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EXPLORING THE ASSOCIATION BETWEEN INFLAMMATORY BOWEL DISEASES AND ACUTE PANCREATITIS

Ph.D. thesis

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List of abbreviations

5-ASA	- 5-aminosalicylic-acid; mesalamine
6-MP	- 6-mercaptopurine
6-TG	- 6-thioguanine
6-TGN	- 6-thioguanine-nucleotide
AP	- acute pancreatitis
AZA	- azathioprine
BCECF-AM	- 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester
$[Ca^{2+}]_i$	- cytosolic calcium levels
CCK	- cholecystokinin
CD	- Crohn's disease
CFTR	- cystic fibrosis transmembrane conductance regulator
DIAP	- drug-induced acute pancreatitis
DNA	- deoxyribonucleic acid
EIM	- extraintestinal manifestation
EPS	- 4,6-ethylidene(G1)-4-nitrophenyl(G7)-a-(1-4)-D-maltoheptaoside
ER	- endoplasmic reticulum
FURA2-AM	- Fura-2-acetoxymethyl ester
GI	- gastrointestinal
HA	- hyperamylasaemia
HGPRT	- hypoxanthine-guanosine phosphoribosyltransferase
HL	- hyperlipasaemia
HR	- hazard ratio
IBD	- inflammatory bowel disease
IP	- intraperitoneal
MQAE	- N-(Ethoxycarbonylmethyl)-6-Methoxyquinolinium Bromide
NHE	- Na^+/H^+ exchanger
NHERF1	- Na^+/H^+ exchanger regulatory factor (SLC9A3R1)
NBC	- Na^+-HCO_3^- co-transporter
OR	- odds ratio

PDEC - pancreatic ductal epithelial cells
PICO - patients, interventions, comparison, and outcomes
PM - plasma membrane
PY - person-year
QUIPS - Quality In Prognosis Studies
RAC1 - Ras-related C3 botulinum toxin substrate
RoB - risk of bias
RNA - ribonucleic acid
SLC26 - solute carrier family 26
TIP - thiopurine-induced pancreatitis
TPMT - thiopurine S-methyl transferase
UC - ulcerative colitis

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1. Introduction

Inflammatory bowel diseases (IBDs) are chronic, progressive inflammatory diseases of the alimentary tract that impair patients' quality of life and lack a disease-specific cure (Agrawal et al. 2021). The incidence of both two main subtypes, Crohn's disease (CD) and ulcerative colitis (UC) is growing in all age groups, all around the Globe (Gbd 2017 Inflammatory Bowel Disease Collaborators 2020; Mak et al. 2020; Sykora et al. 2018). Although the main symptoms of IBD are connected to the gastrointestinal (GI) tract, IBD is a multi-system disease that can affect many organs of the body creating so-called extraintestinal manifestations (EIMs) that can occur even before the diagnosis of IBD (Greuter and Vavricka 2019; Harbord et al. 2016; Rogler et al. 2021). EIMs have varying frequencies and most commonly they appear as dermatologic, musculoskeletal, oral, ocular, cardiovascular, neurologic, hepatobiliary, or *pancreatic lesions* (Greuter and Vavricka 2019). These can have a substantial impact on IBD patients' quality of life, sometimes even a bigger one than the intestinal disease itself (Rogler et al. 2021). IBDs, with their growing incidence, progressive behavior, lack of a cure, and impaired quality of life create an ever-growing healthcare burden. The latest treatment guidelines for IBD emphasize "360-degree IBD care", which includes preventive strategies and personalized treatment (Agrawal et al. 2021), however, this requires a deep understanding of all aspects of IBD, from epidemiology to the pathomechanism of EIMs.

Recently, pancreatic abnormalities observed in IBD patients have attracted more and more attention from clinicians. Ball et al. were the first to suggest an association between pancreatic involvement and IBD based on autopsy studies of UC patients in 1950 (Ball et al. 1950). Based on the clinical experience gathered in the following decades, asymptomatic serum amylase and/or lipase level elevations are the most common pancreatic alterations associated with IBD (Iida et al. 2019). In the absence of any clinical symptoms, hyperamylasaemia (HA) is reported in 5-14%, while hyperlipasaemia (HL) in 7-14% of all IBD patients (Bokemeyer 2002; Heikius et al. 1999; Katz et al. 1988). The corresponding prevalences in patients with CD are somewhat higher (HA 8-17%, HL 9-11%) than in patients with UC (HA 1-11%, HL 7-11%) (Bokemeyer 2002; Heikius et al. 1999; Katz et al. 1988). This data, however, comes from studies conducted in the 1990s-2000s, and in a more recent cohort of 649 pediatric IBD patients from Italy, the reported

prevalence of HA and/or HL without clinical symptoms was only 2.4% (Martinelli et al. 2015). Nevertheless, even if these abnormalities are relatively common, they are usually harmless (Massironi et al. 2022).

The suggested etiologies of pancreatic hyperenzymaemia include altered pancreatic circulation, colonization by amylase-producing bacteria, or increased amylase absorption of the inflamed gut, supporting its clinical harmlessness, but secondary causes such as renal impairment, familiar pancreatic hyperenzymaemia, or macroamylasemia should be ruled out before confirming the diagnosis of asymptomatic pancreatic hyperenzymaemia (Iida et al. 2019). Another possible cause of elevated pancreatic enzyme levels in IBD is the presence of pancreatic autoantibodies (PAbs), which can be observed in up to 39% of CD and 4% of UC patients, and is in close connection with the primary immune dysregulation leading to the development of IBD (Massironi et al. 2022; Stocker et al. 1987). In contrast to asymptomatic enzyme elevations, which showed no correlation with disease activity or other clinical factors, PAbs correlate with disease location (presence of ileitis), stricturing behavior, and early-onset IBD (Massironi et al. 2022). Also, in a Hungarian cohort, CD patients with PAbs had a higher probability to have resective surgery or developing perianal disease (Papp et al. 2015). Although they could be used to predict disease outcomes, their presence is not an IBD-specific phenomenon, since PAbs have been also reported in other autoimmune diseases, and they cannot predict the development of more serious pancreatic complications such as autoimmune pancreatitis or *acute pancreatitis* (AP) (Massironi et al. 2022).

AP is a potentially serious, sterile inflammation of the pancreatic tissue characterized by the activation of pancreatic enzymes inside the pancreas, and is associated with a possibly high mortality rate (Miko et al. 2019; Parniczky et al. 2016). The diagnosis of AP resides on three pillars: typical acute abdominal pain, pancreatic enzyme elevations more than 3-times above the upper limit, and imaging findings consistent with AP (Lee P. J. and Papachristou 2019). The diagnosis is made when at least two of these criteria are met. In IBD, however, abdominal pain is a frequent part of the clinical picture and – as we discussed above – enzyme elevations without pancreatitis can co-occur, therefore, diagnosing AP in IBD can set a great challenge for clinicians (Massironi et al. 2022). In the last decades, an emerging number of case reports and clinical studies suggested that acute pancreatitis (AP) was more frequent in IBD, however, at the time of the initiation

of our research no firm evidence was available confirming this association. Therefore, we aimed to explore the *association between AP and IBD* – with a special focus on *thiopurine-induced pancreatitis* – to increase our understanding of this important EIM.

1.1. Acute pancreatitis and inflammatory bowel diseases

The most common etiologies of AP in the general population are biliary obstruction and ethanol abuse (Dimagno and Dimagno 2007; Gullo et al. 2002). Although these non-IBD-specific etiologies can be the cause of AP in IBD patients as well, two main IBD-specific categories of AP have been also suggested. The first comprises the consequences of the management of IBD (eg. thiopurine-induced-AP) and IBD-associated diseases (eg. biliary stones) as the primary cause of AP, while the other is suggested to be closely related to the pathogenesis of IBD (often termed as idiopathic pancreatitis) and is, therefore, considered a true EIM of IBD (Harbord et al. 2016). This, however, requires the often not executable exclusion of all other possible causes, resulting that the exact distribution of etiologies in IBD-associated AP not being fully explored.

One explanation of the frequently observed AP cases in IBD is that IBD would increase the risk of the classic etiologies of AP. In the general population, cholelithiasis leading to biliary obstruction is the most common cause of AP, contributing to up to 65% of all cases (Roberts et al. 2017; Zilio et al. 2019), and multiple pieces of evidence suggest that the risk of biliary stone development is increased in IBD (Massironi et al. 2022). In a meta-analysis, the risk of gallstone disease was increased by a 2.05 odds ratio (OR) in CD than in the general population, while UC patients did not have an elevated risk for cholelithiasis (Zhang et al. 2015). Also, a recent nationwide study from Taiwan reported an adjusted hazard ratio (HR) of 1.9 in CD and 1.5 in UC for cholelithiasis, and 2.8 and 1.7 HR for choledocholithiasis for CD and UC, respectively (Chen C. H. et al. 2018). Consistent with the fact that gallstone development is considered to be a result of ileal malabsorption of bile acids leading to impaired enterohepatic circulation, the ileocolonic location of CD and ileal resections were shown as independent factors in gallstone development, respectively (Massironi et al. 2022). Excessive ethanol consumption is the second most frequent cause of AP, contributing to 20-40% of all cases in the general population, however, it seems that in IBD alcoholic AP is less frequent. A study from the US showed that alcohol abuse was recorded in only 12% of hospital admissions with a primary diagnosis of AP and a co-diagnosis of IBD, 11.6% in CD, and 12.1% in UC,

while this proportion was 21% in healthy controls (Alexoff et al. 2016). Moolsintong et al. found that only 15% of AP was attributed to alcohol abuse in a cohort of CD patients, whereas biliary and idiopathic AP were equally frequent, each contributing to 21% of cases (Moolsintong et al. 2005). They also reported 13% thiopurine-induced AP. In a large Spanish cohort, Bermejo et al. found that most AP cases were attributed to drug exposure (64%), whilst 20% were idiopathic, 12% biliary and 4% of miscellaneous etiology (Bermejo et al. 2008). In summary, IBD does increase the risk of bile stone formation, but neither cholelithiasis nor alcohol abuse seems to be the leading cause of AP in IBD.

Another proposed reason for increased pancreatitis risk in IBD can be the higher frequency of interventions with a known risk for pancreatitis, such as taking pancreato-toxic medications or having a balloon-assisted enteroscopy (Iida et al. 2019). While numerous case reports and small-scale studies confirm AP in IBD patients who underwent medical procedures (Massironi et al. 2022), such as small bowel enteroscopy (single or double-balloon) or endoscopic retrograde cholangiopancreatography, these are relatively infrequent procedures even in IBD and most probably have only a minor contribution to the total number of AP cases associated with IBD (Bermejo et al. 2008). On the contrary, *drug exposure* was named as the supposed etiology in larger proportions of IBD-linked AP cases. In the general population, drug-induced acute pancreatitis (DIAP) is considered an uncommon condition, reported in only 0.1-2% of the cases (Nitsche et al. 2010). The latest systematic review separates the culprit drugs of DIAP into 4 main classes (Wolfe et al. 2020). In the treatment of IBD patients, several pancreato-toxic drugs are used regularly. In the early treatment of mild UC, aminosalicylates (5-ASA, Class Ia) alone can be the first-line maintenance therapy, while both in UC and CD, thiopurines, such as *azathioprine* (Class Ia) are often used as long-term maintenance therapy. Furthermore, other medications, such as vedolizumab (Class Ic), TNF-alpha-inhibitors (Class IV), corticosteroids (Class Ia-Ib), and metronidazole (Class Ia) are also used regularly in IBD (Agrawal et al. 2021; Montenegro et al. 2022; Wolfe et al. 2020).

The occurrence of thiopurine-induced pancreatitis (TIP) has been described since the 1970s (Massironi et al. 2022). This adverse event occurs in up to 1.5-7.3% of IBD patients taking thiopurine medications (Bermejo et al. 2008; Chang and Cheon 2019; Chaparro et al. 2013; Kim et al. 2017; Ledder et al. 2015; Teich et al. 2016; Tominaga et al. 2020;

Zabala-Fernandez et al. 2011), and the incidence rate ratio of AP was shown to be 5.82 during AZA treatment compared to no treatment (Wintzell et al. 2019). A recent meta-analysis of randomized trials confirmed that AZA elevates the odds of developing acute pancreatitis in CD, however, they did not assess the risk of AP in UC (Gordon et al. 2021). TIP usually occurs in the *first 30-60 days after initiation of treatment* and has a mild course with a rapid improvement in clinical symptoms upon withdrawal of the drug (Eskazan et al. 2021; Massironi et al. 2022; Teich et al. 2016; Wintzell et al. 2019). Although TIP is mostly considered a mild complication of thiopurine treatment, it requires the cessation of the thiopurine therapy and the change of medication, often to a less effective drug (Ledder et al. 2015), and might be associated with a higher risk of surgery (Kim et al. 2017). Female sex and smoking seem to be the strongest risk factors for TIP, and also its incidence seems to be higher in CD than in UC (Massironi et al. 2022).

1.2. Pathomechanism of acute pancreatitis

The central mechanisms in the development of AP mainly involve intracellular events of *pancreatic acinar cells*, such as pathologic calcium (Ca^{2+}) signaling, mitochondrial dysfunction, endoplasmic reticulum (ER) stress, and premature intracellular/intraductal activation of pancreatic enzymes (Lee P. J. and Papachristou 2019). In acinar cells, one of the major functions of ER and mitochondria is the regulation of Ca^{2+} signaling, as they rapidly take up Ca^{2+} after transient increases in cytosolic Ca^{2+} levels ($[Ca^{2+}]_i$) through their transporters (Habtezion et al. 2019). One of the most widely used animal models, *cerulein-induced pancreatitis* utilizes the sensitivity of acinar cells to a sustained increase in $[Ca^{2+}]_i$, leading to mitochondrial Ca^{2+} overload and mitochondrial dysfunction and consequent cellular damage (Habtezion et al. 2019). Cerulein is a cholecystokinin (CCK) 8 analog that acts on both CCK receptor subtypes leading to inositol triphosphate-dependent Ca^{2+} release from the ER (Archer-Lahlou et al. 2005). In rodents, repeated intraperitoneal (IP) doses of cerulein lead to a sustained increase in $[Ca^{2+}]_i$ with consequent pancreatic necrosis and a biphasic elevation in serum amylase levels (Gress et al. 1994; Yamaguchi et al. 1989). Another important role of ER in acinar cells is protein secretion (in particular, *digestive enzyme secretion* such as amylase and trypsinogen), and $[Ca^{2+}]_i$ is the main mediator of this complex secretory pathway (Gukovskaya et al. 2019). Altered Ca^{2+} homeostasis leads to a multiplex

dysregulation of intracellular organelles in the acinar cells, resulting in altered secretion and premature trypsinogen activation, which ultimately causes cellular destruction and pancreatic inflammation (Gukovskaya et al. 2019; Habtezion et al. 2019; Lee P. J. and Papachristou 2019). Many risk factors can trigger this above-described *central acinar dysregulation* leading to the initiation of AP. For instance, genetic mutations of many pancreatic enzymes and their inhibitors, such as cationic trypsinogen, carboxypeptidase A1, and the serine protease inhibitor Kazal-type 1 are associated with increased AP risk (Gukovskaya et al. 2019). Also, the majority of the most common risk factors of AP, such as smoking, hypertriglyceridemia, ethanol, or bile acids – which can reach pancreatic tissue in case of biliary obstruction – have been shown to trigger acinar Ca^{2+} dysregulation (Habtezion et al. 2019). Furthermore, recent studies shed light on the important role that *disturbances in pancreatic ductal HCO_3^- and fluid secretion* can play in triggering the central pathophysiology of AP (Habtezion et al. 2019; Lee P. J. and Papachristou 2019).

The primary role of *pancreatic ductal epithelial cells* (PDECs) is fluid and HCO_3^- secretion, mediated through the interplay between basolateral and luminal ion channels and transporters. The basolateral Na^+-HCO_3^- co-transporters (NBCe1-B), the luminal Cl^-/HCO_3^- exchangers (SLC26A3,6) and *cystic fibrosis transmembrane conductance regulator* (CFTR) channels, and the ubiquitous Na^+/H^+ exchanger proteins (NHE1-4) are all needed for optimal pancreatic ductal secretory functions (Lee M. G. et al. 2012). Of these pancreatic ductal transporter proteins, CFTR seems to play a key role in pancreatic diseases (Angyal et al. 2021; Mayerle et al. 2019). Previously, our group has demonstrated in mice, that ethanol and fatty-acid treatment could reduce the expression and activity of pancreatic ductal CFTR, impair the ductal HCO_3^- secretion, and could also lead to increased severity of experimental AP (Maleth et al. 2015). Several other studies confirmed that any disturbance in the ductal secretion (that often involves CFTR inhibition) can lead to a slowed ductal clearance of digestive enzymes, to the alkalinization of the pancreatic juice, and thus to premature, intraductal activation of trypsinogen, which both in itself and through triggering acinar calcium dysregulation can initiate pancreatitis (Lee P. J. and Papachristou 2019; Pallagi et al. 2022; Pallagi et al. 2011; Venglovecz et al. 2008).

1.3. Pathomechanism of thiopurine-induced acute pancreatitis

After their development in the 1950s, the three thiopurines – *azathioprine* (AZA), 6-mercaptopurine (6-MP), and 6-thioguanine (6-TG) – became the cornerstones of treatment in organ transplantation, and a wide range of inflammatory, autoimmune, and hematological diseases, but the most experience has been gathered in the field of gastroenterology and the management of IBD (Bayoumy et al. 2021; Sahasranaman et al. 2008). Thiopurines are pro-drugs and have to be enzymatically converted into the active 6-thioguanine-nucleotides (6-TGNs) by xanthine-oxidase, thiopurine S-methyl transferase (TPMT), hypoxanthine-guanosine phosphoribosyltransferase (HGPRT), and inosine monophosphate dehydrogenase or, in the case of 6-TG, only by HGPRT (Tominaga et al. 2020). 6-TGNs are cytotoxic purine nucleotide analogs that can be incorporated into the DNA and RNA as fraudulent bases, activate the mismatch repair pathway, inhibit the small GTPase, Ras-related C3 botulinum toxin substrate 1 (RAC1), and ultimately trigger apoptosis (Bayoumy et al. 2021; Marinkovic et al. 2014; Sahasranaman et al. 2008). These effects are more pronounced in the rapidly dividing cells, such as lymphocytes, which leads to the immunosuppressive effect of thiopurines.

Although they are widely administered medications, thiopurines have a narrow therapeutic window and adverse events can occur in up to 26% of patients receiving treatment, ranging from digestive intolerance, alopecia, leukopenia, and hepatotoxicity to pancreatitis (Chaparro et al. 2013; Tominaga et al. 2020). Myelosuppression and hepatotoxicity are well-recognized dose-dependent side-effects of thiopurines (Gordon et al. 2021), and the cessation of the applied therapy is usually not necessary, since these adverse events tend to improve with the dose reduction of the applied thiopurine drug. They have also been shown to have a strong association with TPMT polymorphisms (Liu et al. 2015).

On the other hand, AP observed in association with thiopurine treatment is a rather dose-independent adverse event, usually requiring the complete withdrawal of the drug (Cucinotta et al. 2021; Iida et al. 2019). Accordingly, studies investigating the background of TIP found no association with TPMT polymorphisms (Ramos et al. 2016). It is of interest that TIP is rarely seen in medical conditions other than IBD, despite receiving similar doses and lengths of thiopurine therapy (Weersma et al. 2004), which suggests some association between TIP and the IBD phenotype. Interestingly, the human leukocyte

antigen (HLA) allele HLA-DRB1*07 is overrepresented in European CD patients (Ahmad et al. 2002; Ahmad et al. 2006; As et al. 2022), and recently, an association between this HLA gene region and TIP has been identified. Namely, an OR 2.59 of AZA-induced AP was seen in a genome-wide association study with single nucleotide polymorphism rs2647087 that maps to the HLADQA1*02:01-HLA-DRB1*07:01 haplotype (Heap et al. 2014). This has been later confirmed by a Canadian (Wilson et al. 2018) and a Swedish study (As et al. 2022), and also with a pre-treatment HLA screening, the AP incidence could be decreased 11-fold compared to no screening (Wilson et al. 2021). Although HLA-DRB1 and DQA1 expression are limited in pancreatic exocrine tissue, immune cells in the pancreatic tissue express these HLA types extensively (As et al. 2022). Although these findings support the most frequently suggested immune-mediated mechanisms in the background of TIP (Heap et al. 2014; Ledder et al. 2015), direct toxic mechanisms have also been proposed in the etiopathogenesis of TIP – a field that remained, however, basically unexplored so far (Ledder et al. 2015).

Currently, we have also very limited knowledge of the direct cellular effects of AZA on pancreatic ductal transporters. A preliminary study performed on dogs in the 1970s suggested that AZA could inhibit pancreatic secretion *in vivo* (Dreiling and Nacchiero 1978), but this effect was left unexplored in the following decades. More recently, a group investigated the cellular effect of AZA on basolateral NHE activity in dendritic cells, and their result suggested that AZA might have an inhibitory effect (Bhandaru et al. 2012). Also, the genetic deletion of Na^+/H^+ exchanger regulatory factor-1 (NHERF1) has been shown to cause mislocalization of CFTR in PDEC with a decreased ductal secretory activity, and it led to a more severe experimental AP (Pallagi et al. 2014). NHERF1 is a member of a protein complex tethering CFTR – a key member of ductal secretion – to the apical membrane. This complex is comprising NHERF1, ezrin (an adaptor protein), CFTR, and actin (Short et al. 1998). The regulation and membrane trafficking of CFTR is also investigated extensively in bronchial epithelia, and the inhibition of ezrin was shown to reverse the NHERF1-induced increase in the CFTR functions of human bronchial cells (Favia et al. 2010). Since ezrin is a downstream target of RAC1 (Moniz et al. 2013), a known target inhibited by AZA in human leukocytes (Tiede et al. 2003), we aimed to explore the potential role of CFTR, ezrin, and RAC1 in the AZA-induced effects on PDEC.

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2. Objectives

During the research projects of my doctoral thesis, I conducted an extensive literature search and a comprehensive meta-analysis regarding the association between IBD and AP. Also, – steered by the clinical observation and conclusions of my literature search that TIP is responsible for a major proportion of AP seen in IBD – I carried out experiments in mice by combining oral thiopurine treatment and the common cerulein-induced pancreatitis model. Finally, I 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 HCO_3^- secretion?
- Does azathioprine affect pancreatic ductal CFTR functions and distribution?
- What molecular mechanism lies behind the pancreatic ductal effects of azathioprine in mice?

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3. Methods

Ethics

The meta-analysis was reported according to the preferred reporting items for systematic review and meta-analysis (PRISMA) guidelines (Moher et al. 2009). No ethical approval was required. All experiments were performed in compliance with the Hungarian regulations and EU directive 2010/63/EU for the protection of animals used for scientific purposes and the study was approved by the National Scientific Ethical Committee on Animal Experimentation (approval nr: CS/I01/2233-4/2018).

Search strategy

A systematic search was conducted in four major electronic databases (MEDLINE via PubMed, Embase, Web of Science, and Scopus) up to 19 June 2019 (the date of the last search), without any search restrictions. The search strategy used comprehensive strings of words with variations of the terms “pancreatitis” in combination with term variations for “inflammatory bowel diseases”, “Crohn’s disease” or “ulcerative colitis”. The exact search query used for the search can be found in **Table 1.** The reference lists of selected articles were also checked.

Table 1. – **The Search Query Template Used for Database Search on Four Major Electronic Databases.**

(pancreatitis)
AND
(IBD OR (inflammatory bowel disease) OR (inflammatory bowel diseases)
OR
(crohn disease) OR (crohn's disease) OR (crohns disease) OR (enteritis regionalis) OR (regional enteritis) OR (morbus crohn) OR (regional enterocolitis)
OR
(ulcerative colitis) OR (chronic ulcerative colitis) OR (colitis ulcerativa) OR (colitis ulcerosa) OR (colitis ulcerosa chronica) OR (mucosal colitis) OR (ulcerous colitis) OR (colonic chronic ulceration) OR (ulcerative colorectitis) OR (ulcerative procto colitis) OR (ulcerative proctocolitis) OR (ulcerous proctitis) OR (ulcerative proctitis))

On PubMed/MEDLINE and Embase we used plain text search in *all fields* (including MeSH/Emtree headings search). On Web of Knowledge and Scopus, we searched in *Topic* and *Article Title, Abstract, Keyword* fields, respectively. (The formulation was adapted for each database)
Period: from inception until 19 June 2019.

Study selection and eligibility criteria

The selection of the studies and screening was conducted by two investigators (Bence Stubnya and myself) independently, to conform to the PRISMA guidelines. The screening was performed through a review of the titles and abstracts of the records. Studies that met the inclusion criteria and those with abstracts that lacked crucial information for the decision regarding their exclusion were retrieved for full-text evaluations. Decisions of eligibility and disagreements were resolved through discussion or by a third reviewer (Gábor Veres). To be included in the review, studies had to meet all of the following criteria:

i. completed and published observational studies with human subjects
ii. the use of objective definitions of IBD and AP,
iii. the provision of quantitative reports of IBD and AP, and
iv. all participants had been counted only once in the published report.

Two different clinical questions were evaluated in two different analyses.

First, to appraise the odds of AP in IBD we used the modified "PICO" for observational studies (PECO) as follows:

P (population) – general population
E (exposure) – IBD patients
C (comparison) – non-IBD population
O (outcome) – odds of AP.

The modified "PICO" used in the second analysis appraising the annual incidences (PO) of AP in IBD was:

P (population) – IBD population
O (outcome) – reported incidence rates of AP over an observation period given in person-years (PYs).

Studies were excluded if they did not report the incidence of AP cases either in the IBD or in the non-IBD population, or if the reported data was redundant (ie. a participant could be counted more than once in the final report). Studies were not included if they reported only mean follow-up data or reported redundant data. Conference abstracts were excluded from both analyses. When studies reported the same population (database) and period, the most recent study was selected for inclusion.

Data extraction

Name of the first author, date of publication, geographical location, study type, study period, the age range of included individuals, the subtype(s) of IBD, number of IBD patients, crude incidence of AP cases (for IBD and non-IBD population respectively), adjusted relative measures and the observation period of the study in PYs were extracted using a data extraction table, if applicable.

Data synthesis and analysis

Odds ratios were calculated from the crude incidences and pooled odds ratios (ORs) or event rates with 95% confidence intervals (CIs) were calculated. We applied the random-effects model with the DerSimonian-Laird estimation (Dersimonian and Laird 1986). Cochrane's Q, I^2, and Chi-square tests were used to quantify statistical heterogeneity and gain probability values, respectively. Based on Cochrane's handbook $I^2 = 100\% \times (Q-df) / Q$, and represents the magnitude of the heterogeneity (I^2 = 30-60% – moderate; 50-90% – substantial; 75-100% – considerable heterogeneity) and $p < 0.1$ indicated significant heterogeneity (Higgins Jpt 2019). All statistical analyses were performed using STATA 16.0 (Stata Corporation, College Station, TX, USA).

Risk of bias and quality of evidence

Following the Cochrane Prognosis Methods Group recommendation (Hayden et al. 2013; Iorio et al. 2015) the quality assessment of prognostic studies was made using the Quality In Prognosis Studies (QUIPS) tool. First, six important domains were critically appraised to evaluate validity and bias in the studies: (1) study participation, (2) study attrition, (3) prognostic factor measurement, (4) outcome measurement, (5) study confounding, and (6) statistical analysis and reporting (Hayden et al. 2013). Each domain

contained between three and seven prompting items to be rated on a four-grade (yes/ partial/no/unsure), or two-grade scale (yes/no). In the final stage, the overall judgment of the risk of bias (RoB) within each domain was made based on the rated items; all of the responses to the prompting items were taken together when judging a domain's overall RoB which was expressed on a three-grade scale (high, moderate or low RoB). Hence, the QUIPS assessment results in six ratings of RoB, one for each domain. The final RoB of each study was decided by the number of domains with high and low RoB: studies were considered to have low overall RoB if none of the six domains had high and most of the domains had low RoB; high overall RoB was judged when two or more domains had high RoB, or less than half of the domains had low RoB; otherwise, the overall RoB was judged to be moderate. To examine small study effects we used the visual assessment of a funnel plot since tests for funnel plot asymmetry are not advised in analyses with fewer than ten studies (Sterne et al. 2011).

Table 2. **A list of used chemicals and materials.** *Based on (Tél et al. 2023)*

Name	Provider	Cat. No.:
Azathioprine	ThermoFisher Scientific	*J62314*
6-thioguanine	ThermoFisher Scientific	*B21280*
6-Mercaptopurine monohydrate	ThermoFisher Scientific	*A12197*
Stainless steel feeding tubes, 22ga x 25mm, straight	Instech Laboratories	*FTSS-22S-25*
Human secretin	Sigma-Aldrich	*S7147*
Harrys Hematoxylin	Leica	*3801560E*
Eosin Y alcoholic solution	Leica	*3801600E*
DMEM/F-12 Ham	Sigma-Aldrich	*D6421*
collagenase	Worthington	*LS005273*
Soybean trypsin inhibitor	Gibco	*17075029*
bovine serum albumin (BSA)	Sigma Aldrich	*A8022*
CELLSTAR 48-well culture plates, clear	Greiner Bio-One	*M8937-100EA*
8-well cell culture chamber, on a glass slide	Sarstedt	*94.6170.802*
cover glass	VWR	*ECN 631-1583*
poly-L-lysine	Sigma-Aldrich	*P4707-50ML*
BCECF, AM	Invitrogen	*B1170*
MQAE	Invitrogen	*E3101*
FURA-2, AM	Invitrogen	*F1201*

Table 2, continued

Pluronic F-127	Invitrogen	*P3000MP*
Shandon Cryomatrix	Thermo Scientific	*6769006*
Triton-X-100	Reanal labor	*32190-1-99-33*
Sodium Citrate	Sigma Aldrich	*C8532*
PFA	Alfa Aesar	*43368*
PBS	Sigma Aldrich	*P4417-100TAB*
goat serum	Sigma Aldrich	*G9023*
ProLong™ Gold Antifade Mountant	Invitrogen	*P36930*
Anti-CFTR antibody, polyclonal, rabbit	Alomone Labs	*ACL-006*
Goat anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 488	Invitrogen	*A-11034*
Recombinant Anti-Ezrin antibody [EPR23353-55], monoclonal, rabbit	Abcam	*ab270442*
Anti-CFTR antibody [CF3]	Abcam	*ab2784*
Donkey anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 647	Invitrogen	*A-31571*
Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 568	Invitrogen	*A-11011*
Collagenase IV	Worthington	*LS004188*
Dispase	Sigma-Aldrich	*D4693*
corning matrigel	Corning	*354234*
glucose oxidase	Sigma Aldrich	*G2133-50KU*
6-well plates	Greiner BioOne	*657160*
catalase	Sigma Aldrich	*C100*
glucose	Sigma Aldrich	*G8270*
cysteamine hydrochloride	Sigma Aldrich	*M6500*
Ehop-016	Sigma Aldrich	*SML0526*
NSC668394	Sigma Aldrich	*341216*
RAC1 G-LISA Activation Assay	Cytoskeleton	*BK128*
Alpha-Amylase Assay	Diagnosticum	*47462*
Cerulein	Tocris Bioscience	*6264*
Apoptosis-Necrosis Detection Kit (blue, green, red)	Abcam	*ab176749*
CellTiter-Glo® 3D Cell Viability Assay	Promega	*G9682*

Materials

All materials used for the experiments can be found with catalog numbers in **Tables 2 and 3**.

Animals

The C57BL6 mice used in this study were 8-12 weeks old and weighed 20-25 grams. The gender ratio was 1:1 for all groups. Animals were allowed to have free access to chow and water, kept at a constant 24°C room temperature and humidity, and under a 12-hour light-dark cycle. Animals received VRF1(P) standard rodent food (Special Diets Services, UK, Cat.No.: 801900), and standard bedding (JRS, Germany; REHOFIX MK2000 corn cob) was purchased from Akronom, Hungary.

Table 3. **Composition of solutions used during experiments.**

HCO_3^- containing solutions were gassed with 2 L/min carbogen. Concentrations are shown in millimolar (mM). *Based on (Tél et al. 2023)*

	Standard HEPES	**Standard HCO_3^-**	**20 mM NH_4Cl-HCO_3^-**	**Cl^--free HCO_3^-**	**Cat. No.: *(Sigma-Aldrich)***
NaCl	130	115	95		*S9625*
KCl	5	5	5		*P4504*
$MgCl_2 \cdot 6H_2O$	1	1	1		*M0250*
$CaCl_2 \cdot 2H_2O$	1	1	1		*C3881*
HEPES	10				*H3375*
D-(+)-Glucose	10	10	10	10	*G8270*
$NaHCO_3$		25	25	25	*S8875*
NH_4Cl			20		*A4514*
Na-gluconate				115	*S2054*
K_2-sulfate				2.5	*P0772*
Ca-D-gluconate • H_2O				6	*G4625*
Mg-D-gluconate • xH_2O				1	*G9130*

In vivo experimental pancreatitis

Moderate experimental pancreatitis was induced with 8 × 50 μg/BWkg IP cerulein injections administered hourly, as described previously (Madacsy et al. 2022; Pallagi et

al. 2022). Sham control animals received IP physiologic saline injections. Mice were sacrificed one or four hours after the last injection. For histologic evaluation, pancreata were removed, washed with physiologic saline, cleaned from lymph nodes and fat, and stored at +4°C in 4% formaldehyde. Paraffin-embedded sections of the pancreas were stained with hematoxylin-eosin following standard protocol. The severity of pancreatitis was assessed independently by three observers, blinded for the experimental setup, based on the histologic scores of 3 random fields per slide. The extent of the tissue edema, and leukocyte infiltration, were scored from 0 to 5 and the proportion of acinar cell necrosis was evaluated as previously described (Maleth et al. 2015). Images were captured with a Zeiss AxioImager.M2 with 5x and 10x objectives (N-Achroplan 5x/0.15 M27 and Plan-Apochromat 10x/0.45 M27; Zeiss, Germany). To determine serum amylase activity, serum samples were centrifuged at 2000 × g for 20 minutes at 4ºC, then the serum alpha-amylase activity was measured using the alpha-amylase kit on a CLARIOStar plate reader following the manufacturer's protocol.

Per os treatment of mice with thiopurines

Based on previous pharmacokinetic studies, the usual therapeutic serum concentrations of AZA were below 1 μg/mL in human subjects treated with conventional oral AZA doses (0.5-2.5 mg/kg/day) (Voogd 1989b), and the maximal serum levels in mice were observed to be 11.3 μg/mL after a single oral dose of 33.3 mg/kg AZA (Lewis 1996). To test both therapeutic and toxic concentrations of thiopurines, we chose to use the 1 and 10 μg/mL *ex vivo* concentrations and the 1.5 and 15 mg/kg *in vivo* doses in our experiments. 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 through a 22 G gastric feeding tube (Instech Laboratories, PA, USA), respectively. After the oral treatment, mice were selected either for further experiments or immediate pancreas collection and cell isolation.

Measurement of *in vivo* pancreatic fluid secretion

The stimulated *in vivo* pancreatic juice-secretion was measured in ketamine-xylazine sedation, as previously described (Perides et al. 2010). Briefly, through a narrow laparotomy, the duodenum and the head of the pancreas were exposed and the main

pancreatic duct was cannulated with a blunted 26 G needle and fixated with a vascular clip. To avoid measuring the bile secretion, the ductus choledochus was also clipped carefully. Total pancreatic juice secretion was stimulated using IP 0,75 U/kg human secretin and the secreted volume was measured after 30 minutes with a 100 μL pipette and normalized to the animals' body weight and time.

Isolation of pancreatic acinar cells and ductal segments

Mouse pancreatic cells were isolated as previously described (Geron et al. 2014; Gout et al. 2013), with some adaptations. Briefly, the collected pancreas was minced into 1-3 mm^3 pieces and placed in ice-cold HBSS. Lipids and fats were removed by centrifugation at 450×*g* for 2 minutes. Tissue pieces were digested in 5 mL freshly prepared HBSS solution containing 200 units/ml collagenase, 10 mM HEPES and 0.25 mg/ml trypsin inhibitor at 37°C for 20-30 minutes. Digested tissue was washed with HBSS of 4°C with 10 mM HEPES, 0.25 mg/ml soybean trypsin inhibitor, and 5% FBS, centrifuged 3x for 2 minutes at 450 RCF (Rotor radius: 180 mm), and the pellet was filtered through a 100 μM cell mesh and resuspended in Medium-199 with 2.5% FBS and 0.25 mg/ml soybean trypsin inhibitor. Pancreatic ductal segments were isolated as described previously (Gray et al. 1994; Molnar et al. 2020). In summary, mice were euthanized with pentobarbital, and the pancreas was removed, and placed into ice-cold DMEM/F12. The pancreas was injected with a digestion medium (DMEM/F12 containing 100 U/ml collagenase, 0.1 mg/ml trypsin inhibitor, and 1 mg/ml bovine serum albumin) and was incubated in a shaking water bath at 37ºC for 30 minutes. Small intra- and interlobular ducts were identified and were mechanically dissected from the acini under a stereomicroscope.

Preparation Mouse Pancreatic Organoid Culture

Mouse pancreatic ductal organoids were generated as previously described (Molnar et al. 2020; Pallagi et al. 2022). Briefly, the mouse pancreas was minced into small segments and incubated for 1 h at 37°C in a digestion media containing collagenase IV and dispase. After digestion, the tissue was washed and resuspended in Matrigel Basement Membrane Matrix and plated in domes and kept in culture (at 37°C 95% relative humidity, and 5% CO_2) by changing media every other day. Organoids were used for experiments between passage numbers 2 and 5. For the experiments, organoids were

digested into single cells TrypLE Express and after 60 minutes of recovery time, they were incubated in 6-well plates with drug/inhibitor-containing or control culture media for another 60 minutes.

Measurement of intracellular pH, Ca^{2+}, and Cl^- changes by fluorescence microscopy

Fluorescence microscopy measurements were conducted as previously described (Madacsy et al. 2022; Maleth et al. 2015; Pallagi et al. 2022). Isolated pancreatic acini or ductal segments were attached to a poly-L-lysine coated cover glass, placed in a perfusion chamber, and incubated with MQAE (2 μM), Fura 2-AM (5 μM), or BCECF-AM (2 μM) in HEPES solution for 30 minutes at 37 °C with 5% CO_2. After incubation, chambers were mounted on an Olympus IX73 inverted microscope (Olympus, Japan) and were perfused continuously at a constant speed with extracellular solutions heated to 37°C. Microscopes were equipped with LED illumination systems (Olympus CoolLED PE-4000 and pE-340^{Fura2}) and matching dichroic filters. A Hamamatsu Orca-Flash 4.0 CCD camera (Hamamatsu Photonics, Japan) and an Olympus 20 × water immersion objective (NA: 0.8) were used for capturing fluorescent signals with a temporal resolution of 1 sec using the Olympus Excellence software. To test the effect of thiopurines and inhibitors we changed the prefusion from control solutions to thiopurine and/or inhibitor-containing buffered solutions for 10 minutes before and during performing the stimulations described below.

To estimate the Cl^- secretory activity of PDEC, we continuously monitored the intracellular Cl^- concentration ($[Cl^-]_i$) of the ductal segments with the help of the Cl^--sensitive fluorescent dye MQAE, during continuous perfusion with HCO_3^-/CO_2^--buffered solutions. The removal of extracellular Cl^- induces a rapid elevation in fluorescence intensity equivalent to a decrease in $[Cl^-]_i$, and the relative amplitude of this change is proportionate to the luminal Cl^- efflux mediated by CFTR. Intracellular Ca^{2+} signals ($[Ca^{2+}]_i$) were measured as described previously (Molnar et al. 2020; Pallagi et al. 2022). The fluorescence signals from MQAE and FURA-2 measurements were normalized and expressed as relative fluorescence (F/F_0). The constitution of applied buffered solutions is listed in **Table 3**.

RECOVERY FROM AN ALKALINE LOAD. During continuous perfusion with HCO_3^-/CO_2^--buffered solutions, we monitored the changes in intracellular pH (pH_i). During alkaline

loading, ductal segments were exposed to 20 mM NH_4Cl from the basolateral side, where the passive influx of NH_3 triggered a rapid alkalosis of the pH_i, followed by a decaying recovery created by the active efflux of HCO_3^- (as the combined effect of mainly the luminal SLC26A and CFTR activity). The initial rates of recovery (dpH_i/dt) over 30 seconds from the highest pH_i levels (downward-tilted arrows in **panel A of Figures 10-12**) were calculated and converted to base flux values (B^-/min) using calibration data from previously published measurements (Hegyi et al. 2004; Maleth et al. 2015; Molnar et al. 2020).

RECOVERY FROM AN ACID LOAD. Ductal segments were loaded with NH_4Cl as described above. The removal of the NH_4Cl from the extracellular solution triggers the so-called *acid loading*, where the unfacilitated efflux of NH_3 generates rapid acidification in the pH_i, which is followed by a decaying increase in the pH_i created by the active influx of HCO_3^- (effect of mainly the basolateral NBCe1 activity) and the active efflux of H^+ (a result of the basolateral NHE1&4 activity). The initial rates of recovery (dpH_i/dt) over 60 seconds from the lowest pH_i levels (upward-tilted arrows in **panel A of Figures 10-12**) were calculated and converted to base flux values (B^-/min) using calibration data from previously published measurements (Hegyi et al. 2004; Maleth et al. 2015; Molnar et al. 2020).

Immunostaining

Immunofluorescent labeling on sectioned ductal segments was performed as previously described (Molnar et al. 2020). Briefly, isolated pancreatic ductal segments and organoids were frozen in Shandon Cryomatrix at -20 °C. The 7 μm thick sections were placed on microscope slides, and fixation was performed with 4% PFA-PBS for 20 minutes followed by washing for 3x5 minutes with PBS. After permeabilization in citrate/Triton-X 100, the sections were blocked with 0.1% goat serum and 10% BSA in PBS for 2 h. The overnight incubation with primary CFTR (rabbit, monoclonal) antibodies at 4°C was followed by repeated washing steps after which secondary (goat anti-rabbit Alexa Fluor 488) antibodies were added for 2h at room temperature. Nuclear staining and mounting were carried out simultaneously by ProLong™ Gold Antifade mounting medium with DAPI. Images were captured with a Zeiss LSM880 confocal microscope using a 40X oil immersion objective (Plan-Apochromat 40x/1.4 Oil DIC

M27; Zeiss, Germany). For the semi-quantitative analysis, images were captured with the same setup from 5 different ductal fragments per group, derived from at least 2 different mice. On each image, CFTR fluorescence intensity profiles were determined over 4.5 μm long vectors at every 30 μm all around the identified luminal cavities, starting from the luminal side. Linear profiles were determined using the FIJI software (NIH, USA), peak intensity was noted in arbitrary units (AU), and peak distance from the lumen, as well as peak width (measured at half-maximal intensity), were calculated.

Direct Stochastic Optical Reconstruction Microscopy (dSTORM)

Primary mouse pancreatic ductal cells were generated for dSTORM by digesting and plating mouse pancreatic ductal organoids on cover glass followed by incubation for 60 minutes with 1 μg/mL AZA-containing media. Organoids were fixed with 4% PFA in PBS for 10 minutes and antigen retrieval was performed with 0.01% Triton-X-100 in PBS for 10 minutes. Aspecific binding sites were blocked by applying 10% BSA in PBS for 2 h at 37 °C. CFTR and Ezrin primary antibodies (1:100) were applied during overnight incubation at 4 °C and fluorophore-conjugated secondary antibodies were applied (mouse anti-rabbit Alexa Fluor 647 and goat anti-rabbit Alexa Fluor 568). Cover glasses were placed in blinking buffer (which contains 100 U glucose oxidase, 2000 U catalase, 55.5 mM glucose, and 100 mM cysteamine hydrochloride in 1 mL final volume with sterile PBS) and dSTORM images were captured by Nanoimager S (Oxford Nanoimaging ONI Ltd., UK). Cluster analysis of dSTORM images was evaluated by CODI (Oxford Nanoimaging ONI Ltd., UK).

Biochemical assays

RAC1 Activity. Mouse pancreatic organoids were digested into single cells using TrypLE™ Express and incubated at 37 °C with 1 μg/mL AZA, 10 μM Ehop-016, or both for 60 minutes. Cell lysates were harvested and RAC1 activity was determined with the RAC1 G-LISA kit (Cytoskeleton, USA) following the manufacturer's protocol.

Amylase release assay. Freshly isolated acinar cells were seeded to 48-well plates and incubated for 30 minutes at 37 °C with supplemented Medium-199, then 1 μg/mL AZA and/or 100 nM cerulein-containing media was added, and cells were incubated for another 60 minutes. After incubation, aliquots were taken from the supernatant and frozen

instantly in liquid nitrogen to calculate the released amylase activity. Then, Triton X-100 was added in a final concentration of 1% to the remaining cell suspension, incubated for 15 minutes, centrifuged at 450 × g for 3 minutes, 4°C, and a second aliquot was frozen in liquid nitrogen to calculate the total amylase activity. Both activities of released and total amylase were determined for each sample using an EPS method-based(International Federation of Clinical et al. 2006) Alpha-Amylase Kit (Diagnosticum Zrt., Hungary) on a CLARIOStar plate reader at 405 nm (BMG Labtech, Germany) following the manufacturer's protocol, and the percentage of released amylase was calculated.

Measurement of cell viability

Freshly isolated acinar cells were incubated for 30 minutes with supplemented Medium-199 in 48-well plates (Greiner Bio-One, Hungary), then AZA containing (1-10-100-1000 μg/mL) media was added, and cells were incubated for another 60 min at 37°C. Cerulein (100-1000 nM) was applied as a positive control. We determined the intracellular ATP content, which is proportional to the number of viable cells, using the CellTitre-Glo 3D (Promega) luminometric assay on a CLARIOStar plate reader. In addition, living, apoptotic, and necrotic cells were stained in 8-well chamber-slides (Sarstedt, Germany) with Apoptosis-Necrosis Kit (Abcam) and were visualized under a Zeiss LSM880 confocal microscope using a 40X oil immersion objective (Zeiss, NA: 1.4). The number of cells was calculated in FIJI (NIH, USA) using the built-in Cell Counter plugin.

Statistical analysis, post-tests.

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.

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4. Results

4.1. The risk of acute pancreatitis in inflammatory bowel diseases

To comprehensively describe the association between acute pancreatitis and inflammatory bowel diseases we conducted a systematic literature search in four major electronic databases followed by a screening, data extraction, and meta-analysis (Tel et al. 2020). **Figure 1** gives an overview of the screening and study selection as recommended by PRISMA guidelines (Moher et al. 2009).

4.1.1. Search and study selection

The search in the four electronic databases yielded a total of 9178 entries (EMBASE 3540, Scopus 3132, Web of Science 1198, and PubMed 1308). **Table 1** shows the search protocol used in the literature search. After excluding duplicate entries, 3627 articles were screened by title and abstract. In total 143 full-text articles were evaluated, handling the screening for the two analyses together. An additional article (Thisted et al. 2006) was identified through the reference lists of the studies screened by full text. A total of eight articles were included for qualitative analysis, six in the first (Chen Y. T. et al. 2016; Munk et al. 2004; Rasmussen et al. 1999; Sundstrom et al. 2006; Thisted et al. 2006; Yang et al. 2018) and four in the second analysis, respectively (Chen Y. T. et al. 2016; Kim et al. 2017; Mcauliffe et al. 2015; Rasmussen et al. 1999). The basic characteristics and main findings of the eight included articles are shown in **Tables 4 and 5**.

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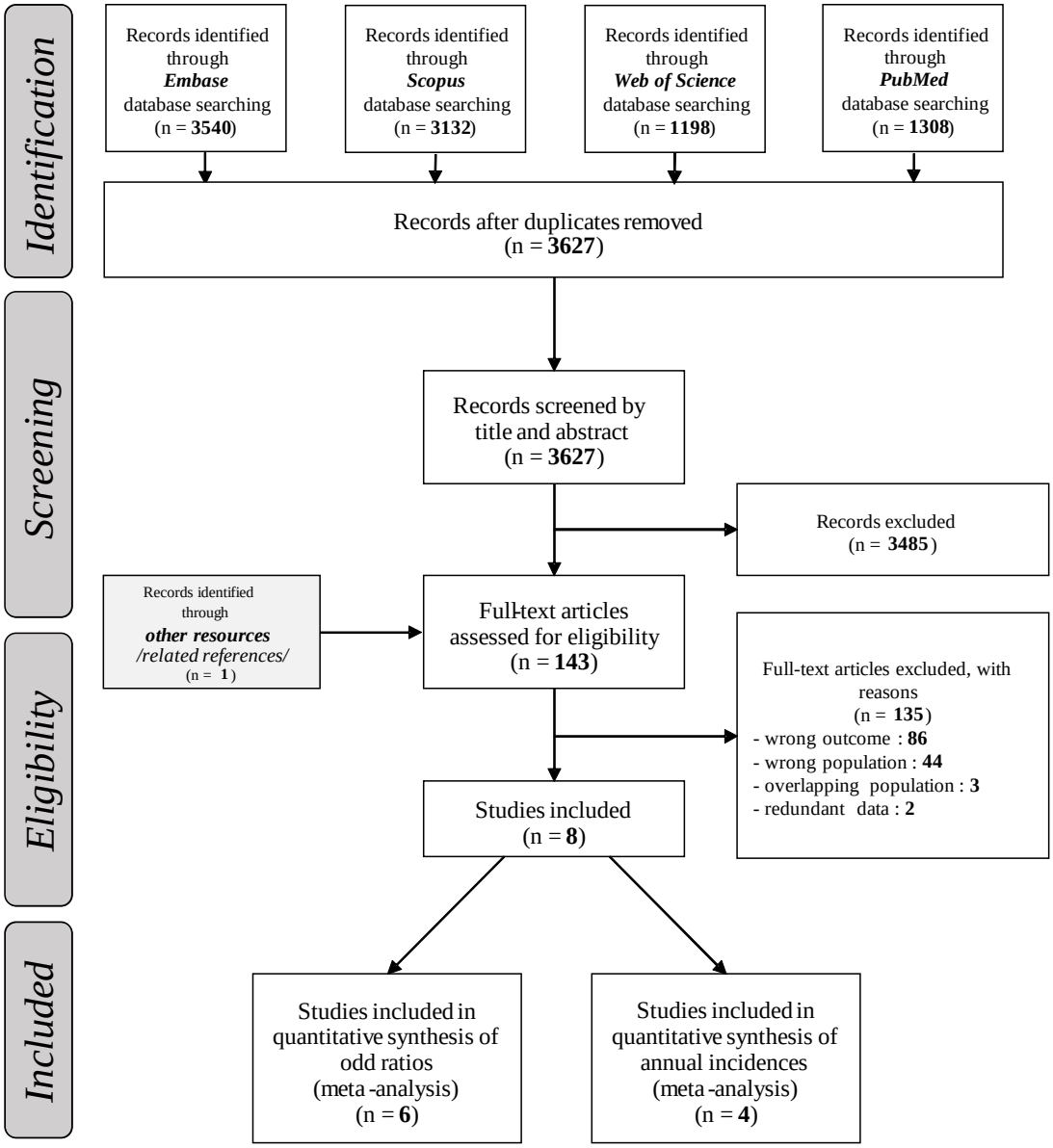

Figure 1. – **PRISMA Flowchart** (Moher et al. 2009) *Based on (Tel et al. 2020)*

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Table 4. – Basic Characteristics Of The Eight Included Studies

N/A = no data published, AP = acute pancreatitis, IBD = inflammatory bowel disease, ICD = international coding of diseases, OR = odds ratios, PYs = person-years, UC = ulcerative colitis, yrs = years. *Based on (Tel et al. 2020)*

Author & Year	Country	Study period	Study design	IBD subgroup	basis of diagnosis	Age range	Observation period (PYs)	No. of IBD patients	No. of controls (non-IBD)
Chen Y. T. et al. 2016	Taiwan	*2000-2010*	prospective cohort	all IBD patients	ICD9 codes	≥20 yrs	63,532	11,909	47,636
Kim et al. 2017	South-Korea	*1989-2015*	retrospective cohort	UC patients only	medical records	9-90 yrs	33,355	33,386	*N/A*
McAuliffe et al. 2015	USA	*2004-2011*	retrospective cohort	all IBD patients	ICD 9 codes	18-80 yrs	59,148	3307	*N/A*
Munk et al. 2004	Denmark	*1991-2002*	case-control	all IBD patients	ICD8 & 10 codes	*N/A*	*N/A*	94	17,409
Rasmussen et al. 1999	Denmark	*1977-1992*	prospective cohort	all IBD patients	ICD8 codes	*N/A*	112,824	15,526	15,526
Sundstrom et al. 2006	Sweden	*1995-1998*	case-control	all IBD patients	medical records	20-85 yrs	*N/A*	28	2215
Thisted et al. 2006	Denmark	*1996-2003*	case-control	all IBD patients	ICD8 & 10 codes	*N/A*	*N/A*	129	28,264
Yang et al. 2018	South-Korea	*2014*	cross-sectional	all IBD patients	ICD 10 codes	19-75 yrs	*N/A*	43,281	1,127,261

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Table 5 – Effect Estimates Of The Eight Included Studies

N/A = no data published, AP = acute pancreatitis, CI = confidence interval, IBD = inflammatory bowel disease, OR = odds ratios, PYs = person-years

* crude ORs, calculated based on published data. *Based on (Tel et al. 2020)*

Author & Year	OR * (overall)	OR * (Crohn's disease)	OR * (ulcerative colitis)	Annual incidence per 100 000 PYs	Adjusted measures reported	Measures are adjusted for
Chen Y. T. et al. 2016	**3.24**	*N/A*	*N/A*	**318**	**Hazard ratio:** CD 3.4 (95 % CI, 2.7-3.26) UC 2.49 (95 % CI,1.91-3.26)	age, sex, alcohol-related disease, biliary stone, hypertension, hyperlipidemia, diabetes mellitus, obesity, hepatitis B & C, COPD, hypertriglyceridemia, cardiovascular disease, chronic kidney disease, hypercalcemia
Kim et al. 2017	*N/A*	*N/A*	*N/A*	**153**		
McAuliffe 2015	*N/A*	*N/A*	*N/A*	**360**		
Munk et al. 2004	**2.90**	**4.28**	**1.77**	*N/A*		
Rasmussen et al. 1999	**2.73**	**4.33**	**2.09**	**76**	**Standardized incidence ratio:** CD 4.3 (95 % CI, 2.9-6.2)	sex, age
Sundstrom et al. 2006	**3.94**	*N/A*	*N/A*	*N/A*	**Odds Ratio:** IBD 4,7 (95 % CI, 2.2-10)	sex, age
Thisted et al. 2006	**2.80**	*N/A*	*N/A*	*N/A*		
Yang et al. 2018	**3.11**	**4.12**	**2.64**	*N/A*	**Standardized prevalence ratio:** CD 4.94 (95 % CI, 4.47-5.40)	sex, age

4.1.2. Analysis of the odds of AP in IBD

We first assessed studies that reported appropriate data on the odds of AP in IBD. We found six observational studies to be eligible: one cross-sectional, two prospective cohort, and three case-control studies. The six articles altogether included data from 1,309,278 people. The studies originated from Denmark (3), Sweden (1), South Korea (1), and Taiwan (1), respectively. In the case-control studies, the definition of cases was set to be AP (based on ICD codes) and the control groups had been selected accordingly (Munk et al. 2004; Sundstrom et al. 2006; Thisted et al. 2006). We extracted the number of events and created (new) contingency tables per article and calculated crude ORs. Pooling the crude ORs using the random-effects model yielded an OR of 3.11 (95% CI, 2.93–3.30; I^2 = 0.0%; **Figure 2.**) meaning that the odds of AP are three times higher in IBD compared to the non-IBD population. The I^2 statistics showed no significant heterogeneity, making this observation robust.

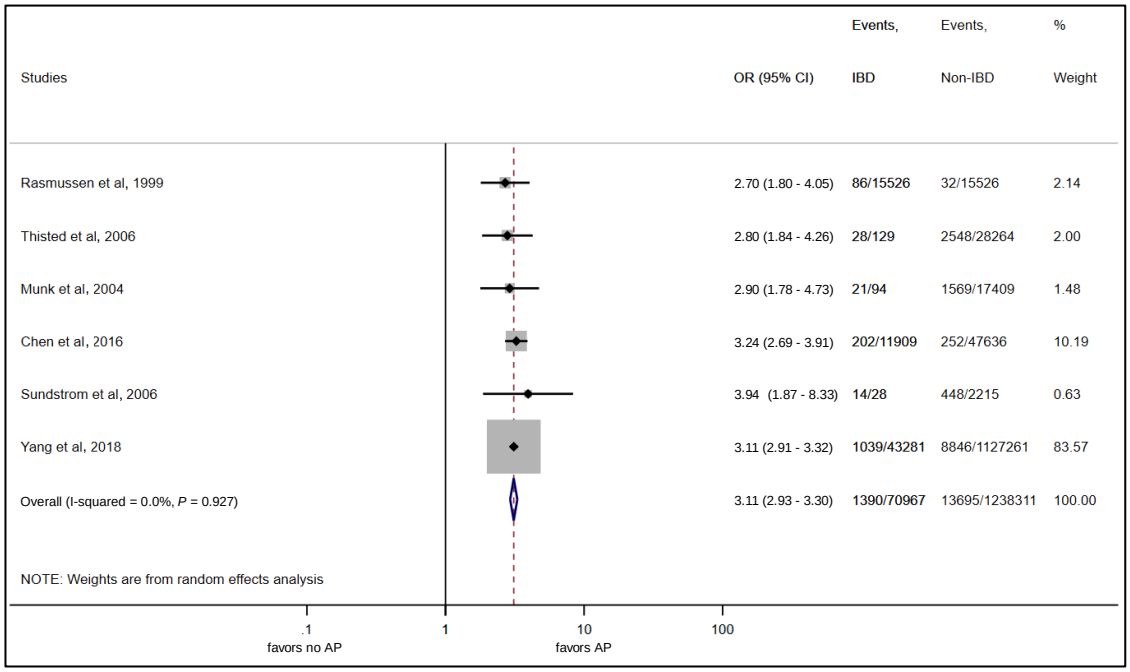

Figure 2. – Pooled Odds Ratios Of AP In IBD (vs. Non-IBD Population)

Dots represent individual odds ratios (ORs) and lines represent 95% confidence intervals (CI). The grey boxes denote relative study weights using random-effects models with the DerSimonian-Laird estimation (Dersimonian and Laird 1986). The blue diamond represents the pooled odds ratio and CI. *Based on (Tel et al. 2020)*

4.1.3. Analysis of the odds of AP in CD and UC

Of the six eligible studies, we could create contingency tables of AP events broken down to CD and UC subpopulations individually, therefore, making them eligible for subgroup analysis. This analysis found the pooled OR of AP in CD patients to be 4.12 (95% CI, 3.75–4.54; $I^2 = 0.0\%$), which was significantly higher than in UC patients (OR, 2.61; 95% CI, 2.40–2.83; $I^2 = 0.0\%$; $P < .0001$; **Figure 3.**). The I^2 statistics also showed no significant heterogeneity.

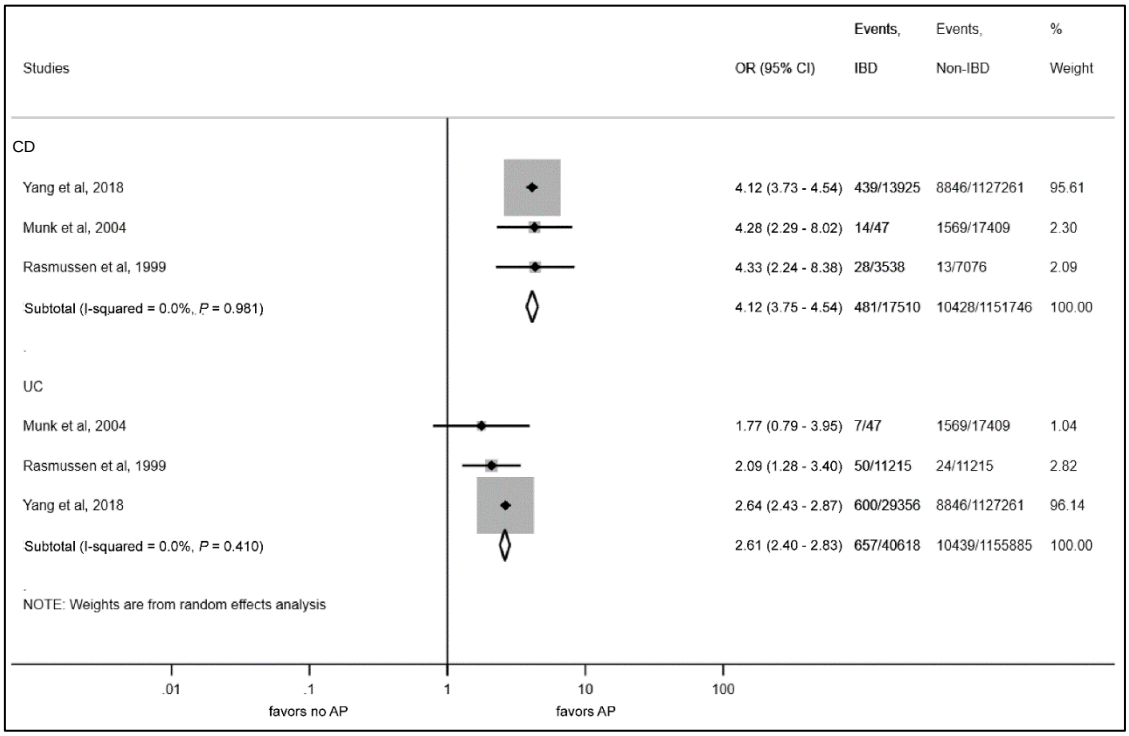

Figure 3. – Pooled Odds Ratios Of AP In CD And UC

Dots represent individual odds ratios (ORs) and lines represent 95% confidence intervals (CI). The grey boxes denote relative study weights using random-effects models with the DerSimonian-Laird estimation (Dersimonian and Laird 1986). Blue diamonds represent the pooled odds ratio and CI. *Based on (Tel et al. 2020)*

4.1.4. Analysis of the annual incidence of AP in IBD

In total four studies reported the incidence rates of AP among IBD patients as events/person-years (PY) or equivalent. Of the four observational studies, two were prospective and two were retrospective cohort studies. Two of these four were also included in the previous analysis (Chen Y. T. et al. 2016; Rasmussen et al. 1999). Three

studies reported the annual incidences of AP in all IBD patients (ie. both CD and UC patients), while one reported only in UC patients. Altogether, the included studies covered 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%), in other words, 210 AP cases per 100,000 PYs (95% CI, 84–392 cases /100,000 PY). The forest plot of the analysis is shown in **Figure 4**. The I^2 statistics detected a significant heterogeneity (I^2 = 98.66 %, P < .001) however, due to the low number of studies, the source of heterogeneity could not be investigated by any further subgroup analysis.

The reported incidences of AP in only UC patients were 0.061% (Rasmussen et al. 1999), 0.153% (Kim et al. 2017), and 0.282% (Chen Y. T. et al. 2016), respectively. The annual incidence of AP in CD in the studies was somewhat higher, 0.116% (Rasmussen et al. 1999) and 0.388% (Chen Y. T. et al. 2016), however, since only these two studies reported the incidences for CD, a subgroup analysis could not be made.

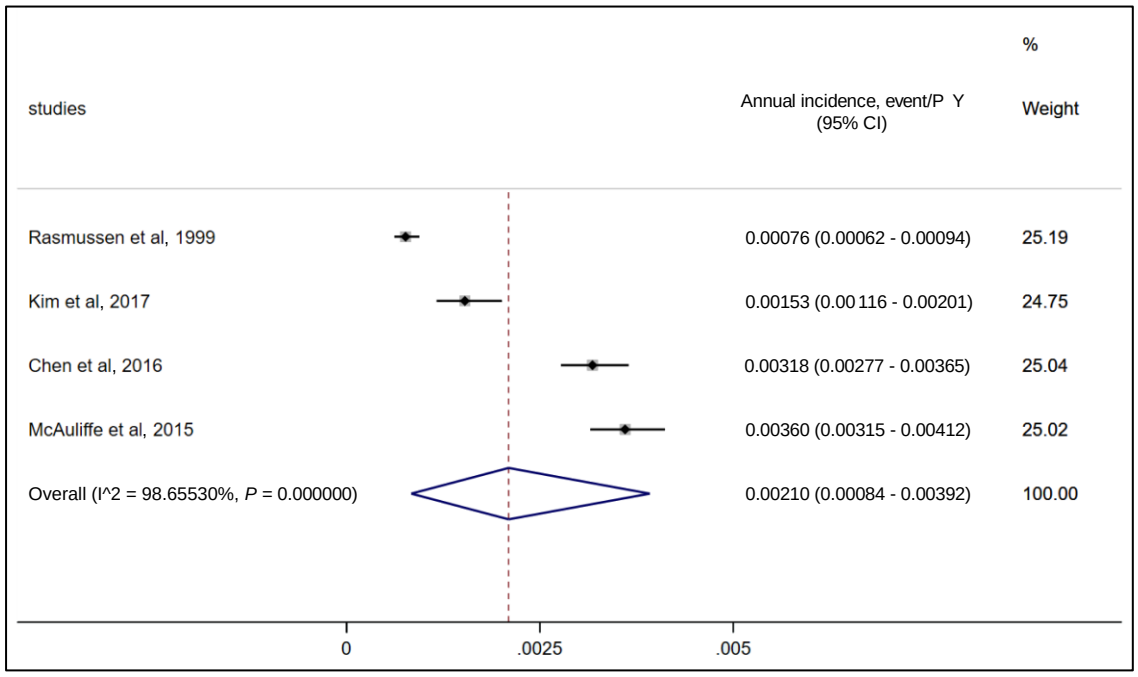

Figure 4. – **The pooled annual incidences of acute pancreatitis in IBD**

Dots represent individual annual incidences (as events/person-years (PY)) and lines represent 95% confidence intervals (CI). The grey boxes denote study weights using random-effects models with the DerSimonian-Laird estimation (Dersimonian and Laird 1986). The blue diamond represents the pooled annual incidence and CI. *Based on (Tel et al. 2020)*

4.1.5. Quality assessment of data

Quality assessments of the studies were made using the Quality In Prognosis Studies (QUIPS) tool, in compliance with the Cochrane Prognosis Methods Group recommendations (Hayden et al. 2013; Iorio et al. 2015).

In the first analysis (of the ORs), the overall quality of the included studies was high: the risk of bias (RoB) was judged to be low in five and moderate in one of the six articles (**Table 6/A**). The *Study participation*, *Outcome* (AP) *measurement*, and *Statistical analysis & reporting* domains were found to have low RoB in all included studies. The *Study attrition* domain was assessed only in the prospectively recruiting studies (Chen Y. T. et al. 2016; Rasmussen et al. 1999; Sundstrom et al. 2006) and concluded in moderate RoB in all three. One study had moderate RoB in the *Measurement of the prognostic factor* (IBD) domain, while two studies in *Study confounding* domains, respectively; all other studies had low RoB in these domains. To examine small study effects we used the visual assessment of a funnel plot since tests for funnel plot asymmetry are not advised in analyses with fewer than ten studies (Sterne et al. 2011). The visual assessment of the outcomes of the six studies on a funnel plot suggested no serious small study effects.

In the second analysis (of the annual incidences), the overall quality of the included articles was moderate: the RoB was judged to be low in one, and moderate in three of the four studies (**Table 6/B**). The *Measurement of the prognostic factor* (IBD) and *Statistical analysis* domains had low RoB in all four studies, while the *Study participation* and the *Study confounding* domains were judged to be low once and moderate thrice, respectively. The *Study attrition* domain was assessed only in the prospective studies and concluded in moderate RoB in both cases. The *Outcome* (AP) *measurement* domain was of moderate RoB in one study and low in the other three studies. Furthermore, due to the type of outcome measure (ie. event rates), the presence of publication bias could not be ruled out.

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Table 6. – Quality Assessment Of The Included Studies With The QUIPS Tool

Based on (Tel et al. 2020)

A) Quality Assessment Of The Studies In The Analysis Of The Odds Of AP In IBD.

Authors	Domains						Overall judgment (RoB)
	1. Study participation	2. Study attrition	3. IBD measurement	4. AP measurement	5. Study confounding	6. Statistical analysis & reporting	
Chen Y.T. et al, 2016	Low	Moderate	Low	Low	Low	Low	**Low**
Munk et al, 2004	Low		Low	Low	Low	Low	**Low**
Rasmussen et al, 1999	Low	Moderate	Low	Low	Moderate	Low	**Moderate**
Sundstrom et al, 2006	Low	Moderate	Low	Low	Low	Low	**Low**
Thisted et al, 2006	Low		Moderate	Low	Low	Low	**Low**
Yang et al, 2018	Low		Low	Low	Moderate	Low	**Low**

B) Quality Assessment Of The Studies In The Analysis Of The Annual Incidences Of AP In IBD

Authors	Domains						Overall judgment (RoB)
	1. Study participation	2. Study attrition	3. IBD measurement	4. AP measurement	5. Study confounding	6. Statistical analysis & reporting	
Kim et al, 2017	Moderate		Low	Moderate	Moderate	Low	**Moderate**
McAuliffe et al, 2015	Moderate		Low	Low	Moderate	Low	**Moderate**
Chen et al, 2016	Low	Moderate	Low	Low	Low	Low	**Low**
Rasmussen et al, 1999	Moderate	Moderate	Low	Low	Moderate	Low	**Moderate**

In summary, with a comprehensive literature search, we identified 8 studies that could be used to describe the risk of AP in patients with IBD. The meta-analysis of six studies yielded an OR of 3.11 for all types of IBD, while a subgroup analysis had an OR of 4.12 for CD and an OR of 2.61 for UC. Also, pooling the reported incidence rates from four studies suggested that in 10,000 IBD patients 21 AP cases are to be expected annually. 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 insufficient number of studies and lack of corresponding data in the included studies. However, it was clear from some of the evaluated studies (Bermejo et al. 2008; Chaparro et al. 2013; Teich et al. 2016; Zabala-Fernandez et al. 2011) 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

To explore the pancreatic effects of thiopurines we treated animals *in vivo*, as well as mouse pancreatic ductal segments and acinar cells *ex vivo* with thiopurines *(Tél et al. 2023)***.** We used 2-3 months old C57BL6 mice, with a gender ratio of 1:1 in all experiments. To test both therapeutic and toxic concentrations of thiopurines, we choose 1 and 10 μg/mL *ex vivo* concentrations and the 1.5 and 15 mg/kg *in vivo* doses for our experiments, based on previous pharmacokinetic studies (Lewis 1996; Voogd 1989a). After the oral treatment, mice were selected either for further experiments or immediate pancreas collection and cell isolation, or mouse pancreatic ductal organoids were generated

4.2.1. AZA treatment increases pancreatic damage in the early phase of cerulein-induced pancreatitis in mice

As previously no preclinical model of TIP was described, we aimed to set up an *in vivo* mouse model. To mimic the clinical situation, mice received a daily dose (150 μL) of 1.5 mg/kg AZA (a conventional human daily dose) through a 22G gastric feeding needle for 1 week. The control (sham) group received sterile physiologic saline (PS) doses. As shown earlier (**Figure 4** in section **4.1.4**), the expected incidence of AZA-induced AP in humans is below 0.3% and, accordingly, no spontaneous pancreas injury or pancreatitis was observed in the AZA-treated mice (**Figures 5 and 6/A-C**).

Then, to test whether AZA treatment modifies the outcomes of cerulein-induced model, moderate acute pancreatitis was induced in both AZA-treated and sham animals by intraperitoneal (IP) 8 x 50 μg/kg cerulein (or in the case of control mice, PS) injections. In rodents, the repeated IP administration of the secretagogue cerulein induces diffuse pancreatic necrosis with a subsequent biphasic elevation in the serum amylase levels (Yamaguchi et al. 1989). 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 (Gress et al. 1994).

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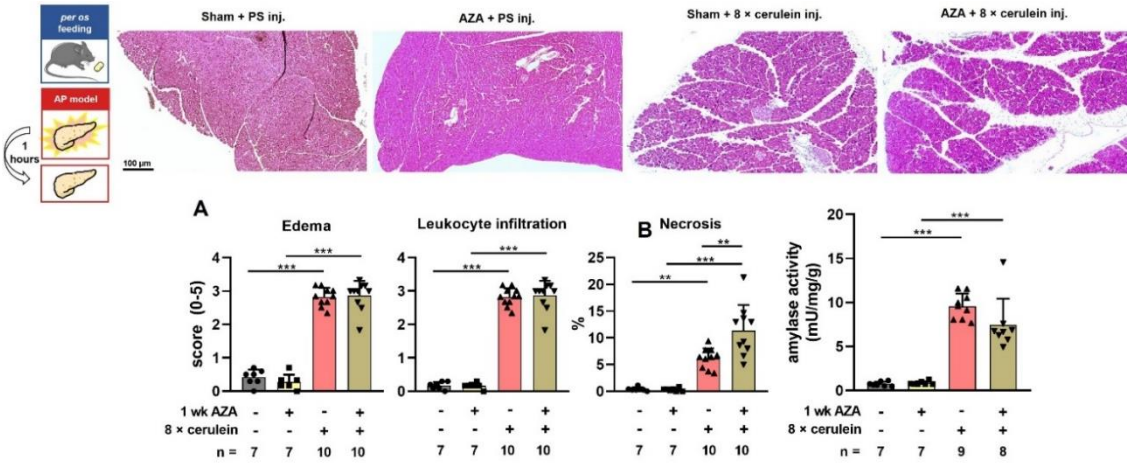

Figure 5. – AZA increases early pancreatic damage in cerulein-induced pancreatitis

Mice received either 1.5 mg/BWkg daily oral doses of azathioprine (AZA) or *per os* physiologic saline (Sham) for one week, followed by induction of moderate experimental pancreatitis through the administration of 8 × cerulein IP injections. Controls received **8** × IP physiologic saline (PS) injections. Animals were sacrificed 1 hour after the last cerulein injection.
A, Representative hematoxylin-eosin (H-E) stained slides of formaldehyde-fixed pancreata captured with 100× total magnifications and corrected for background illumination. Images were captured with a Zeiss Axio Imager.M2 microscope. Scale bar: 100 µ. **B**, Histology scores and proportion of necrosis of H-E slides in moderate experimental pancreatitis (n = 7, sham + PS; n =7, AZA+ PS; n= 10, sham + cerulein; n =10, AZA + cerulein groups). Each slide was analyzed in at least 3 different fields of view, by three independent observers. **C**, Activity of serum amylase in moderate experimental pancreatitis (n = 7, sham + PS; n =7, AZA+ PS; n= 9, sham + cerulein; n =8, AZA + cerulein groups.). The data are shown as mean ± SD, *P< 0.05, ***P < .001, one-way ANOVA with Sidak's multiple comparisons tests. *AZA = azathioprine, PS = physiologic saline. Based on (Tél et al. 2023)*

To examine both phases of pancreatic cell injury, first, mice were terminated 1 hour after the last cerulein injection. Interestingly, the extent of interstitial edema, leukocyte infiltration, average pancreas weight/body weight ratio, or serum amylase activity was not significantly different in the AZA + cerulein-co-treated group compared to cerulein-only controls. However, the extent of necrosis was found to be significantly higher in the AZA + cerulein-treated mice (**Figure 5**). 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.

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, and surprisingly, although the extent of necrosis increased in both cerulein-only and AZA+cerulein-co-treated groups, the previously observed significant differences

disappeared and neither of the other histological scores nor the average serum amylase activity differed significantly (**Figures 6**).

Taken together we created the first *in vivo* mouse model of TIP and utilizing this model we confirmed the clinical suspicion 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.

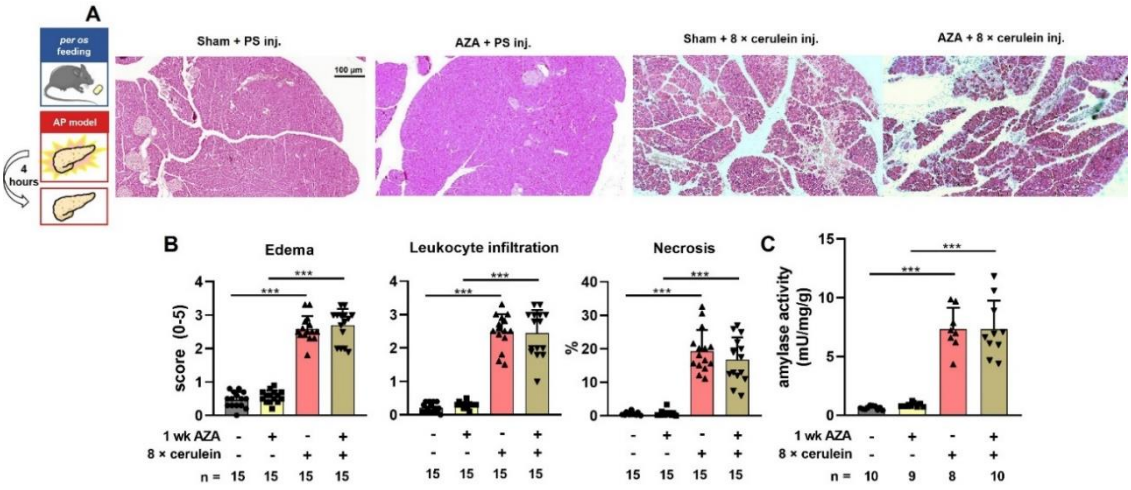

Figure 6. – AZA does not alter the overall severity of cerulein-induced pancreatitis

Mice received either 1.5 mg/BWkg daily oral doses of azathioprine (AZA) or *per os* physiologic saline (Sham) for one week, followed by induction of moderate experimental pancreatitis through the administration of 8 × cerulein IP injections. Controls received **8** × IP physiologic saline (PS) injections. Animals were sacrificed 4 hours after the last cerulein injection.

A Representative hematoxylin-eosin (H-E) stained slides of formaldehyde-fixed pancreata captured with 100× total magnifications and corrected for background illumination. Images were captured with a Zeiss Axio Imager.M2 microscope. Scale bar: 100 µ. **B**, Histology scores and percentage of necrosis of H-E slides in moderate experimental pancreatitis (*n = 15, in all groups*). Each slide was analyzed in at least 3 different fields of view, by three independent observers. C, Activity of serum amylase in moderate experimental pancreatitis (*n = 10, sham + PS; n =9, AZA+ PS; n= 8, sham + cerulein; n =10, AZA + cerulein groups. The lower samples size is due to the inability to analyze several samples because of serious hemolysis*). The data are shown as mean ± SD, **P*< 0.05, ****P* < .001, one-way ANOVA with Sidak's multiple comparisons tests. *AZA = azathioprine, PS = physiologic saline. Based on (Tél et al. 2023)*

4.2.2. AZA does not alter the viability of pancreatic acinar cells in mice

A well-described hallmark of AP with different etiologies – including DIAP – is the premature activation of digestive enzymes due to pancreatic acinar cell injury and necrosis (Gerasimenko et al. 2011; Lee P. J. and Papachristou 2019). Therefore, we investigated the effects of AZA on acinar viability and functions. First, freshly isolated primary mouse acinar cells were incubated *ex vivo* for 60 minutes with clinically relevant (0.1-1 μg/mL) and toxic (10-1000 μg/mL) concentrations of AZA, and cell viability was measured. In line with our findings observed in the *in vivo* TIP model, none of the applied AZA concentrations were found to cause any significant change in the proportion of the living acinar cells, evaluated with a luminescence-based ATP assay (CellTitre^{Glo} 3D) compared to the positive control cerulein (**Figure 7**).

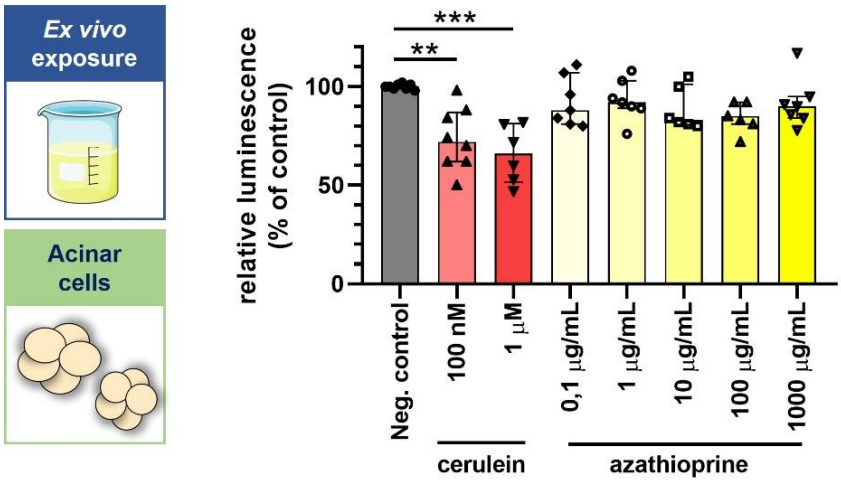

Figure 7. – Azathioprine Does Not Alter The Viability Of Mouse Pancreatic Acinar Cells

The proportion of viable mouse pancreatic acinar cells was measured after incubation with different concentrations of either AZA or cerulein for 60 minutes in 48 well plates *in vitro.* The relative luminescence of wells was measured with CellTitre Glo 3D luminometric assay. Recorded luminescence of wells relative to control wells is shown as mean ± SD, n = 6-8 wells, measured in triplicates, from at least 3 animals for each group; ** $P < .01$, ***$P < .001$, one-way ANOVA with Sidak's multiple comparisons tests. *Based on (Tél et al. 2023)*

Next, we compared the effects of both acute (*ex vivo*) and chronic (*in vivo*) AZA exposure on cerulein-induced acinar cell death, as AZA is known to induce apoptosis in immune cells via a caspase-9-dependent pathway (Tiede et al. 2003). To do so, the proportions of viable, apoptotic, and necrotic/late apoptotic acinar cells were determined after *ex vivo* incubation with 1 μg/mL AZA and/or 100 nM Cerulein for 1 h at 37ºC, using

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a fluorescence-based apoptosis/necrosis detection kit. The same apoptosis-necrosis assay was also performed on acinar cells from previously *in vivo* AZA-treated (1 week of 1.5 mg/kg/die) mice.

Upon either the *ex vivo* AZA exposure, the chronic *in vivo* AZA treatment, or the AZA-cerulein co-stimulation, we observed no significantly altered proportions of living, necrotic, and apoptotic acinar cells compared to no treatment or cerulein-only treatment, which further confirmed the previous results (**Figure 8/A-B**).

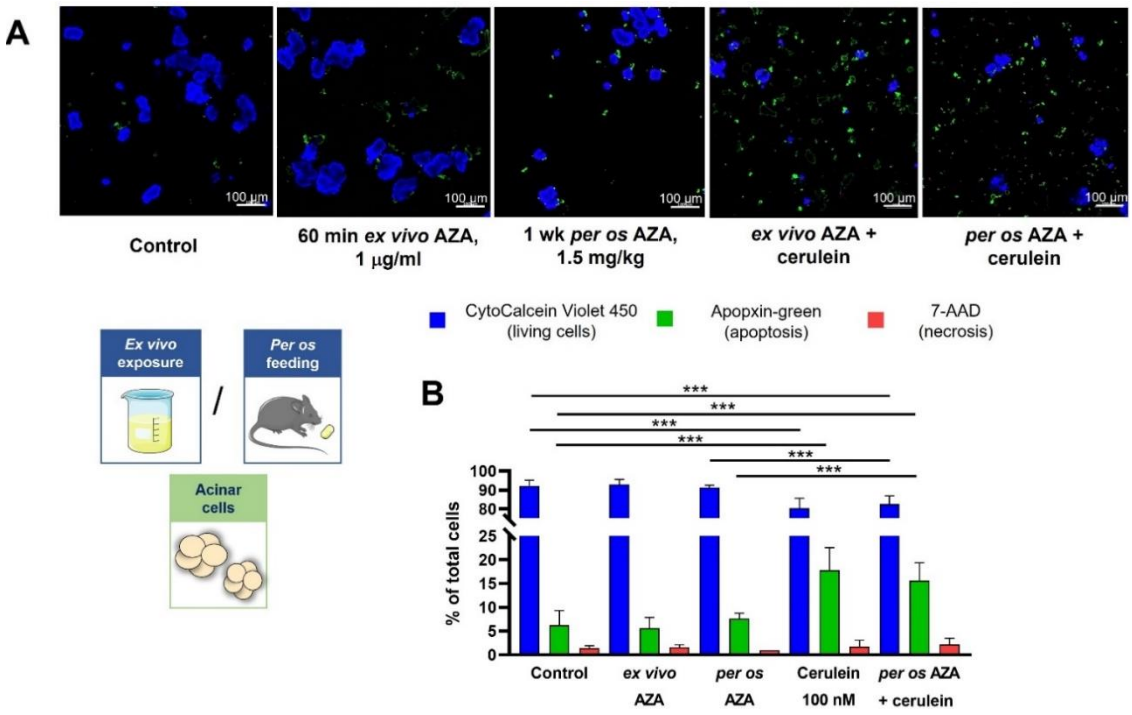

Figure 8. – Azathioprine Does Not Alter The Viability Of Mouse Pancreatic Acinar Cells

Cell death and viability were assessed on acinar cells from azathioprine (AZA) treated or control animals, with or without 60 minutes of incubation with 100 nM cerulein and/or AZA in 8-well chamber slides, using a fluorescent staining kit. **A**, Representative fluorescent images of apoptosis-necrosis staining of acinar cells, captured with an LSM880 confocal microscope using a 40X oil immersion objective. **B**, Average proportion of living, necrotic and apoptotic cells in the different treatment conditions, shown as a percentage of total cells (n = 5 fields of view, in total ca. 1500-2000 cells, from 2 animals per group). The data are shown as mean ± SD, ***$P < .001$, two-way ANOVA with Tukey's multiple comparisons tests. *Based on (Tél et al. 2023)*

4.2.1. AZA does not alter the Ca^{2+} homeostasis in the murine exocrine pancreas.

The majority of pancreato-toxic agents trigger acinar injury through a sustained elevation of $[Ca^{2+}]_i$ (Habtezion et al. 2019; Lee P. J. and Papachristou 2019), whereas AZA was also found to increase $[Ca^{2+}]_i$ in blood cells (Geiger et al. 2008). To test, whether AZA also influences the Ca^{2+} homeostasis in pancreatic acinar cells, the 100 nM carbachol-induced $[Ca^{2+}]_i$ changes were monitored with a Ca^{2+} sensitive fluorescent dye (FURA 2-AM) during the *ex vivo* perfusion of acinar cells with AZA-containing media. However, we could not observe significant changes either in the baseline $[Ca^{2+}]_i$ levels or in the response to 100 mM carbachol stimulation upon 10 minutes of perfusions with 1 μg/mL AZA (**Figure 9/A-C**). Similarly, we also couldn't observe any altered acinar Ca^{2+} signals after a 1 week-long *in vivo* AZA treatment (**Figure 9/A-C**) upon carbachol stimulation.

Next, to investigate the possible effects of AZA also on Ca^{2+}-dependent acinar functions, we calculated the proportion of released amylase in the supernatant of acinar cells. For this, 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 (**Figure 9/D).** 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 (**Figure 9/D)**. 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 (section 4.2.2 and **Figures 7 and 8**), nor the severity of cerulein-induced pancreatitis (section 4.2.1 and **Figures 5 and 6**).

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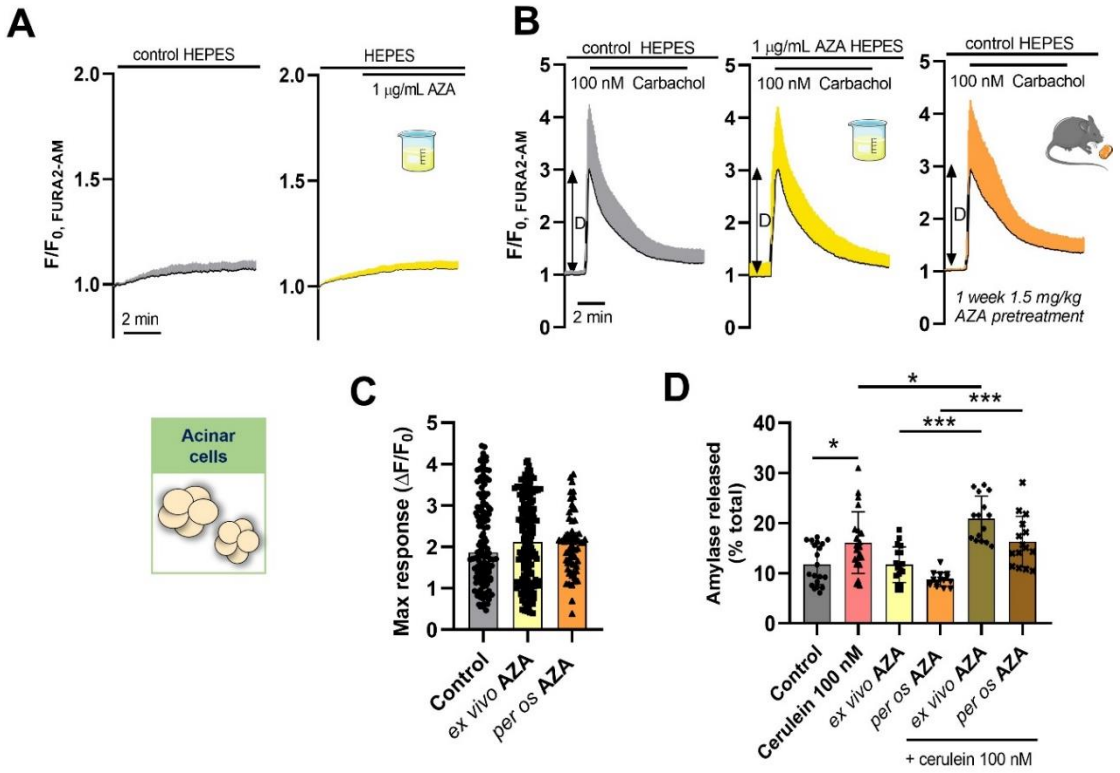

Figure 9. – Azathioprine Does Not Alter The Function Of Mouse Pancreatic Acinar Cells

For Ca^{2+} measurements, mouse pancreatic acinar cells, either from untreated animals or from *in vivo* AZA-treated animals, were *ex vivo* stimulated with carbachol, with or without a previous 10-minute long perfusion with AZA. The response to carbachol stimulation in the intracellular Ca^{2+} concentration was monitored by recording the normalized FURA2 fluorescence. **A**, Average traces of normalized FURA2 fluorescence intensity during normal conditions and AZA exposure of pancreatic acinar cells. **B**, Average traces of normalized FURA2 fluorescence intensity during carbachol stimulation of pancreatic acinar cells. The double-sided arrows mark how the maximal response values plotted on the C panel were calculated. **C**, The magnitude of the maximal Ca^{2+} response to carbachol stimulation. The data are shown as mean ± SD, $P = .076$, one-way ANOVA. **D**, Amylase release was measured after *in vitro* 60-minutes incubation of pancreatic cells in 48-well plates with cerulein or cerulein + AZA, and amylase activity of the supernatant and the total well after lysis of cells were measured with a colorimetric assay, in triplicates. The amylase release was measured also on acinar cells from *in vivo* AZA-treated mice. The proportions of released amylase of acinar cells are plotted (n = 15-20 wells, from 3 animals in each group). The data are shown as mean ± SD, $*P < .05$, $***P < .001$, one-way ANOVA with Sidak's multiple comparisons tests. *Based on (Tél et al. 2023)*

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

Pancreatic ductal cells secrete HCO_3^--rich, alkaline fluid, which was shown to play a major role in exocrine pancreatic homeostasis (Pallagi et al. 2014; Pallagi et al. 2022). As AZA was found not to affect acinar cells, we wanted to see whether it does affect ductal functions. For this, freshly isolated mouse pancreatic ductal segments were perfused with standard or 1 and 10 μg/mL AZA-containing solutions for 10 minutes, and the intracellular pH (pH_i) changes were monitored with a pH-sensitive fluorescent dye (BCEFC-AM) during so-called *alkaline* and *acid loading*, where ductal segments are exposed to and relieved from 20 mM NH_4Cl from the basolateral side, respectively.

Interestingly, the *ex vivo* 10-minute perfusion, both with 1 and 10 μg/mL AZA, significantly decreased the base flux values during recovery from alkaline loading compared to control base flux values (observed on the same ductal segment before the AZA treatment). This indicates that AZA impairs the luminal HCO_3^- transport (**Figure 10/A-B**). The base flux values during the recovery from an acid load were also significantly lower after the AZA treatment with both concentrations (**Figure 10/C**), which suggests that AZA also inhibits the activity of the basolateral transporters of pH_i regulation (ie. NBC and/or NHE). As neither of the base flux values showed significantly greater inhibition upon perfusion with higher AZA concentrations, we used only the – clinically more relevant – 1 ug/mL concentration in the further experiments.

Not only AZA, but the other two commercially available thiopurines (6-MP and 6-TG) were also reported to induce DIAP (Haber et al. 1986; Herrlinger et al. 2003), therefore, to test whether these thiopurines would also have an inhibitory effect on ductal HCO_3^- secretion, we performed the above-described experiments were also performed after perfusion of ductal segments with 1 μg/mL 6-MP and 6-TG, respectively. Similarly to AZA treatment, the base flux values during recovery from alkaline loading were significantly reduced by both 6-MP and 6-TG perfusions compared to controls (**Figure 10/A-C**). Furthermore, the inhibition of the base flux values in the recovery from acid loading, caused by either 6-MP or 6-TG, were both significantly higher than the inhibition caused by AZA (**Figure 10/A-C**).

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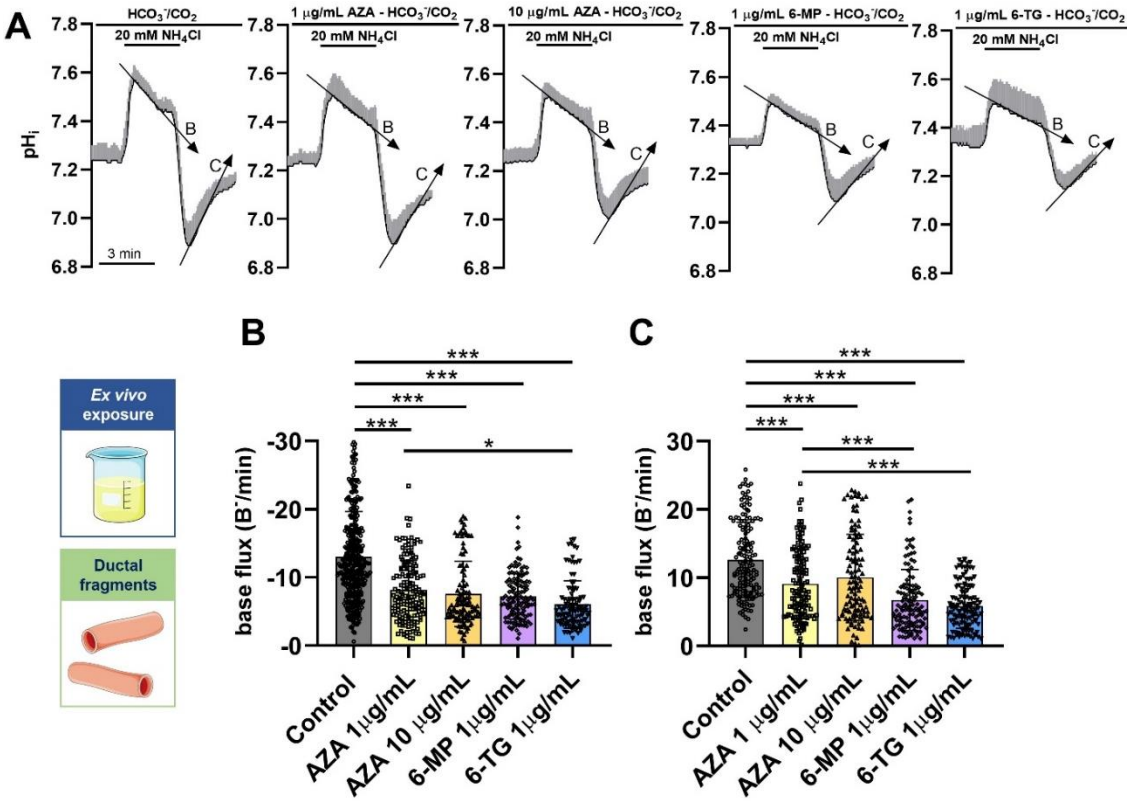

Figure 10. – *Ex vivo* Perfusion Of Mouse Pancreatic Ductal Segments With Thiopurines Inhibits HCO_3^- Secretion

Untreated mouse pancreatic ductal segments were stimulated *ex vivo* with 20 mM NH_4CL (controls) then they were perfused for 10 minutes with 1 μg/mL or 10 μg/mL azathioprine (AZA), or 1 μg/mL 6-mercaptopurine (6-MP) or 6-thioguanine (6-TG), and the NH_4CL stimulation was repeated. BCECF-AM fluorescence signals were recorded and converted into intracellular pH (pH_i) values **A**, The average traces (± SD) of intracellular pH, recorded during the NH_4Cl stimulation. Downward and upward-tilted arrows on the traces mark the slope of recoveries, which made the basis when calculating the base flux values displayed on B and C panels, respectively. **B-C,** The base flux values during recovery from alkaline (**B**) and acid loading (**C**). The number of animals (N), ductal segments (n), and regions of interest (ROIs) used in the experiments: 1 μg/mL AZA (N = 5, n = 15, ROIs = 157), 10 μg/mL AZA (N = 4, n = 12, ROIs = 111), 1 μg/mL 6-MP (N = 4, n = 12, ROIs = 129), or 1 μg/mL 6-TG (N = 4, n = 12, ROIs = 104). The data are shown as mean ± SD, **P* < .05, ****P* < .001, one-way ANOVA test with Sidak's multiple comparisons tests. *Based on (Tél et al. 2023)*

Then, to investigate whether the *ex vivo* observed effects of thiopurines on ductal secretion could also be seen in a clinically more relevant setup, mice received *in vivo* either therapeutic (1.5 mg/kg/die) or non-lethal toxic (15 mg/kg/die) AZA doses for one and four weeks, respectively. Also, another group of mice was selected to receive one of the three thiopurines for one week (1.5 mg/kg/die).

As expected, when conducting the above-described (*ex vivo*) experiments on ductal segments isolated from *in vivo* treated animals, the one-week oral AZA treatment also significantly impaired the base-flux values during recovery from alkalosis in both (1.5 and 15 mg/kg) doses (**Figure 11/A-B**). There was no significant difference between the effects of the therapeutic and toxic doses (**Figure 11/A-B**). These signs of inhibited luminal HCO_3^- secretion could also be observed after 4 weeks of AZA treatment, but the longer treatment time did not cause greater inhibition (**Figure 11**). The recovery from acid loads (which correlates with the activity of basolateral transporters) was also significantly inhibited compared to untreated mice and, interestingly, the lower (therapeutic) doses had significantly higher inhibitory effects than the higher toxic doses, regardless of the length of the treatment (**Figure 11/B-C**).

Animals treated for 1 week with 1.5 mg/kg daily doses of 6-MP and 6-TG also recapitulated the effects of the AZA treatment: both recoveries, ie. from alkaline or acid loads, were significantly decreased, and there was no significant difference in the magnitude of inhibition between the three thiopurines (**Figure 12/A-C**). 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 our observations, we also measured the total *in vivo* pancreatic juice secretion rate of anesthetized mice by the cannulation of the main pancreatic duct, after 1 week of *in vivo* treatment with either 1.5 mg/kg/die AZA or 6-MP. In harmony with the previous findings, the secretion rates were significantly reduced in both AZA and 6-MP treated mice compared to sham controls (**Figure 12/D**), supporting that thiopurine's inhibitory effects on PDEC also have an impact *in vivo*.

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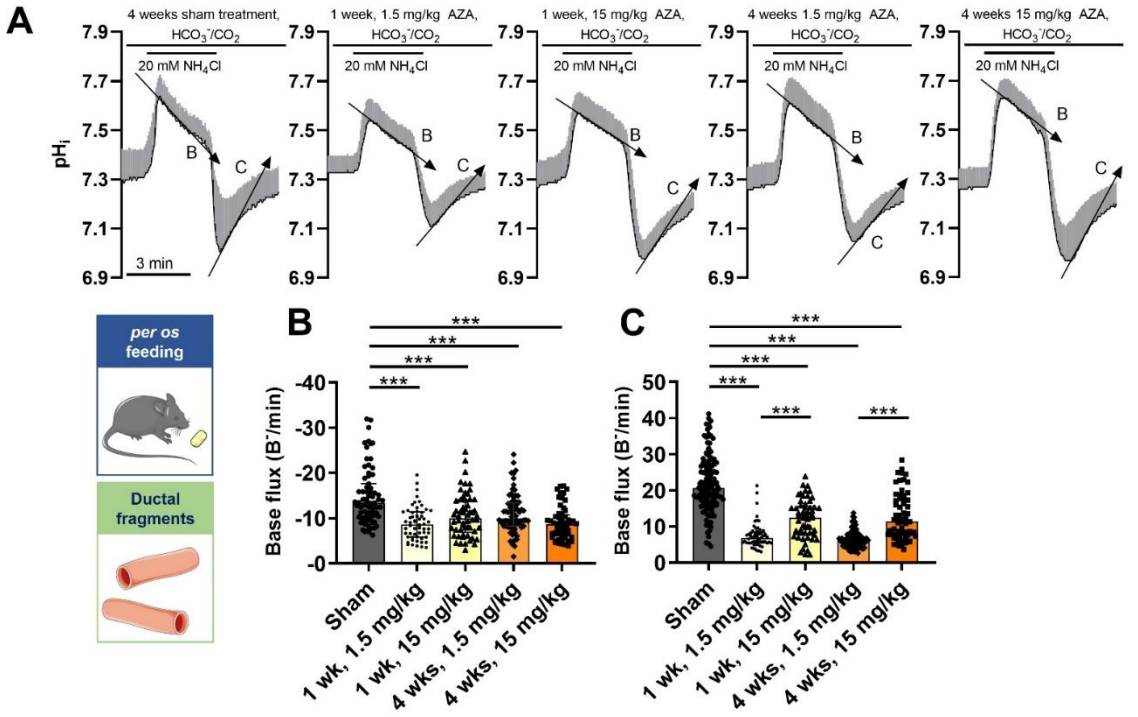

Figure 11. – *In vivo* Treatment With Azathioprine Inhibits Mouse Pancreatic Ductal HCO_3^- Secretion

Mice received either 1.5 or 15 mg/kg daily oral doses of azathioprine (AZA) or 50 mL/kg/day physiologic saline (Sham). Isolated pancreatic ductal segments were stimulated *ex vivo* with 20 mM NH_4CL while BCECF-AM fluorescence signals were recorded and converted into intracellular pH (pH_i) values. **A**, The average traces (± SD) of pH, recorded during the NH_4Cl stimulation of ductal segments from only AZA-treated-treated animals. **B-C,** The base flux values during recovery from alkalosis (**B**) and acidosis (**C**), after treatment with different doses and durations of thiopurine, respectively. The number of animals (N), ductal segments (n), and regions of interest (ROIs) used in the experiments **A-C**: 4 weeks physiologic saline (N = 4, n = 12, ROIs = 71), 1 week 1.5 mg/kg AZA (N = 3, n = 10, ROIs =56), 1 week 15 mg/kg AZA (N = 3, n = 11, ROIs =58), 4 weeks 1.5 mg/kg AZA (N = 4, n = 10, ROIs =74), 4 weeks 15 mg/kg AZA (N = 3, n = 10, ROIs =57). The data are shown as mean ± SD, ***P < .001, one-way ANOVA with Sidak's multiple comparisons tests. 6-MP = 6-mercaptopurine, 6-TG = 6-thioguanine. *Based on (Tél et al. 2023)*

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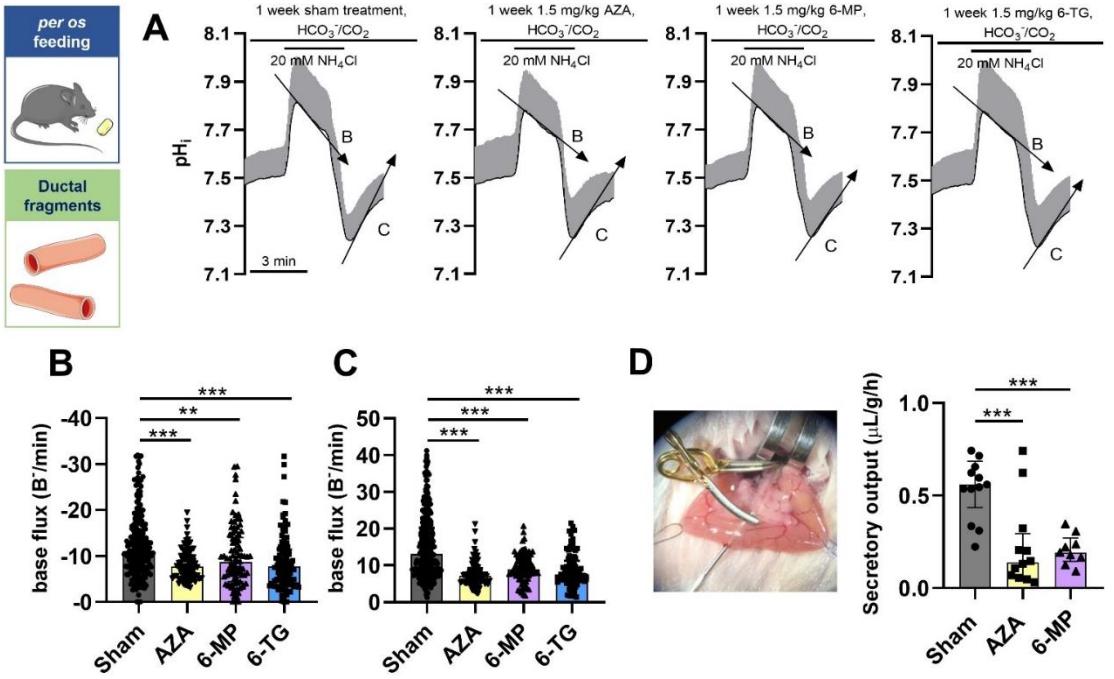

Figure 12. – *In vivo* Treatment With Thiopurines Inhibit Mouse Pancreatic Ductal HCO_3^- Secretion And Total Pancreatic Juice Secretion

Mice received either 1.5 mg/kg daily oral doses of either azathioprine (AZA), 6-mercaptopurine (6-MP), or 6-Thioguanine (6-TG), or 50 mL/kg/day physiologic saline (Sham). Isolated pancreatic ductal segments were stimulated *ex vivo* with 20 mM NH_4CL while BCECF-AM fluorescence signals were recorded and converted into intracellular pH (pH_i) values. **A**, The average traces (± SD) of pH, recorded during the NH_4Cl stimulation of ductal segments from thiopurine-treated animals. **B-C,** The base flux values during recovery from alkalosis (**B**) and acidosis (**C**), after treatment with different doses and durations of thiopurine, respectively. The number of animals (N), ductal segments (n), and regions of interest (ROIs) used in the experiments **A-C**: 1-week physiologic saline (N = 4, n = 21, ROIs = 121), 1.5 mg/kg AZA (N = 3, n = 13, ROIs =89), 1.5 mg/kg 6-MP (N = 3, n = 16, ROIs = 104) or 1.5 mg/kg 6-TG (N = 3, n = 17, ROIs = 90). **D**, Animals treated for 1 week either with saline (N = 13), 1.5 mg/kg AZA (N = 12), or 6-MP (N = 9) were anesthetized with ketamine-xylazine and the secretin stimulated *in vivo* pancreatic juice secretion was measured by canulation of the main pancreatic duct and quantified as µL/body weight (g)/hour. The image depicts the canulation and clipping of the main pancreatic duct during the operation procedure. The data are shown as mean ± SD, ** $P < .01$, ***$P < .001$, one-way ANOVA with Sidak's multiple comparisons tests. 6-MP = 6-mercaptopurine, 6-TG = 6-thioguanine. *Based on (Tél et al. 2023)*

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

Previously pancreatitis-inducing agents, such as bile acids or non-oxidative ethanol metabolites, were demonstrated to inhibit the pancreatic ductal HCO_3^- secretion via sustained intracellular Ca^{2+} elevation (Maleth et al. 2015; Venglovecz et al. 2008). 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 (**Figure 13**). This suggests that AZA doesn't have a major influence on ductal Ca^{2+} homeostasis either.

Also, many substances commonly involved in the development of pancreatitis, can inhibit pancreatic ductal CFTR functions and, therefore, increase the severity of experimental pancreatitis (Maleth et al. 2015; Maleth et al. 2011; Pallagi et al. 2011). After confirming that AZA impairs pancreatic ductal HCO_3^- secretion without altering the Ca^{2+} homeostasis, we wanted to test, whether these effects of AZA are dependent on CFTR functions.

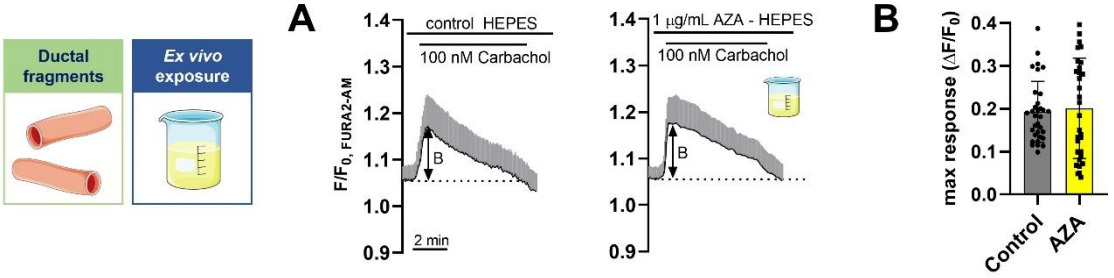

Figure 13. – Azathioprine Does Not Alter The Pancreatic Ductal Ca^{2+} homeostasis in mice

For the measurement of ductal Ca^{2+} signals, ductal segments from untreated animals were isolated and stimulated with 100 nM Carbachol with a prior *ex vivo* perfusion with 1 μg/mL azathioprine (AZA), and without (controls). **A**, Average traces of normalized FURA2 fluorescence intensity during carbachol stimulation are shown. The double-sided arrows mark how the maximal response values plotted on the B panel were calculated. **B,** The magnitude of the maximal Ca^{2+} response to carbachol stimulation. 33 ROIs on 10 ductal segments, isolated from 3 animals were used per group. The data are shown as mean ± SD, P = .073, unpaired t-test. Ductal segments isolated from untreated mice were either incubated *in vitro* for 60 minutes with AZA, as well as controls, and ductal segments from *in vivo* AZA-treated mice were incubated for 60 minutes in normal culture media. CFTR was labeled with fluorescent antibodies and images were captured with an LSM880 confocal microscope using a 40X oil immersion objective. *Based on (Tél et al. 2023)*

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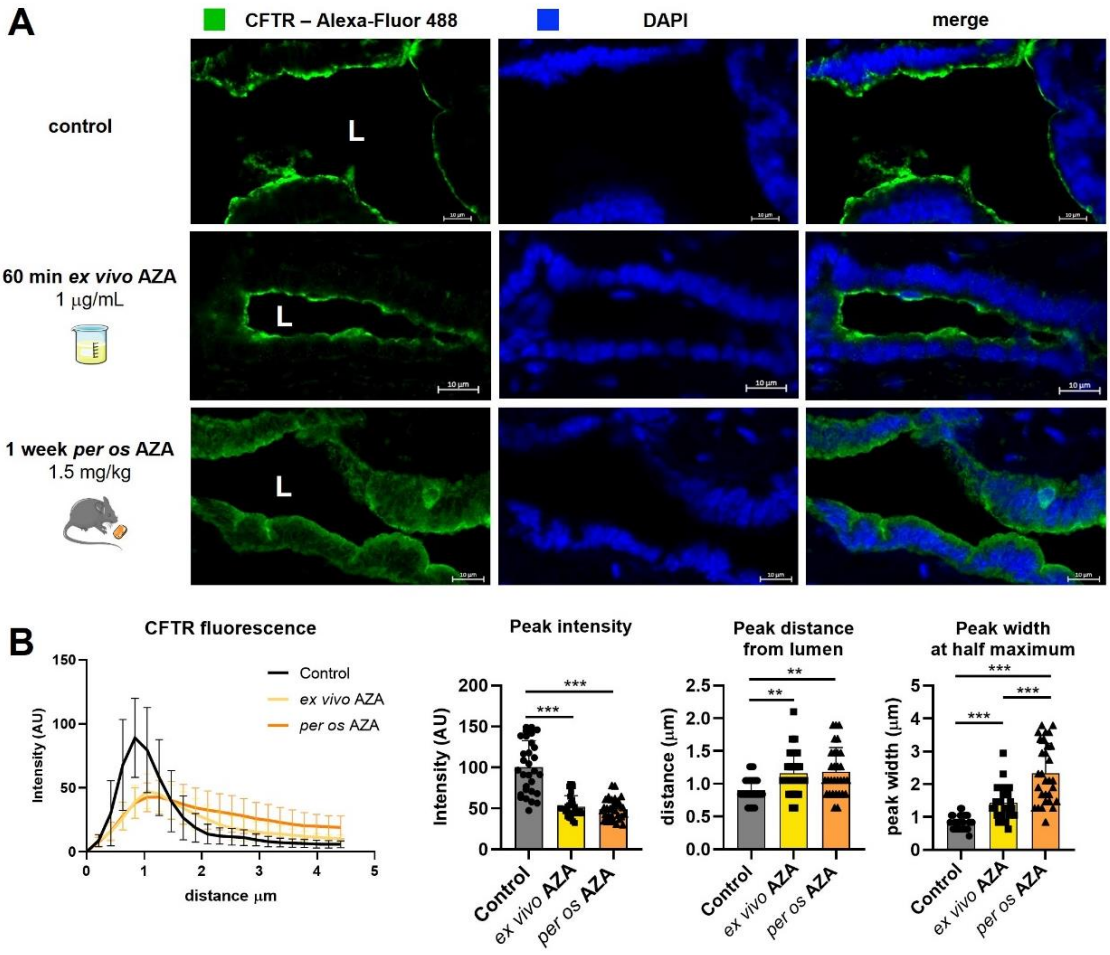

Figure 14. – Azathioprine Impairs Pancreatic Ductal CFTR Expression

A, Representative images of immunofluorescent staining of cystic fibrosis transmembrane conductance regulator (CFTR) on mouse pancreatic ductal fragments. The white "L" marks the luminal cavity. **D**, Average intensity profiles of luminal CFTR fluorescence, average peak fluorescence intensity, average peak distance from the lumen, and average peak width at half-maximal intensity (N = 5 ducts per group, n = 30 profiles). The data are shown as mean ± SD, **P < .01, ***P < .001, one-way ANOVA with Sidak's multiple comparisons tests. AZA = azathioprine. *Based on (Tél et al. 2023)*

To test this, the effects of AZA treatment on the cellular localization of CFTR were examined using immunofluorescent staining. Interestingly, both the 60 minutes *ex vivo* incubation of ductal segments with 1 μg/mL of AZA and the 1 week of *in vivo* oral treatment with 1.5 mg/kg AZA, indeed significantly altered the predominantly apical distribution of CFTR and triggered the retention of the protein into the cytosol (**Figure 14**). Next, we wanted to see, whether the altered apical CFTR localization also manifests in an altered CFTR function. For this, the CFTR-dependent Cl^- secretion of the ductal epithelia was examined by determining the maximal MQAE intensity signal in ductal

segments upon the withdrawal of the extracellular Cl^- (from 1 to 0 mM), both in the absence and presence of the CFTR inhibitor CFTR(inh)-172.

In these experiments, the maximal intensity signal – indicating the luminal Cl^- transport – was significantly impaired upon the 10-minute *ex vivo* perfusion with 1 μg/mL AZA compared to untreated controls, similar to the inhibition caused by the selective CFTR inhibitor (**Figure 15**). The 1-week-long 1.5 mg/kg AZA treatment also significantly impaired the luminal Cl^- efflux, however, the effect of the *ex vivo* AZA perfusion was significantly greater than the *in vivo* treatment (**Figure 15**). 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*.

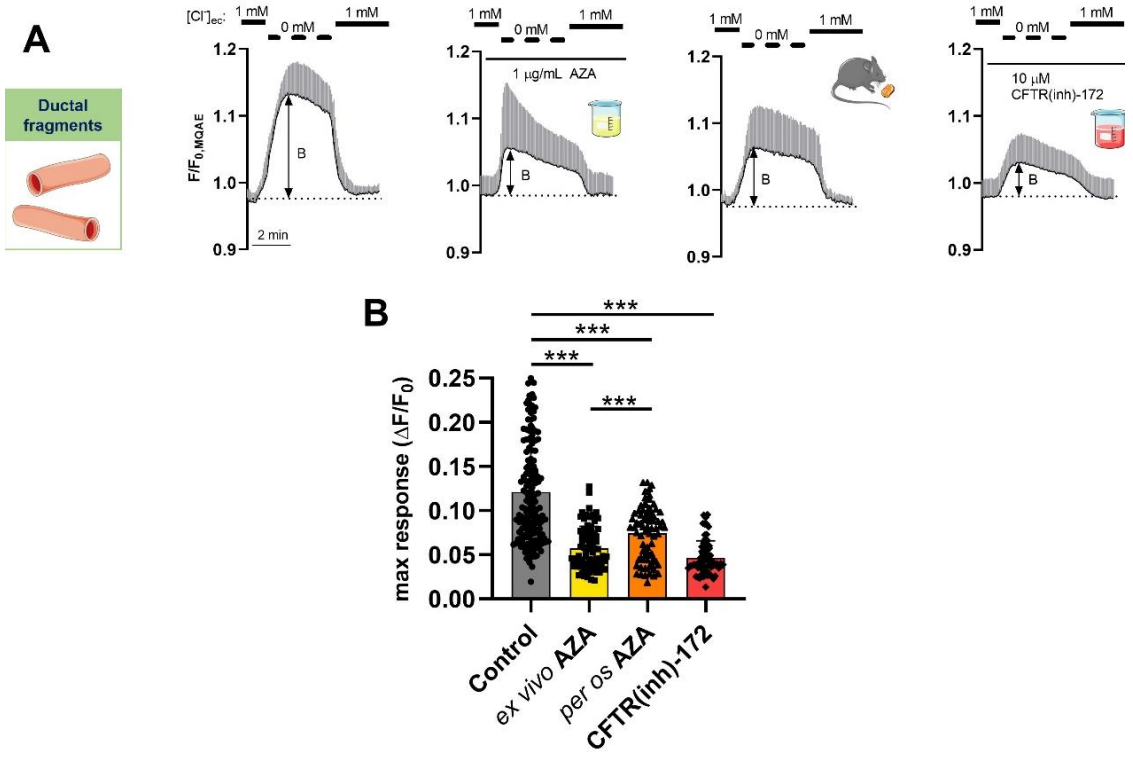

Figure 15. – Azathioprine Impairs Pancreatic Ductal Cl^- Secretion

A, The average traces of normalized MQAE fluorescence intensity (± SD) during extracellular Cl^- depletion are shown. The double-sided arrows mark how the maximal response values plotted on the B panel were calculated. **B**, Magnitude of Cl^- response to Cl^- depletion measured in different conditions. The number of animals (N), ductal segments (n), and regions of interest (ROIs) used in the experiments: controls (N = 4, n = 19, ROIs = 174) 1 μg /mL AZA (N = 4, n = 15, ROIs = 83), 1 week 1.5 mg/kg AZA (N = 4, n = 13, ROIs = 75) 10 mM CFTR(inh)-172 (N = 3, n = 12, ROIs = 72). The data are shown as mean ± SD, ***$P < .001$, one-way ANOVA with Sidak's multiple comparisons tests. AZA = azathioprine. *Based on (Tél et al. 2023)*

4.2.3. AZA disrupts CFTR plasma membrane retention in PDEC by inhibiting RAC1 and ezrin in mice

The tethering of CFTR to the plasma membrane by a scaffolding protein complex is well-described (Short et al. 1998). This is comprised of NHERF family members and the actin cytoskeleton adaptor protein, ezrin (Moniz et al. 2013; Moyer et al. 1999), a protein that was also shown to play a role in the Protein-kinase-A-mediated regulation of CFTR (Sun et al. 2000). As previously AZA was suggested to inhibit RAC1 (Seinen et al. 2016; Tiede et al. 2003), an activator of ezrin (Moniz et al. 2013), investigating this pathway promised a better understanding of the mechanism of the AZA-induced disturbance in CFTR localization and functions.

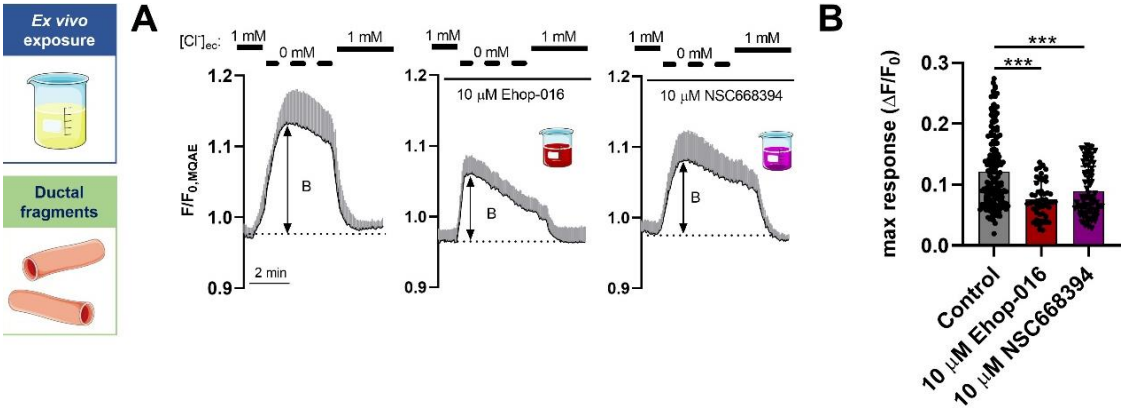

Figure 16. –RAC1 inhibition alters pancreatic ductal CFTR functions recapitulationg the effects of azathioprine

A-B, Ductal segments from untreated mice were perfused *ex vivo* either with 10 mM RAC1 inhibitor (Ehop-016) or 10 mM ezrin inhibitor (NSC668394). The Cl^- secretion was measured upon the withdrawal of extracellular Cl^- by determining the amplitude of change in the normalized MQAE fluorescence intensity. **A**, The average traces of normalized MQAE fluorescence intensity (± SD) during extracellular Cl^- depletion are shown. The double-sided arrows mark how the maximal response values plotted on the B panel were calculated. **B**, Magnitude of Cl^- response to Cl^- depletion measured in different conditions. The number of animals (N), ductal segments (n), and regions of interest (ROIs) used in the experiments: controls (N = 4, n = 19, ROIs = 174) 10 mM Ehop-016 (N = 3, n = 9, ROIs = 59), or 10 mM NSC668394 (N = 3, n = 11, ROIs = 78). The data are shown as mean ± SD, ***P < .001, one-way ANOVA with Sidak's multiple comparisons tests. RAC1 = Ras-related C3 botulinum toxin substrate. *Based on (Tél et al. 2023)*

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^- efflux was measured as described above (**Figure 16**). Interestingly, both ezrin and RAC1 inhibitors recapitulated the effect of AZA on Cl^- secretion, and the observed Cl^- efflux was significantly inhibited in both

cases confirming our suspicion 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. As organoids contain epithelial cells only, no contamination of other cell types is to be expected (Molnar et al. 2020). To test the effects of AZA, the organoids were digested into single cells as previously described (Madacsy et al. 2022) and were incubated *ex vivo* with 1 μg/mL AZA for 60 min. Not surprisingly, the amount of active RAC1 significantly decreased after AZA treatment in a G-LISA assay (**Figure 17**), while the RAC1 inhibitor Ehop-016 was not able to further decrease the activity of RAC1 in the presence of AZA.

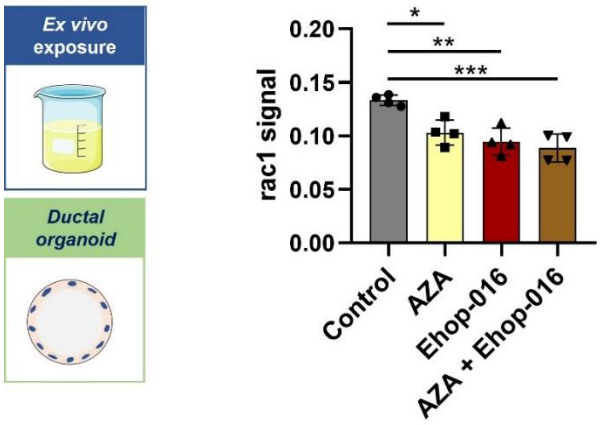

Figure 17. – Azathioprine Inhibits RAC1 activity in mouse pancreatic organoids
Mouse pancreatic ductal organoids digested into single cells were incubated *in vitro* with either 1 μg /mL azathioprine (AZA), 10 mM RAC1 inhibitor (Ehop-016), or both for 60 minutes. Then cells were lysed on ice and RAC1 activity was measured with a G-LISA assay following the manufacturer's protocol. RAC1 activity signals were measured with the G-LSIA assay (n = 4 per group, from 2 different cultures). The data are shown as mean ± SD, **P* < .05, ** *P* < .01, ****P* < .001, one-way ANOVA with Sidak's multiple comparisons tests. *Based on (Tél et al. 2023)*

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. 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 (**Figure 18**), 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.

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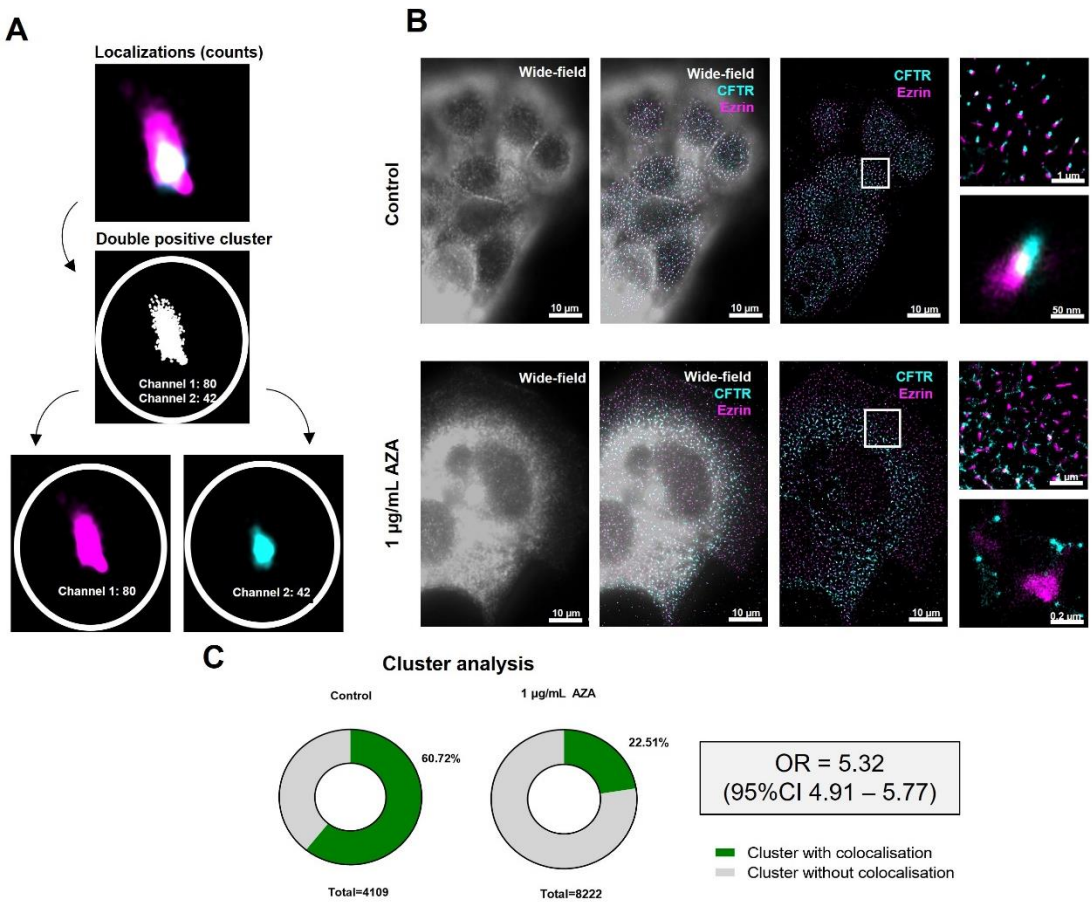

Figure 18. – Azathioprine alters CFTR-ezrin colocalization in mouse pancreatic organoids

Mouse pancreatic ductal organoids were incubated for 60 minutes with 1 µg/mL azathioprine (AZA) or normal culture media. CFTR and ezrin were labeled with fluorescent antibodies and visualized on Direct Stochastic Optical Reconstruction Microscopy (dSTORM). **A**, Schematic diagram of the cluster analysis process. A channel-independent cluster analysis was performed based on the collected and individually recorded blinking events. Clusters were defined using a diameter of 120 nm from the center. The number of homogeneous and double-positive clusters was determined by examining the composition of individual clusters In the figure, the number after the channel name for the example cluster indicates the recorded blinking events of the fluorophores. **B**, Representative dSTORM images of CFTR-ezrin clusters in control and AZA-treated organoids. **C**, Results of a cluster analysis of CFTR-ezrin colocalization. OR and 95% CI are calculated with Chi-Square test, $P < .001$. *Based on (Tél et al. 2023)*

5. Discussion

The quality of life of patients with IBD can be impaired on multiple levels: intestinal inflammation is a common cause of regular abdominal pain and disturbed bowel movements; severe inflammation can lead to bowel resection and stoma formation; the medical treatment with potent immunomodulators, such as azathioprine and biologics, can lead to iatrogenic infections parallel to commonly occurring adverse events, and the frequent extraintestinal manifestations can further influence both their quality of life and medical treatment (Agrawal et al. 2021; Rogler et al. 2021). AP – a potentially fatal inflammatory disease – is the most frequently mentioned pancreatic lesion in association with IBD (Bregenzer et al. 2006; Harbord et al. 2016; Ramos et al. 2016; Seyrig et al. 1985), that can lead to recurrent episodes of pancreatitis, as well as to chronic pancreatitis and exocrine pancreatic insufficiency (Iida et al. 2019). The clinical evidence, suggesting that AP would occur more frequently in IBD, has been emerging in the last 50 years, however, to the best of our knowledge, no comprehensive meta-analysis was available on this topic at the time of publication of our work.

To fill this gap, we aimed to synthesize the available findings of large-scale studies in a meta-analysis and we were able to confirm the supposed association between IBD and AP. We found the pooled odds of AP in IBD – extracted from six large-scale observational studies – to be three times higher (OR 3.11) than in the non-IBD population (Section 4.1.2, **Figure 2**.). Furthermore, we could perform a subgroup analysis and found significantly higher odds of AP in CD than in UC patients (Section 4.1.3, **Figure 3.**). We also conducted a second analysis of four eligible studies and pooled the annual incidences of AP in IBD and received a 210/100.000PYs pooled annual incidence (Section 4.1.4, **Figure 4**).

IBD is a heterogeneous disease, comprising CD, UC, and also unclassified IBD (ie. IBD-U), therefore, treating it as a homogeneous cohort can oversimplify results. In our main analysis, we did treat IBD as one homogenous patient group, however, we were able to also conduct a subgroup analysis of the IBD subtypes which provided a clinically more relevant outcome (Section 4.1.3, **Figure 3.**). As much as it would be more helpful for clinical decision-making, subgroup analyses based on different anatomic locations or clinical behavior of CD or UC (eg. as per Montreal classification), treatment regimes, or

the etiology of AP could not be made, since most of the studies did not report the occurrence, nor the chance of AP for these subgroups. Despite these limitations, we observed no heterogeneity in the analysis of ORs, making our observations to be of high certainty. On the other hand, in the analysis of annual incidences of AP a significant heterogeneity was found ($I^2 = 98.67\%$). This can be partly because prognostic studies with large sample sizes can produce high heterogeneity (Iorio et al. 2015). Another reason might be the different geographical and temporal distribution of the included studies as we included studies originating from Taiwan (Chen Y. T. et al. 2016), South Korea (Kim et al. 2017), the USA (Mcauliffe et al. 2015), and Denmark (Rasmussen et al. 1999), and from the 1990s (Rasmussen et al. 1999), as well as from the late 2010s. Also, most studies reported aggregate data of adult IBD patients (ie. both CD and UC), but one study included in this analysis (Kim et al. 2017) reported aggregate data of 9-90 years old UC patients. As the results from this study are in between the results of the other three included studies, it is not the main source of heterogeneity and, therefore, we decided to include it in the analysis.

Parallel to our work, a Danish group also published a meta-analysis on the same topic and found a similar pooled risk ratio of 2.78 (95% CI, 2.40-3.22) for AP in IBD based on three studies, and also confirmed that the risk of AP seems to be higher in CD than in UC (Pedersen et al. 2020). However, methodological differences from our work can be identified in multiple aspects. First, while we conducted a literature search on three major databases, Pedersen et al. searched only PubMed/MEDLINE and Embase databases, retrieved only 895 non-duplicate entries, and included only four studies in the final analysis, in contrast to the six included studies of our analysis. Also, Pedersen et al. did not pool the annual incidence of AP in IBD. Another difference is while they pooled the risk ratios of the included studies as they were published, we calculated crude ORs based on the published incidences of AP in the groups of interest (IBD and non-IBD population), and we pooled the crude ORs calculated from contingency tables. While both of these methods are suboptimal, pooling the sex, age, and comorbidity-adjusted ORs was not feasible. Furthermore, while Pedersen et al. included the study of Blomgren et al., we excluded this because the study population was exactly the same as in the work of Sundstrom et al., and in such cases, we decided to include the one published later (Blomgren et al. 2002; Sundstrom et al. 2006). Last but not least, the robustness of a meta-

analysis is guaranteed if a pre-study protocol of the conducted literature search, inclusion and exclusion criteria, and PICOs are published and reviewed as a PROSPERO registration. This, we had done beforehand (under registration number CRD42017080464) while Pedersen et al. did not mention such in their publication. Nevertheless, the work of Pedersen et al. is a well-performed meta-analysis, with a similarly low heterogeneity (I^2 = 0%, P = .605) in their findings, which are fully compatible with the results of our work.

Although in our analysis we originally aimed to also determine the proportion of the different etiologies of the AP in IBD, this we could not reach due to the lack of published data on etiologies in the eligible studies. However, – in agreement with later reviews of the topic (Massironi et al. 2022; Montenegro et al. 2022) – we have found many studies suggesting that DIAP – especially TIP – can be the cause of a major proportion of AP cases in IBD (Bermejo et al. 2008; Chaparro et al. 2013; Teich et al. 2016; Zabala-Fernandez et al. 2011).

Thiopurines, especially AZA are the leading cause of DIAP worldwide (Sanchez-Aldehuelo et al. 2021), with a cumulative incidence between 1 and 6% of exposed patients (Van Geenen et al. 2010). So far, mostly clinical studies tried to investigate the pathophysiology of TIP, which suggested mainly immune-mediated and genetics-associated mechanisms (Heap et al. 2014; Ledder et al. 2015). Later, the potential role of direct toxic mechanisms was also suggested (Ledder et al. 2015), however, the molecular background of TIP is essentially unexplored. Although early studies from the late 1970s - early 1980s on dogs have tried to investigate the pancreatic toxic effects of AZA, their results were ambiguous. Dreiling et al. in a preliminary study found that co-administration of IV 100 mg AZA and 1 mg hydrocortisone significantly decreased the pancreatic juice flow of Thomas fistula dogs (Dreiling and Nacchiero 1978), while Broe et al. reported that infusions with both 0,5 and 5 mg/kg AZA increased the pancreatic flow and HCO_3^- output in *ex vivo* canine pancreas preparations (Broe and Cameron 1983). Nevertheless, the results from both studies demonstrated that AZA can have an impact on pancreatic secretory functions, but this possible effect has not yet been further explored. Therefore, we aimed to explore the effects of thiopurines on pancreatic secretory functions to get a better understanding of the pathomechanism of TIP. Using different *ex vivo* and *in vivo* experiments in mice, we demonstrated that *in vivo* AZA treatment sensitized mice to early

cerulein-induced pancreatic injury without increasing the severity of the ultimately developed AP. We also demonstrated that the *ex vivo* administration of AZA impaired the expression and function of CFTR in the apical plasma membrane of pancreatic ductal cells via RAC1 inhibition and disruption of CFTR-ezrin interaction, leading to a consequent decrease in the exocrine pancreatic secretion.

Clinical studies usually report TIP during the first 30 days of treatment, although not immediately on the first days of exposure (Haber et al. 1986; Weersma et al. 2004; Wintzell et al. 2019). Therefore, we first demonstrated that the regular, daily administration of AZA for a week had no visible effect on the exocrine pancreatic morphology in mice (Section 4.2.1, **Figures 5 and 6/A-C**). Then, mice were challenged with cerulein-caused hyperstimulation, and when we focused on the early phase of pancreatitis, which portrays mostly hyperstimulation-related cell injury, we found a significantly higher proportion of pancreatic necrosis in AZA + cerulein-treated mice compared to cerulein-only controls (**Figure 5**). However, when we observed the fully developed AP, the previously observed difference in the extent of necrosis disappeared and none of the parameters differed significantly upon AZA treatment (Section 4.2.1, **Figure 6**). Kerstein et al. previously reported ameliorative effects of AZA treatment in experimental pancreatitis induced in dogs (Kerstein and Grabman 1979). In our results, the extent of necrosis showed a further increase from the early to later phase of pancreatitis in cerulein-only controls (**Figures 5/A** and **6/A**). This phenomenon could be the result of the immunosuppressant property of AZA, as in this later phase of cerulein-induced pancreatitis supposedly immune cell-mediated injury dominates the picture (Gress et al. 1994). The further increase of necrosis in the cerulein-only group confirms that further pancreatic injury developed between the 1st and 4th post-treatment (ie. cerulein) hours, but the extent of this increase was observed to be lower in the AZA-treated group, suggesting a relationship with the immunomodulatory effects of AZA. Our results demonstrate that AZA increases the sensitivity of the murine pancreas to cerulein-induced early cellular damage, while the overall severity of the disease remains unchanged.

Next, we assessed the mechanism behind the increased sensitivity of the exocrine pancreas to pancreato-toxic stimuli. The most common culprits of AP, such as ethanol and bile acids, can affect the pancreatic acinar cells (Satoh et al. 2006). Previously ethanol

and non-oxidative ethanol metabolites, as well as bile acids were shown to trigger sustained intracellular $[Ca^{2+}]_i$ and mitochondrial damage both in pancreatic acinar and ductal cells, triggering intra-acinar trypsinogen autoactivation and cellular necrosis (Maleth et al. 2015; Venglovecz et al. 2008). More importantly, similar intracellular events were described in another form of DIAP, asparaginase-induced AP (Peng et al. 2016). As Foitzik et al. showed that oral treatment either with supra-clinical doses of AZA (10 mg/kg/day) caused more extensive acinar cell necrosis in rats (Foitzik et al. 1998), one would suggest that AZA might affect the viability of the pancreatic epithelia. In addition, Geiger et al. did prove that toxic concentrations (5 and 10 μg/mL, respectively) of AZA elevated $[Ca^{2+}]_i$ and triggered phosphatidylserine exposure – a hallmark of apoptosis – of human erythrocytes *in vitro* (Geiger et al. 2008).

On the contrary, in our experiments, the *ex vivo* administered, non-toxic 1 μg/mL AZA had no detectable effect on the viability of pancreatic acinar cells when used in a clinically relevant concentration range (Section 4.2.2, **Figures 7 and 8**). Also, no significant alterations were observed either in the intracellular Ca^{2+} signaling or in the (highly Ca^{2+} dependent) amylase release of pancreatic acinar cells (Section 4.2.3, **Figure 9**). These data suggest that AZA, in clinically relevant concentrations, does not affect pancreatic acinar cells. This aligns well with our results from cerulein-induced pancreatitis (**Figure 6**) as well as the clinical observation, that the majority of TIP cases are mild to moderate (Sanchez-Aldehuelo et al. 2021).

Pancreatitis-inducing agents not only target acinar cells but similarly, they can affect PDECs (Habtezion et al. 2019; Lee P. J. and Papachristou 2019). The role of PDEC is to secrete an alkaline, HCO_3^--rich pancreatic juice that washes the digestive enzymes out from the pancreatic tree, preventing the premature autoactivation of trypsinogen and, thus, cellular damage (Pallagi et al. 2011). Disturbances of PDEC-mediated secretion can trigger acinar stress and therefore it can make the pancreas susceptible to pancreatitis (Habtezion et al. 2019). Transcellular ductal HCO_3^- transport in the pancreas is a delicate process requiring multiple transporters and ion channels on both the luminal and basolateral membrane of the PDEC, where CFTR has an important role (Angyal et al. 2021; Mayerle et al. 2019). Among others, ethanol was previously shown to reduce both the expression and activity of CFTR, with a consequently impaired ductal HCO_3^- secretion (Maleth et al. 2015). Additionally, genetic disturbance in the protein complex

tethering CFTR to the apical membrane has also been shown to cause CFTR mislocalization in PDEC and decreased ductal secretory activity (Pallagi et al. 2014).

Similarly, in this work we clearly demonstrate that both *ex vivo* (Section 4.2.4, **Figure 10/B**) and *in vivo* thiopurine treatment decrease the pancreatic ductal luminal HCO_3^- (Sections 4.2.4, **Figures 11/B and 12/B**) and fluid (**Figure 12/D**) secretion in mice, in clinically relevant concentrations. Since in the *in vivo* experiments, drugs were administered using oral gavage feeding, the pharmacokinetics of the usual clinical (ie. oral) administration of AZA was completely recapitulated. Furthermore, as TIP is usually reported within 28 days, we also tested, whether a longer (4 weeks) AZA treatment would result in more pronounced effects than the shorter (1 week) treatment but could not find such effects (**Figure 11**). In addition, both *in vivo* and *ex vivo* thiopurine treatment significantly impaired the basolateral (ie, NHE and NBCe1; **Figures 10/C, 11/C, and 12/C)** ion transport activities. This is surprising, as in a previous study on dendritic cells, Bhandaru et al. found no effect of 2.77 μg/mL AZA on the basolateral NHE activity. This may suggest that the impaired basolateral transport activities may be secondary – a result of the damaged apical transport processes, however, this phenomenon was not pursued further in the current study.

To further characterize the AZA-induced inhibition of HCO_3^- secretion, the apical CFTR expression, as well as CFTR activity was analyzed in pancreatic ductal cells. PDECs express CFTR channels almost exclusively on the apical membrane (Lee M. G. et al. 2012). CFTR controls transepithelial fluid secretion and the hydration of the epithelial luminal surfaces, as the CFTR-mediated Cl^- and HCO_3^- transport represents a rate-limiting step in epithelial anion secretion (Hegyi et al. 2016). Several mutations of the CFTR gene lead to the well-known disease, cystic fibrosis, which is characterized by impaired ion channel function and consequently impaired epithelial fluid transport in organs such as the lung and exocrine glands, including the pancreas. However, other mutations can exhibit only an increased risk for pancreatitis or associated pancreatic damage, with elevated mucus levels, fibrosis, and cyst formation within the pancreas (Hegyi et al. 2016). In addition, recently, other pancreatitis-causing insults, such as alcohol, smoking, or bile acids, were shown to inhibit CFTR-dependent epithelial functions, which makes one expect similar mechanisms with AZA as well. And indeed, experiments shown in this work confirm, that AZA exposure – applied either *ex vivo* or

in vivo – not only inhibits the apical HCO_3^- secretion (as shown above) but also decreases the apical plasma membrane expression of CFTR (Section 4.2.5, **Figure 14**) and the (heavily CFTR-dependent) Cl^- secretion of ductal segments (Section 4.2.5, **Figure 15**).

As in multiple studies, the inhibited ductal secretory functions and CFTR activity were found to be at least partly the result of a toxic Ca^{2+} overload in ductal epithelial cells (Madacsy et al. 2018; Maleth et al. 2015; Pallagi et al. 2022; Venglovecz et al. 2008), we also examined the Ca^{2+} signaling of PDEC to investigate their role in the observed CFTR-inhibiting property of AZA. However, similar to acinar cells, no altered Ca^{2+} signals were to be observed in PDEC during *ex vivo* AZA treatment, suggesting a Ca^{2+}-independent inhibitory effect of AZA (Section 4.2.5, **Figure 13**), suggesting that AZA disturbs CFTR functions through other pathways. CFTR is known to be a member of a plasma membrane protein complex (Kunzelmann and Mehta 2013) that maintains the apical localization of CFTR in secretory epithelial cells. This protein complex comprises CFTR, NHERF1, ezrin, and actin (Matos et al. 2018; Pallagi et al. 2014), and ezrin is regulated by RAC1 (Moniz et al. 2013). Interestingly, AZA was suggested to inhibit RAC1 in T lymphocytes (Poppe et al. 2006), and the stimulation of RAC1 was shown to restore F508del-CFTR expression and functions in human bronchial epithelial cells (Matos et al. 2018), suggesting that AZA could act by disturbing the tethering protein complex via RAC1-ezrin inhibition. Here, as the first confirmation of this theory, RAC1 and ezrin inhibitors were shown to recapitulate the effects of the AZA exposure, as they both impaired the Cl^- secretion of PDEC (Section 4.2.6, **Figure 16**). Furthermore, the *ex vivo* AZA exposure also inhibited RAC1 activity in the pancreatic ductal epithelia (Section 4.2.6, **Figure 17**). And last but not least, incubation of ductal epithelia with AZA resulted in a lower colocalization probability of ezrin with CFTR (Section 4.2.6, **Figure 18**), which ultimately caused the disturbed PM localization and impaired function of CFTR in mouse ductal segments, and can explain the impaired pancreatic HCO_3^- and fluid secretion observed in AZA-treated animals.

To our knowledge, these observations are the first to provide mechanistic insight into thiopurine-induced acute pancreatitis, in a clinically relevant setting. The described impairment in the apical CFTR expression and activity potentially contributes to the increased sensitivity of the exocrine pancreas to other stress factors, that result in the development of TIP, however, most clinical studies highlighted other, immunologic and

genetic, mechanisms of TIP (Heap et al. 2014; Iida et al. 2019). Other, well-known, serious adverse events of thiopurines, such as myelosuppression, were associated with TPMT polymorphisms (Zabala-Fernandez et al. 2011). In the case of TIP, such an association has not been shown, however, Heap et al. identified a strong association between Class II HLA variants and susceptibility to TIP in IBD patients (Heap et al. 2014). Another interesting observation is, that in some studies, TIP occurred in a higher proportion of IBD patients – especially in CD – than in any other patients treated with AZA (Bermejo et al. 2008; Sanchez-Aldehuelo et al. 2021; Weersma et al. 2004). Although the above-mentioned studies are strongly suggestive of immunologic processes in TIP, the exact mechanisms that would support this hypothesis were not yet found, but the direct effects of AZA on CFTR described in our work may open new directions in the prevention of TIP. Personalized evaluation of thiopurines based on the pharmacogenetics of TPMT was successful to prevent myelotoxicity in a recent study, but it failed in the prevention of TIP (Bangma et al. 2020). CFTR functions, however, can be assessed in a personalized manner using rectal organoid cultures derived from rectal biopsies, an approach that already offers personalized treatment options in patients with cystic fibrosis (Beekman 2016). Given that colonoscopy is routinely performed in the clinical management of IBD patients, a similar approach might prove useful to prevent TIP in the future. In addition, novel drugs that modify CFTR activity – one of the areas driving intense research in the pharmaceutic industry – also hold the potential to be utilized in the prevention of TIP.

Taken together, in this work, a meta-analysis showed that IBDs, especially CD, to increase the risk of AP more than 3-fold compared to non-IBD populations. The analysis could not reach the goal to describe different etiologies of AP in IBD, however, the qualitative review suggested that TIP contributes to a large part of AP cases. Therefore, the mechanism of action of thiopurines, especially AZA, was explored. When assessing the effects of AZA on the exocrine pancreas of mice, it exclusively affected the secretory activity of pancreatic ductal cells but not the acinar cells. This is explained by AZA inhibiting RAC1, which disturbs the ezrin-CFTR interaction, leading to altered CFTR localization on the apical PM of the pancreatic ductal cells. These observations might open new directions for research aiming at the prevention of TIP.

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6. Conclusions

The main conclusions of my research are summarised in the following statements:

1. Acute pancreatitis is found to be more frequent in inflammatory bowel diseases than in the non-inflammatory bowel disease population with a pooled OR of 3.11 (**Section 4.1.1**). A clinically more relevant finding is that acute pancreatitis occurs more frequently in Crohn's disease (OR of 4.12) than in UC (OR of 2.61, **Section 4.1.2**). My meta-analyses are based on homogenous results, with zero heterogeneity indicated by the I^2 statistics, making these results highly certain.
2. Based on my meta-analysis, approximately 210 AP case per 100.000 IBD patient is anticipated annually, which emphasizes the importance of the thorough surveillance of pancreatic involvement in patients with inflammatory bowel disease (**Section 4.1.3**). The analysis indicated significant heterogeneity, therefore, its results are of moderate certainty.
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 acute pancreatitis (**Section 4.2.1**). This suggests an increased susceptibility to developing AP but not to having a more severe disease upon azathioprine exposure.
4. Azathioprine is found not to alter pancreatic acinar viability or cell death, or the Ca^{2+}-dependent acinar functions in mice, neither upon short-term direct thiopurine stimulation nor after one week of *in vivo* azathioprine treatment (**Sections 4.2.2-4.2.3**).
5. Murine pancreatic ductal HCO_3^- secretion is found to be inhibited by short-term *ex vivo* thiopurine perfusion, just as well as it is inhibited by one week of *in vivo* treatment with the three thiopurines (azathioprine, 6-mercaptopurine, and 6-thioguanine), respectively, while the pancreatic ductal 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 shown in immunofluorescent labeling experiments (**Section 4.2.4-4.2.5**).

7. The azathioprine-induced inhibition of CFTR functions (ie. Cl^- secretion) is recapitulated by both ezrin and RAC1 inhibitors in mouse pancreatic ductal segments, while azathioprine can also inhibit the RAC1 activity in mouse pancreatic ductal organoid cells. Azathioprine exposure also leads to a significantly decreased colocalization probability of ezrin with CFTR in ductal organoids. These are suggestive of an impaired RAC1-ezrin-CFTR interaction leading to the disturbed ductal functions triggered by azathioprine exposure (**Section 3.2.6**).

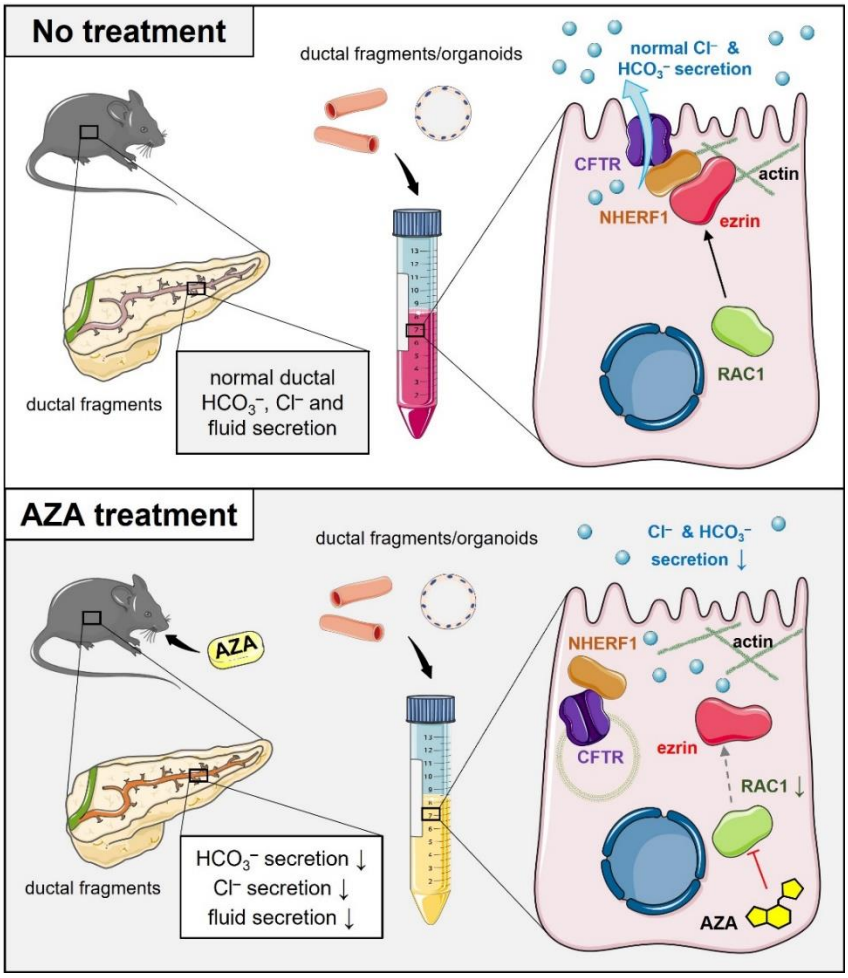

Figure 19. – A graphical summary of findings

Under physiologic circumstances, pancreatic ductal (HCO_3^-, Cl^-, and fluid) secretion is maintained by intact cystic fibrosis transmembrane conductance regulator (CFTR) plasma membrane (PM) tethering complex, consisting of actin, ezrin, CFTR, and Na+/H+ exchanger regulatory factor (NHERF1). Upon azathioprine (AZA) exposure, CFTR function and, therefore, pancreatic ductal secretion are impaired due to the lowered RAC1 activity, leading to the disassembly of the PM tethering complex and internalization of CFTR.

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7. Summary

AP is one of the most frequently observed pancreatic involvement of IBD. Although AP is associated with a possibly high mortality rate, its etiology and epidemiology in the subpopulation of IBD patients are not settled. Therefore, in the first part of my research, a literature search with two meta-analyses was conducted. Altogether eight eligible studies describing the risk of AP in patients with IBD were identified. The first meta-analysis of six studies yielded an OR of 3.11 for all types of IBD, while a subgroup analysis yielded a significantly higher OR (of 4.12) for CD than for UC (OR of 2.61). The second meta-analysis pooled the reported incidence rates extracted from four eligible studies, suggesting that 21 AP cases per 10,000 IBD patients are to be expected annually. These analyses were the first worldwide to confirm the clinical experience that the risk of AP would be higher in patients with IBD.

The etiology of AP in IBD patients is different from that in the general population and medical treatment – especially with thiopurines – seems to contribute to a major proportion of pancreatitis cases. In the general pathomechanism of AP, the impairment of pancreatic ductal secretory functions by CFTR inhibition is a confirmed phenomenon. CFTR functions depend on the integrity of a protein complex that anchors CFTR to the PM, with ezrin being one of the key proteins in this complex. Ezrin was also shown to be regulated by RAC1, a small GTPase protein that can be inhibited by the major thiopurine, AZA, suggesting that AZA might influence pancreatic fluid secretion. As neither the mechanism of thiopurine-induced AP nor the direct effects of thiopurines on pancreatic epithelial tissue have been described in detail, the second part of my research aimed to explore the effects of thiopurines, especially AZA, on mouse pancreatic cells.

Oral AZA treatment significantly increased pancreatic necrosis in the early, but not in the later phase of the cerulein-induced pancreatitis model, which. This was explained by the exclusive inhibitory effect of AZA on the secretory activity of pancreatic ductal segments both upon *ex vivo* and *in vivo* exposure. This AZA-mediated inhibition of secretory functions, especially Cl^- and HCO_3^- secretion was a result of inhibited RAC1 activity, which disturbed the ezrin-CFTR interaction, leading to altered CFTR localization on the apical PM of the pancreatic ductal cells (**Figure 19**). These observations might open new directions for research aiming at the prevention of TIP.

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9. Bibliography of the candidate's publications

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