# CFTR DEFICIENCY CAUSES PMCA4 DYSFUNCTION, INTRACELLULAR $CA^{2+}$ ELEVATION AND PROMOTES EPITHELIAL CELL DAMAGE IN ALCOHOLIC PANCREATITIS AND HEPATITIS

Ph.D. Thesis

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## LIST OF PUBLICATIONS:

### Pulications related to this thesis:

I. **Tamara Madacsy**, Árpád Varga, Noémi Papp, Bálint Tél, Petra Pallagi, Viktória Szabó, Aletta Kiss, Júlia Fanczal, Zoltan Rakonczay Jr, László Tiszlavicz, Zsolt Rázga, Meike Hohwieler, Alexander Kleger, Mike Gray, Péter Hegyi, József Maléth; Impaired Regulation of PMCA Activity by Defective CFTR Expression Promotes Epithelial Cell Damage in Alcoholic Pancreatitis and Hepatitis; CELLULAR AND MOLECULAR LIFE SCIENCES (2022) D1 **IF: 9.261**

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II. Balla, Z; Kormanyos, ES; Kui, B; Balint, ER; Für, G; Orján, ME; Ivanyi, B; Vecsei, L; Fulop, F; Varga, G, **Madacsy, T,** et al. Kynurenic acid and its analogue SZR-72 ameliorate the severity of experimental acute necrotizing pancreatitis FRONTIERS IN IMMUNOLOGY: Q1 DOI: 10.3389/fimmu.2021.702764 **IF: 7,561**

III. Breunig, Markus; Merkle, Jessica; Wagner, Martin; Melzer, Michael K; Barth, Thomas F E; Engleitner, Thomas; Krumm, Johannes; Wiedenmann, Sandra; Cohrs, Christian M; Perkhofer, Lukas, **Madacsy, Tamara** et al. Modeling plasticity and dysplasia of pancreatic ductal organoids derived from human pluripotent stem cells. CELL STEM CELL (2021) D1 DOI: 10.1016/j.stem.2021.03.005 **IF: 24,633**

IV. Déri, Szilvia; Borbás, János; Hartai, Teodóra; Hategan, Lidia; Csányi, Beáta; Visnyovszki, Ádám; **Madácsy, Tamara**; Maléth, József; Hegedűs, Zoltán; Nagy, István et al. Impaired cytoplasmic domain interactions cause co-assembly defect and loss of function in the p.Glu293Lys KNCJ2 variant isolated from an Andersen-Tawil Syndrome patient CARDIOVASCULAR RESEARCH (2021: D1 DOI: 10.1093/cvr/cvaa249 **IF: 10,787**

V. Kata Judit, Szántó; **Tamara, Madácsy**; Diána, Kata; Tamás, Ferenci; Mariann, Rutka; Anita, Bálint; Renáta, Bor; Anna, Fábián; Ágnes, Milassin; Boldizsár, Jójárt et al. Advances in the optimisation of therapeutic drug monitoring using serum, tissue and faecal anti-tumour necrosis factor concentration in patients with inflammatory bowel disease treated with TNF-α antagonists EXPERT OPINION ON BIOLOGICAL THERAPY (2021) Q1 Folyóirat szakterülete: Scopus - Drug Discovery SJR Q1 DOI: 10.1080/14712598.2021.1890712 **IF: 4,388**

VI. Bálint, Anita; Farkas, Klaudia; Méhi, Orsolya; Kintses, Bálint; Vásárhelyi, Bálint Márk; Ari, Eszter; Pál, Csaba; **Madácsy, Tamara**; Maléth, József; Szántó, Kata Judit et al. Functional Anatomical Changes in Ulcerative Colitis Patients Determine Their Gut Microbiota Composition and Consequently the Possible Treatment Outcome PHARMACEUTICALS (2020) D1 DOI: 10.3390/ph13110346 **IF: 5,863**

VII. Fanczal, Júlia; Pallagi, Petra *; Görög, Marietta; Diszházi, Gyula; Almássy, János; **Madácsy, Tamara**; Varga, Árpád; Csernay-Biró, Péter; Katona, Xénia; Tóth, Emese et al. TRPM2-mediated extracellular Ca2+ entry promotes acinar cell necrosis in biliary acute pancreatitis JOURNAL OF PHYSIOLOGY-LONDON (2020) D1 DOI: 10.1113/JP279047 **IF: 5,182**

VIII. Molnár, Réka; **Madácsy, Tamara**; Varga, Árpád; Németh, Margit; Katona, Xénia; Görög, Marietta; Molnár, Brigitta; Fanczal, Júlia; Rakonczay, Zoltán; Hegyi, Péter et al. Mouse pancreatic ductal organoid culture as a relevant model to study exocrine pancreatic ion secretion LABORATORY INVESTIGATION (2020) Q1 DOI: 10.1038/s41374-019-0300-3 **IF: 5,662**

IX. Pallagi, Petra; **Madácsy, Tamara***; Varga, Árpád; Maléth, József Intracellular Ca2+ Signalling in the Pathogenesis of Acute Pancreatitis: Recent Advances and Translational Perspectives INTERNATIONAL JOURNAL OF MOLECULAR SCIENCES (2020) D1 DOI: 10.3390/ijms21114005 **IF: 5,924**

X. 11. Bakondi, Edina; Singh, Salam Bhopen *; Hajnády, Zoltán; Nagy-Pénzes, Máté; Regdon, Zsolt; Kovács, Katalin; Hegedűs, Csaba; **Madácsy, Tamara**; Maléth, József; Hegyi, Péter et al. Spilanthol Inhibits Inflammatory Transcription Factors and iNOS Expression in Macrophages and Exerts Anti-inflammatory Effects in Dermatitis and Pancreatitis. INTERNATIONAL JOURNAL OF MOLECULAR SCIENCES (2019) D1 DOI: 10.3390/ijms20174308 **IF: 4,556**

XI. **Madacsy, Tamara***; Pallagi, Petra *; Maleth, Jozsef Cystic Fibrosis of the Pancreas: The Role of CFTR Channel in the Regulation of Intracellular Ca2+ Signaling and Mitochondrial Function in the Exocrine Pancreas FRONTIERS IN PHYSIOLOGY (2018): Q2 DOI: 10.3389/fphys.2018.01585 **IF: 3,201**

XII. Szentesi, A; Toth, E; Balint, E; Fanczal, J; **Madacsy, T**; Laczko, D; Ignath, I; Balazs, A; Pallagi, P et al. Analysis of Research Activity in Gastroenterology: Pancreatitis Is in Real Danger PLOS ONE (2016) Q1 DOI: 10.1371/journal.pone.0165244 **IF: 2,806**

XIII. **Madácsy, T;** Maléth, J New findings in the pathogenesis of acute pancreatitis. CENTRAL EUROPEAN JOURNAL OF GASTROENTEROLOGY AND HEPATOLOGY / GASZTROENTEROLÓGIAI ÉS HEPATOLÓGIAI (2015)

**XIV.** Maleth, J *; **Madacsy, T** *; Pallagi, P; Balazs, A; Venglovecz, V; Rakonczay, Z Jr; Hegyi, P Pancreatic epithelial fluid and bicarbonate secretion is significantly elevated in the absence of peripheral serotonin. GUT (2015) D1 DOI: 10.1136/gutjnl-2015-309776 **IF: 14,921**

XV. Bereczki, Zsolt; **Madácsy, Tamara**; Király, Kitty; Sóskuti, Kornél; Paja, László Szarmata sebészi trepanációk a Kárpát-medencében **ANTHROPOLOGIAI KÖZLEMÉNYEK** 61pp.25-32.,8p.(2020)DOI: 10.20330/AnthropKozl.2020.61.25

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## INTRODUCTION

Annually 3 million deaths result from excessive alcohol consumption worldwide representing 5.3% of all deaths, whereas in the age group 20–39 years approximately 13.5 % of the total deaths are attributed to alcohol. Among alcohol-related disorders the diseases of the liver and pancreas emerge due to their therapeutic challenges and socioeconomic burden. Alcohol induced acute pancreatitis (AP) is one of the most frequent form of AP associated with high mortality in severe cases. Our group evidenced previously that ethanol and fatty acid-mediated reductions of cystic fibrosis transmembrane conductance regulator (CFTR) expression and activity in pancreatic ductal cells cause decreased $HCO_3^-$ secretion and increased severity of alcohol-induced AP. We also showed that ethanol and fatty acids impair CFTR folding and reduce the plasma membrane stability of the protein. Whereas alcoholic hepatitis (AH) is a potentially lethal complication of alcoholic liver disease, which has been attributed to hepatocellular damage in the past. Recent studies showed that cholestatic liver injury can be involved in the pathogenesis of AH, moreover, impaired secretion by cholangiocytes, or cholestasis, results in a worse outcome. Takeuchi et al. highlighted that integrin beta-1 (ITGB1)-mediated binding of neutrophils to cholangiocytes in AH results in the development of cholestasis. It is well-established that cholangiocyte secretion largely depends on the proper function of the apically expressed CFTR. Therefore, considering that the alcohol-mediated effect on CFTR expression has been observed in other organs, such as the sweat glands, it is tempting to speculate that impaired CFTR expression might contribute to AH-related cholestasis.

In general, lack of ion conductance and impaired fluid secretion, ultimately leading to morphological changes and atrophy of the organ, is considered central to the pathogenesis of CFTR-related disorders. However, a more complex, integrative role of CFTR was recently suggested in the regulation of intracellular signaling events, which are potentially involved in the development of CFTR-related cell- and tissue damage. For example, cystic fibrosis (CF)-associated increased $IP_3R$-dependent $Ca^{2+}$ release, elevated activity of the sarco/endoplasmic reticulum $Ca^{2+}$-ATPase (SERCA) pump, and enhanced mitochondrial $Ca^{2+}$ uptake paralleled with decreased $Ca^{2+}$ ATPase (PMCA) function was described in cultured airway epithelial cells. Notably, alterations of intracellular $Ca^{2+}$ signaling –such as sustained intracellular $Ca^{2+}$ overload mediated by impaired SERCA- and PMCA activity– in pancreatic acinar and ductal cells is a well-established hallmark of AP. Recently, Bozoky *et al.* showed that calmodulin interacts with CFTR in the alternative binding conformation through independent binding of

the two calmodulin lobes to two separate sequences allowing it to bridge larger distances. The authors suggested that calmodulin binding by CFTR may allow CFTR to recruit calmodulin and subsequently determine the activity of other calmodulin-regulated proteins, such as proteins involved in the regulation of the intracellular $Ca^{2+}$ homeostasis. However, if and how these subcellular changes impact gastrointestinal tract epithelial cell function in alcoholic pancreatitis and hepatitis, is currently unknown.

Thus, we aimed to investigate the effect of ethanol on CFTR activity and expression in cholangiocytes and analyse the impact of ethanol mediated CFTR damage on intracellular $Ca^{2+}$ homeostasis in pancreatic ductal cells and cholangiocytes in multiple independent model systems.

## MATERIALS AND METHODS

### Ethics.

Animals were used with adherence to the NIH guidelines and the EU directive 2010/63/EU. The study was approved by the National Scientific Ethical Committee on Animal Experimentation under license number XXI. /2523/2018. The collection and use of human samples including cadaver donor pancreas and liver samples were executed in adherence with the EU standards and approved by the Regional Committee of Research Ethics of the Hungarian Medical Research Council under license number 37/2017-SZTE.

### Cell Lines and Animals

HeLa cells were cultured according to the manufacturer's protocol. CFPAC-1 cells were a generous gift of Michael Gray. *Cftr* KO mice were originally generated by Ratcliff *et al*.

### Isolation of Pancreatic Ductal Fragments and Acinar Cells

Pancreatic ductal fragments were isolated under stereomicroscope. For pancreatic acinar cell isolation, the tissue was injected with type 4 collagenase and vigorously shaken followed by centrifugation after which the pellet was resuspended in Media 199.

### Mouse and Human Organoid Cultures

Mouse pancreatic ductal and liver organoids were generated according to the protocol of Clevers and Tuveson. Human pancreatic tissue samples were collected from transplantation donors.

### Generation of Human CF-specific Induced Pluripotent Stem Cells

Reprogramming of keratinocytes isolated from plucked hair of a CF patient or a healthy donor was performed to generate CF-specific and control induced pluripotent stem cells. The CF patient harbored the compound heterozygous mutations p.F508del and p.L1258Ffs*7.

**Constructs and Transfection**

HeLa cells were transfected with plasmids coding EGFP-hPMCA4b, mCherry-CFTR-3xHA, and mCherry-CFTR-3xHA(S768A) using Lipofectamine2000. Site-directed mutagenesis using Q5 Site-Directed Mutagenesis Kit was carried out according to the manufacturer's protocol. For *Cftr* silencing ducts were transfected with 50 nM siCFTR or siGLO-Green transfection indicator in Opti-MEM.

**Fluorescent Microscopy**

$[Ca^{2+}]_I$, intracellular pH and $Cl^-$ level were measured with Fura-2-AM, BCECF-AM, or MQAE, respectively, as described earlier. Changes in mitochondrial membrane potential ($\Delta\Psi_m$) were followed with tetramethylrhodamine-methyl ester (TMRM). Ducts, acini, or organoids were mounted on an Olympus IX71 fluorescent microscope equipped with an MT-20 illumination system.

**Gene Expression Analysis**

Total RNA was isolated from whole pancreatic tissue, isolated ductal fragments, and pancreatic or liver organoids. Relative gene expression analysis was performed by ΔΔCq technique. RNA sequencing was carried out by an Illumina NextSeq 500 instrument on mouse and human pancreatic ductal organoids. The pattern of gene expression was determined by TPM (transcript/million) values.

**Immunofluorescent- and Immunohistochemical Labelling and Duolink® Proximity Ligation Assay**

Isolated pancreatic ducts or organoids were frozen, sectioned, and labelled for immunofluorescent microscopy. Cell lines were grown on cover glass and fixed without sectioning. Immunohistochemical labelling of CFTR was carried out with a Leica Bond-MAX Fully Automated IHC and ISH Staining System. Duolink® assay was performed on a humidified chamber after antigen retrieval according to the manufacturer's protocol. Images were captured with a Zeiss LSM880 confocal microscope using a 40X oil immersion objective (Zeiss, NA: 1.4).

**Direct Stochastic Optical Reconstruction Microscopy (dSTORM)**

HeLa cells grown on cover glass were co-transfected with different plasmids. dSTORM images were captured by Nanoimager S (Oxford Nanoimaging ONI Ltd.).

**Transmission Electron Microscopy**

Pancreatic tissue was fixed in glutaraldehyde and dextran, embedded into Embed812, and ultrathin sections were cut with a Leica ultramicrotome. Images were captured with a Jeol 1400 plus electron microscope at 12000X magnification.

**FAEE-induced Acute Pancreatitis in mice**

FAEE-induced AP was evoked in wild type (WT) FVB/N mice. The animals received two intraperitoneal injections of ethanol (1.35 g/bwkg) and palmitoleic acid (POA;150 mg/bwkg) or equivalent volume vehicle (25% DMSO, 75% sterile water). Treated groups received one intraperitoneal dose of aurintricharboxylic acid (ATA) (5mg/bwkg) in vehicle (25% DMSO, 75% sterile water) 90 min before the first injection of ethanol/POA or vehicle. The severity of pancreatitis was assessed via analysis of histology sections, and the measurement of serum amylase activity.

**Statistical Analysis**

Statistical analysis was performed with Graphpad Prism software. All data are expressed as means ± SEM as well as individual data points. For parametric tests unpaired T-test, for nonparametric tests Mann-Whitney test, Kruskal-Wallis test and Dunn's multiple comparison were used based on the normality of data distribution. P value below 0.05 was considered statistically significant.

## RESULTS

### Absence of CFTR impairs the function of plasma membrane $Ca^{2+}$ pump in pancreatic ductal epithelial cells

Our hypothesis was that the decreased CFTR expression caused by chronic ethanol per se is sufficient to disturb the $Ca^{2+}$ homeostasis of the gastrointestinal epithelial cells. Previously, we established that acute exposure to ethanol releases $Ca^{2+}$ from the ER and activates extracellular $Ca^{2+}$ influx in pancreatic ductal cells. To assess whether the decreased CFTR expression disturbs the intracellular $Ca^{2+}$ homeostasis wild-type (WT) and *Cftr* KO mice pancreatic ducts were challenged with carbachol. The maximal $Ca^{2+}$ release was not different between the two groups, the slope of the $Ca^{2+}$ signal plateau phase –representing the $Ca^{2+}$ extrusion from the cytosol– was significantly higher in *Cftr* KO ductal fragments compared to WT. Next, we utilized mouse pancreatic organoids (MPO) generated from WT and *Cftr* KO mice. WT organoids were treated with 100 mM ethanol (EtOH) and 200 μM palmitic acid (PA) for 12 h, control and *Cftr* KO MPOs received no treatment. Store operated $Ca^{2+}$ influx was activated by re-addition of the extracellular $Ca^{2+}$ after ER depletion (25 μM cyclopiazonic-acid

(CPA) in $Ca^{2+}$-free media). The basal intracellular $Ca^{2+}$ concentrations were significantly higher in the EtOH-treated and *Cftr* KO organoids. As expected, the ER $Ca^{2+}$ release in response to CPA was lower in the EtOH treated organoids and was not changed in *Cftr* KO organoids, whereas both EtOH treated and *Cftr* KO organoids showed a significantly decreased $Ca^{2+}$ extrusion after removal of the extracellular $Ca^{2+}$. The same phenomenon was observed in *Cftr* KO ducts. Next human pancreatic organoids (HPO) were treated with 100 mM EtOH and 200 µM PA overnight. Importantly, compared to untreated HPOs, $Ca^{2+}$ extrusion was significantly decreased after pre-incubation with EtOH/PA. Next, to confirm that the observed difference in $Ca^{2+}$ extrusion was specific to CFTR-expressing cells, we analysed $Ca^{2+}$ signaling in pancreatic acinar cells, which lack CFTR in general and did not detect difference in the carbachol response (maximal intracellular $Ca^{2+}$ release or extrusion) between WT and *Cftr* KO mice acini . Moreover, functional inhibition of CFTR with 10 µM CFTR(inh)-172 –which significantly impaired CFTR activity– had no effect on the carbachol-induced $Ca^{2+}$ extrusion in WT ductal cells. Correction of CFTR expression in CFPAC-1 cells–derived from liver metastasis of a CF patient's pancreatic ductal adenocarcinoma–restored $Ca^{2+}$ extrusion. In contrast, knockdown of CFTR expression in WT ductal fragments with siCFTR impaired $Ca^{2+}$ extrusion compared to control. Considering that both PMCA and $Na^{+}/Ca^{2+}$ exchangers (NCX) can forward $Ca^{2+}$ extrusion in non-excitable cells, we used the pan-NCX inhibitors SEA0400 and CB-DMB to assess the contribution of NCX to the process. None of these inhibitors had any effect on the slope of the decrease**.** Recently, Partner of STIM1 (POST) –an adaptor protein linking STIM1 to other proteins– was shown to enhance the function of PMCA4. However, siSTIM1 treatment had no effect on the $Ca^{2+}$ efflux in WT pancreatic ducts, suggesting that Stim1-POST is not involved in the regulation of PMCA in epithelial cells. Taken together, these results indicate that attenuation of CFTR expression -rather than the lack of activity- by ethanol treatment is sufficient to alter $Ca^{2+}$ homeostasis through limiting PMCA activity.

**Ethanol has no effect on the PMCA4 expression in pancreatic ductal cells**

Currently, four mammalian PMCA genes have been identified which contribute to cytosolic $Ca^{2+}$ extrusion. Using whole transcriptome analysis we revealed the expression of *Pmca1* and *Pmca4* in MPO and *PMCA1* and *PMCA4* in HPO samples, with highest levels of *Pmca1* in mouse and highest levels of *PMCA4* in humans. Of note, expression levels of *Pmca2* and *Pmca3* were below detection limit in all samples. RT-PCR followed by endpoint analysis confirmed the expression of *Pmca1* and *Pmca4* in whole pancreatic tissue as well as isolated mouse pancreatic ducts. Immunofluoresent staining of PMCA1 and PMCA4 in cross sections

of MPOs revealed the apical localization of PMCA4, whereas PMCA1 was evenly distributed over the apical and basolateral membranes. In addition, a strong co-localization of PMCA4 and CFTR at the apical membrane was observed (Mander's correlation coefficient:0.906, therefore in the downstream analysis we focused on PMCA4. In intestinal stem cells, loss of CFTR expression results in alkaline $pH_i$ deriving Wnt/β-catenin-mediated expression of different genes, which may affect the expression of PMCA4 in pancreatic ductal cells. To test this, the relative expression of *Pmca4* was compared with qRT-PCR in control, EtOH/PA-treated and *Cftr* KO MPOs. While, control and EtOH/PA-treated WT MPO showed no significant alteration, *Pmca4* expression was moderately increased in *Cftr* KO ductal organoids compared to WT control suggesting that the difference of $Ca^{2+}$ efflux is not due to reduced gene expression . Next, we wondered whether loss of CFTR due to EtOH treatment would alter the apical membrane-specific localisation of PMCA4. Whereas immunofluorescent microscopy revealed diminished CFTR levels at the apical membrane in EtOH/PA-treated and *Cftr* KO MPOs compared to untreated WT, PMCA4 retained its apical localisation in all samples. Subsequently, the presence of CFTR and PMCA4 on the apical plasma membrane of HPOs was confirmed by immunolabelling. Whereas overnight incubation of HPO with EtOH/PA resulted in a diminished, patchy apical expression pattern of CFTR, PMCA4 retained its apical membrane localisation. Notably, alcohol treatment resulted in a detectable cytosolic shift of PMCA4. These results suggest that the lack of CFTR at the apical membrane of pancreatic ductal cells diminishes the activity but not the expression or localization of PMCA4.

**iPSC-derived organoids from cystic fibrosis patients recapitulate the alteration of PMCA function**

Our results suggest that the diminished CFTR expression caused by genetic mutations in CF may also disturb $Ca^{2+}$ extrusion of pancreatic ductal cells. Therefore, we assessed the relevance of our findings in human iPSC-derived pancreatic organoids generated from CF patients. To establish CF-iPSC lines from donors affected by classical CF, lentiviral reprogramming of patient keratinocytes was used and performed stepwise *in vitro* differentiation to direct the iPSCs towards the pancreatic lineage followed by generation of exocrine pancreatic organoids in 3D-suspension culture. First, immunofluorescent analysis revealed that, while CFTR levels were absent in CF patient-derived organoids, which was markedly restored by 12 h incubation with the CFTR-corrector VX-809 (10 μM), PMCA4 expression was present in control- and CF patient-derived iPSC organoids. Then, $Ca^{2+}$ removal after ER $Ca^{2+}$ store depletion resulted in a significantly decreased $Ca^{2+}$ extrusion in CF

organoids compared to control, further recapitulating our previous observation obtained in other model systems. Importantly, pre-treatment with 10 μM VX-809 for 12 h significantly improved $Ca^{2+}$ extrusion indicating that CFTR corrector treatment can restore decreased PMCA activity and thus the $Ca^{2+}$ extrusion in CF organoids.

**Ethanol reduces CFTR expression and PMCA activity in cholangiocytes**

Although the cholangiocyte secretory function greatly depends on CFTR activity, alcohol-related changes in CFTR function or expression were never analysed in alcoholic hepatitis (AH). Immunohistochemistry on formalin-fixed paraffin-embedded liver samples revealed that the apical CFTR distribution in cholangiocytes was significantly impaired in patients with AH compared to controls. Next, we recapitulated this phenomenon *in vitro* in WT mouse-derived liver organoids (MLO) positive for the epithelial cell lineage marker KRT19. CFTR showed a luminal membrane localisation in untreated MLOs, which was significantly decreased and shifted towards the cytosol in EtOH-treated MLOs without biologically relevant changes in *Cftr* gene expression levels. Subsequent functional analysis of MLOs revealed a significantly impaired apical $Cl^-/HCO_3^-$ exchange activity in EtOH/PA-treated MLOs compared to control. Also, whereas extracellular $Cl^-$ removal resulted in CFTR-dependent increase in MQAE fluorescence –used as a marker of intracellular $Cl^-$– in control MLOs, alcohol treatment resulted in a significant decrease of CFTR-dependent $Cl^-$ extrusion. Finally, $Ca^{2+}$ measurements revealed significantly decreased PMCA activity in ethanol pre-incubated- as well as *Cftr* KO organoids compared to WT control, suggesting that decreased apical distribution of CFTR impairs PMCA function in cholangiocytes. Changes of *Pmca4* gene expression didn't achieve a biologically relevant level in MLOs**.** Importantly, these results highlight that EtOH exposure alters CFTR localization and activity in cholangiocytes leading to decreased ion secretion and disturbed intracellular $Ca^{2+}$ homeostasis.

**PMCA4 interacts with CFTR at the apical membrane of pancreatic ductal epithelial cells**

Our observations suggesting that proper PMCA4 activity requires a close connection with CFTR. Therefore, we performed Duolink proximity ligation assay (PLA) between endogenous PMCA and CFTR. Of note, to avoid non-specific antibody binding, guinea pig pancreatic ductal fragments were used, which recapitulated the colocalization of PMCA4 and CFTR. Duolink PLA suggested that PMCA4 and CFTR are in a proximity of <40 nm. Then, we used dSTORM to visualize this interaction at even higher resolution. In HeLa cells co-

transfected with plasmids encoding CFTR and PMCA4, we observed a perfect overlap (<20 nm) between the two proteins in the plasma membrane suggesting physical proximity.

**Calmodulin binding by CFTR regulates PMCA4 activity in pancreatic ductal cells and in cholangiocytes**

Next, we wanted to provide mechanistic insight into the regulation of PMCA4 activity by CFTR. The recently described alternative calmodulin binding of CFTR has been suggested to allow the regulation of other proteins. Thus, we hypothesized that such type of calmodulin-CFTR interaction might subsequently influence the activity of the calmodulin-regulated PMCA4. First, we evidenced strong co-localization of calmodulin with CFTR and PMCA4 at the apical membrane of ductal epithelial cells with dSTORM on cross-sections of MPOs. Next, whereas calmodulin strongly associated with the apical membrane in WT MPOs and MLOs, it dissociated from the apical membrane and diffused throughout the cytosol –as suggested by the line intensity profiles– in EtOH-treated or *Cftr* KO MPOs and MLOs. A similar localisation pattern was observed in *Cftr* KO ductal fragments. Then, we wanted to analyse the effect of impaired calmodulin-CFTR interaction on PMCA4 activity in epithelial cells. As general knockdown or inhibition of calmodulin can have multiple downstream effects, we co-transfected HEK-293 cells with PMCA4 and CFTR or CFTR harboring a mutation in the calmodulin binding site (CFTR(S768A)). Of note, both CFTR and CFTR(S768A) localised to the plasma membrane and co-localized with PMCA4. While co-transfection of PMCA4 and CFTR markedly increased the slope of $Ca^{2+}$ extrusion, PMCA4 alone showed moderate activity. However, more importantly, cells transfected with CFTR(S768A) showed a significantly impaired PMCA4 activity compared to cells transfected with CFTR. Moreover, dSTORM cluster analysis revealed a 34% reduction of the co-localization ratio between PMCA4-CFTR(S768A) compared to PMCA4-CFTR suggesting that the lack of calmodulin/CFTR interaction is sufficient to decrease PMCA4 activity as well as the stability of the protein nanodomain on the apical plasma membrane.

**Inhibition of PMCA4 impairs mitochondrial function, increases apoptosis, and results in more severe ethanol-induced acute pancreatitis**

Sustained intracellular $Ca^{2+}$ elevation is known to impair mitochondrial function and trigger apoptosis. In the next step we wanted to assess the role of impaired CFTR expression in this phenomenon. Transmission electron microscopy showed no difference in the mitochondrial volume/cell ratio between *Cftr* KO and WT pancreatic ductal cells. Next, administration of 100

μM carbachol resulted in a significant decrease in mitochondrial membrane potential ($\Delta\psi_m$) in EtOH/PA pre-treated and *Cftr* KO–but not in WT– MPOs, suggesting that a sustained intracellular $Ca^{2+}$ elevation impairs mitochondrial function. To function properly, the ATPase PMCA4 relies on ATP generated by oxidative phosphorylation and glycolysis. As EtOH decreases the mitochondrial ATP production, we inhibited the F1F0-ATPase by oligomycin, which had no effects on the PMCA function in ductal cells. However, the intracellular distribution of cytochrome c released from the mitochondria –a hallmark of apoptosis– significantly increased in *Cftr* KO compared to WT pancreatic ductal cells, suggesting that sustained $Ca^{2+}$ elevation and disturbed mitochondrial function leads to apoptosis. Additionally, *Cftr* KO pancreatic ductal cells had higher cytoplasmic levels of the initiator caspase 9 compared to WT pancreatic ductal cells, further confirming the increased rate of apoptos. Finally, by using the PMCA4 inhibitor aurintricarboxylic acid (ATA) in an alcohol-induced pancreatitis mouse model, we aimed to analyse if impaired PMCA4 function could independently enhance the severity of pancreatic and liver diseases. Incubation of pancreatic ductal organoids with 10 μM ATA for 30 min before *in vitro* $Ca^{2+}$ measurements resulted in significantly decreased PMCA4 activity compared to controls, confirming the inhibitory effect of ATA on PMCA4 function. Next, a single injection of ATA (intraperitoneally, 5 mg/kg) was administered to WT FVB/N mice 90 min before the first EtOH/POA injection. Compared to vehicle control, ATA pre-treated animals had significantly elevated pancreatic oedema and necrosis scores paralleled with significantly elevated serum amylase activities. Taken together, these results indicate that impaired PMCA4 activity diminish mitochondrial function, augments apoptosis, and potentially increases the severity of CFTR-related pancreatic- and presumably liver diseases.

## DISCUSSION

CFTR is generally considered as a cAMP-activated $Cl^-$ channel, although it may be regulated by $[Ca^{2+}]_i$, or by possible interactions with other components involved in $Ca^{2+}$ homeostasis. However, the downstream effects of these interactions on the subcellular signaling events or on the activity of CFTR is not well understood. In our study, we first demonstrated that $Ca^{2+}$ extrusion is significantly impaired in ethanol treated and *Cftr* KO pancreatic ductal cells due to diminished PMCA activity. Using several independent *in vitro* model systems, including HPOs and iPSC-derived organoids generated from CF patients, we revealed that the lack of apical CFTR –rather than its function– affects PMCA activity. The fact that diminished CFTR

expression did not influence PMCA4 expression or cellular distribution implied a functional interaction between the two proteins. Importantly, correction of CFTR expression with VX-809 in this model seemed to restore the intracellular $Ca^{2+}$ signaling alterations. Besides increased intracellular $Ca^{2+}$ release and impaired $Ca^{2+}$ clearance, increased activity of extracellular $Ca^{2+}$ influx is described in CF. On the other hand, sustained intracellular $Ca^{2+}$ overload is a hallmark of AP. The most frequent pathogenic factors –including bile acids or non-oxidative EtOH metabolites– trigger the release of ER $Ca^{2+}$ stores and activate extracellular $Ca^{2+}$ influx leading to impaired fluid and $HCO_3^-$ secretion. The subsequent sustained intracellular $Ca^{2+}$ overload triggers mitochondrial damage with consequent ATP depletion and cell damage, further impairing ATP-dependent $Ca^{2+}$ extrusion.

AH is a potentially lethal complication of alcoholic liver disease, which has been attributed to hepatocellular damage in the past. Recently, binding of neutrophils to ITGB1 expressed on the cell surface of cholangiocytes was shown to contribute to epithelial cell damage and the development of cholestasis in AH. Other studies showed that cholestatic liver injury can be involved in the pathogenesis of AH, moreover, impaired secretion by cholangiocytes, or cholestasis, results in a worse outcome. This previously unrecognised contribution of cholangiocyte damage in AH altered our understanding of the disease pathogenesis and offers novel therapeutic strategies. Proper cholangiocyte secretion largely depends on efficient functioning of CFTR located at the apical membrane of these cells. As EtOH and its metabolites damage the expression and function of CFTR in pancreatic ductal epithelial cells, it seems plausible that alcohol induced CFTR damage may contribute to the development of cholangiocyte dysfunction and cholestasis in AH. In this study, we demonstrated significantly decreased apical plasma membrane CFTR expression accompanied with a significantly impaired CFTR activity in post-mortem AH patient liver samples and apical diminished $Cl^-/HCO_3^-$ exchange upon alcohol exposure in mouse liver organoids. In addition, both EtOH pre-incubated WT and *Cftr* KO liver organoids displayed impaired $Ca^{2+}$ extrusion due to decreased PMCA activity. The lack of CFTR-mediated secretion as well as altered intracellular $Ca^{2+}$ homeostasis can damage the cholangiocyte secretion contributing to the development of AH-related cholestasis and potentially CF-related liver disease (CFLD) and severely worsen the clinical outcome.

In our experiments, we demonstrated that PMCA4 co-localizes with CFTR at the apical membrane of ductal cells in multiple models. Moreover, using super-resolution dSTORM, we highlighted that CFTR and PMCA4 are within 20 nm distance suggesting a physical interaction of the two proteins. Previously, the co-immunoprecipitation experiments of Philippe *et al.*

suggested that CFTR interacts with SERCA and PMCA in airway epithelial cells, although the nature of this interaction was not revealed. Recently, Bozoky *et al.* demonstrated increased open probability of CFTR due to direct binding of calmodulin to its R domain, which provided a novel mechanism for the regulation of intracellular $Ca^{2+}$-mediated CFTR activity. This binding conformation may allow CFTR to recruit calmodulin and subsequently determine the activity of other calmodulin-regulated proteins such as PMCA isoforms in a macromolecular complex at the apical plasma membrane. Binding of $Ca^{2+}$-calmodulin with the calmodulin binding domain of PMCA competitively antagonizes the autoinhibitory domain leading to PMCA activation. In untreated WT pancreatic- and liver organoids, calmodulin was associated with the apical membrane and strongly co-localized with CFTR and PMCA4. In *Cftr* KO- and ethanol-treated organoids, this apical localization was lost suggesting that the presence of CFTR at the apical membrane is necessary to recruit calmodulin. To verify that interaction of calmodulin with CFTR is required for PMCA activation, we overexpressed a calmodulin-binding site mutant of CFTR (CFTR(S768A)) and PMCA4 in HEK-293 cells and showed significantly impaired $Ca^{2+}$ extrusion, whereas the interaction between CFTR and PMCA4 also remarkably decreased. During the pathogenesis of alcohol-induced AP, it is well-established that acute exposure of acinar and ductal cells to ethanol or ethanol metabolites induce sustained elevation of $[Ca^{2+}]_i$. In our experiments, instead of acute administration, we incubated the ductal cells overnight, which may model the effects ethanol consumption better. Although we found no evidence for mitochondrial morphological damage but detected a remarkable drop of $\Delta\Psi_m$ when challenging the ethanol treated and *Cftr* KO MPOs with carbachol. In addition, increased cytosolic staining for cytochrome c and caspase 9 in *Cftr* KO ductal cells suggested increased apoptosis. Finally, PMCA4 inhibition in an *in vivo* model of alcoholic AP significantly increased disease severity suggesting that impaired PMCA4 function due to impaired CFTR expression by itself can contribute to cell damage in alcoholic pancreatitis and hepatitis. Importantly, our results highlight that restoration of PMCA activity or enhancement of the $Ca^{2+}$ extrusion can have potential therapeutic benefit not only in alcoholic pancreatitis and hepatitis, but also in cystic fibrosis related liver disease and pancreatitis. We demonstrated that restoration of CFTR expression with correctors can also improve the function of PMCA. Effectivity of triple combination therapy for CF patients was shown recently and persistent improvement of pancreatic function was reported in CF patients receiving ivacaftor. Taken together, in this study we identified a novel regulatory interaction based on the apical recruitment of calmodulin by CFTR determining the activity of PMCA4 and intracellular $Ca^{2+}$ extrusion in polarized epithelial cells.

**NOVEL OBSERVATIONS:**

- We have provided evidence that impaired CFTR expression –induced by ethanol exposure or genetic mutations and subsequently decreased CFTR-mediated recruitment of calmodulin to the apical membrane attenuates PMCA4 activity and $Ca^{2+}$ extrusion in pancreatic ductal cells and cholangiocytes.
- The consequent disturbed $Ca^{2+}$ homeostasis leads to damaged mitochondrial function and enhanced apoptosis and ultimately results in increased disease severity. Thus, our results shed light on a novel regulatory mechanism of intracellular $Ca^{2+}$ signaling that might contribute to severity of alcoholic pancreatitis- and hepatitis and potentially to the development of CF-related liver and pancreatic damage.

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