FACTORS INFLUENCING THE SEVERITY OF ACUTE PANCREATITIS: EXPERIMENTAL AND CLINICAL STUDIES

Ph.D. Thesis

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I. PUBLICATIONS

I.1. Publications related to the subject of the thesis

Fűr G, Bálint ER, Orján EM, Balla Z, Kormányos ES, Czira B, Szűcs A, Kovács DP, Pallagi P, Maléth J, Venglovecz V, Hegyi P, Kiss L, Rakonczay Z Jr (2021). Mislocalization of CFTR expression in acute pancreatitis and the beneficial effects of VX-661/VX-770 treatment on disease severity. J Physiol. 599, 4955-4971. IF_{2020}: 5.182, Q1, D1

Bálint ER, **Fűr G**, Kui B, Balla Z, Kormányos ES, Orján EM, Tóth B, Horváth G, Szűcs E, Benyhe S, Ducza E, Pallagi P, Maléth J, Venglovecz V, Hegyi P, Kiss L, Rakonczay Z Jr (2022). Fentanyl but not morphine or buprenorphine improves the severity of necrotizing acute pancreatitis in rats. Int J Mol Sci. 23, 1192. IF_{2020}: 5.924, Q1, D1

Kiss L*, **Fűr G***, Mátrai P, Hegyi P, Iványi E, Cazacu IM, Szabó I, Habon T, Alizadeh H, Gyöngyi Z, Vigh É, Erőss B, Erős A, Ottoffy M, Czakó L, Rakonczay Z Jr (2018). The effect of serum triglyceride concentration on the outcome of acute pancreatitis: systematic review and meta-analysis. Sci Rep. 8, 14096. IF_{2018}: 4.011, Q1, D1 *Authors share a co-first authorship

I.2. Publications not related to the subject of the thesis

Balla Z, Kormányos ES, Kui B, Bálint ER, **Fűr G**, Orján EM, Iványi B, Vécsei L, Fülöp F, Varga G, Harazin A, Tubak V, Deli MA, Papp C, Gácser A, Madácsy T, Venglovecz V, Maléth J, Hegyi P, Kiss L, Rakonczay Z Jr (2021). Kynurenic acid and its analogue SZR-72 ameliorate the severity of experimental acute necrotizing pancreatitis. Front Immunol. 12, 702764. IF_{2020}: 7.561, Q1

Gróf I, Bocsik A, Harazin A, Santa-Maria AR, Vizsnyiczai G, Barna L, Kiss L, **Fűr G**, Rakonczay Z Jr, Ambrus R, Szabó-Révész P, Gosselet F, Jaikumpun P, Szabó H, Zsembery Á, Deli MA (2020). The effect of sodium bicarbonate, a beneficial adjuvant molecule in cystic fibrosis, on bronchial epithelial cells expressing a wild-type or mutant CFTR channel. Int J Mol Sci. 21, 4024. IF_{2020}: 5.923, Q1, D1

Bálint ER, **Fűr G**, Kiss L, Németh DI, Soós A, Hegyi P, Szakács Z, Tinusz B, Varjú P, Vincze Á, Erőss B, Czimmer J, Szepes Z, Varga G, Rakonczay Z Jr (2020). Assessment of the course of acute pancreatitis in the light of aetiology: a systematic review and meta-analysis. Sci Rep. 10, 17936. IF_{2020}: 4.379, Q1, D1

Szakács Z, Hegyi PJ, Farkas N, Hegyi P, Balaskó M, Erős A, Szujó S, Pammer J, Mosdósi B, Simon M, Nagy A, **Fűr G**, Hussain A (2020). Pregnancy outcomes of women whom spouse fathered children after tyrosine kinase inhibitor therapy for chronic myeloid leukemia: A systematic review. PLoS One. 15, e0243045. IF_{2020}: 2.740, Q1, D1

I.3. Scientometrics

Number of publications:	7
Publications with first authorship:	2
Cummulative impact factor:	35.72
Number of independent citations (MTMT2):	37
Hirsch index:	3

II. INTRODUCTION

II.1. The physiological functions of the pancreas

The exocrine pancreas mainly consists of acinar and ductal cells. The acinar cells secrete inactive digestive enzymes in a NaCl-rich isotonic fluid. The pancreatic ductal epithelium secretes 2.5 litres of alkaline fluid daily that may contain up to 140 mM HCO_3^-. The secretion of HCO_3^- across the apical membrane into the lumen of the ducts occurs mainly via four transporters: cystic fibrosis transmembrane conductance regulator Cl^- channel (CFTR), solute carrier family 26 (SLC26) anion exchangers (SLC26A3 and SLC26A6) and Anoctamin-1. Among these ion transporters, CFTR is the most prominent and acts as a signalling hub due to its numerous roles in secretion and regulation of other ion channels, like SLC26A6.

II.2. Acute pancreatitis

II.2.1. Epidemiology and diagnostic criteria

Acute pancreatitis (AP) is the sudden inflammation of the pancreas and one of the most common gastrointestinal diseases requiring hospitalisation. Its incidence shows increasing tendency, and it is more than 30 per 100 000 population in Europe. The diagnosis of AP includes epigastric upper abdominal pain, more than 3 times elevated serum or urinary amylase/lipase activity, and imaging consistent with the diagnosis. Meeting two of these three criteria helps ensure appropriate diagnosis. Notably, pain is present in 90-95% of AP patients.

II.2.2. Aetiological factors

Massive alcohol consumption and gallstone disease are responsible for about 60–80% of AP cases, whereas 1–9% of the cases are hypertriglyceridemia (HTG)-induced. Patients with HTG-AP are characterized by younger age and predominantly male gender, but higher fat intake or higher body mass index may also contribute to this aetiology. Furthermore, complications, comorbidities or mortality and the need for hospitalisation seem to be more common in HTG-AP than in other aetiologies.

II.2.2.1. Effect of different serum triglyceride levels

The reference value of triglyceride (TG) in the blood serum is below 1.7 mM. The extent of HTG has been classified by the Endocrine Society into the following groups based on fasting serum TG (seTG): mild (1.7 to 2.3 mM), moderate (2.3 to 11.2 mM), severe (11.3 to 22.4 mM) and very severe HTG (>22.4 mM). It is widely accepted that severe and very severe HTG markedly increase the risk for AP. However, some authors define HTG-AP when seTG is >5.6 mM. There is no significant evidence for HTG-AP at <5.6 mM seTG. Beyond the increased risk for AP in severe HTG, previous publications have indicated that there is a relationship between seTG and the severity of AP, even in the case of mild or moderate HTG. Others have shown no relationship between seTG and the severity of AP. These discrepancies urge further analyses of the effect of seTG on the severity of AP.

II.2.3. Pathomechanism

The mechanisms underlying the development of AP are complex and not fully understood. AP pathogenesis includes toxic intracellular Ca^{2+} overload which induces premature activation of digestive enzymes, activation of the nuclear factor kappa B, impairment of autophagy, mitochondrial dysfunction, as well as impairment of ductal function. The aetiology of the disease can also determine the pathogenesis

of AP.

II.2.3.1 Impact of aetiological factors

In HTG-AP, one of the possible processes is that pancreatic lipases metabolize seTG to non-esterified fatty acids (NEFA). NEFA induces sustained elevation of Ca^{2+} concentration in pancreatic acinar cells and inhibits mitochondrial function and ATP production. Our earlier studies have also indicated that fatty acids inhibit CFTR activity and decrease the HCO_3^- and fluid secretion of pancreatic ductal cells.

II.2.3.2. The role of pancreatic ducts and cystic fibrosis transmembrane conductance regulator

Pancreatic ductal cell damage can occur during AP which is associated with impaired ductal secretion. Moreover, the impaired ductal function due to CFTR mutations in cystic fibrosis or CFTR inhibition in AP decreases HCO_3^- and fluid secretion and lead to aggravated AP. CFTR activity contributes significantly to proper channel function. Disturbance or loss of ductal function is a key factor in the development of AP. Several drugs have recently been clinically approved to improve CFTR expression, localisation, and function by correcting the folding of the protein or potentiating its activity in cystic fibrosis. Of these, the CFTR corrector lumacaftor (VX-809), tezacaftor (VX-661), elexacaftor (VX-445) and the CFTR potentiator ivacaftor (VX-770) have been shown to be the most effective.

II.2.4. Treatment

The therapy of AP is only supportive and there is no specific drug against it. Nowadays, the opportunities for early management of AP are analgesia, Ringer's lactate solution-based fluid resuscitation, and early oral refeeding, or if it this not tolerated enteral nutrition should be applied. Unfortunately, recent guidelines for AP treatment do not have clear recommendations for the types of analgesics to be used. Opioids, like fentanyl (FE), buprenorphine or morphine (MO) are the most effective pain killers. There is a scientific debate on the use of opioids due to their side effects such as constipation or immunosuppression. FE and MO administration is less preferred in humans due to the spasm of sphincter of Oddi which might worsen the outcome of AP.

III. AIMS

Our overall goal was to investigate the effects of different factors on AP by using experimental animal models or processing clinical data. Based on these, our detailed aims were the following:

a. to reveal how the disease course affects pancreatic ductal functions and the expression of transporters involved in HCO_3^- secretion
b. to investigate how correction and stimulation of CFTR affect the disease severity
c. to study the effect of the pain reliever fentanyl on the severity of experimental biliary AP
d. to evaluate and compare the effects of normal or elevated seTG on the severity, mortality and other complications of AP in humans

IV. MATERIALS AND METHODS

IV.1. Animal experiments

IV.1.1. Ethics

Animal experiments were implemented in compliance with the European Union Directive 2010/63/EU and the Hungarian Government Decree 40/2013 (II.14.). Mice or rats were sacrificed via intraperitoneal (i.p.) injection of 200 or 85 mg/kg pentobarbital (Bimeda MTC, Cambridge, Canada), respectively.

IV.1.2. Chemicals

Most of the chemicals were obtained from Merck Life Science Kft. (Budapest, Hungary). Cerulein (Cer) was acquired from Glentham Life Sciences (Corsham, United Kingdom); VX-661 (tezacaftor) and VX-770 (ivacaftor) were obtained from Cayman Chemical (Ann Arbor, MI, USA) Cer, VX-661 and VX-770 were dissolved in dimethyl sulfoxide (DMSO) and Cer was further diluted in physiological saline (PS) before injection.

IV.1.3. Animals

8-10 week-old male FVB/n mice from Charles River Laboratories Inc. (Wilmington, MA, USA) or female Wistar rats weighing 200-250 g were used for experiments.

IV.1.4. *In vivo* experiments: acute pancreatitis induction and treatments

Necrotizing AP was induced by hourly i.p. injection of 6 or 10 × 50 μg/kg Cer (5 μg/ml) in FVB/n mice or intraductal (i.d.) administration of 1 ml/kg Na-taurocholate solution (NaTc; 40 mg/ml) in rats. VX-661 and VX-770 were administered i.p. at 2 mg/kg once a day before and during AP. FE was administered i.p. at doses of 0.1 and 0.2 mg/kg based on the literature data. FE was used as pre- or post-treatment in NaTc model. Mice were sacrificed at 0, 6, 12, 24, 48, and 72 h, but in case of VX-661+VX-770 combination, the first termination time was at 24 h and the second at 48 h. Rats were sacrificed between 16-24 h. Control groups were given PS instead of Cer or NaTc, and DMSO instead of VX compounds.

IV.1.5. Histological examination

Formalin-fixed and paraffin embedded pancreatic tissues were sectioned to 3 μm. These sections were stained with hematoxylin and eosin (H&E) and were scored by independent experts blinded to the experimental protocol. To quantify cellular damage, leukocyte infiltration, vacuolisation and oedema grades, a semiquantitative scoring system was used.

IV.1.6. Laboratory measurements

Pancreatic myeloperoxidase activity (normalized to total protein content) was measured, which is a hallmark of leukocytic infiltration. To evaluate pancreatic water content, the wet weight (WW) and the dry weight (DW) of the pancreata was also measured and the following calculation was used: [(WW-DW)/WW]×100. Serum amylase activity was determined with a colorimetric kinetic method.

IV.1.7. mRNA extraction and reverse transcription

A small piece of pancreas was placed on ice in 1 ml TRIzol reagent and was homogenised

immediately. Total RNA purification was performed in three steps. Phase separation was performed by chloroform. The top aqueous phase with RNAs was aspirated into an empty tube. RNAs were vortexed and centrifuged with isopropanol, then RNA precipitated in the tubes. The supernatant was removed and RNAs were washed with 75% alcohol. The excess ethanol was evaporated briefly and then the RNA was redissolved in 70 µl of RNAse-free water. RNA was stored at –80 °C until use. The RNA concentration in the solution and RNA integrity were investigated. 2 µg of total RNA was used for reverse transcription. cDNA was used for real-time PCR measurements.

IV.1.8. Immunohistochemistry

The pancreatic tissues were sliced by a Leica Cryostat at 7 µm thickness. Sections were fixed in 2 % paraformaldehyde, then antigen retrieval was performed. After the blocking step, sections were incubated with anti-CFTR antibody overnight. The following day, the samples were incubated with Alexa488-conjugated secondary antibody in the dark at room temperature. Co-immunostaining was performed with the AlexaFluor647-conjugated cytokeratin-19 antibody. Nuclei were counterstained with Hoechst 33342. Tissue slices were analysed using a Zeiss LSM 880 confocal laser scanning microscope (Carl Zeiss Technika Kft., Budapest, Hungary). To quantify pancreatic ductal CFTR and CK19 expression, three or four representative large tile scan images (in average 1500 x 1000 µm) were taken from each group. Image J software (National Institutes of Health, Bethesda, MD, USA) was used to convert images to grey scale (16 bit), and threshold function was used to select the positively stained area based on the fluorescence intensities.

IV.1.9. Fluid secretion and intracellular pH measurement in cultured pancreatic ducts

Intra-/interlobular pancreatic ducts were isolated after collagenase digestion by microdissection. In case of fluid secretion measurements, ducts were cultured for 6-14 h. Some ducts were treated with 3 µM VX-661 and 1 µM VX-770 during incubation, while others subjected to the vehicle (0.5% DMSO) or only the medium. Fluid secretion into the closed luminal space of the cultured pancreatic ducts was analysed using a swelling method. The ducts were perfused with different solutions in the following order: 1) standard HEPES, 2) standard HEPES with 5 µM forskolin 3) standard HCO_3^-/CO_2 with 5 µM forskolin. Bright-field images were acquired at 1-min intervals using a Zeiss Axio Observer 7 with CMOS camera. Digital images of the ducts were analysed using ImageJ software.

Intracellular pH (pH_i) measurements were started immediately after isolation and were carried out within 8 h thereafter using IX71 live cell imaging fluorescence microscope and CellR imaging system from Olympus (Budapest, Hungary). The alkali load method was applied to determine pancreatic ductal HCO_3^- secretion. The HCO_3^- secretion was estimated by the rate of pH_i recovery from alkalization. The isolated ducts were loaded with the pH sensitive BCECF-AM fluorescent dye in standard HEPES solution. After that, ducts were perfused with solutions in the following order: 1) standard HEPES, 2) standard HCO_3^-/CO_2, 3) NH_4Cl in HCO_3^-/CO_2, 4) standard HCO_3^-/CO_2, 5) standard HEPES. Exposing ducts to 20 mM NH_4Cl caused alkalization of pH_i. Four to ten small areas (region of interests, ROIs) of 5-10 cells in each intact duct were monitored. The ducts were excited with light at wavelengths of 490 and 440 nm, and

the 490/440 fluorescence emission ratio was measured at 535 nm. One pH_i measurement was obtained per second. The extent of pH_i change ($\Delta pH/\Delta t$) was calculated by linear regression analysis.

IV.1.10. Statistical analysis

Graphs were generated by GraphPad Prism 9.2.0 (GraphPad Software, San Diego, CA, USA) or by Microsoft Excel and PowerPoint (Redmond, WA, USA). Experiments were evaluated by one- or two-way ANOVA followed by the Tukey HSD post hoc test in Cer-AP or Holm–Sidak post hoc tests in NaTc-AP (SPSS, IBM, Armonk, NY, USA). $P<0.05$ was accepted as statistically significant.

IV.2. Systematic review and meta-analysis

IV.2.1. Registration and PICO

Our systematic review and meta-analysis was conducted according to the protocol previously registered in the PROSPERO database (https://www.crd.york.ac.uk/PROSPERO/, ID: CRD42017071264). The analysis was based on the Problem, Intervention, Comparison intervention, and Outcome (PICO) model. The problem was AP. The intervention was HTG with various groups formed for the analysis: >1.7, 1.7–5.6, 1.7–11.3, >5.6 and >11.3 mM seTG. The comparison interventions were normal (<1.7), <5.6, 1.7–5.6, 1.7–11.3 and <11.3mM seTG. Different outcomes were investigated: AP severity, mortality, pancreatic necrosis, persistent organ failure (POF) and multi-organ failure (MOF), pulmonary and renal failure, and admission to an intensive care unit (ICU).

IV.2.2. Article search strategy

The search was carried out in late August 2017. Observational prospective and retrospective cohorts, and case control studies were identified in Embase (published from 1948 to July 2017) and PubMed Library (published from 1961 to July 2017). Furthermore, ClinicalTrials.gov was also screened for additional unpublished data.

IV.2.3. Eligibility criteria

Articles were included if they fulfilled the following criteria: 1) case control or cohort studies; 2) studies involving AP patients; 3) HTG (>1.7mM) was present in at least one of the groups under investigation; 4) seTGs were defined; 5) outcome data were provided for at least one of the following: severity of AP according to the Revised Atlanta Classification, mortality, pancreatic necrosis, POF, MOF, pulmonary failure, renal failure, and intensive care unit (ICU) admission; 6) written in English.

The seTG in different groups used as comparisons were below 1.7, 5.6 or 11.3 mM as well as within the 1.7–5.6 and 1.7–11.3 mM ranges.

IV.2.4. Study selection and data extraction

Relevant studies were manually screened by two independent authors. The investigators after that extracted the characteristics of proper studies and also the data, which was then statistically analysed. Discrepancies were resolved by discussion with other two authors.

IV.2.5. Quality assessment of the articles

The Newcastle–Ottawa Scale (NOS) was used to assess the quality of the articles included. Since

seTG decreases rapidly when food intake is restricted, the NOS was supplemented with another scoring system in which the articles were also evaluated based on the timing of the seTG measurement.

IV.2.6. Data analyses

The statistical analysis was performed with Stata 11 SE (StataCorp LLC, College Station, TX, USA. Odds ratios (ORs) were pooled using the random effects model with the DerSimonian–Laird estimation and displayed on forest plots. Summary OR estimation, p value and 95% confidence interval (CI) were calculated. $P<0.05$ was considered a significant difference from summary OR=1. Statistical heterogeneity was analysed using the I^2 statistic and the chi-square test to acquire probability values; $p<0.05$ was defined to indicate significant heterogeneity. The small-study effect was visually investigated on funnel plots.

V. RESULTS

V.1. Animal experiments

V.1.1. The time course of cerulein-induced acute pancreatitis severity

The progression of Cer-induced AP was followed from 0 to 72 h. Cer injection caused the greatest degree of cell damage at 24 h. This was adequately supported by the scoring results of vacuolization. Cell damage and vacuolization were significantly decreased at 48 and 72 h compared to 24 h groups. Leukocyte infiltration was significantly increased at 6, 12, 24, and 72 h after the first Cer injection compared to control (0 h). Changes in pancreatic MPO activity closely followed leukocyte infiltration and showed marked increases at 12 and 24 h. AP evoked significant elevations in pancreatic water content and serum amylase activity at 12 and 24 h compared to the control group. Serum amylase activity was highest at 12 h, which then decreased back to control levels after 48 h. At 48-72 h, almost all histological and laboratory parameters showed decreased tendency compared to the 12 or 24 h groups.

V.1.2. Changes in CFTR expression and staining morphology during acute pancreatitis

Cftr mRNA expression was markedly increased during AP from 24 h compared to the control. The peak was detected at 48 h and almost 20-fold increase was measured in *Cftr* mRNA amount compared to the healthy group. This tendency was also observed in case of the ductal marker *Ck19*, but its mRNA had less marked increased expression than *Cftr*. *Ck19* mRNA expression was highest at 12 and 24 h after the initiation of AP. *Slc26a3* mRNA expression was also increased at 24 h, while the mRNA of *Slc26a6* was decreased between 24 and 72 h.

The percentage of area staining of CFTR and CK19 proteins was determined by fluorescent immunostainings. Six percent of pancreatic tissue in untreated group stained for CFTR. At the beginning of AP (6 and 12 h), the CFTR staining area showed decrease in tendency, but at 24 h the protein expression significantly increased compared to the control or 6-12 h treatment groups. The increase of CFTR protein expression is in accordance with the results of the mRNA measurements. CK19 staining area in the control group was approximately 20 %. This area of CK19 decreased in pancreatic tissue at the beginning of AP (6 h), while later on CK19 expression did not differ from the control group.

Detailed ductal structures were also captured after CFTR and CK19 immunostaining. The physiological location of CFTR is in the apical membrane of the pancreatic ducts. Clearly, detectable ductal morphology was observed in the control animals in cases of both CFTR and CK19. The lumens of the stained intralobular ducts were approximately 2-3 μm in diameter, which could be followed through several μm. The nuclei were located close to the ductal lumen. Bigger interlobular ducts did not stain for CFTR. Notably, AP even from 6 h disturbed the characteristic structure of the ductal tree. CFTR was mislocalized and both CFTR and CK19 proteins showed diffuse and perinuclear appearance. At 72 h after the initiation of the disease, some duct-like structures appeared in stained tissue slices.

V.1.3. Pancreatic ductal HCO_3^- secretion during the course of acute pancreatitis

Alkali load method and measurement of pH_i changes during the cellular regeneration phase provided estimation of the HCO_3^- secretory function of isolated pancreatic ducts. This is mainly carried out by apical transporters (CFTR, SLC26A3, SLC26A6). When cellular alkalosis was stopped by discontinuing NH_4Cl administration, the cells shortly became acidotic. The cellular regeneration from acidosis can activate basolateral transporters (e.g. Na^+/HCO_3^- co-transporter, Na^+/H^+ exchanger or H^+-ATPase). Therefore, the regeneration rate from acidosis refers to the activity of basolateral transporters. At the early phase (6 h) of AP, HCO_3^- secretion by apical transporters was significantly increased as demonstrated by regeneration from alkali load. Basolateral transporter activity was also significantly elevated when regeneration from acidosis was measured. However, from 12 to 72 h, the response to alkalosis and acidosis were not significantly different vs. the control group.

V.1.4. The combination of CFTR corrector VX-661 and CFTR potentiator VX-770 decreased the severity of acute pancreatitis

The combination of VX-661 and VX-770 by itself did not induce any gross adverse effects. In fact, the morphology of the pancreas was normal after administration of VX-661+VX-770, and the histological and laboratory parameters were also similar to the non-treated group. Almost all measured parameters were increased in AP groups compared to the control group. Representative histological sections showed that pre-treatment with VX-661+VX-770 could ameliorate the extent of AP-induced pancreatic cell damage. We could not observe any significant difference in vacuolization over time or treatment in the AP groups. Measurements of pancreatic leukocyte infiltration and MPO activity showed similar kinetics, with no significant difference in the AP groups. No change was observed in pancreatic water content when the AP group was compared with the AP+VX-661+VX-770 group. In case of serum amylase activity, no significant difference was measured between the control and treated groups.

V.1.5. The effect of CFTR corrector VX-661 and CFTR potentiator VX-770 on ductal morphology and protein expression in acute pancreatitis

CFTR and CK19 co-immunostainings showed normal ductal structures at 24 and 48 h in control groups. AP disturbed the staining morphology of CFTR and CK19 at both 24 and 48 h. VX-661+VX-770 pre-treatment could not restore or improve the damaged ductal structure as demonstrated by CFTR or CK19 staining. CFTR protein expression was increased by AP while CK19 expression was unchanged. VX-661+VX-770 pre-treatment had no effect on AP-induced alterations of CFTR and CK19 protein expressions.

V.1.6. VX-661 and VX-770 enhance fluid secretion in isolated pancreatic ducts from mice with acute pancreatitis

To investigate if fluid secretion is influenced by the treatment with VX-661 and VX-770, isolated ducts (treated with or without 0.5 % DMSO/VX-661 and VX-770) from control and AP mice were used, and their swelling was followed. The cAMP agonist forskolin treatment significantly enhanced the swelling of ducts from control animals, especially in HCO_3^-/CO_2 containing buffer. DMSO administration

did not influence changes in relative ductal luminal volume. Therefore, the corresponding groups treated with or without DMSO were combined (i.e., Cer and Cer+DMSO). Ducts isolated from Cer-treated animals showed tendency towards increased swelling rates compared to the PS-treated control mice, but this did not reach statistical significance. Interestingly, VX-661 and VX-770 treated ducts showed significantly increased fluid secretory rate compared to the non-VX treated ducts derived from AP animals.

V.1.7. The effect of fentanyl post-treatment on acute pancreatitis

Intraductal infusion of NaTc induced AP in rats and increased the extent of pancreatic necrosis, leukocyte infiltration and oedema. Both necrosis and immune cell infiltration were decreased by the higher dose of FE, while the score of oedema did not change in the AP groups after FE treatment. Serum amylase activity also decreased in the NaTc+3x0.2 mg/kg FE group versus the AP group without FE treatment.

V.2. Meta-analysis of the effect of serum triglyceride concentration on the outcome of acute pancreatitis

V.2.1. Study selection

The search for articles in three databases resulted in 2261 records. After removing duplicates and screening titles and abstracts, 90 articles were assessed in full text for eligibility. Of these manuscripts, 29 prospective and retrospective cohorts seemed to be suitable for data collection. However, in 13 publications, seTGs were defined inappropriately (e.g. <1.88 mM was identified as normal) or the outcome data could not be used. Therefore, these 13 publications were removed from the assessment, and only 16 articles were included in the statistical analysis (in which the seTG ranges or the outcome data were appropriate). These studies were published between January 2000 and March 2016.

V.2.2. Characteristics of studies

Both single- (13) and multicentre (3) cohort studies were included. Population sizes ranged from 43 to 3203, and six trials involved over 300 patients. The aetiology of AP was noted in all the studies, eight comprised HTG-AP (>11.3 mM seTG), and twelve contained alcoholic and biliary pancreatitis patients. Other aetiologies, such as post-ERCP, idiopathic, mixed and drug-induced AP, were also included in some articles. The studies were performed in the following countries: China (nine cohorts), the USA (three cohorts), Hungary (two cohorts), the UK (one cohort) and Spain (one cohort). During the quality assessment, we evaluated patient selection, comparability of the groups, outcome data, and the timing of the seTG measurement.

V.2.3. Clinical outcomes

V.2.3.1. Comparing the effects of hypertriglyceridemia vs. normal serum triglyceride on the severity of acute pancreatitis

Different groups were created based on the extent of HTG, and the outcomes for AP were compared with those in the normal (<1.7 mM) seTG group. HTG significantly increased the number of severe AP cases (severity), pancreatic necrosis, POF and renal failure compared to the non-HTG group. However, HTG did not significantly increase the odds for mortality and pulmonary failure compared to the <1.7 mM

group. Analysing the effect of seTG in the range from 1.7 to 11.3 mM showed results similar to the previous comparison. The severity of AP and the incidence of POF significantly increased in the 1.7–11.3 mM range compared to the <1.7 mM seTG group, while it had no significant effect on the mortality of the patients.

HTG was further divided into ranges of 1.7–5.6, >5.6 and >11.3 mM seTG. The severity of AP was not significantly different in patients with 1.7–5.6 mM seTG compared to the <1.7 mM group. However, seTGs >5.6 mM significantly increased the risk for severe AP in patients with OR of 2.01 compared to seTG <1.7 mM. The presence of severe and very severe HTG (>11.3 mM) markedly increased the severity of AP, POF and ICU admission, but there was no significant elevation in mortality compared to the normal seTG group.

V.2.3.2. The effect of different ranges of hypertriglyceridemia on acute pancreatitis

If seTG is elevated, the extent of the increase could also have an impact on the course of AP. Comparing the effect of seTG below and above 5.6 mM showed that seTG higher than 5.6 mM significantly increased the risk for severe AP, mortality, and pulmonary and renal failure. However, the severity of AP was not significantly different in HTG patients with seTG of 1.7–5.6 mM vs. >5.6 mM.

Severe and very severe HTG significantly increased the OR of AP severity, mortality, pancreatic necrosis and ICU admission compared to group with seTG <11.3 mM, but it did not influence the occurrence of MOF.

Interestingly, when the effects of severe and very severe HTG were compared with mild and moderate HTG (seTG 1.7–11.3 mM), no significant difference was revealed between the two groups with regard to AP severity, mortality and POF.

VI. DISCUSSION

VI.1. Animal experiments

VI.1.1. CFTR and its restored function, as a central player in ameliorating acute pancreatitis severity

The pathomechanism of AP is complex and the underlying processes are not completely understood. However, the important role of ductal impairment and CFTR function in the pathomechanism of the disease is already known.

Pallagi et al. (2011) have shown that activation of trypsin causes ductal CFTR inhibition through proteinase-activated receptor 2 and elevation of intracellular Ca^{2+} concentration. Our study demonstrates that not just functional inhibition, but also mislocalization of CFTR may cause the decrease of ductal function. Our results demonstrate that AP induces the loss of CFTR staining along the ductal lumen, and CFTR staining was observed in the perinuclear region. Presumably, the inflammation and cellular stress direct CFTR proteins into proteosomes for degradation. Interestingly, the mRNA expression of *Cftr* was unchanged in the beginning of AP (6-12 h) and was significantly increased from 24 h. The protein expression of CFTR followed the mRNA changes and increased after 24 h. Consequently, the results of the present study and our earlier investigation showed that two different animal models of AP cause CFTR mislocalization, suggesting that this adverse effect is independent of the disease aetiology.

The *ex vivo* HCO_3^- secretion of isolated mouse interlobular ducts (with a luminal diameter of 20-130 μm) was increased at 6 h after AP induction, whereas at later time points it was similar to that of the non-AP group. However, CFTR expression was not observed in these ducts, only smaller (2-3 μm luminal diameter) intercalated ducts were stained for CFTR, as it was also shown in human samples. However, in earlier studies and this work demonstrated functional CFTR activity in interlobular ducts by fluid secretion measurement. Therefore, it is likely that the CFTR expression in mouse pancreatic interlobular ducts is lower compared with intercalated ducts. Based on our results, the measured increase in ductal HCO_3^- secretion at 6 h after AP initiation mainly relates to activation of transporters other than CFTR, e.g. SLC26A3, SLC26A6, ANO1, NBC, NHE or H^+-ATPase. Previous publications and the present work suggest that etiological factors of AP or the disease itself initiates CFTR mislocalization or degradation, and inhibition of fluid secretion. These factors possibly contribute to increased pancreatic inflammation. Therefore, we hypothesized that pharmacological correction of ductal function should reduce pancreatic damage and acinar necrosis/apoptosis in AP. For this reason, the combination of CFTR corrector, VX-661 and potentiator, VX-770 was used. Pre-treatment of mice with VX-661+VX-770 significantly decreased the pancreatic tissue damage during AP; however, other inflammatory parameters were similar to the AP group. Interestingly, the expression and localization of CFTR protein was not changed by the VX-661+VX-770 treatment. Since the expression of CFTR was unchanged after VX-661+VX-770 treatments, we suppose that the residual and functional CFTR proteins in pancreatic ducts were activated, and this could lead to the observed decrease in acinar damage. As localization of CFTR protein was unchanged,

we think that the beneficial effect in AP was mainly related to the use of CFTR potentiator (VX-770). We could demonstrate that CFTR correction and potentiation by VX molecules increases fluid secretion in pancreatic ducts isolated from AP mice. We hypothesize that ductal secretion defends the pancreas by washing out toxic agents like activated digestive enzymes. If this defence mechanism is insufficient, the harmful agents cannot be eliminated from the pancreas, and this can result in tissue damage.

VI.1.2. The effect of fentanyl on the severity of acute pancreatitis

Opioids are used to relieve the pain in AP, but the literature is divided on whether administration of these drugs is beneficial or detrimental. Opioids exert their effects on mu, kappa, or delta opioid receptors, but their affinity or specificity are different to them. In an earlier work described that FE is 80 times more potent than MO and is a highly selective full mu opioid receptor agonist ligand. Our published study is a multi-objective comparative work, but my thesis focused on the results about FE in the NaTc-AP model in rats.

FE pre-treatment in the NaTc model greatly exacerbated the condition of the animals, therefore after humanely terminating the experiments this type of treatment was discontinued. FE pre-treatment in L-ornithine-induced AP increased the severity of the disease but the animals survived, whereas in Cer-AP model, the drug had no effect (other parts of our work are published in Bálint et al., 2022). The post-treatment of FE in necrotizing models of AP (NaTc and L-ornithine) ameliorated the disease. In NaTc-AP, the higher dose (0.2 mg/kg) of FE, whereas in the L-ornithine model even the lower dose (0.1 mg/kg) of FE significantly reduced the severity of AP. This leads to the conclusion that in mild form of the disease, FE treatment does not worsen the outcome, but in severe necrotizing pancreatitis, the timing its administration is important.

Experimental and clinical studies also reported the beneficial effects of FE. Others have tested the effect of intravenous administration of FE on NaTc-induced AP. They injected FE 23–23.5 h after AP induction and sacrificed the animals 24 h after the induction of the disease. Surprisingly, FE reduced pancreatic inflammation and AP-induced myocardial damage within that really short time (30–60 min). However, the potential problems related to opioid use include their inhibitory effects on intestinal motility with resultant anorexia, constipation, nausea and vomiting, also habituation and abuse. A recent experimental trial demonstrated that administration of MO, exacerbated the severity of AP and increased the risk for bacterial translocation. MO also delayed macrophage migration and caused a persistence of inflammation.

VI.2. Meta-analysis of the effect of serum triglyceride concentration on the outcome of acute pancreatitis

HTG (>1.7 mM) on admission worsens the course of AP compared to the normal seTG group. However, increase in seTG up to 5.6 mM did not significantly influence the severity of AP compared to normal seTG. Selecting subgroups within HTG (>5.6; >11.3 mM) resulted in a significant elevation of ORs (2.01 and 3.08, respectively) for severity when all the groups were compared with the effect of normal seTG. Our findings are in line with the results of earlier animal studies, in which hyperlipidaemia increased the severity of AP.

Interestingly, the mortality of patients did not show statistically significant differences between >1.7; 1.7-11.3; 11.3 mM groups compared to the normal, which is likely to be the result of the small number of patients with this outcome. The odds for complications (systemic inflammatory response syndrome, POF, involving pulmonary, renal, and circulatory failure) were significantly increased in groups with HTG. Although mortality is related to disease complications, the results from the statistical analysis for mortality were not in line with the outcomes for AP (e.g. severity, POF, necrosis). Therefore, further investigation would be beneficial for the relation of HTG and AP with respect to mortality.

Although there is no unanimous definition for HTG-AP, it is widely accepted that AP with seTG >11.3 mM is HTG-related. However, some researchers consider HTG-AP to be defined by a seTG threshold >5.6 mM. Therefore, this encouraged us to investigate the relationships between the extent of HTG and the outcome of AP. SeTG >5.6 mM significantly worsened the outcomes for AP when compared with the seTG <5.6 mM group, while there was no difference when seTG >5.6 mM was compared with seTG in the1.7–5.6 mM range. Similar results were seen at a cut-off seTG of 11.3 mM. SeTG >11.3 mM caused more severe AP than seTG <11.3 mM, but when the effect of seTG >11.3 mM was compared with that of seTG in the 1.7–11.3 mM range, no significant difference was seen between the two groups. These comparisons also support our previous assumption that compared to normal seTG, HTG is associated with an increased risk for severe AP and complications. Further studies would be important to clarify the relationship between the extent of HTG and the severity of AP.

Notably, seTG changes dynamically, which relates to food intake and fasting. Current treatment protocols for most AP patients include fasting at the beginning of hospitalization, except for suspected severe AP cases where early enteral feeding (within 48 hours) is recommended. Fasting results in a rapid (within 48 h) drop of seTG and measuring seTG 48 or 72 h after the admission might underrepresent levels at the onset of AP. Others demonstrated a dramatic decrease in seTG during fasting: seTG falls from approx. 30 mM to 5 mM within three days. To take this bias into account, we scored the articles based on the timing of the seTG measurement. Having high scores for NOS and seTG measurement timing represents good quality for the selection of articles for this meta-analysis.

VI.3. Conclusions

We demonstrated that AP markedly affects the expression and function of the CFTR channel in pancreatic ducts. Correction and stimulation of the CFTR channel was shown to be beneficial, as the VX drug combination significantly improved pancreatic cell damage and ductal bicarbonate secretion rates in pre-treatment of AP mice compared to control groups. We also showed that FE treatment could effectively decrease the severity of necrotizing AP, but the timing of drug administration is important. Our meta-analysis of clinical studies confirmed that HTG worsens the severity of AP and increases the odds of complications. Overall, it seems that numerous factors have roles in the development and aggravation of AP. Therefore, an appropriate combination of treatments can be the answer to curing the disease.

VII. SUMMARY OF NEW FINDINGS

- Experimental AP causes mislocalization of CFTR protein, while it increases *Cftr* mRNA expression.
- The CFTR corrector and potentiator, VX-661 and VX-770, significantly reduces the extent of pancreatic tissue damage possibly via increased fluid secretion, but the CFTR protein expression was unchanged.
- NaTc-AP combined with FE post-treatment reduced, while pre-treatment exacerbated the severity of the disease. Therefore, the timing is crucial in the case of FE administration.
- Elevated seTG concentrations significantly increase the severity of AP in human patients, with a higher likelihood of death, organ damage and hospitalisation. This aetiological factor of AP deserves particular attention.

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"The researcher knows what frustration is, knows how many months of working in the wrong direction, and knows the failures. But failures are also useful, because if you analyse them correctly, they can lead to success." — Sir Alexander Fleming