Final work report for grant K-119938 entitled “Investigation of therapeutic possibilities in acute pancreatitis”

Acute pancreatitis (AP) is a sudden inflammation of the pancreas which usually develops due to biliary disease or moderate to heavy ethanol consumption (Pandol et al., Gastroenterology 2007). The incidence of AP is increasing and has recently become the most common reason of hospitalization amongst gastrointestinal diseases in the United States (Peery et al., Gastroenterology 2012). The disease causes huge financial burden, the annual aggregate inpatient costs in the United States alone add up to about $2.6 billion (Peery et al., Gastroenterology 2012). Although about 80% of the cases is mild and self-limiting, the overall mortality of AP remains 5-10%, and may increase to 30-50% if complications develop (in the necrotizing form of the disease) (Banks et al., Gut 2013, Pandol et al., Gastroenterology 2007). Unfortunately, there is no reliable way to predict the severity of AP. Another pressing problem is that our understanding of the disease is still far from complete as its pathomechanism is rather complex. Initial therapy of AP is only supportive with fluid resuscitation, pain control, and enteral nutrition.

The main aim of this project was to investigate some potential therapeutics in experimental AP. We also determined the effects of altering pancreatic ductal secretion in various AP models. In addition, we performed two meta-analyses related to the topic of the project.

The effects of some potential therapeutics in experimental pancreatitis models

The tryptophan metabolite L-kynurenic acid (KYNA) and its synthetic analogue SZR-72 are antagonists of the N-methyl-D-aspartate receptor (NMDAR) and have immune modulatory roles in several inflammatory diseases. mRNA and protein expression of NMDAR1 was detected in pancreatic tissue. We found that both KYNA and its synthetic analogue SZR-72 significantly reduced the severity of L-ornithine-induced AP in rats. To investigate the pathomechanism of these compounds, we aimed to examine their effects on microcirculation, acid-base balance, pancreatic acinar cell viability, and leukocyte function.

Pancreatic water content was significantly higher in animals with L-ornithine-induced AP compared with controls due to tissue oedema. However, pancreatic water content was significantly lower if 300 mg/kg KYNA or SZR-72 was given besides L-ornithine. Similar results were found when measuring pancreatic myeloperoxidase activity, which was significantly reduced as a result of KYNA or SZR-72 treatment. Furthermore, histological

parameters of inflammation in AP groups were reduced in response to KYNA or SZR-72 treatment.

The changes in microcirculation were measured by orthogonal polarization spectral (OPS) imaging 24 hours after the induction of AP. Both KYNA and SZR-72 treatment significantly improved microcirculation which was markedly reduced by L-ornithine induced AP. KYNA and SZR-72 treatment restored arterial blood pH to control levels whereas in pancreatitis group animals were acidotic.

Isolated rat pancreatic acinar cells were subjected to L-ornithine, KYNA and/or SZR-72 in vitro. Cell viability was checked with propidium-iodide for 24 hours. L-ornithine administration significantly reduced the viability of acinar cells, whereas when combined with KYNA or SZR-72 treatment, the survival of acinar cells was significantly enhanced. Treatment of acini with NMDA (25, 250, 2000 μM) did not influence the effects of KYNA or SZR-72.

To determine leukocyte function, we performed oxidative/respiratory burst assays (H_2O_2 production) and bacterial killing on isolated neutrophil granulocytes. We found detectable H_2O_2 production in neutrophil granulocytes isolated from both control (physiological saline-treated) and pancreatitis (AP) groups. However, there was no significant difference between the two groups. Interestingly, *in vitro* L-ornithine administration to the suspension of neutrophil granulocytes significantly increased H_2O_2 production. Treatment with 1x300 mg/kg SZR-72 caused no significant alteration in H_2O_2 levels compared to physiological saline and AP groups. Bacterial killing efficiency of isolated neutrophil granulocytes from control (physiological saline-treated), pancreatitis (AP) and SZR-72 (SZR-72 and AP+SZR-72) treated groups did not significantly differ. Overall, the results of our study suggest that administration of KYNA and its derivative could be beneficial in AP (**Balla et al., Front Immunol 2021, IF: 7.561**)

Mu opioid receptor (MOR) expression and function was assessed in control and AP animals. MOR was expressed in both the pancreas and brain. Interestingly, the pancreatic expression and function of MOR were reduced in AP. The appropriate doses and timing of major analgetics were determined by performing the Writhing test in rats. In case of morphine, the proper dose was 5 mg/kg injected every 6 hours. In case of fentanyl, the dose of 0.1-0.2 mg/kg administered every 10 hours seemed to be the most adequate.

Intraperitoneal injection of 8x5 mg/kg or 9x10 mg/kg morphine significantly improved the severity of L-ornithine-induced experimental AP. In case of cerulein-induced experimental AP, morphine treatment at the dose of 3x5 mg/kg did not significantly affect laboratory or histological parameters. 4x5 mg/kg morphine significantly reduced vacuolization, but it did not

influence any other parameters. The overall effects of morphine on disease severity were negligible.

L-ornithine-induced AP resulted in about 60% pancreatic necrosis and intensive leukocyte infiltration. These signs even worsened due to fentanyl pre-treatment. The extent of tissue necrosis significantly increased when the higher dose (3×0.2 mg/kg) of fentanyl was applied, whereas the level of leukocyte infiltration was higher in the 3×0.1 mg/kg fentanyl and AP group compared to the AP group not receiving fentanyl. Fentanyl treatment did not cause any change in pancreatic water content in the AP groups. Serum amylase activity markedly increased in the AP groups versus the control group. Importantly, 3×0.1 mg/kg fentanyl significantly increased serum amylase activity during AP. Myeloperoxidase activity was greatly elevated in the AP groups compared to the control group, and the dose of 3×0.2 mg/kg fentanyl further increased myeloperoxidase activity in AP. Fentanyl post-treatment decreased the extent of histopathological changes (pancreatic tissue necrosis and leukocyte infiltration) caused by L-ornithine-induced AP. On the other hand, fentanyl administration did not alter pancreatic water content in the AP groups. L-ornithine-induced AP increased pancreatic myeloperoxidase and serum amylase activities, which were decreased by both fentanyl doses tested. Pancreatic IL-1β levels only decreased significantly in case of the L-ornithine + 3×0.2 mg/kg fentanyl group. Compared to controls, the administration of fentanyl increased serum amylase activity in the L-ornithine-induced pancreatitis group. However, no significant difference was detected in any other parameters (pancreatic myeloperoxidase activity, water content, edema, leukocyte infiltration and necrosis). Overall, fentanyl post-treatment reduced necrotizing AP severity, whereas pre-treatment exacerbated it. Fentanyl did not affect the outcome of cerulein-induced mild edematous AP in rats.

In conclusion, the type, dosing, administration route, and timing of opioid treatment can influence the effects of opioids on AP severity (**Bálint et al., Int J Mol Sci 2022, IF: 6.208**).

We set out to investigate and characterize the potential role of mesenchymal stem cells (MSCs) in experimental AP induced in male SPRD rats (200-220g) with 3g/kg, 30% L-ornithine-HCl. MSCs were periodontal ligament cells (PDL) and dental pulp stem cells (DPSC) that were administered intravenously through the tail vein of rats.

First, we tested whether MSCs reduce the severity of experimental AP, given 1 hour before or 6 hours after AP induction. We administered $1x10^6$ DPSC and PDL cells. Animals were sacrificed 24 hours after AP induction. Pancreas samples were fixed in formalin, sectioned and

stained with hematoxilin and eosin to evaluate pancreatic oedema, leukocyte infiltration and cell damage. We found no significant effects of MSCs on AP severity.
To test for the dose (cell number) dependence of MSC treatment on experimental AP, we administered $5x10^5$ or $4.5x10^6$ PDL and DPSC cells i.v. simultaneously with the L-ornithine-HCl injection (i.p.). The animals were sacrificed 24 hours after the AP induction. Unfortunately, there was no difference between the AP and the AP+MSC groups.
The possible immune-modulatory role of MSCs was tested by administering $2x10^6$ MSCs 24, 48, 72, 96 hours after the AP induction and the animals were sacrificed 1 week after the L-ornithine injection. The histological examination showed that there were significantly more intact acini in the AP+MSC group compared to the AP group. Furthermore, MSC treatment significantly increased serum amylase activity which correlates with the number of functioning acini. Overall, it seems that long-term MSC treatment promotes the recovery after L-ornithine-induced AP in rats.

It has been shown that mitochondrial dysfunction plays a crucial role in the development of AP. We investigated the effects of a novel mitochondrial transition pore inhibitor, N-methyl-4-isoleucine cyclosporin (NIM811), in AP. *In vivo* experiments revealed that *per os* administration of NIM811 has a protective effect in experimental AP models by reducing pancreatic oedema, necrosis, leukocyte infiltration and serum amylase activity (**Tóth et al., J Physiol 2019, IF: 4.547**).

We also investigated the effects of transient receptor potential melastatin 2 (TRPM2), a non-selective cation channel, on acute biliary pancreatitis in mice. Bile-acid-induced experimental pancreatitis was less severe in TRPM2 knockout mice, whereas the lack of TRPM2 had no protective effect in cerulein-induced AP (**Fanczal et al., J Physiol 2020, IF: 5.182**).

With repeated bouts of AP, damage to the pancreas can lead to chronic pancreatitis (CP). Defective mucus production in the pancreas may be an important factor in the initiation and progression of CP, therefore we aimed to (i) investigate the qualitative and quantitative changes of mucus both in human CP and in an experimental pancreatitis model and (ii) to correlate the mucus phenotype with epithelial ion transport function. We demonstrated increased mucus content in the small pancreatic ducts in CP. Secretory mucins MUC6 and MUC5B were upregulated in human, Muc6 in mouse CP. *In vivo* and *in vitro* fluid secretion was decreased in

cerulein-induced CP. Analysis of time course changes showed that impaired ductal ion transport is paralleled by increased Muc6 expression (**Balázs et al., Front Physiol 2018, IF: 3.201**).

In a collaborative study, we aimed to identify blood-brain barrier (BBB) changes in L-ornithine-induced AP and to determine whether L-ornithine, a cationic amino acid, used to induce AP has a direct effect on brain endothelial cells *in vitro* contributing to the increased permeability of blood-brain barrier (BBB). We demonstrated BBB damage in the L-ornithine-induced rat AP model suggesting a general, AP model independent effect. L-ornithine induced oxidative stress, decreased barrier integrity and altered BBB morphology in a culture BBB model. These data suggest a direct effect of the cationic L-ornithine on brain endothelium. Endothelial surface glycocalyx injury was revealed both *in vivo* and *in vitro*, as an additional novel component of the BBB-related pathological changes in AP (**Walter et al., Fluids Barriers CNS 2022, IF: 6.961**).

The effects of altering pancreatic ductal secretion in acute pancreatitis models

We investigated the localization and expression of cystic fibrosis transmembrane conductance regulator (CFTR) during cerulein-induced AP in mice and determined the effects of a CFTR corrector (VX-661) and potentiator (VX-770) on disease severity. Immunohistochemistry demonstrated disturbed staining morphology of CFTR and CK19 proteins in AP. Mislocalization of CFTR protein was observed from 6 hours, while expression increased at 24 hours compared to control. Ductal HCO_3^- transport activity was significantly increased 6 hours after AP induction. Pre-treatment of AP mice with VX-661 + VX-770 significantly reduced the extent of tissue damage by about 20-30%, but other parameters were unchanged. Interestingly, *in vitro* administration of VX-661 + VX-770 significantly increased the fluid secretion of ducts derived from AP animals. Our results published in The Journal of Physiology (**Fűr et al., 2021, IF: 6.228**) suggest that the beneficial effects of CFTR correctors and potentiators should be further investigated in AP.

Accumulating evidence indicate that decreased pancreatic ductal fluid secretion plays an essential role in AP; therefore, we aimed to investigate the physiological and pathophysiological role of aquaporins (AQPs) in the pancreas. The expression of AQP1 was detected throughout the whole plasma membrane of mouse ductal cells and its expression

highly depended on the presence of CFTR chloride channel. In contrast, the expression of AQP1 was mainly localized to the apical membrane of ductal cells in the human pancreas. HCO_3^- and fluid secretion significantly decreased in AQP1 KO versus WT mice and the absence of AQP1 also worsened the severity of pancreatitis. 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 (**Venglovecz et al., Front Physiol, IF: 3.201**).

Pancreatic organoid cultures may help to overcome shortcomings of the current secretory models. We provided side-by-side comparison of gene expression, morphology, and function of epithelial cells in primary isolated pancreatic ducts and organoids (**Molnár et al., Lab Invest 2020, IF: 5.662**).

Meta-analyses investigating the effects of hypertriglyceridemia and aetiological factors on the severity of acute pancreatitis

Since it has been proposed that elevated serum triglyceride concentration (seTG, >1.7 mM) or in other words hypertriglyceridemia (HTG) could worsen the course of AP, we performed a meta-analysis to compare the effects of various seTGs on the severity, mortality, local and systemic complications of AP, and on intensive care unit admission. 16 eligible studies, including 11,965 patients were retrieved from PubMed and Embase. The results showed that HTG significantly elevated the odds ratio (OR=1.72) for severe AP when compared to patients with normal seTG (<1.7 mM). Furthermore, a significantly higher occurrence of pancreatic necrosis, persistent organ failure and renal failure was observed in groups with HTG. The rates of complications and mortality for AP were significantly increased in patients with seTG >5.6 mM or >11.3 mM versus <5.6 mM or <11.3 mM, respectively. These results have been published in Scientific Reports (**Kiss et al., 2018, IF: 4.011**).

In another meta-analysis, we evaluated the effects of major aetiological factors like biliary disease, alcohol consumption, HTG and endoscopic retrograde cholangiopancreatography (ERCP) on the severity and outcome of AP. Data were extracted from 72 eligible studies (1,194,353 patients). The risk of non-mild (moderately severe and severe) AP was highest in HTG-induced AP (HTG-AP) followed by alcoholic AP (AAP), whereas disease course of biliary AP (BAP) and post-ERCP AP were milder. The ORs of multiple OF and recurrence rate

were also significantly lower among BAP vs. HTG-AP patients (OR=0.26 and 0.56, 95% CI=0.08-0.89 and 0.41-0.75, respectively). Mortality rate was significantly greater in HTG-AP vs. BAP (OR=0.39, 95% CI=0.24-0.63), but aetiology did not influence the occurrence of pancreatic necrosis. This manuscript by **Bálint et al.** was published in **Scientific Reports** (**2020, IF: 4.379**).

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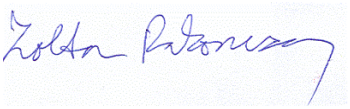

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