EXPLORING THE ASSOCIATION BETWEEN INFLAMMATORY BOWEL DISEASES AND ACUTE PANCREATITIS

Ph.D. thesis - short version

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1. Introduction

Inflammatory bowel diseases (IBDs) are chronic, progressive inflammatory diseases of the gastrointestinal tract that impair patients' quality of life and have no cure. Although the main symptoms of both two main subtypes – Crohn's disease (CD) and ulcerative colitis (UC) – are connected to the gastrointestinal tract, IBD is a multi-system disease that can affect many organs of the body creating so-called extraintestinal manifestations, including pancreatic processes. The first suggestion of an association between pancreatic involvement and IBD has been raising awareness among clinicians since the 1950s and, in the last decades, multiples studies showed that besides the harmless asymptomatic serum enzyme level elevations, *acute pancreatitis* (AP) is the most commonly seen pancreatic pathology in IBD. AP is an often serious, sterile inflammation of the pancreatic tissue characterized by the premature activation of pancreatic enzymes inside the pancreas. Although AP is associated with a possibly high mortality rate, its etiology and epidemiology in the subpopulation of IBD patients are not settled.

In the general population, biliary obstruction and excessive ethanol consumption are the most common etiologies of pancreatitis, however, these "classical" etiologies seem not to be the primary cause of AP in IBD. In the treatment of IBD patients, several pancreato-toxic drugs are used regularly, for example, *thiopurines* are often used as long-term maintenance therapy both in UC and CD. Interestingly, *thiopurine-induced AP* (TIP) is reported almost exclusively in IBD patients, and a recent meta-analysis confirmed that the mainly used thiopurine, *azathioprine* (AZA) elevates the risk of AP in CD patients.

The central mechanisms in the general development of AP mainly involve acinar cellular events, such as pathologic calcium (Ca^{2+}) signaling and premature intracellular/intraductal activation of pancreatic enzymes. However, many factors can trigger this central dysregulation leading to the initiation of AP, including disturbances in the secretory functions of *pancreatic ductal epithelial cells* (PDECs). Importantly, multiple substances (e.g. ethanol) were shown to inhibit PDEC fluid secretion leading to consequent pancreatic injury through the inhibition of *Cystic Fibrosis Transmembrane Conductance Regulator* (CFTR) luminal expression and function.

Although mostly genetic and immunologic mechanisms are mentioned in the pathogenesis of TIP, the exact mechanism remains unknown and some early studies on dogs also suggested that AZA treatment could directly disturb *pancreatic fluid secretion*. The regulation of CFTR involves a protein complex that anchors CFTR to the plasma membrane, involving the adaptor protein *ezrin*. Previously AZA was also shown to inhibit *Ras-related C3 botulinum toxin substrate 1* (RAC1) in leukocytes, a known regulator of ezrin. Therefore, one can hypothesize that AZA might influence pancreatic ductal secretory and CFTR functions by inhibiting RAC1.

As a better understanding of the pathomechanism of AP-associated IBD could make the basis for preventing this disease, I focused on both settling the epidemiology of AP and IBD, and on exploring the effects of thiopurines on pancreatic secretory functions.

2. Objectives

During the research projects of my doctoral thesis, I aimed to provide firm evidence regarding the association between IBD and AP with an extensive literature search and a comprehensive meta-analysis. Also, I aimed to explore the pathomechanism of AZA-induced pancreatitis and carried out experiments in mice by combining oral thiopurine treatment and the common cerulein-induced pancreatitis model and examined the cellular effects of thiopurines on the murine pancreatic epithelial cells. The principal questions I aimed to answer are as follows:

2.1. The risk of acute pancreatitis in inflammatory bowel diseases

- Based on large-scale observational studies, what are the pooled odds of AP in IBD in general, as well as in Crohn's disease and ulcerative colitis, respectively?
- Based on large-scale observational studies, what is the pooled annual incidence of AP in IBD?

2.2. The effects of thiopurines on pancreatic functions and experimentally induced pancreatitis outcomes in mice

- How does azathioprine treatment influence the histologic parameters of cerulein-induced experimental acute pancreatitis in mice?
- What are the direct effects of azathioprine on mouse pancreatic acinar cells?
- What are the direct and indirect effects of thiopurines on mouse pancreatic ductal $HCO3–$ secretion?
- Does azathioprine affect pancreatic ductal CFTR functions and distribution?
- What molecular mechanism lies behind the pancreatic ductal effects of azathioprine in mice?

3. Methods

3.1. The risk of acute pancreatitis in inflammatory bowel diseases

To comprehensively describe the association between AP and IBD, a systematic literature search in four major electronic databases (MEDLINE via PubMed, Embase, Web of Science, and Scopus) was conducted, followed by a screening by title and abstract, full-text screening, data extraction, and meta-analysis. Two different clinical questions were evaluated in two different analyses, one to appraise the odds of AP in IBD and the other to determine the annual incidence of AP in IBD.

Besides bibliographic data, crude incidences of AP cases and the observation period of the study in PYs were extracted. Odds ratios were calculated from the crude incidences, and pooled odds ratios (ORs) or event rates with 95% confidence intervals (CIs) were calculated. The random-effects model with the DerSimonian-Laird estimation was applied. The I^2, and Chi-square tests were used to quantify statistical heterogeneity and gain probability values.

3.2. The effects of thiopurines on pancreatic functions and experimentally induced pancreatitis outcomes in mice

For the experiments, 8-12 weeks-old C57BL6 mice were used with a gender ratio of 1:1 for all groups.

To test both *therapeutic and toxic concentrations of thiopurines*, we chose to use the 1 and 10 μg/mL concentrations *ex vivo*. For *in vivo* treatment, animals received a daily dose (150 μL) of either thiopurine (1.5 or 15 mg/kg/day AZA, 6-MP, or 6-TG) or sterile physiologic saline as sham control, for one or four weeks, respectively

Moderate *experimental pancreatitis* was induced with 8 × 50 μg/bwkg IP cerulein injections administered hourly. Sham control animals received IP physiologic saline injections. Mice were sacrificed one or four hours after the last injection. The severity of pancreatitis was determined by evaluating histological and laboratory parameters.

The stimulated in vivo *pancreatic juice secretion* was measured in mice after making a narrow laparotomy, exposing the duodenum and the head of the

pancreas, and cannulation of the main pancreatic duct following published protocols.

Mouse pancreatic *acinar cells* or *ductal segments* were isolated by mechanical (mincing - acini, microdissection – ducts) and enzymatic (collagenase) digestion following published protocols. To prevent autodigestion of the cells, 0.25 mg/ml trypsin inhibitor was added to the culture media.

Mouse *pancreatic ductal organoids* were generated after mincing of the mouse pancreas, enzymatic digestion, and washing steps, cells were resuspended in Matrigel Basement Membrane Matrix and plated in domes and kept in culture by changing media every other day. Organoids, digested into single cells with TrypLE Express, were used for experiments between passage numbers 2 and 5.

For *fluorescence microscopy* measurements isolated pancreatic acini or ductal segments were transferred to the perfusion chamber, incubated with fluorescent dyes, then chambers were mounted on an inverted microscope. To test the effect of thiopurines and inhibitors we changed the perfusion from control solutions to thiopurine and/or inhibitor-containing buffered solutions for 10 minutes before and during performing the stimulations described below.

Intracellular *Ca^{2+} signals* were measured using the Ca^{2+}-sensitive dye FURA-2 and stimulation with 100 nM of the acetylcholine agonist, carbachol.

To calculate *HCO_3^--secretion* of PDEC, changes in the intracellular pH were monitored with the pH-sensitive dye, BCECF-AM, and the application of alkaline and acid loading by adding and removing 20 mM NH_4Cl, respectively. *Base flux values*, representing transmembrane HCO_3^- transport, were determined from the slopes of recovery observed after the alkaline and acid loading, respectively.

To estimate the *Cl^- secretory* activity of PDEC, the Cl^--sensitive fluorescent dye MQAE was used, and tested by the removal of extracellular Cl^-. Besides AZA treatment, PDEC were pre-treated either with a RAC1 inhibitor Ehop-016 or an ezrin inhibitor NSC668394.

Immunofluorescent labeling on sectioned ductal segments was performed after permeabilization in citrate/Triton-X 100 with primary monoclonal, rabbit anti-CFTR, and secondary goat anti-rabbit Alexa Fluor 488 antibodies. Nuclear staining and mounting were carried out simultaneously by ProLong™ Gold Antifade mounting medium with DAPI and images were captured with a Zeiss LSM880 confocal microscope.

Direct Stochastic Optical Reconstruction Microscopy (dSTORM) was performed on mouse pancreatic ductal organoids, followed by incubation for 60 minutes with 1 μg/mL AZA-containing media. After antigen retrieval with 0.01% Triton-X-100, CFTR and Ezrin primary antibodies (1:100) were applied during overnight incubation and fluorophore-conjugated secondary antibodies were applied. Cover glasses were placed in a specific buffer and dSTORM images were captured by Nanoimager S. The cluster analysis of dSTORM images was evaluated by CODI software.

RAC1 Activity was measured on mouse pancreatic organoids with a commercially available RAC1 G-LISA kit following protocol. Cells were incubated for 60 minutes with control media or with media containing either 1 μg/mL AZA, 10 μM Ehop-016, or both.

Cell viability was measured with CellTitre-Glo 3D assay and the Abcam Apoptosis-Necrosis Kit for confocal microscopy. Freshly isolated acinar cells were incubated for 60 minutes with either AZA-containing media, negative control media, or Cerulein (100-1000 nM) containing media as the positive control. The luminometric assay was performed on a CLARIOStar plate reader. The living, apoptotic, and necrotic cells were visualized under a Zeiss LSM880 confocal microscope. The number of cells was calculated in FIJI (NIH, USA) using the built-in Cell Counter plugin.

All experimental data were expressed as means ± SD. One-way ANOVA followed by Sidak's post hoc test was used for multiple group comparisons. The Chi-square test was used for the comparison of frequencies. T-test was used for pairwise comparisons. Two-way ANOVA with Tukey's post hoc test was used for comparisons of multiple groups split by independent variables. $P < .05$ was accepted as being significant

4. Results

4.1. The risk of acute pancreatitis in inflammatory bowel diseases

Following the removal of duplicates, the literature search yielded a total of 3627 articles. After screening by title and abstract, 143 full-text articles were evaluated. Finally, a total of eight articles were included for qualitative analysis, six in the first and four in the second analysis, respectively

4.1.1. The odds of AP is elevated in patients with IBD

The six articles, that reported appropriate data on the *odds of AP in IBD*, included data altogether from 1,309,278 people. Pooling of the data yielded an OR of 3.11 (95% CI, 2.93–3.30; $I^2 = 0.0\%$). A subgroup analysis in four of the above-mentioned six studies was eligible, to analyze the odds of AP broken down for *CD and UC subpopulations* individually. This analysis found that the pooled OR of AP in CD (OR 4.12; 95% CI, 3.75–4.54; $I^2 = 0.0\%$) to be significantly higher than in UC patients (OR, 2.61; 95% CI, 2.40–2.83; $I^2 = 0.0\%$; $P < .0001$). The I^2 statistics showed no significant heterogeneity, making this observation robust.

4.1.2. The pooled annual incidence of AP in IBD is 0.21%

A total of four studies were eligible to include in the second analysis, covering a sum of 268,859 PYs observation time. The meta-analysis resulted in a *pooled incidence rate* of 0.21% (95% CI, 0.084%–0.392%). As the I^2 statistics detected a significant heterogeneity ($I^2 = 98.66$ %, $P < .001$), the certainty of this analysis is moderate.

In summary, with a comprehensive literature search, eight studies were included in two analyses describing the risk of AP in patients with IBD, and these confirmed that the *odds of AP is three times higher in the IBD population* compared to the non-IBD population, and it is even is *significantly higher in CD than in UC*. This work was the first meta-analysis to confirm the clinical experience that the risk of AP is higher in patients with IBD. Although initially aimed at, summarizing the underlying etiologies of IBD-associated AP cases failed due to the lack of corresponding data in the included studies. However, it was clear from the evaluated studies that a large part of the AP cases observed in IBD patients is related to medical treatment, especially thiopurine medications.

4.2. The effects of thiopurines on pancreatic functions and experimentally induced pancreatitis outcomes in mice

4.2.1. AZA treatment makes the murine pancreas more sensitive to cerulein-induced injury

First, to test the effects of thiopurines on pancreatic secretory functions, I aimed to set up an *in vivo* mouse model of TIP. To mimic the clinical situation, mice received a daily dose of 1.5 mg/kg AZA orally for 1 week, while the control (sham) group received sterile physiologic saline (PS) doses. As AP during AZA treatment occurs quite rarely, it was not surprising that *no spontaneous pancreas injury or pancreatitis was observed* in the AZA-treated mice during 1 week of AZA treatment.

Then, to test whether AZA treatment would modify the histologic parameters of the cerulein-induced pancreatitis model, moderate pancreatitis was induced in both AZA-treated and sham animals by 8 × intraperitoneal cerulein injections, while controls received PS. First, to examine the early phase[1] of cerulein pancreatitis, mice were terminated 1 hour after the last cerulein injection. Interestingly, the extent of necrosis was found to be significantly higher in the AZA + cerulein-treated mice, while there was no difference in interstitial edema, leukocyte infiltration, or serum amylase activity. This suggests that AZA makes the murine pancreas susceptible to secretagogue hyperstimulation-induced pancreas necrosis, ie. *increases the pancreatic damage in the early phase* of experimental pancreatitis. Next, to evaluate the effects of AZA on the later, immune cell-related phases of pancreatitis, mice were sacrificed 4 hours after the last cerulein injection but surprisingly, although the extent of necrosis increased in both cerulein-only and AZA+cerulein-co-treated groups, the *previously observed significant*

[1] In rodents, the repeated IP administration of the secretagogue cerulein induces diffuse pancreatic necrosis with a subsequent biphasic elevation in the serum amylase levels. The first peak in serum amylase is seen approximately one hour after the last cerulein injection and is considered to be the consequence of the direct secretagogue and/or toxic effect of cerulein, while the second peak, 4 hours after the last injection, is thought to be the result of immune cell activation.

difference in the extent of necrosis disappeared and neither the histological scores nor the average serum amylase activity differed significantly.

Taken together the clinical suspicion was confirmed, that *AZA treatment* would increase the sensitivity of the exocrine pancreas to harmful stimuli in the early phases of pancreatic injury, therefore *making the pancreas more susceptible to developing AP but not to developing more severe disease*.

4.2.2. AZA does not alter the viability, nor the Ca^{2+} homeostasis of pancreatic acinar cells in mice

To investigate the effects of AZA on acinar viability and functions, freshly isolated primary mouse acinar cells were incubated *ex vivo* for 60 minutes with clinically relevant different concentrations of AZA, but *none of the applied AZA concentrations* (1-1000 μg/mL) *were found to cause any significant change* in the proportion of the living acinar cells (CellTitreGlo 3D assay) compared to the positive control cerulein. Also, when assessing the cell death of acinar cells with confocal microscopy (Abcam Apoptosis Necrosis kit), neither *ex vivo* treatment (60 minutes, 1 μg/mL AZA), nor the previous *in vivo* AZA treatment (1 week, 1.5 mg/kg/die) would alter the proportions of viable, apoptotic, or necrotic/late apoptotic acinar cells significantly.

As the central pathophysiology of AP involves altered acinar Ca^{2+} signaling, it was important to test, whether AZA would influence the Ca^{2+} homeostasis in pancreatic acinar cells. To do so, 100 nM carbachol-induced intracellular Ca^{2+} changes were monitored with a FURA2-AM, during the *ex vivo* perfusion of acinar cells with AZA-containing media of 10 minutes. Importantly, *no significant changes were to be observed* either in the baseline Ca^{2+} levels or in the response to 100 mM carbachol stimulation upon AZA treatment. Similarly, 1 week-long *in vivo* AZA treatment did not change the intracellular Ca^{2+} homeostasis of acinar cells.

Next, cells were incubated for 60 minutes *in vitro* with 1 μg/mL AZA or 100 nM cerulein. In this series of experiments, neither the *in vitro* incubation for 60 minutes, nor a previous 1-week-long *in vivo* AZA treatment was proven sufficient to increase the proportions of released amylase. And while the *in vitro* co-stimulation of acinar cells with AZA and cerulein caused a moderate, but significantly higher amylase release than cerulein stimulation alone, the

cerulein stimulation of acini from *in vivo* AZA-pretreated mice did not recapitulate this effect.

Altogether, these results confirm that *pathologic Ca^{2+} signals play no major role in the adverse effects of AZA,* which sits well with our observation that AZA does not alter acinar cell viability, nor the overall severity of cerulein-induced pancreatitis.

4.2.3. Thiopurines impair pancreatic ductal HCO_3^- secretion both *ex vivo* and *in vivo* in mice

Early studies on cannulated dog pancreas suggested that AZA treatment could directly influence *pancreatic fluid secretion.* To see whether AZA would indeed affect pancreatic ducal functions, freshly isolated mouse pancreatic ductal segments were *ex vivo* perfused with 1 or 10 μg/mL AZA-containing solutions for 10 minutes, and base-flux values (ie. HCO_3^- transport) were calculated by monitoring the intracellular pH (pH_i) changes during the so-called alkaline and acid loading.

Interestingly, upon *ex vivo* stimulation (both with 1 and 10 μg/mL AZA) a significant decrease in the base flux values was to be observed after both acid and alkaline loading. This indicates that *AZA impairs both the luminal HCO_3^- transport* (e.g. CFTR) *and the activity of the basolateral transporters* of pH_i regulation. As neither of the base flux values showed significantly greater inhibition upon perfusion with higher AZA concentrations, only the – clinically more relevant – 1 μg/mL concentration was used in the further experiments.

Furthermore, when conducting the above-described (*ex vivo*) experiments on ductal segments isolated from animals previously treated *in vivo* with oral doses of AZA, the one-week *oral AZA treatment also significantly impaired the base-flux values*, regardless of whether therapeutic (1.5 mg/kg/die) or toxic 15 mg/kg) doses were applied. Also, 4 weeks of AZA treatment had the same effects, but the longer treatment time did not cause greater inhibition of HCO_3^- secretion.

Importantly, when tested, the base flux values were *also significantly reduced by the two other clinically applied thiopurines*, 6-mercaptopurine (6-MP) and 6-thioguanine (6-TG) compared to controls, both upon *ex vivo* and *in vivo*

exposure. This confirms that the inhibitory effect of thiopurines is not dependent on the specific conformational differences between the drugs, but it is rather a common property of all thiopurines.

To further validate these observations, the total *in vivo* pancreatic juice secretion rate of anesthetized mice was measured by the cannulation of the main pancreatic duct, after a previous *in vivo* treatment with either 1.5 mg/kg/die AZA or 6-MP for 1 week, respectively. In harmony with the previous findings, the *in vivo* pancreatic secretion rates were significantly reduced in both AZA and 6-MP treated mice compared to sham controls, supporting that thiopurine's inhibitory effects on PDEC also have an impact *in vivo*.

4.2.4. AZA inhibits CFTR-mediated Cl^- secretion in murine PDEC

Multiple substances were demonstrated to inhibit the pancreatic ductal HCO_3^- secretion via sustained intracellular Ca^{2+} elevation. To test whether this mechanism plays a role in the observed inhibitory effects of AZA, the intracellular Ca^{2+} homeostasis of pancreatic ductal cells was assessed in the presence of AZA. In harmony with the observations with acinar cells, no significant changes were to be observed neither in the baseline $[Ca^{2+}]_i$ levels during the 10-minute perfusion with 1 μg/mL AZA nor in the maximal response to 100 nM carbachol stimulation after the AZA perfusion, compared to controls. This suggests that AZA doesn't have a major influence on ductal Ca^{2+} homeostasis.

After confirming that AZA impairs pancreatic ductal HCO_3^- secretion, without altering the Ca^{2+} homeostasis, I wanted to test, whether these effects of AZA are dependent on CFTR functions. To test this, first, the effects of AZA treatment on the cellular localization of CFTR were examined using immunofluorescent staining. Interestingly, AZA treatment indeed altered the predominantly apical distribution and resulted in a diffuse intracellular distribution of CFTR, both upon 60 minutes of *ex vivo* incubation with 1 μg/mL of AZA, as well as after 1 week *in vivo* oral treatment with 1.5 mg/kg AZA. This suggests that *the plasma membrane retention of CFTR might be disturbed*, which can be the cause of the altered HCO_3^- secretion upon AZA exposure.

Next, as the luminal HCO_3^- secretion depends not only on CFTR activity, we wanted to see, whether the altered apical CFTR localization also manifests in an altered CFTR function. As the Cl^- secretion of the ductal epithelia is solely CFTR-dependent, this was performed by measuring Cl^- transport, both in the absence and presence of the CFTR inhibitor CFTR(inh)-172 to increase specificity. In harmony with the previous results, pancreatic ductal Cl^- transport was significantly impaired upon both *ex vivo* (10 minutes, 1 μg/mL) and *in vivo* (1 week, 1.5 mg/kg/die) AZA exposure compared to untreated controls, which was similar to the inhibition caused by the selective CFTR inhibitor, however, the effect of the *ex vivo* AZA perfusion was significantly greater than the *in vivo* treatment. These results revealed that AZA treatment diminishes the apical CFTR expression and significantly impairs the function of CFTR in pancreatic ductal cells both *ex vivo* and *in vivo*.

4.2.5. AZA disrupts CFTR plasma membrane retention in PDEC by inhibiting rac1 and ezrin in mice

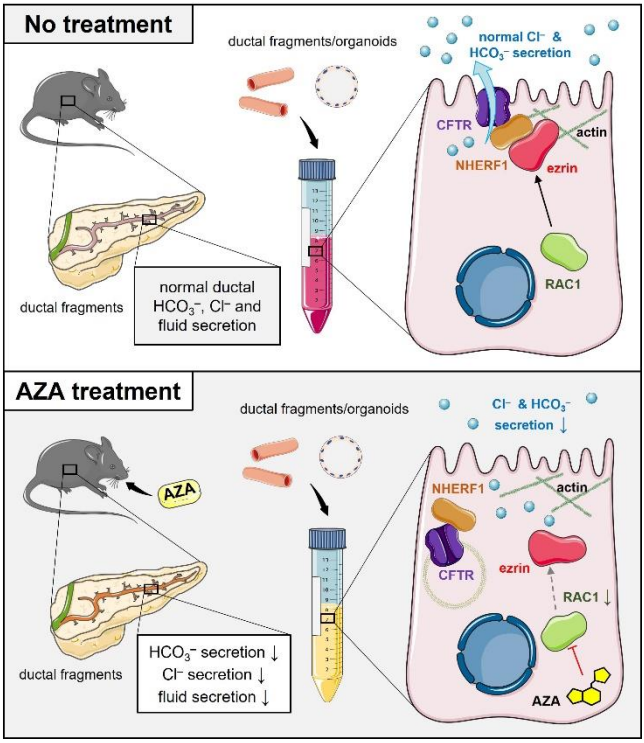
CFTR is anchored to the plasma membrane by a scaffolding protein complex that includes the cytoskeleton adaptor protein, *ezrin*. Also, previously AZA was suggested to inhibit *RAC1*, a small GTPase protein that is an activator of ezrin. To confirm the role of RAC1 and ezrin in the AZA-induced CFTR dysfunction, first RAC1 (Ehop-016) and ezrin (NSC668394) inhibitors were administered *ex vivo* for 10 minutes to isolated ductal segments, and the Cl^- transport was measured. Interestingly, both ezrin and RAC1 inhibitors recapitulated the inhibitory effect of AZA on Cl^- secretion, confirming that AZA might act through the inhibition of ezrin and RAC1.

To further validate this hypothesis, RAC1 activity was measured in mouse pancreatic ductal organoids and not surprisingly, the amount of active RAC1 significantly decreased after AZA treatment in a G-LISA assay, while the RAC1 inhibitor Ehop-016 was not able to further decrease the activity of RAC1 in the presence of AZA.

Finally, after confirming, that AZA could act through the inhibition of RAC1, Direct Stochastic Optical Reconstruction Microscopy (dSTORM) was utilized to quantify the colocalization of CFTR and ezrin in adherent epithelial cells derived from mouse pancreatic ductal organoids. And indeed, the performed cluster analysis of ezrin-CFTR clusters revealed, that the *in vitro* incubation with 1 μg/mL AZA remarkably decreased the colocalizing

probability of ezrin and CFTR and confirmed that the impaired localization and function of CFTR in AZA-treated ductal cells is caused by the inhibition of RAC1-ezrin-CFTR axis.

In summary, oral AZA treatment significantly increased pancreatic necrosis in the early phase of the cerulein-induced pancreatitis model, but not in the later phase. This was explained by the exclusive inhibitory effect of thiopurines, including AZA on the secretory activity of pancreatic ductal segments both upon ex vivo and in vivo exposure while not affecting acinar cells. This AZA-mediated inhibition of secretory functions, especially Cl^- secretion was a result of inhibited RAC1 activity, which disturbed the ezrin-CFTR interaction, leading to altered CFTR localization on the apical plasma membrane of the pancreatic ductal cells. These observations are the first to provide mechanistic insight into thiopurine-induced acute pancreatitis, in a clinically relevant setting that might open new directions for research aiming at the prevention of TIP.



5. Conclusions

I summarize the main results of my studies in the following points:

1. AP is found to be more frequent in IBD than in the non-IBD population. My meta-analysis yielded a pooled OR of 3.11 based on homogenous results, making this observation highly certain. A clinically more relevant, highly certain finding is that the odds of AP in CD are significantly higher (OR 4.12) than in UC (OR 2.61).
2. Based on my meta-analysis (of moderate certainty), approximately 210 AP cases per 100.000 IBD patients are anticipated annually.
3. In cerulein-induced murine pancreatitis, one week of oral treatment with azathioprine causes increased pancreatic damage in the early, but not in the late phases of pancreatitis. This suggests an increased susceptibility to developing AP but not to having a more severe disease.
4. Azathioprine is found not to alter pancreatic acinar viability, cell death, or Ca^{2+}-dependent functions in mice, neither upon short-term direct stimulation nor after one week of *in vivo* treatment.
5. Murine pancreatic ductal HCO_3^- secretion is found to be inhibited by short-term *ex vivo* perfusion, as well as by one week of in vivo treatment with the three thiopurines (azathioprine, 6-mercaptopurine, and 6-thioguanine), respectively, while the Ca^{2+} homeostasis remains unaffected.
6. Azathioprine also inhibits mouse pancreatic ductal CFTR functions indicated by the inhibited Cl^- secretion, both upon *ex vivo* and *in vivo* stimulations, which is a result of disturbed plasma membrane localization showed on immunofluorescent labeling.
7. The disturbed CFTR functions in mouse pancreatic ductal segments are recapitulated by both ezrin and RAC1 inhibitors, while azathioprine can also inhibit RAC1 activity and leads to a significantly decreased colocalization probability of ezrin with CFTR. These are suggestive of an impaired RAC1-ezrin-CFTR interaction leading to the disturbed ductal functions triggered by azathioprine exposure.

6. Bibliography of the candidate's publications

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