** the current and former head of the First Department of Medicine, University of Szeged who gave me the opportunity to work at the Department. I also thank **Prof. Dr. András Varró** (former head of Department of Pharmachology and Pharmachotherapy, University of Szeged) his support. I owe special thanks to **Dr. Viktória Venglovecz** (Department of Pharmachology and Pharmachoterapy, University of Szeged) for her scientific advices and kind support throughout the years. I also would like to thank **Dr. Andrea Szentesi** (First Department of Medicine University of Szeged and Institute for Translational Medicine, University of Pécs), **Dr. Petra Pallagi** (First Department of Medicine University of Szeged) and **Prof. Dr. Zoltán Rakonczay Jr.** (Department of Pathophysiology, University of Szeged) for the scientific advices during these years.

Thank you to all the members of the Cell and Physiology Laboratory at the First Department of Medicine, University of Szeged and the Hungarian Pancreatic Study Group. This dissertation would not have been possible to achieve without a great team and the assistance of **Edit Magyarné Pálfi, Rea Fritz, Zsuzsanna Miklósné Árva, Tünde Pritz Béláné Horesnyi, Brigitta Kazi** and **†Erzsébet Zoltánné Fuksz**. Special thanks to my great lab colleagues and friends (in alphabetical order**)** for their advices, assistance and the fun times we had together: **Emese Réka Bálint, Zsolt Balla, Attila Ébert, Júlia Fanczal, Gabriella Fűr, Anett Lőrincz, Tamara Madácsy, Réka Molnár** and **Noémi Závogyán.** I am thankful to my husband **János Tóth** for his exceptional patience and for being my greatest cheerleader all along these years. I am beyond grateful to my brother **Máté Gergely Tóth** for always brighten my days; to all my family and friends for their emotional support. You are awesome! **I dedicate my dissertation work to my parents and grandparents.** To my parents; **Éva** and **Tibor Tóth,** who taught me about the importance of diligence, education, hard work and integrity. To my grandfather; **†Imre Tóth**, who believed in my scientific research career even when I did not know what even science meant.

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

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