INVESTIGATION OF THE PATHOMECHANISM OF DIARRHOEA-RELATED DISEASES

Ph.D. THESIS

ÉVA PALLAGI

First Department of Medicine,

University of Szeged

Szeged, Hungary

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LIST OF ABBREVIATIONS

$(\Delta\psi)_m$: mitochondrial transmembrane potential

$[Ca^{2+}]_i$: intracellular Ca^{2+}-concentration

6-MP: 6-mercaptopurine

ATI: antibody to infliximab

ATP_i: intracellular ATP-concentration

AZA: azathioprine

BA: bile acid

BAM: bile acid malabsorption

BAPTA-AM: 1,2-bis(o-aminophenoxy)ethane-N,N,N9,N9-tetraacetic acid

BCECF-AM: 2',7'-biscarboxyethyl-5(6)-carboxyfluorescein-acetoxymethylester

CBE: Cl^-/HCO_3^--exchanger

CCCP: mitochondrial toxin carbonyl cyanide m-chlorophenyl hydrazone

CD: Crohn's disease

CDAI: Crohn's Disease Activity Index

CDC: non-conjugated bile acid chenodeoxycholic acid

CFTR: cystic fibrosis transmembrane conductance regulator Cl^--channel

D: ileum-resected/cholecystectomised patients suffering from diarrhoea

DOG: glycolysis inhitbitor deoxyglucose

DRA: Cl^-/HCO_3^--exchanger downregulated in adenoma

ELISA: enzyme-linked immunosorbent assay

ER: endoplasmic reticulum

ESR: erythrocyte sedimentation rate

FURA-2-AM: 2-(6-(bis(carboxymethyl)amino)-5-(2-(2-(bis(carboxymethyl)amino)-5-methylphenoxy)-ethoxy)-2-benzofuranyl)-5-oxazolecarboxylic acetoxymethyl ester

GCDC: glycine-conjugated bile acid glycochenodeoxycholic acid

HOE-642: 4-isopropyl-3-methylsulphonylbenzoyl-guanidin methanesulphonate, cariporide

IAA: glycolysis inhitbitor idoacetamide

IBD: inflammatory bowel disease

IFN-γ: interferon gamma

IFX: infliximab

IL-2: interleukin-2

IP$_3$R: inositol triphosphate receptor

IQR: interquartile range

NHE: Na^+/H^+-exchanger

NON-D: ileum-resected/cholecystectomised patients without diarrhoea

pH$_i$: intracellular pH

ROI: region of interest

RR: ruthenium red

RyR: ryanodin receptor

SERCA: sarcoplasmic/endoplasmic reticulum calcium ATPase

TEM: transmission electron microscopy

Tg: thapsigargin

TGR5: G-protein-coupled bile acid receptor

TMRM: tetramethylrhodamine methyl ester

TNF-α: tumor necrosis factor alpha

UC: ulcerative colitis

ΔpH$_{max}$: maximal change of intracellular pH

LIST OF FULL PAPERS RELATED TO THE SUBJECT OF THE THESIS

I. A. Bálint[*], K. Farkas[*], **É. Pallagi-Kunstár**, G. Terhes, E. Urbán, M. Szűcs, T. Nyári, Zs. Bata, F. Nagy, Z. Szepes, P. Miheller, K. Lőrinczy, P.L. Lakatos, B. Lovász, T. Szamosi, A. Kulcsár, A. Berényi, D. Törőcsik, T. Daróczi, Z. Saródi, T. Wittmann, T. Molnár. Antibody and cell-mediated immune response to whole virion and split virion influenza vaccine in patients with inflammatory bowel disease on maintenance immunosuppressive and biological therapy. *Scand J Gastroenterol* 2015 Feb;50(2):174-81

II. É. Pallagi-Kunstár, K. Farkas, J. Maléth, Z. Rakonczay Jr, F. Nagy, T. Molnár, Z. Szepes, V. Venglovecz, J. Lonovics, Z. Rázga, T. Wittmann, P. Hegyi Bile acids inhibit Na^+/H^+ exchanger and Cl^-/HCO_3^- exchanger activities via cellular energy breakdown and Ca^{2+} overload in human colonic crypts. *Pflugers Arch - Eur J Physiol* 2014 Jul 13. [Epub ahead of print]

III. É. Pallagi-Kunstár, K. Farkas, Z. Szepes, F. Nagy, M. Szűcs, R. Kui, R. Gyulai, A. Bálint, T. Wittmann, T. Molnár. Utility of serum TNF-α, infliximab trough level, and antibody titers in inflammatory bowel disease. *World J Gastroenterol* 2014 May 7;20(17):5031-5:5031-5.

IV. É. Kunstár, P. Hegyi, Z. Rakonczay Jr, K. Farkas, F. Nagy, T. Wittmann, T. Molnár. Is Bile Acid Malabsorption Really a Common Feature of Crohn's Disease or is It Simply a Consequence of Ileal Resection? *Front Physiol*. 2011;2:28

LIST OF FULL PAPERS NOT RELATED TO THE SUBJECT OF THE THESIS

I. K. Farkas, P.L. Lakatos, M. Szűcs, **É. Pallagi-Kunstár**, A. Bálint, F. Nagy, Z. Szepes, N. Vass, L. S Kiss, T. Wittmann, T. Molnár. Frequency and prognostic role of mucosal healing in patients with Crohn's disease and ulcerative colitis after one-year of biological therapy. *World J Gastroenterol* 2014 Mar 21;20(11):2995-3001

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SUMMARY

Background. Chronic diarrhoea is a common unpleasant concomitant of gastrointestinal disorders, such as inflammatory bowel diseases (IBDs) or bile acid malabsorption (BAM). Its exact pathomechanism is still not completely understood, thus, its successful management is a great challenge for clinicians. Recent animal and cell-culture studies underline the crucial role of colonic epithelial ion transporters in the development of bile-induced diarrhoea, but there are just a few human data from the last century available about the influence of bile acids on human colonic ion-transport mechanisms. The exact treatment of IBD represents another unsolved problem. Nowadays biological therapy with anti-tumour necrosis factor alpha (TNF-α)-agents opened new perspectives in the fight against IBD. However, beside the promising results, the loss of response, the presence of anti-drug-antibodies, low drug serum concentrations, hypersensitivity and allergic reactions during the administration of anti-TNF-α are proved to be predisposing factors for therapeutic failure. Thus, therapeutic drug monitoring may be a useful opportunity in order to optimise the treatment of IBD-patients. Moreover, IBD-patients on immunosuppressive therapy are at increased risk for infectious diseases, thus influenza vaccination is strongly recommended. Our **aims** were to investigate the effects of bile acids on the ion-transporter activities (namely Na^+/H^+ exchangers [NHE1-3], and Cl^-/HCO_3^--exchanger CBE) of human colonic epithelial cells and to characterise the cellular pathomechanism of bile-induced diarrhoea. In the second part of the study, we aimed to assess tumor necrosis factor-a (TNF-a), infliximab (IFX) concentrations, and antibodies against IFX molecules in patients with inflammatory bowel disease (IBD) who develop loss of response, side effects, or allergic reaction during anti TNF-a therapy. We also evaluate the antibody and cell-mediated immune response to the split and whole virion influenza vaccine in patients with IBD treated with anti-TNF-α and/or immunosuppressive therapy. However, immunosuppressive and biological therapies seem to influence the immune response to vaccinations. **Materials and methods.** In the first part of our study, patients with negative colonoscopic finding were involved. They were divided into three groups. The first group contained patients having diarrhoea after ileum-resection/cholecystectomy (Diarrhoea). In the second group ileum-resected/cholecystectomised patients were involved who did not develop diarrhoea (NON-Diarrhoea). The control group included patients without any surgical

intervention in the gastrointestinal tract. Primary colonic crypts were isolated from human biopsy samples. The functional characteristics of NHE1-3 and CBE, intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), ATP level (ATP_i) and mitochondrial transmembrane potential ($(\Delta\psi)_m$) were determined using fluorescence technique. The morphology of the intracellular organelles was investigated with transmission electronmicroscopy. In the second part of the study, 67 IBD-patients, receiving the anti-TNF-α agent infliximab (IFX), were enrolled and categorized into two groups. Blood samples of 36 patients with loss of response, side effects, or hypersensitivity to IFX therapy (Group I) and 31 patients in complete clinical remission (Group Ⅱ) selected as a control group were collected. Enzyme-linked immunosorbent assay (ELISA) was applied to determine the serum levels of TNF-α, IFX trough levels, and anti-IFX-antibody (ATI). We examined the correlation between loss of response, the development of side effects or hypersensitivity, and serum TNF-α, IFX trough levels, and ATI concentrations. In an additional part of our study, 156 immunocompromised influenza-vaccinated IBD-patients and 53 non-vaccinated patients (control group) were involved. Split virion vaccine and whole virion vaccine were used. Serum samples were obtained pre- and postimmunisation. TNF-α, interferon-γ (IFN-γ) and interleukin-2 (IL-2) serum levels were measured with ELISA. **Results**. In vitro measurement of colonic epithelial ion transporter activities revealed impaired NHE and CBE activities in cholecystectomised/ileum-resected patients suffering from diarrhoea, compared to control patients. Acute treatment of colonic crypts with 0.3 mM chenodeoxycholate (CDC) caused dose-dependent intracellular acidosis; moreover, the activities of acid/base transporters (NHE and CBE) were strongly impaired. This concentration of CDC did not cause morphological changes of the intracellular organelles of colonic epithelial cells, although significantly reduced the ATP_i, decreased $(\Delta\psi)_m$ and caused sustained elevation of $[Ca^{2+}]_i$. We also showed that CDC induced Ca^{2+}-release from the endoplasmic reticulum and extracellular Ca^{2+}-influx contributing to the $[Ca^{2+}]_i$-elevation. The CDC-induced inhibition of NHE activities was ATP-dependent, whereas the inhibition of CBE activity was mediated by the sustained $[Ca^{2+}]_i$-elevation. We did not observe any inhibitory effect on the functions of ion transporters in case of the administration of a conjugated bile acid. In IBD-patients, receiving IFX-therapy, serum TNF-α level was shown to be correlated with the presence of ATI; ATI positivity was significantly correlated with low trough levels of IFX. ATIs were detected in 25% of IBD patients with loss of response, side effects, or hypersensitivity, however no association was revealed between these patients and antibody positivity or lower serum IFX levels. Previous use of IFX correlated with the development of ATI, although concomitant immunosuppression did not

have any impact on them. Neither TNF-α, nor INF-γ levels changed significantly after influenza vaccination; however, a significant decrease was observed in the level of IL-2 after vaccination with split vs. whole virion vaccine. **Discussion.** Our results suggest that bile acids inhibit the function of human colonic epithelial ion transporters via cellular energy breakdown and Ca^{2+}-overload, which can reduce fluid and electrolyte absorption in the colon and promote the development of diarrhoea. Furthermore, on the basis of our study, we propose that the simultaneous measurement of serum TNF-α level, serum anti TNF-α concentration, and antibodies against anti TNF-α may further help to optimize the therapy in critical situations. IBD-patients on immunosuppressive therapy are recommended to be immunised with influenza-vaccines and measuring the cytokine-responses in patients treated with immunosuppressants may help to determine the efficacy of influenza vaccination.

I. INTRODUCTION

1. The role of human colonic epithelial ion transporters in bile-acid induced diarrhoea

The colon plays a fundamental role in the maintenance of the water balance of the body by absorbing 1.5 to 1.9 liters of electrolyte-rich fluid daily, leaving just 0.1 to 0.5 liters to lose with the stool. The adequate activity of ion transporters localized on the apical and basolateral membrane of polarised epithelial cells is essential to keep the precise balance between absorption and secretion. The critical role of Na^+/H^+-exchangers (NHEs) in this process is well-known such as their involvement in intracellular pH (pH_i) and cell volume regulation. The functionally coupled NHE3 and Cl^-/HCO_3^--exchanger (CBE) downregulated in adenoma (DRA) are most probably responsible for the majority of electroneutral NaCl absorption in the colon [1-3]. So far, nine different NHE isoforms (NHE1-9) have been identified in mammalian cells with a specific tissue and membrane distribution [4]. These are transmembrane proteins which replace one extracellular Na^+ for one intracellular H^+. NHE1 is constitutively expressed on the basolateral membrane of the epithelial cells. Although it does not play a role in the absorption of Na^+, it fulfils housekeeping functions, regulating cell volume and pH_i. The presence of NHE2 and NHE3 on the apical membrane of colonic epithelial cells has been confirmed [5], however, only the prominent role of NHE3 in colonic Na^+ absorption has been demonstrated by previous studies. The occurrence of diarrhoea in NHE3 knockout mice further supports the idea that this is the dominant NHE isoform responsible for Na^+ uptake in the intestine [6-8]. Together with NHE3, the Cl^-/HCO_3^- exchanger DRA, a member of the SLC26 gene family, maintains the absorption of NaCl in the colon. Despite that other anion exchangers are also found in the gastrointestinal tract (AE2, putative anion transporter-1 (PAT-1)), there are evidences that DRA is the most important mediator of electroneutral Cl^- uptake in the large intestine [9]. Mutation of this transporter results in congenital chloride-losing diarrhoea [10], moreover, similar conditions develop in DRA-deficient mice [9]. Disturbances in colonic epithelial Na^+ and/or Cl^- transport have shown previously to be involved in the development of diarrhoea in ulcerative colitis (UC) and secretory diarrhoea [9, 11-13] because of the increased secretion and/or decreased reuptake of water and electrolytes. This fact further verifies the importance of the NHE3 and DRA in colonic electrolyte and water absorption.

Bile acids (BAs) are natural detergents that participate in the solubilisation and absorption of dietary lipids. They are excreted into the small intestine, and most of them (90-95%) are reclaimed in the distal ileum then return to the liver through the portal vein. When

this precisely regulated enterohepatic circulation impairs, bile acid malabsorption (BAM) occurs, thus BAs are allowed to enter the colon in higher concentration and can induce diarrhoea through unidentified mechanisms. BAM and diarrhoea are well-known clinical complications after ileal resection or after cholecystectomy [14-16]. According to the study of Smith et al. about the occurrence of BAM, the numbers are depressive. The prevalence of BAM is 97% in Crohn's patients with resection, 58% of patients with gastric surgery and/or cholecystectomy, and in 33% of patients with unexplained, idiopathic chronic diarrhoea [17] . Since the diagnosis is not available everywhere, the disease is under-recognised, therefore managing bile-induced diarrhoea is a great challenge for gastroenterologists [18]. Further complicates the situation that the applicable therapy with the bile-acid sequestrant cholestyramine does not solve the problem in every case [19, 20]; on the other hand, a life-long medication of the patients is not supportable. Moreover, cholestyramine treatment can reduce the bioavailability of co-administered drugs as well. During the past decades, it became more and more clear that the disturbed colonic absorptive and/or secretory functions, including Na^+ and Cl^- transport, must play a critical role in BAM associated diarrhoea. One of the earliest studies revealed the prosecretory and antiabsorptive actions of bile acids on the human colon, providing the first explanation for bile-induced diarrhoea [21]. Keely et al. also demonstrated electrolyte secretion in cultured colonic epithelial cells induced by bile salts [22], not to mention their sophisticated role in the overall regulation of ion and water movement in the colon [23]. In a recent study, Ao et al. revealed that the non-conjugated bile acid chenodeoxycholic acid (CDC) stimulates Cl^- secretion in colonic epithelial cells via activation of cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channel [24]. Notwithstanding, BAs are also recognised as hormones, regulating numerous intestinal and extra-intestinal processes [25] thus, can no longer be considered exclusively as a component of digestion. Their regulatory function and hormone-like properties exerted through either nuclear receptors or G-protein-coupled receptor (TGR5) [26] have been recognised in the last few years and been under a great interest. A number of elegant studies indicate the influence of TGR5 not only in the gastrointestinal tract (in colonic secretory responses [27], acute pancreatitis [28]) but in various other organ systems as well [29-32].

Nevertheless, the mechanism, by which BAs induce diarrhoea, is not fully elucidated yet, and there are just a few *in vitro* human study available about the influence of bile acids on colonic ion transport processes. Therefore, to understand to pathogenesis of bile-induced diarrhoea and the development of new therapeutical approaches are extremely necessary.

2. Biological therapy with the anti-tumor necrosis factor-α (TNF-α) infliximab (IFX) in the management of inflammatory bowel diseases (IBD)

Crohn's disease (CD) and ulcerative colitis (UC) are the two types of inflammatory bowel diseases (IBDs), defined as chronic disorders of the gastrointestinal tract. The most frequent symptom in IBD is the so-called exudative diarrhoea, defined as the presence of blood and/or pus in the bowel. Exudative diarrhoea is caused by an extensive injury of the mucosa as a result of inflammation or ulceration, leading to a loss of mucus, and the disability of the epithelium to fulfil its crucial absorptive and barrier function. However, diarrhoea in IBD is usually multifactorial. It is commonly exacerbated by other concomitants of IBD, such as bacterial overgrowth or infections. An additional BAM, being a common concomitant feature of IBD [33], may also participate in the constant existence of diarrhoea. Last, but not least, role of colonic epithelial ion transporters in the development of diarrhoea has to be mentioned as well. The decreased activity of NHE3 and damaged Na^+ and Cl^- absorptions in UC patients have already been demonstrated [12, 13]. Moreover, there is a recently discovered relationship between cytokine-mediated immune responses and disturbance of electroneutral sodium absorption. It is well known that the overexpression of proinflammatory cytokines, leading to a chronic systemic inflammation, is another characteristic of IBD, mainly that of CD. Interestingly, high levels of proinflammatory cytokines are linked to the pathogenesis of diarrhoea in IBD. It has been demonstrated both in animal studies and in cultured colonic epithelial cells, that TNF-α and interferon-γ (IFN-γ) downregulate mRNA and protein expression and repress the activities of NHE2 and NHE3, the key mediators of intestinal salt and water absorption [34, 35].

Although IBD is one of the most investigated topics and it is under the spotlight of numerous work groups, its pathophysiology is still not completely understood. Genetic, environmental and immunological factors are thought to be involved in the development of the disease. IBD is characterized by alternating periods of relapse and remission. In case of active disease, signs and symptoms beside diarrhoea may include abdominal pain and cramping, ulcers in the mouth, perianal fistulas, weight loss and fever. The severity and the causing factors are demonstrating a wild range of variety between patients.

Proinflammatory cytokines, such as TNF-α, IFN-γ and the members of interleukin (IL) -family, have been directly implicated in the pathogenesis of IBD and they seem to have a crucial role in the control of intestinal inflammation and associated clinical symptoms. TNF-α is an outstanding component of the inflammatory processes. It is produced in lamina propria

by CD14+ macrophages, adipocytes, dendritic cells, fibroblasts and effector T-cells. Indeed, its concentration is elevated in the stool, mucosa, and blood of IBD patients [36]. TNF-α has been shown to induce various pro-inflammatory functions in the inflamed mucosa, such as hypervascularization and angiogenesis, it augments pro-inflammatory cytokine production by macrophages and T-cells, causes barrier alterations and promotes cell death of intestinal epithelial cells. TNF-α also promotes tissue destruction and drives T cells to be resistant to apoptosis [37]. The complexity of IBD makes a lot of difficulties in the treatment and an established standard therapy is lacking. In some of the cases, surgery is unavoidable; however, resection is not curative in the majority of the CD cases. Beside 5-aminosalicylic acid compounds, corticosteroids and immunosuppressive drugs, biological therapies are most commonly used therapeutic methods. Proinflammatory cytokine TNF-α is currently the key target for the management of different inflammatory diseases including IBD. Infliximab (IFX), a chimeric monoclonal anti-TNF-α-antibody, has been approved for the induction and maintenance of remission in both CD and UC. In the past decade, IFX provided new perspectives for the management of the diseases resulted in marked clinical improvement and macroscopic healing of the inflamed mucosa. However, loss of response, presence of antibodies against IFX, low drug serum concentrations, hypersensitivity and allergic reactions during the administration of anti-TNF-α are proved to be predisposing factors for therapeutic failure. Approximately 40% of patients will subsequently lose response, thus requiring dose intensification or drug change [38]. Dose intensification may be a solution in case of low anti-TNF-α drug trough levels, while switching to another drug could be useful if antibodies are developed against the biological agents [39]. Immunogenicity (the formation of antibodies to the biological agents) is the major cause of loss of response and adverse reactions. Scheduled maintenance therapy, concomitant immunomodulators therapy, and pretreatment with high-dose corticosteroids may help to reduce immunogenicity [40]. Many observational studies have linked low serum drug levels to a higher risk of the development of anti-drug-antibodies, and/or loss of response to biologics in IBD. In response, reactive measurement of anti-drug-antibodies and serum drug levels using Enzyme Linked Immunosorbent Assay (ELISA), and appropriate adjustment of drug regimen, has been utilized in practice to optimize clinical outcomes [41]. Although the role of TNF-α measurement, together with antibody and drug serum concentration, has not previously been investigated in everyday practice, there are more and more studies emphasizing the importance of pharmacokinetic monitoring of IFX and anti-IFX-antibody (ATI) in order to prevent side-effects and to predict the clinical

response to IFX and endoscopic improvement. In this regard, therapeutic drug monitoring may help to optimise the treatment of IBD-patients.

Although patients with IBD should not be routinely considered to have altered immunocompetence per se, there is currently no method of evaluating the effects of immunosuppression on the immune system. IBD-patients, receiving biological therapy and/or immunmodulators (azathioprine (AZA) or 6-mercaptopurine (6-MP)) are exposed to an increased hazard for infectious diseases, with an incremental elevation in the relative risk of opportunistic infection: three fold increased risk (OR 2.9, 95% CI 1.5–5.3) if any one immunomodulator was used, increasing substantially (OR 14.5, 95% CI 4.9–43) if two or more drugs were used concomitantly [42].

Some of the infections occurring more frequently in IBD patients can be prevented with immunisation. Since influenza is one of the most common vaccine-preventable illnesses in adults, influenza vaccination is recommended for all IBD-patients on biological therapy and/or immunmodulators. Inactivated, split virion vaccine and inactivated whole virion vaccine are applied in order to precede influenza. The experimental use of the whole virion vaccine, which is the first inactivated vaccine formulation, dates back to the 1940s. Split virion is derived by disrupting whole virus particles with detergents and is thus less immunogenic than whole virion vaccines [43]. Whole virion vaccine is administered intramuscularly; split virion vaccine is administered intradermally. The types of immunosuppressive and biological therapies seem to affect the immune response to vaccinations, but it remains unclear, whether vaccination has an impact on the cytokine profile of IBD-patients, by which it may influence the process of the disease.

II. AIMS

1. To investigate the influence of bile acids on the ion-transporter activities of human colonic epithelial cells and to characterise the cellular pathomechanism of bile-induced diarrhoea.

2.1. To assess tumor necrosis factor-a (TNF-a), infliximab (IFX) concentrations, and antibodies against IFX in patients with inflammatory bowel disease (IBD) who develop loss of response, side effects, or allergic reaction during anti TNF-α therapy.

2.2. To evaluate the cell-mediated immune response to split and whole virion influenza vaccines in patients with IBD treated with anti-TNF-α and/or immunosuppressive therapy.

III. MATERIALS AND METHODS

1. The role of human colonic epithelial ion transporters in bile-acid induced diarrhoea

1.1. Patients enrolled in the study

Patients were divided into three groups. The first group contained patients having diarrhoea after ileum-resection/cholecystectomy (Diarrhoea). In the second group ileum-resected/cholecystectomised patients were involved who did not develop diarrhoea (NON-Diarrhoea). The control group included patients without any surgical intervention in the gastrointestinal tract. The patients enrolled in this study were between the age of 25-55 years. Informed consent was obtained prior to endoscopy. Protocols of the study were approved by the regional ethical committee at the University of Szeged, Szeged, Hungary. 3-6 colonic biopsies were obtained from the proximal colon (cecum, colon ascendens) from each patient undergoing colonoscopy at the First Department of Medicine. In none of the patients were macroscopic (by endoscopy) or microscopic (by histology) signs of the presence of inflammation in the colon. Patients with normal endoscopic findings were examined because of colorectal cancer screening or different abdominal complaints.

1.2. Materials and solutions for the experiment

The compositions of the solutions used are shown in Table 1. The pH of HEPES-buffered solutions was set to 7.4 with NaOH at $37^{o}C$. HCO_3^--buffered solutions were gassed with 95% O_2/5% CO_2 to set the pH to 7.4 at $37^{o}C$.

General laboratory chemicals were obtained from Sigma-Aldrich. Collagenase A was obtained from Roche Diagnostic (Mannheim, Germany). HOE-642 (4-isopropyl-3-methylsulphonylbenzoyl-guanidin methanesulphonate) was provided by Sanofi Aventis (Frankfurt, Germany) and was dissolved in dimethyl sulfoxide (DMSO). BCECF-AM (20,70-biscarboxyethyl-5(6)-carboxyfluorescein-acetoxymethylester), FURA-2-AM (2-(6-(bis(carboxymethyl)amino)-5-(2-(2-(bis(carboxymethyl)amino)-5-methylphenoxy)-ethoxy)-2-benzofuranyl)-5-oxazolecarboxylic acetoxymethyl ester), BAPTA-AM (1,2-bis(o-aminophenoxy)ethane-N,N,N9,N9-tetraacetic acid), Magnesium-green-AM and TMRM (tetramethylrhodamine methyl ester) were obtained from Invitrogen (Eugene, OR); cell and tissue adhesive from Becton Dickinson Bioscience (Cell Tak, Bedford, MA). BCECF-AM, BAPTA-AM and TMRM were dissolved in DMSO, FURA-2-AM, and Magnesium-green-

AM were dissolved in pluronic acid and DMSO. Thapsigargin was obtained from Merck (Darmstadt, Germany) and it was dissolved in DMSO.

	Standard HEPES	Ca^{2+}-free HEPES	Na^+-free HEPES	NH_4Cl in HEPES	Standard HCO_3^-	Cl^--free HCO_3^-
NaCl	130	130		110	115	
KCl	5	5	5	5	5	
MgCl$_2$	1	1	1	1	1	
CaCl$_2$	1		1	1	1	
Na-HEPES	10	10				
Glucose	10	10	10	10	10	10
NaHCO$_3$					25	25
Na-gluconate						115
Mg-gluconate						1
Ca-gluconate						6
K$_2$-sulfate						2.5
EGTA		0.1				
NH$_4$Cl				20		
NMDG-Cl			140			
HEPES acid			10	10		

Table 1. Composition of the solutions for in vitro studies. Values are concentrations in mmol/L

1.3. Isolation of colonic crypts

Colonic crypts were isolated from three human biopsy specimens obtained from the proximal (cecum or colon ascendens) part of the large intestine. Only one segment of the colon was investigated in each patient. The tissue samples were placed immediately in ice-cold NaHCO$_3$ containing Hank's balanced salt solution (HBSS). The samples were washed three times with HBSS, cut into small pieces with a razor blade and incubated in 1mM dithiothreitol (DTT) in HBSS for 15 minutes followed by 2x30 minutes enzymatic digestion with 0.38mg/mL collagenase A at 37°C and continuously gassed with 5% CO$_2$/95% O$_2$. The small fragments were mixed with a Pasteur pipette, the large fragments were allowed to settle down to the bottom of the flask under gravity for 35–40 seconds, and the supernatant removed and visualised under a Nikon stereo microscope (Jencons-PLS, Grinstead, UK). The crypts (200–300 crypts/isolation) were aspirated into a micropipette and transferred into a Petri dish. For fluorescent measurements the crypts were kept in a culture solution for 3 hours at 4°C before the experiments. The culture solution contained Dulbecco's Modified Eagle's Medium (DMEM), 10% fetal bovine serum (FBS; Sigma-Aldrich, Budapest, Hungary), 2mM L-glutamine, 100U/mL penicillin, and 100µg streptomycin. An isolated colonic crypt is shown in Figure 1.
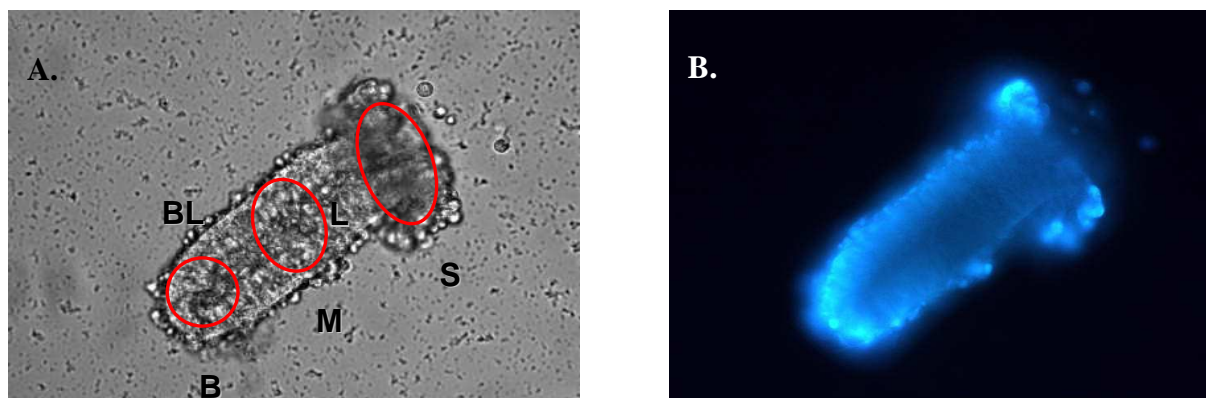
Figure 1. Phase contrast (A) and fluorescent (B) pictures of an isolated human colonic crypt. Crypts were fixed on glass coverglass. Three regions of interests (ROIs) of each crypt were excited with lights at different wavelengths and the fluorescence emissions were measured. B: base, M: middle, S: surface, L: lumen of the crypt, BL: basolateral membrane.

1.4. Measurement of intracellular pH (pH_i), Ca^{2+} concentration ($[Ca^{2+}]_i$) and ATP level (ATP_i) by microfluorometry

Colonic crypts were attached to 24mm glass coverslips covered with CellTak 3h after isolation and placed in a perfusion chamber mounted on the stage of an inverted fluorescent microscope linked to an excellence imaging system (Olympus, Budapest, Hungary).

During the microfluorometry experiments, colonic crypts were incubated in standard HEPES solution at $37^{o}C$ and loaded with the appropriate fluorescent dye. Crypts were continuously perfused with different solutions at a rate of 9-10ml/min. Two to three small areas (region of interest (ROIs)) from the surface of each crypt were investigated (Fig. 1).

pH_i was estimated with the pH-sensitive fluorescent dye BCECF-AM. After incubating with BCECF-AM ($2\mu mol/l$) for 20-30 minutes, colonic crypts were excited with light at wavelengths of 495nm and 440nm and the 495/440 fluorescence emission ratio were measured at 535nm [44, 45].

For the measurement of $[Ca^{2+}]_i$ the cells were loaded with the Ca^{2+} sensitive fluorescent dye FURA-2-AM ($5\mu mol/l$) for 60 min. For excitation, 340 and 380nm filters were used, and the changes in $[Ca^{2+}]_i$ were calculated from the fluorescence ratio (F340/F380) measured at 510nm.

To determine changes of ATP_i the fluorescent dye Mg-green-AM was used, which has been shown to indirectly reflect the changes in ATP_i. Colonic crypts were incubated with Mg-

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Green (4μmol/L) for 60 minutes than were excited with 476nm light and emission was detected at 500–550nm. Because ATP has a 10-fold greater affinity for Mg^{2+} than ADP, and most intracellular Mg^{2+} is present as Mg-ATP [46, 47], the ADP:ATP ratio can be monitored. The elevation of fluorescence intensity caused by the increase in free intracellular Mg^{2+} concentration suggests a reduction of ATP_i [48]. The ATP_i measurements were performed in standard HEPES-buffered solution.

1.5. Determination of NHE activities

During the measurement of pH_i, in order to characterize NHE activity NH_4Cl pulse technique was used in HEPES-buffered solution. Exposure of colonic crypts for 3 minutes to 20mM NH_4Cl induced an immediate rise in pH_i due to the rapid entry of lipophilic base NH_3 into the cells. After the removal of NH_4Cl, pH_i rapidly decreased. This acidification is caused by the dissociation of intracellular NH_4^+ to H^+ and NH_3, followed by the diffusion of NH_3 out of the cell. Under these conditions, the initial rate of pH_i recovery from the acid load reflects the activities of NHEs. $-J(B^-)$ was calculated from the first 60 sec of pH_i recovery from acidification.

Further experiments were done to investigate the activities of the different NHE isoforms. The crypts were acid loaded by exposure to a 3-min-pulse of 20mM NH_4Cl in HEPES solution followed by a 10-min-exposure of Na^+-free HEPES solution. Due to the blocked acid/base transporters (neither sodium nor bicarbonate are present in the solution); the pH_i is set to a stable acidic level. NHE activity was switched on by re-addition of extracellular sodium and the activities of NHEs were determined by measuring the initial rate of pH_i recovery over the first 60 sec. The activities of the different NHE isoforms are extracted by using the isoform selective NHE inhibitor HOE-642. The isoform selectivity of HOE-642 is dose-dependent, 1μM HOE642 inhibits NHE1 whereas 50μM HOE642 inhibits both NHE1 and 2 but not NHE3 [49, 50]. The activities (A) of NHE isoforms can be calculated from the recoveries (R) as follows:

$A_{NHE1} = R_{0\mu M\ HOE\text{-}642} - R_{1\mu M\ HOE\text{-}642}$

$A_{NHE2} = R_{1\mu M\ HOE\text{-}642} - R_{50\mu M\ HOE\text{-}642}$

$A_{NHE3} = R_{50\mu M\ HOE\text{-}642}$

1.6. Measurement of CBE activity

Cl^- withdrawal technique was used to investigate the activity of CBE. Removing Cl^- from the standard HCO_3^-/CO_2 buffered solution caused alkalization due to the reversed activity of the Cl^-/HCO_3^- exchanger. The activity of the exchanger was determined by measuring the initial rate of alkalization over the first 30 sec.

1.7. Determination of buffering capacity and base efflux

The total buffering capacity (β_{total}) of colonic epithelial cells was estimated according to the NH_4^+ pre-pulse technique [51]. Colonic epithelial cells were exposed to various concentrations of NH_4Cl in a Na^+- and HCO_3^--free solution. β_i (which refers to the ability of intrinsic cellular components to buffer changes of pH_i) was estimated by the Henderson–Hasselbach equation. β_{total} was calculated from: $\beta_{total} = \beta_i + \beta_{HCO3-} = \beta_i + 2.3x[HCO_3^-]_i$, where β_{HCO3-} is the buffering capacity of the HCO_3^-/CO_2 system. The measured rates of pH_i change (dpH/dt) were converted to transmembrane base flux $J(B^-)$ using the equation: $J(B^-)=dpH/dtx$ β_{total}. The β_{total} value at the start point pH_i was used for the calculation of $J(B^-)$. We denote base influx as $J(B^-)$ and base efflux (secretion) as $-J(B^-)$.

1.8. Electron microscopy

Morphological changes of the different cell organelles of the colonic epithelial cells were evaluated by transmission electron microscopy (TEM). Biopsy samples were fixed in 2% glutarldehyde (in PBS) overnight at 4°C degree. Samples were cut into small pieces (1X1 mm) than were infiltrated with 2% gelatin (PBS) and the small cubes were made, which were than embedded to Embed 812 (EMS, USA) using a routine TEM embedding protocol. After the semithin sections (1µm), the thin (70nm) sections were cut for TEM examination.

1.9. Measurement of mitochondrial transmembrane potential ($(\Delta\psi)_m$)

Changes of $(\Delta\psi)_m$ were assessed by loading cells with 100nmol/L TMRM for 30 minutes at 37°C to measure fluorescence in the perigranular mitochondrial region. Depolarisation of the mitochondria results in redistribution of TMRM from the mitochondria to the cytosol, causing a decrease in mitochondrial fluorescence [52]. Excitation 488nm, emission was detected at >550nm with Olympus Fluoview FV10i confocal system [48].

1.10. Statistical analysis

Values are means ± SE. Statistical analyses were performed using analysis of variance (ANOVA) with the post-hoc test Dunnett or Bonferroni. $P \leq 0.05$ was accepted as significant.

2. Biological therapy with anti-TNF-α IFX in the management of inflammatory bowel diseases (IBD)

2.1. Study population I.

In the first part of this prospective observational clinical study, 67 patients with CD and UC treated in our centre with IFX between 2011 and 2012 were enrolled and categorized into two groups. Blood samples of 36 patients with response loss, side effects, or hypersensitivity to IFX therapy (Group I) and 31 patients in complete clinical remission (Group Ⅱ) selected as a control group were collected to measure trough serum TNF-α level, IFX, and anti-IFX antibody (ATI) concentration. The study was approved by the Regional and Institutional Human Medical Biological Research Ethics Committee of the University of Szeged. The 3 infusion induction phase was followed by maintenance therapy in every patient. Data on patient demographics, clinical characteristics, concomitant corticosteroid and azathioprine therapies, need of surgery, C-reactive protein level, erythrocyte sedimentation rate (ESR), hematocrit, leukocyte and serum iron levels, and details on biological therapy were prospectively registered. Disease activity was measured by using the Crohn's disease activity index (CDAI) [53] and partial Mayo score [54]. The patients' demographic and clinical data are summarized in Table 2. We examined the correlation between loss of response, side effects, or hypersensitivity and serum TNF-α, IFX trough levels, and ATI concentrations.

Therapy	IBD patients with loss of response, side effects, hypersensitivity (n=36)	Control IBD patients (n=31)
Mean age at diagnosis, yr	34.9 (17-67)	36.4 (17-66)
Mean disease duration at biological therapy, yr	7.1 (1-20)	7.7 (1-21)
CD/UC	19/17	17/14
Male/Female	14/22	14/17
Previous biological therapy	22	15
Concominant steroid therapy	5	3
Concominant thiopurine therapy	18	16
Previous surgery	16	7
Active disease	25	0

Table 2. Demographic and clinical data of patients participating in the study. CD: Crohn's disease; UC: Ulcerative colitis; IBD: Inflammatory bowel disease.

2.2. Study population II.

The second section was a multicentre, prospective cohort study between September 2012 and May 2013 at 4 Hungarian IBD centres (1st Department of Medicine, University of Szeged, 1st Department of Medicine, Semmelweis University, 2nd Department of Medicine, Semmelweis University, Military Hospital, Budapest). Patients with IBD were recruited during outpatient visits at the centres. Inclusion criteria included an age ≥ 18 years, diagnosis of IBD stable for more than 3 months, no signs of activity (biological and clinical) and not requiring any treatment modification for the disease at inclusion. Patients with active IBD were excluded. At inclusion, influenza vaccination was offered to every patient attending the involved centres. Patients were randomised to two groups on the basis of the acceptance of the vaccination. Patients refusing the vaccination served as control subjects. Patients and control subjects were followed up for 4 months to determine the clinical activity and the frequency of influenza infections. Clinical data included age at diagnosis, disease duration, gender, IBD phenotype according to the Montreal classification [53], types of concomitant therapies, and types and dosages of immunomodulator and biological therapy. Immunisation history for the previous 5 years was also obtained.

Patients who received vaccination were divided into two further groups: patients treated with aminosalicylates without immunosuppressive therapy and patients treated with immunomodulator and/or biological therapy for at least three month before the vaccination. Control subjects had received maintenance therapy with immunomodulator and/or biological therapy for at least three month before the vaccination.

The type of vaccine (whole virion or split virion vaccine) was randomly selected. Validated clinical activity indices – CDAI [54] and Partial Mayo Score (pMayo score) [55] were used for CD and UC to assess disease activity. The patients were scored and blood samples were also taken before and after the vaccination. The patients were contacted by phone every week for 16 weeks. During the phone calls, data from each patient were collected using a standardised questionnaire. The patients were asked about any change in clinical activity and the development of local and systemic adverse reactions. Ethical approvals for the study had been obtained from the Scientific and Research Ethics Committee of Hungary. Written informed consent was obtained from each subject.

2.3. Vaccines

Two non-live vaccines directed against the seasonal influenza virus A/California/7/2009 (H1N1), A/Victoria/361/2011 (H3N2), B/Wisconsin/1/2010–like

B/Hubei-Wujiagang/158/2009 were used in the second part of the clinical study. Inactivated, split virion vaccine (IDFlu9) and inactivated, whole virion vaccine (Fluval AB) were administrated depending on a random selection.

2.4. Measurement of serum IFX trough levels, and ATI concentrations, TNF-α, IFN-γ and IL-2 levels

Enzyme-linked immunosorbent assay (ELISA) was applied to determine the serum levels of TNF-α, IFX trough levels, and ATI concentration. Blood samples, from IBD-patients, participating in the first part of our clinical study, were obtained prior to application of IFX infusion. Q-INFLIXI ELISA, Q-ATI ELISA, and Q-TNF-α ELISA kits were obtained from Matriks Biotek, Ankara, Turkey.

In the second part of our clinical study, serum was collected at baseline (pre-vaccination) and 5 to 6 weeks after vaccination and it was stored at -20 ^{o}C until use. From the collected serum samples, we assessed cell-mediated immune response after vaccination and also compared it between patients treated with and without immunosuppressants. The cell-mediated response to influenza A and B vaccines was evaluated using an INF-γ, IL-2, and TNF-α ELISA. Human TNF-α, IFN-γ and IL-2 ELISA kits were obtained from Life Technologies (Hungary). Serum was also obtained to assess leukocyte and lymphocyte levels quantitatively.

2.5. Statistical analysis

In order to examined the correlation between loss of response, the development of side effects or hypersensitivity, and serum TNF-α, IFX trough levels, and ATI concentrations, continuous data were analyzed using medians with an interquartile range (IQR). All categorical data were compared between groups of patients using the Pearson χ 2 statistic. Mann-Whitney U and Fisher's exact tests were used for comparison of infliximab trough levels and ATIs in a subgroup of patients. Relation between laboratory parameters, IFX trough levels, and ATI was analyzed by Mann-Whitney U test. A P value less than 0.05 was considered to be significant.

To the evaluation of cell-mediated immune response to the split and whole virion influenza vaccine in patients with IBD treated with anti-TNF-α and/or immunosuppressive therapy, data were analysed using SPSS version 21 software (SPSS, Chicago, IL). $p<0.05$ was considered significant. Categorical data were analysed using Pearson's chi-square test and Fisher's exact test. The effects of the vaccination on the antibody and cell-mediated immune

response were examined with multivariate analysis of variance (MANOVA) models with time as repeated measures (within-subject) factor and the types of the vaccines, the immunosuppressive status, the vaccinated status, the different therapies and the development of side-effects and influenza-like symptoms as between-subject factors. Pairwise comparisons were performed on estimated marginal means by considering the presence or absence of interaction; p-values were corrected with the Holm-Sidak method.

IV. RESULTS

1. The role of human colonic epithelial ion transporters in bile-acid induced diarrhoea

1.1. Chronic exposure of the colon to bile acids impair the activities of NHEs and CBE of isolated human colonic epithelial cells

Colonic crypts were isolated from patients whose colon is probably exposed to high concentrations of BA. Ileum-resected or cholecystectomised patients were divided into two groups depending on the presence (Diarrhoea; D) or absence (Non-Diarrhoea; NON-D) of diarrhoea after the surgical intervention. The calculated acid/base transporter activities were compared to those measured in control patients to determine the effects of BA on the epithelial ion transport.

Representative curves of the pH_i traces and the summary data of the calculated NHE activities are shown in Fig. 2A, B. The activities of the different NHE isoforms are extracted by using the isoform selective NHE inhibitor HOE-642. 1µM HOE642 inhibits NHE1 whereas 50µM HOE642 inhibits both NHE1 and 2 but not NHE3 [49, 50]. The functions of all examined NHE isoforms were significantly reduced in patients in group D compared to control patients.

The function of CBE was investigated using the Cl^- withdrawal technique (Fig. 2C). Removal of Cl^- from the standard HCO_3^-/CO_2 bath solution caused a marked alkalization in colonic crypt cells suggesting the presence of a functionally active anion exchange mechanism. The activity of the CBE was significantly impaired in D group compared to control patients (Fig. 2D). In colonic crypt cells, isolated from NON-D patients, the activities of the examined acid/base transporters were not changed significantly, compared to the control group, suggesting the significant role of ion transporters in bile-induced diarrhoea.
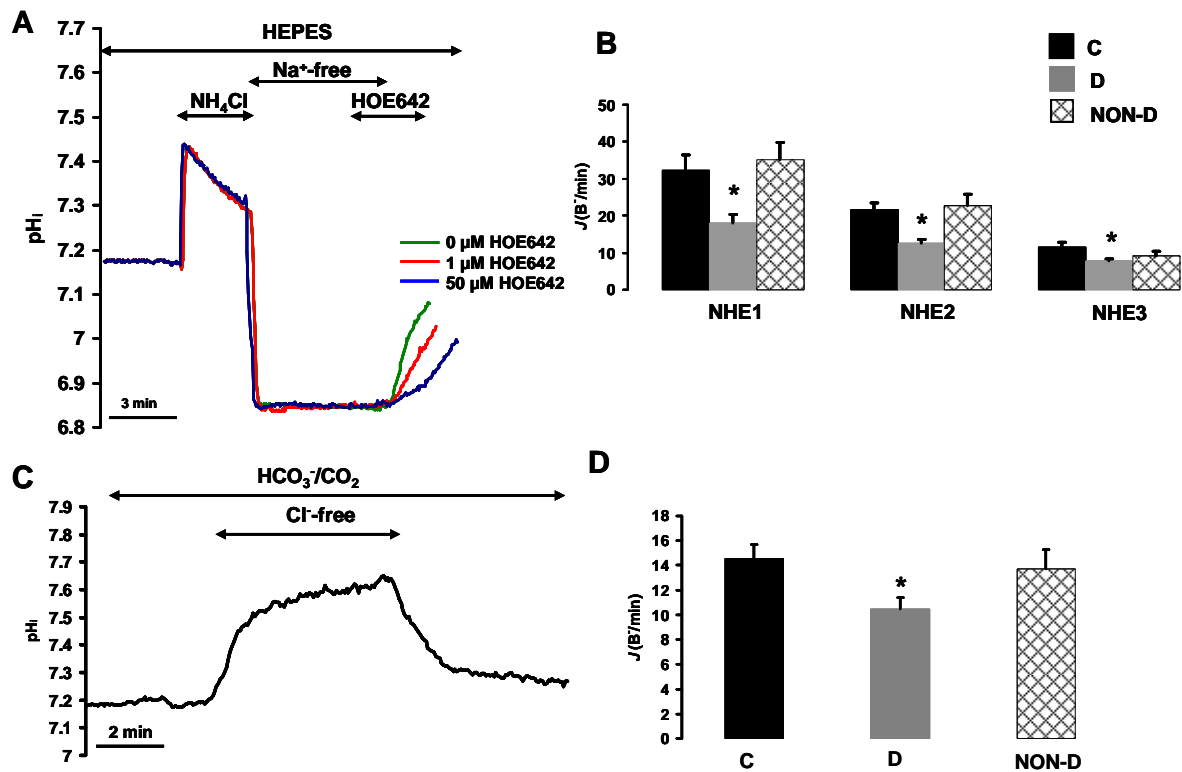
Figure 2. The activities of NHE and CBE were decreased in colonic epithelial cells isolated from ileum-resected/cholecystectomised patients suffering from diarrhoea. (A) The activities of different NHE isoforms were determined by NH_4Cl pulse technique with the isoform-specific NHE inhibitor HOE-642 as described in materials and methods. (B) Summary data of the calculated NHE activities. The activities of NHE1-3 were significantly impaired in patients suffering from diarrhoea. (C) Representative pH_i traces showing the effect of the Cl^- removal on the pH_i of the colonic epithelial cells. (D) Summary data of the calculated CBE activities, which were significantly decreased in patients suffering from diarrhoea. Groups of patients were C: control patients; D: ileum-resected/cholecystectomised patients suffering from diarrhoea and NON-D: ileum-resected/cholecystectomised patients without diarrhoea. Data are presented as means ± SEM. n=5-6 patients/16-24 crypts/32-48 ROIs, *p<0.05 vs. control

1.2. Bile acid administration dose-dependently reduce the pH_i of isolated human colonic epithelial cells

Our next aim was to characterise the basic effects of BAs on healthy colonic epithelial cells. For these experiments, colonic crypts were isolated from control patients. The administration of the non-conjugated chenodeoxycholate (CDC) and the conjugated glycochenodeoxycholate (GCDC) dose-dependently reduced the pH_i of perfused colonic epithelial cells (Fig 3). The characteristic response was a rapid decrease in pH_i which than slowly recovered to a variable degree during continuous exposure to BAs (Fig 3A-D). In HEPES-buffered solution, the ΔpH_{max} was more prominent during CDC administration, compared to those observed in HCO_3^--containing solution, which can be explained by the increased buffering capacity of the colonic epithelial cells in the presence of HCO_3^-/CO_2.

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The summary data of the calculated $J(B^-)$ in Fig 3E demonstrates that the influx of BAs was markedly greater when the non-conjugated CDC was administered compared to GCDC. This could be due to the lipophilic property of non-conjugated BAs which allows them to permeate through the membrane, while conjugated BAs need a BA transporter to enter the cells.
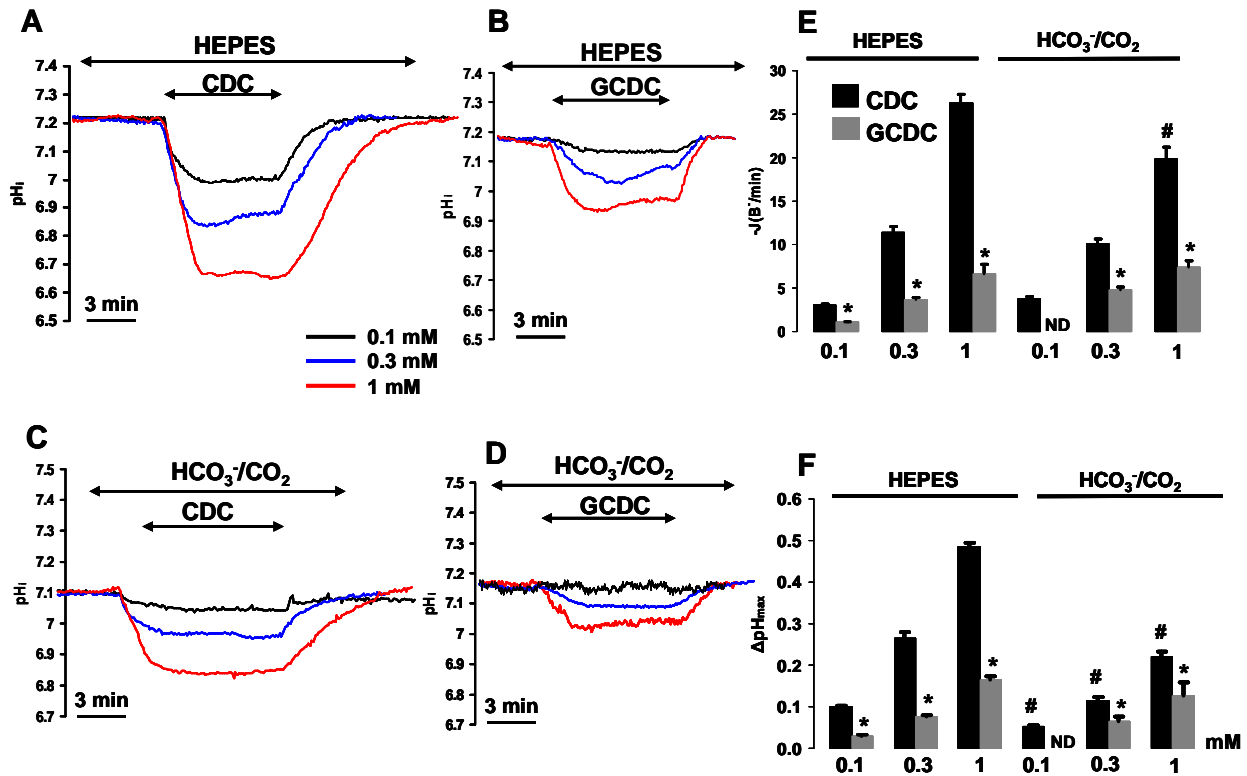

Figure 3. Bile acids induce dose-dependent acidosis in isolated human colonic epithelial cells. Representative pH_i traces **(A-D)** demonstrating the effect of non-conjugated CDC (0.1, 0.3 and 1 mM) and conjugated GCDC (0.1, 0.3 and 1 mM) administered in HEPES- **(A, B)** or HCO_3^-/CO_2-buffered **(C, D)** solution. Summary data of the calculated base flux ($J(B^-/min)$) **(E)** and the maximal pH_i change (ΔpH_{max}) **(F)** induced by bile acids. Data are presented as means ± SEM. n= 4-6 patients/14-18 crypts/28-36 ROIs. *p<0.05 vs. CDC, #p<0.05 vs. HEPES. ND: not detectable.

1.3. High concentration of the non-conjugated CDC in a short term administration inhibits the activities of acid/base transporters of isolated human colonic epithelial cells

Because impaired NHE and CBE activities were observed in patients suffering from diarrhoea, whose colon is probably continuously exposed to high concentration of BAs, we wanted to determine the influence of acute BA administration on the ion transport mechanisms of healthy human colonic epithelial cells. Colonic crypts isolated from control patients were used in these series of experiments. In order to investigate the effects of BAs on the activities of NHEs, we analysed the pH_i recovery from an acid load induced by the removal of NH_4Cl. The representative pH_i traces (Fig 4A) and the summary data of the

calculated NHE activities (Fig 4B) show that 10 min treatment with 0.1 mM CDC or GCDC had no effect on the functions of NHEs. Surprisingly, 0.3 mM GCDC significantly stimulated the activities of NHEs. When the colonic crypts were perfused with 0.3 mM CDC, an inhibition of the activities of NHEs was perceptible. To identify the exact NHE isoform, which is inhibited by 0.3 mM CDC, we used the ammonium pulse technique with the isoform-selective NHE inhibitor HOE-642 during the continuous perfusion with 0.3 mM CDC. According to our results, all of the NHE isoforms were significantly inhibited by 0.3 mM CDC (Fig 4C).
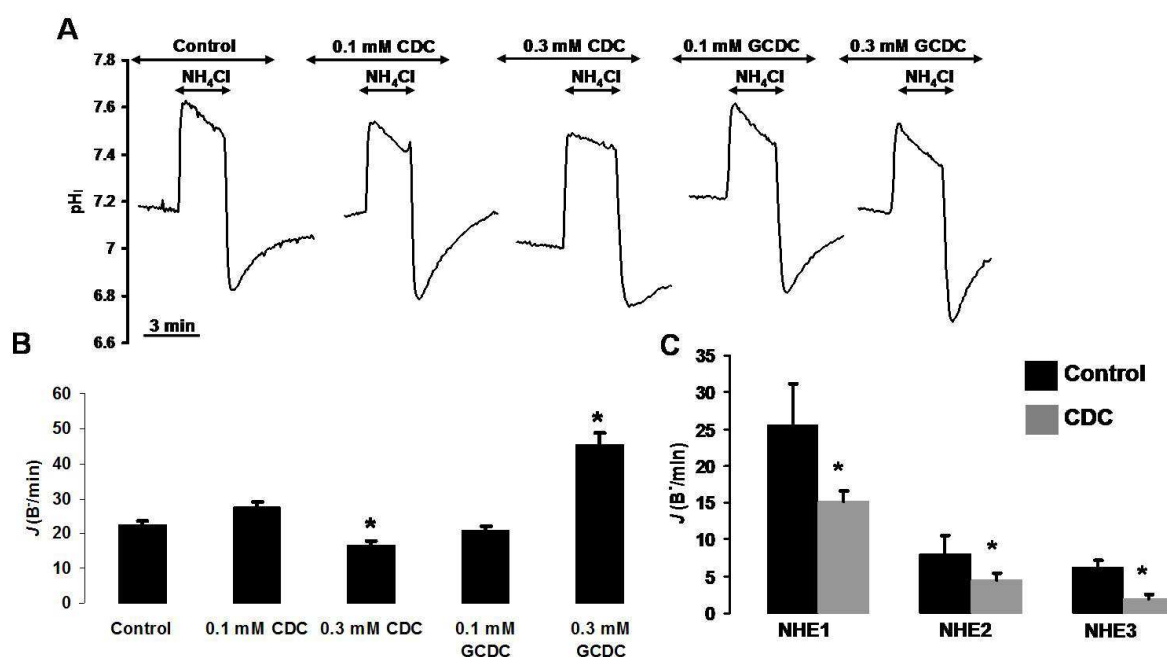

Figure 4. Administration of 0.3 mM CDC significantly inhibites NHE activity in isolated human colonic crypts. **(A)** Representative pH_i curves showing the effects of CDC (0.1 and 0.3 mM) and GCDC (0.1 and 0.3 mM) on the recovery from an acid load induced by removal of 20 mM NH_4Cl. **(B)** Summary data of the initial rate of pH_i recovery from acid load. **(C)** Summary data of the effect of 0.3 mM CDC on the calculated activities of the different NHE isoforms. The isoform-selective NHE inhibitor HOE-642 was administered as described in Matherials and Methods during treatment with 0.3 mM CDC. Data are presented as means ± SEM. n=3-6 patients/6-12 crypts/12-40 ROIs, *p<0.05 vs. control.

We also tested the effects of higher BA concentrations. When 20mM NH_4Cl was applied at the same time with 1 mM CDC, the fluorescent intensities at 440 and 495 nm rapidly decreased causing an elevation of the 440/495 ratio. This must be due to the loss of BCECF and reflects the lysis of the cells. This harmful effect was absent when the crypts were exposed to 1 mM on the conjugated bile acid GCDC (data not shown).

The Cl⁻-withdrawal technique was applied to examine the activity of CBE as well. The apical Cl⁻ removal from the extracellular solution increased the pH_i of the cells by driving HCO_3^- into the cell via the apical CBE, whereas, re-addition of Cl⁻ decreased pH_i inducing secretion of HCO_3^- via the CBE. Treating the crypts with 0.3mM CDC resulted in a strong inhibition in the activity of CBE (Fig 5). Neither low concentration (0.1 mM) of CDC nor 0.1 mM or 0.3 mM GCDC influenced the function of CBE.
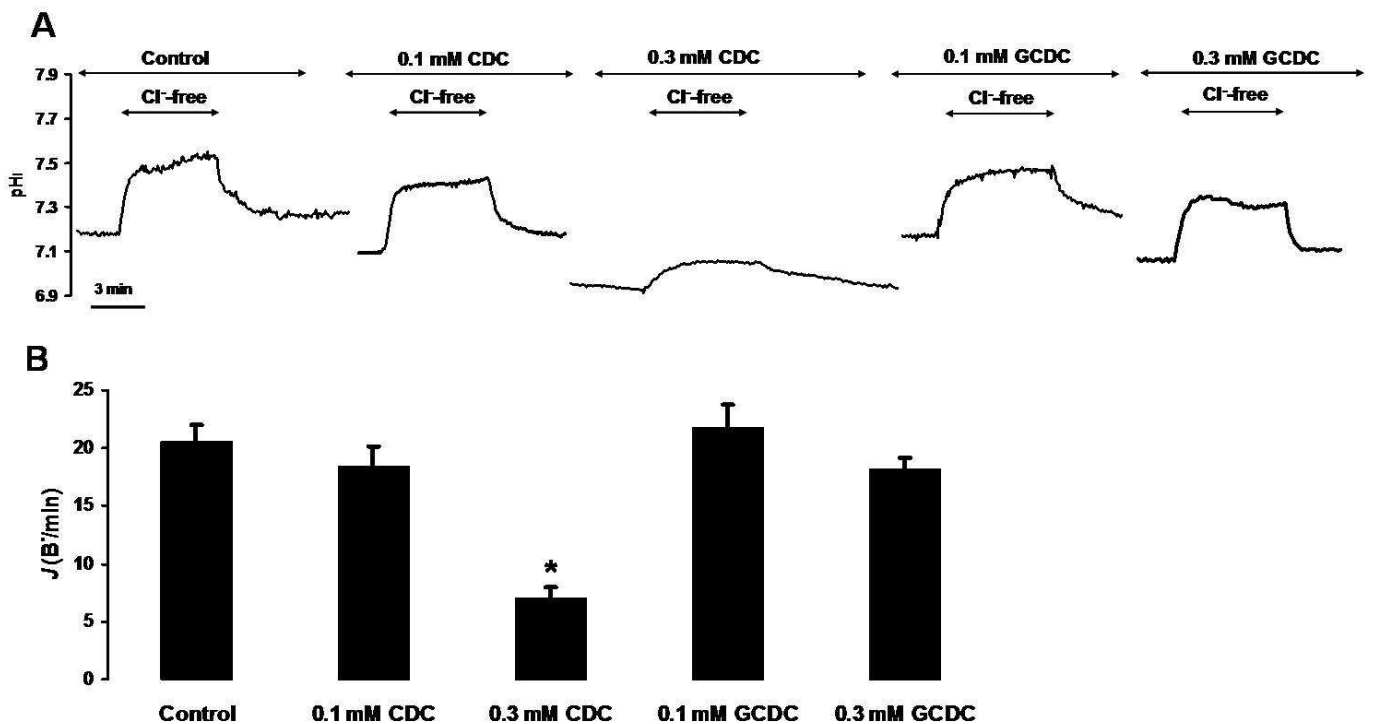

Figure 5. 0.3 mM CDC significantly inhibites CBE activity in isolated human colonic crypts. (A) Representative pH_i traces showing the effect of CDC (0.1 and 0.3 mM) and GCDC (0.1 and 0.3 mM) during removal of extracellular Cl⁻. **(B)** Summary data of the initial rate of pH_i elevation after Cl⁻ withdrawal. Data are presented as means ± SEM. n=3-6 patients/6-12 crypts/12-40 ROIs, *p<0.05 vs. control.

1.4. High concentration of the non-conjugated CDC induces severe mitochondrial damage

Our next aim was to explore the intracellular mechanisms by which BAs exert their inhibitory effect on acid/base transporters. Since previous works have reported that BAs can disrupt intracellular organelles (Golgi, mitochondria) [56-58], we firs analysed the structure of the cell compartments of human colonic epithelial cells following incubation with BAs. TEM (Fig 6) showed that low concentration of CDC (0.1 mM or 0.3 mM) or 1 mM GCDC for 1-10 min had no effect on the structure of intracellular organelles. On the other hand, 10-minute exposure of the human colonic epithelial cells to high concentration (1 mM) of CDC strongly damaged all of the mitochondria. The mitochondria swelled up and the inner membrane

structures were disrupted. We did not observe such alteration in other intracellular organelles, such as endoplasmic reticulum, Golgi apparate or nuclei. For positive control experiments, the mitochondrial toxin carbonyl cyanide m-chlorophenyl hydrazone (CCCP, 100 μM) was applied. The mitochondrial injury was similar to those seen after 1mM CDC treatment.
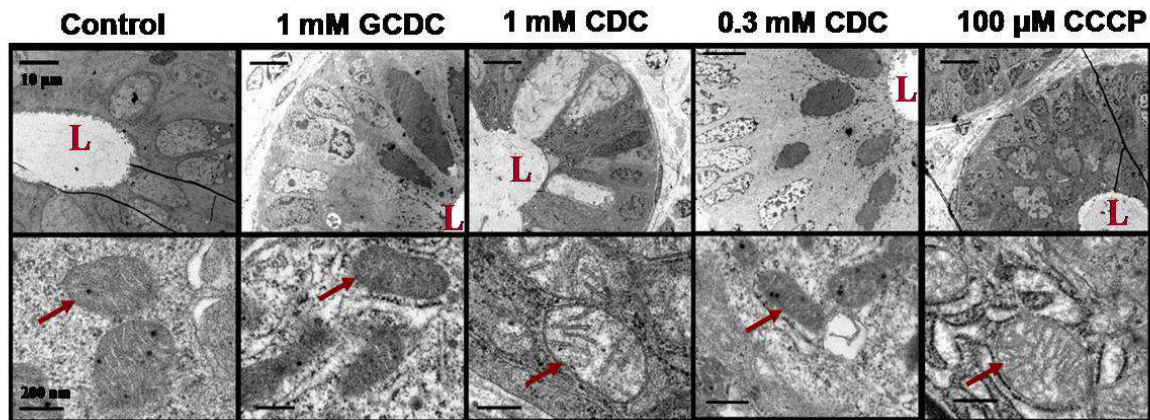

Figure 6. Treatment with 0.3 mM CDC does not alter the structure of intracellular organelles. Transmission electron microscopy. Effects of CDC (0.3 mM and 1 mM) and GCDC (1 mM) on the mitochondria of human colonic epithelial cells. L: crypt lumen, arrow: mitochondria.

1.5. Bile acid treatment decreases ATP_i and $(\Delta\psi)_m$ of isolated human colonic epithelial cells

Since TEM experiments didn't reveal the inhibitory mechanism of 0.3 mM CDC completely, we tried to dissect the mechanism at a functional level. Therefore, in the next step we aimed to find out whether CDC has any influence on the ATP_i level of the human colonic epithelial cells. Using the Mg-Green fluorescent probe, which is indirectly sensitive to ATP_i (see Methods) we showed, that 0.3mM CDC significantly, but reversibly depleted ATP_i of isolated human colonic epithelial cells (Fig. 7A, B). Following administration of 0.1mM CDC the ATP_i was not affected (data not shown). In case of 1mM CDC, not only the structural impairment of the mitochondria was evident but a significant and irreversible decrease of the ATP_i level was perceptible as well. For positive control, the mitochondrial toxin CCCP was applied similarly to the morphological studies. The fact that high concentration of CDC caused more prominent ATP_i depletion than CCCP, suggests that BA have additional effects, which further decreases ATP_i. To investigate the effects of BA on glicolytic ATP-production the combination of deoxyglucose (DOG)/idoacetamide (IAA) was used, which inhibit the glycolytic metabolism of colonic epithelial cells. 10mM DOG + 5mM IAA significantly and irreversibly depleted ATP_i. CCCP and DOG+IAA together mimicked the effect of high conenctrations of CDC on the ATP_i of isolated human colonic epithelial cells.

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Alterations of $(\Delta\psi)_m$ were also examined. Representative traces in Fig. 7C demonstrate that administration of 0.1mM or 0.3mM CDC induced a significant decrease in TMRM fluorescence, which indicates the loss of $(\Delta\psi)_m$. This effect was reversible, $(\Delta\psi)_m$ returned to basal level following removal of CDC. Furthermore 1mM CDC or 100µM CCCP caused a marked and irreversible reduction of $(\Delta\psi)_m$.
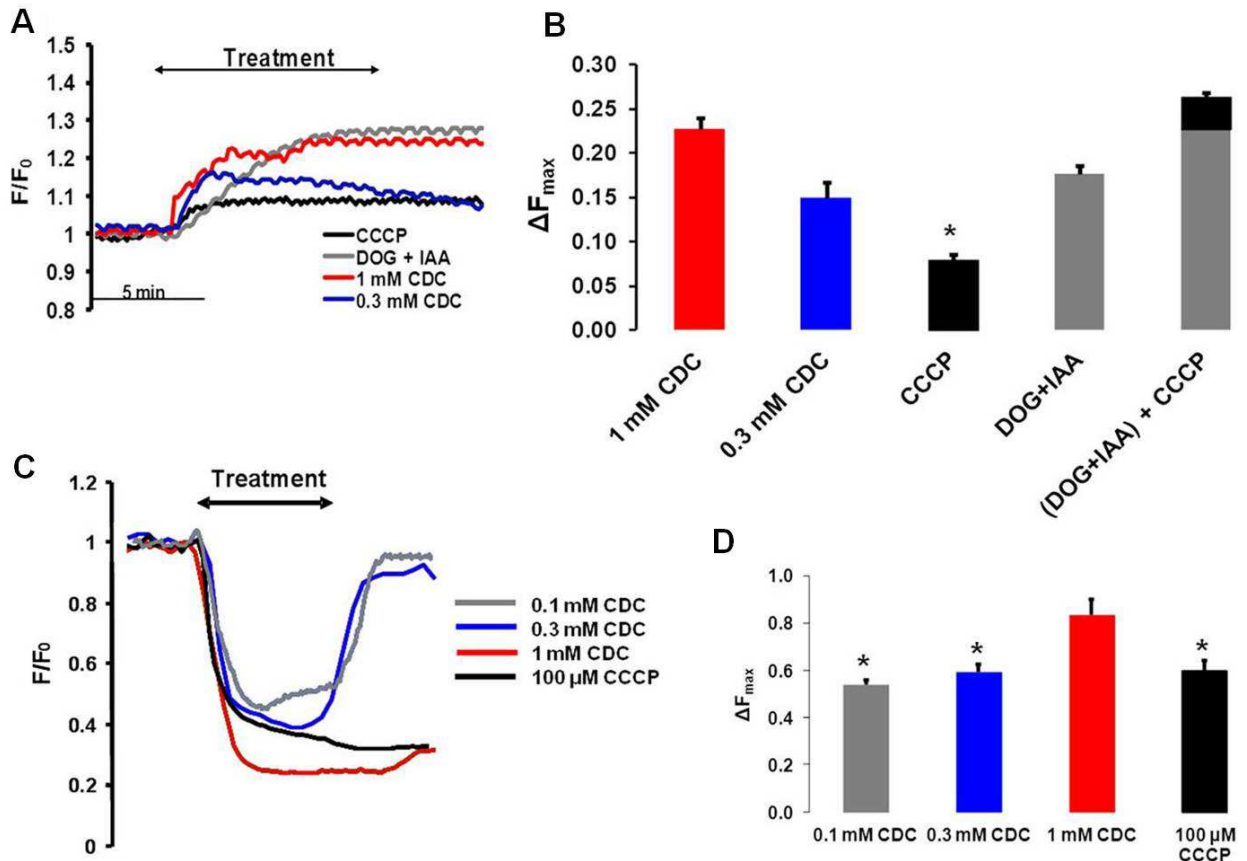

Figure 7. Treatment with 0.3 mM CDC significantly decreases ATP$_i$ and disturbs $(\Delta\psi)_m$. (A) Representative curves of the Mg-green fluorescent experiments. Elevation of fluorescent intensity represents depletion in ATP$_i$. **(B)** Summary data for the maximal fluorescent intensity changes. High concentrations of CDC significantly decreased ATP$_i$. **(C)** Representative traces of the $(\Delta\psi)_m$ measurements. Decrease of fluorescent intensity represents loss of mitochondrial transmembrane potential. **(D)** Summary data for the maximal fluorescent intensity changes. High concentrations of CDC significantly decreased $(\Delta\psi)_m$. All experiments were performed in HEPES-containing solution. Data are presented as means±SEM. n=3 patients/5‑6 crypts/13‑15 ROIs. *p<0.05 vs. 1 mM CDC

1.6. CDC dose dependently increases [Ca^{2+}]$_i$ via endoplasmic reticulum (ER) Ca^{2+} release and extracellular Ca^{2+} influx

To further investigate the intracellular effects of BA, the changes of [Ca^{2+}]$_i$ in isolated human colonic epithelial cells was measured during CDC-treatment. In our experiments, administration of CDC caused a dose-dependent increase in [Ca^{2+}]$_i$ (Fig. 8A-C). The increase in [Ca^{2+}]$_i$ was a sustained, plateau-like pathophysiological signal. The removal of Ca^{2+} from

the extracellular solution significantly decreased this effect. Pretreatment of the colonic crypts with BAPTA-AM (40µM), a fast chelator of $[Ca^{2+}]_i$ abolished the effect of 0.1 and 0.3mM CDC, however, a moderate increase of $[Ca^{2+}]_i$ was still observable when 1mM CDC was applied (Fig. 8D).
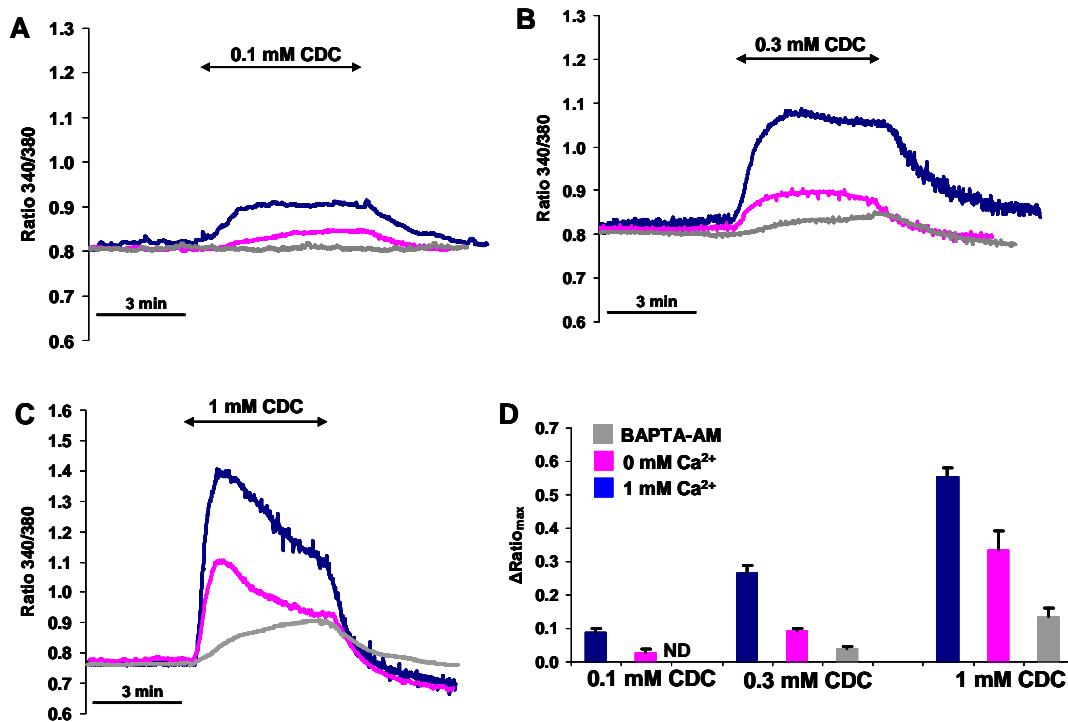

Figure 8. CDC dose-dependently induces an increase in the $[Ca^{2+}]_i$ of isolated human colonic epithelial cells. (A-C) Representative curves showing the effect of CDC (0.1, 0.3 and 1 mM) on $[Ca^{2+}]_i$ of isolated human colonic crypts in Ca^{2+}-free or 1 mM Ca^{2+}-containing solution with/without pretreatment with the $[Ca^{2+}]_i$ chelator BAPTA-AM. **(D)** Summary data for the maximal fluorescent intensity changes. All experiments were performed in HEPES-buffered solution. Data are presented as means ± SEM. n=2-4 patients/5-10 crypts/10-21 ROIs.

We made attempts to identify the source of Ca^{2+}, released during CDC-treatment. Caffeine (20mM) and/or Ruthenium red (RR, 10µM) were utilised in order to antagonise inositol triphosphate receptor (IP_3R) and ryanodin receptor (RyR), which can mediate Ca^{2+} release from the ER. Representative curves and the summary bar chart (Fig. 9A, B) demonstrate that the application of caffeine significantly inhibited the increase in $[Ca^{2+}]_i$ generated by 0.3mM CDC, while the administration of RR had no effect on the Ca^{2+} release. The rate of $[Ca^{2+}]_i$ increase was significantly diminished as well during the administration of caffeine (Fig. 9C).
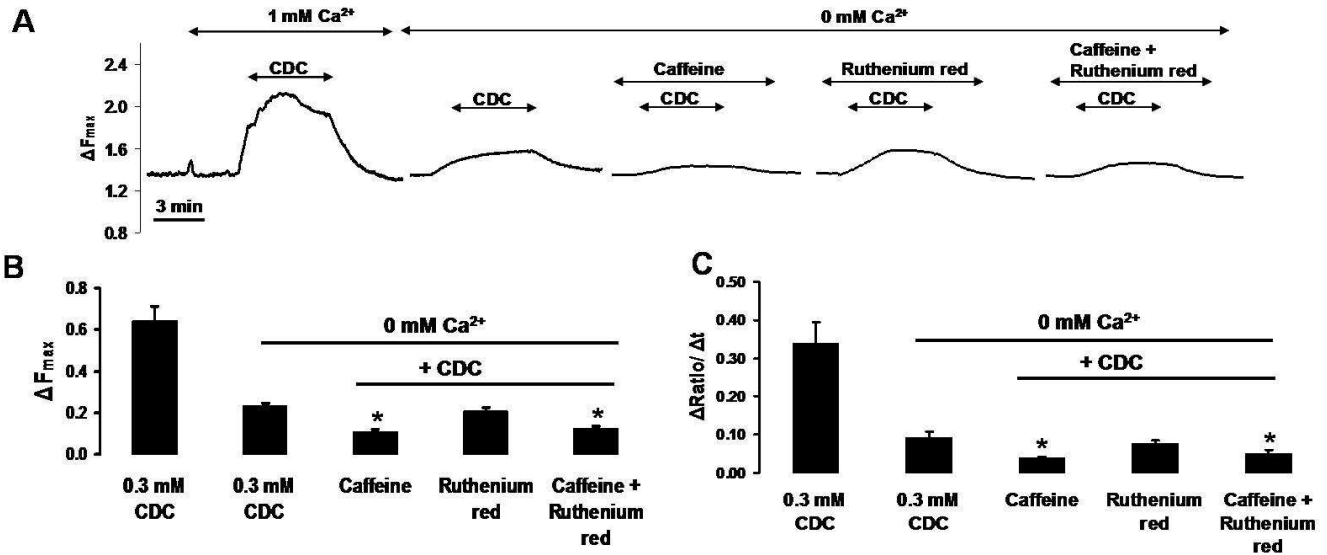
Figure 9. CDC released Ca^{2+} from the ER in isolated human colonic epithelial cells. (A) Representative curves showing the effect of the IP$_3$R inhibitor caffeine (20 mM), RyR inhibitor RR (10 μM) on the increase of [Ca^{2+}]$_i$ induced by 0.3 mM CDC. (B) Summary data of the maximal fluorescent intensity changes. (C) Summary data of the calculated rate of [Ca^{2+}]$_i$ increase. All experiment were performed in HEPES-buffered solution. Data are presented as means ± SEM. n=2-4 patients/5-8 crypts/10-18 ROIs. *p<0.05 vs. CDC in 0 mM Ca^{2+} containing solution.

In the next step gadolinium (Gd^{3+}, 1μM) was applied to block plasma membrane Ca^{2+} entry channels. Gd^{3+} alone was not able to decrease the elevation of [Ca^{2+}]$_i$ induced by 0.3mM CDC, while the simultaneous administration of Gd^{3+}, caffeine and RR significantly reduced it (Fig. 10).

Figure 10. CDC released Ca^{2+} from the ER and induced extracellular Ca^{2+} influx in isolated human colonic epithelial cells. (A) Representative curves showing the effect of the plasma membrane Ca^{2+} channel inhibitor Gd^{3+} (1 μM). (B) Summary data of the maximal fluorescent intensity changes. (C) Summary data of the calculated rate of [Ca^{2+}]$_i$ increase. All experiment were performed in HEPES-buffered solution. Data are presented as means ± SEM. n=2-4 patients/5-8 crypts/10-18 ROIs. *p<0.05 vs. CDC

To further characterize the CDC-induced increase of $[Ca^{2+}]_i$ in colonic epithelial cells, thapsigargin (Tg), the sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA pump) inhibitor was applied. In Ca^{2+}-free solution, Tg (2μM) induced Ca^{2+} store depletion with consequent $[Ca^{2+}]_i$ elevation. This elevation was markedly decreased when Tg was administered after 0.3 mM CDC. Tg, when administered during 0.3mM CDC, further induced a slight increase in $[Ca^{2+}]_i$ (Fig 11). These observations suggest that beside the extracellular Ca^{2+} influx, CDC deplete ER Ca^{2+}-stores via IP_3R mediated processes.
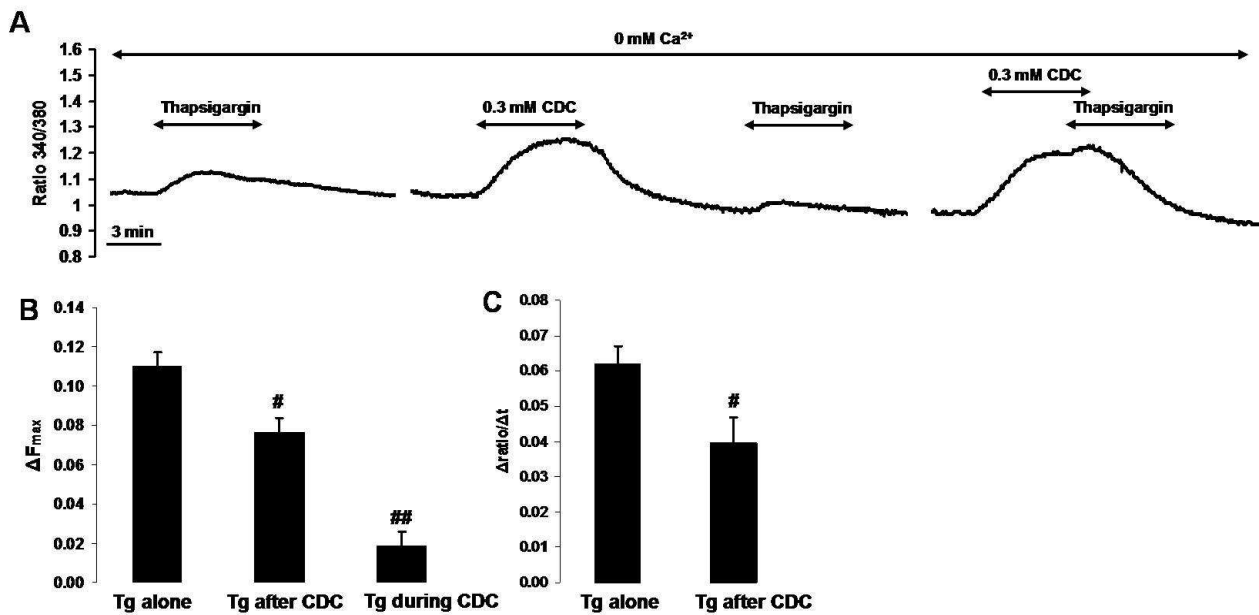

Figure 11. The ER Ca^{2+} pump SERCA inhibitor further increases $[Ca^{2+}]_i$ after CDC treatment in isolated human colonic crypts. (A) Representative traces showing the effects of SERCA-inhibitor Tg (2 μM) administered alone, following or during 0.3 mM CDC on the $[Ca^{2+}]_i$. **(B)** Summary data of the maximal fluorescent intensity changes and **(C)** the calculated rate of $[Ca^{2+}]_i$ increase. All experiments were performed in Ca^{2+}-free HEPES-buffered solution. Data are presented as means ± SEM. n=2-3 patients/5-6 crypts/12-14 ROIs. #p<0.05, ##p<0.01 vs. Tg alone.

1.7. The non-conjugated CDC induces ATP-dependent decrease in the Na^+/H^+-exchange activities and Ca^{2+}-dependent inhibition of CBE activity in isolated human colonic epithelial cells

Next we examined the potential connection between the intracellular effects of 0.3mM CDC (ATP_i depletion and $[Ca^{2+}]_i$ elevation) and the decreased function of acid/base transporters following treatment with the BA. Ammonium pulse technique (Fig. 12A) showed that chelation of intracellular Ca^{2+} with BAPTA-AM did not influence the inhibitory effect of 0.3mM CDC on the NHE activity. In contrast, when the glycolysis inhitbitors DOG+IAA were applied the same inhibition of NHEs was perceptible (Fig. 11B), which suggests that CDC inhibits NHE via ATP_i-depletion. This result confirms the observation of other

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workgroups that NHEs are secondary-active transporters and their function is ATP-dependent [59-61].
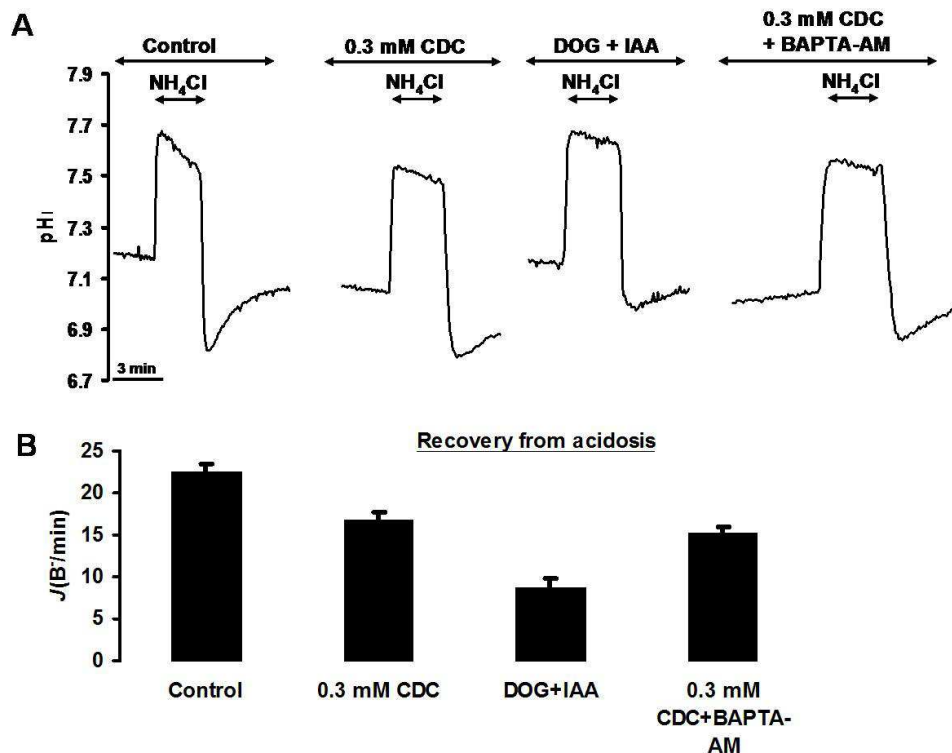

Figure 12. 0.3 mM CDC induced ATP$_i$-dependent inhibition of NHEs in isolated human colonic epithelial cells. **(A)** Representative pH$_i$ curves showing the effect of 0.3 mM CDC, (ATP)$_i$ depletion or pretreatment with the [Ca^{2+}]$_i$ chelator BAPTA-AM on the initial rate of pH$_i$ recovery from an acid load induced by the removal of 20 mM NH$_4$Cl. **(B)** Summary data of the initial rate of pH$_i$ recovery from an acid load. The depletion of ATP$_i$, mimicked, but the prevention of Ca^{2+} elevation did not change the effect of CDC on the NHE activities. The experiments were performed in HEPES-containing solution. Data are presented as means ± SEM. n=3-6 patients/5-12 crypts/8-40 ROIs. *p<0.05 vs. control.

We also investigated the inhibitory effect of 0.3mM CDC on CBE more detailed. We tested the effects of [Ca^{2+}]$_i$ chelation and ATP$_i$ depletion during the Cl$^-$-removal technique in isolated human colonic epithelial cells. In our experiments, the intracellular Ca^{2+}-chelator BAPTA-AM completely abolished the inhibitory effect of 0.3mM CDC on the activity of CBE. In contrast, depletion of ATP$_i$ with DOG+IAA was not able to reduce CBE activity. These results provide evidence for the strong Ca^{2+}-dependence of the transporter. Our observations suggest that bile acids inhibit CBE activity via toxic [Ca^{2+}]$_i$ elevation unlike NHE activity, which is inhibited by ATP$_i$ depletion (Fig. 13).
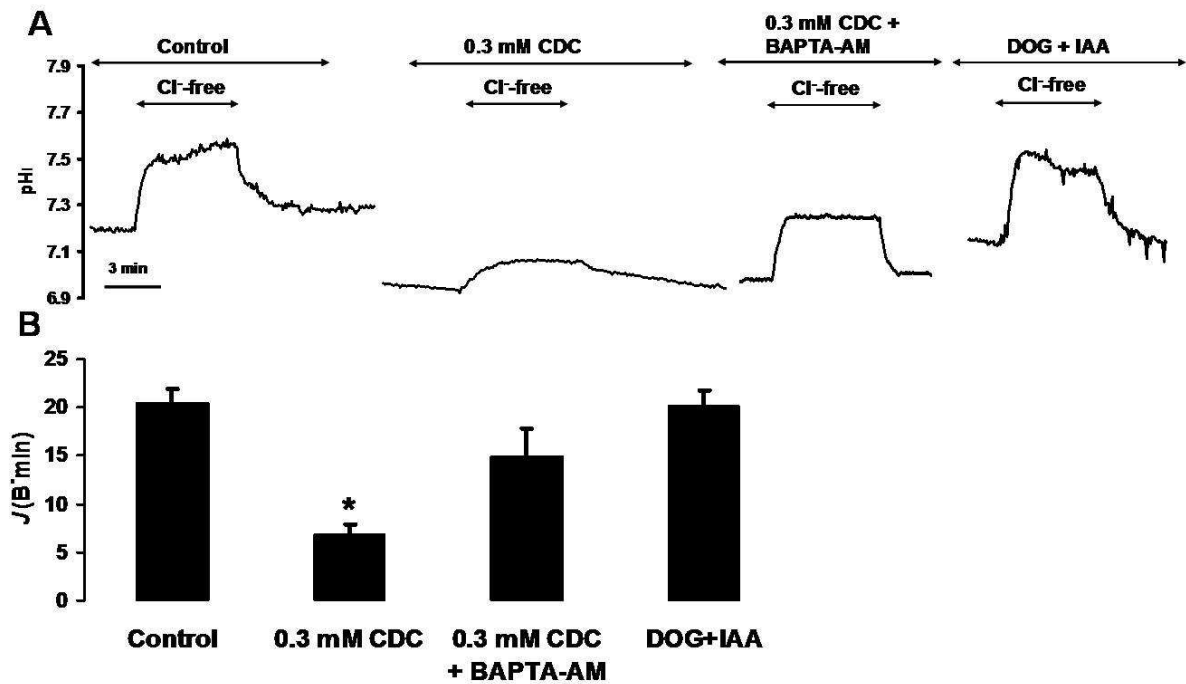
Figure 13. 0.3 mM CDC induced ATP$_i$-dependent inhibition of NHEs and Ca^{2+}-dependent decrease in the activity of CBE in isolated human colonic epithelial cells. (A) Representative pH$_i$ curves demonstrating the effect of 0.3 mM CDC, ATP$_i$ depletion or pretreatment with the [Ca^{2+}]$_i$ chelator BAPTA-AM on the removal of extracellular Cl$^-$. **(B)** Summary data of the initial rate of pH$_i$ elevation after Cl$^-$ withdrawal. The chelation of the [Ca^{2+}]$_i$ elevation abolished the inhibitory effect of CDC. The experiments were performed in HCO$_3^-$-containing solution. Data are presented as means±SEM. n=3‑6 patients/5‑12 crypts/8‑40 ROIs. *p<0.05 vs. Control.

2. Biological therapy with the anti-TNF-α IFX in the management of IBD

2.1. Assessment of TNF-α, IFX concentrations, and antibodies against IFX molecules in IBD-patients who develop loss of response, side effects, or allergic reaction during anti TNF-a therapy

The median CDAI in groups Ⅰ and Ⅱ were 138 (IQR 68-186) and 50 (IQR 34-70), respectively; the partial Mayo score in the two groups were 5 (IQR 3-6) and 1 (IQR 0-1), respectively. The median serum TNF-α levels were 10.5 (IQR 3.2-18-9) and 6.3 (IQR 1.5-15.7) pg/mL in groups Ⅰ and Ⅱ, respectively. The median IFX trough level was 3.1 (IQR 2.6-5.04) and 3.5 (IQR 2.6-4.7) μg/mL in the two groups, respectively. Fourteen patients were found to have ATI positivity with a median of 933 μg/mL (IQR 328-3306). ROC analysis revealed that the cut off value of serum IFX for detecting ATI was 3.01 μg/mL. The serum TNF-α level was significantly higher in the presence of ATI (24.23 pg/mL *vs* 6.28 pg/mL, *P*= 0.005). ATI positivity correlated significantly with low trough levels of IFX (2.66 μg/mL *vs* 3.86 μg/mL, *P* = 0.015). However, no difference was detected in serum IFX and antibody

levels between the two groups (2.67 μg/mL *vs* 2.66 μg/mL, $P = 0.821$). Serum IFX and ATI levels in patients with ATI positivity are summarized in Table 3.

Two of the IBD patients with antibodies against anti TNF-α developed side effects, 5 patients lost response, and an allergic reaction occurred in 3 patients. 37 patients were previously treated with biologicals, with development of ATI being more frequent those patients ($P = 0.048$). Dose intensification was required in 9 patients. No association was found between dose intensification and the development of ATI. Concomitant immunosuppression had no impact on IFX trough levels or on the development of ATI formation. Increased ESR and C-reactive protein correlated significantly with lower serum IFX level ($P = 0.04$ and $P = 0.002$). The serum TNF-α level was higher in patients not treated concomitantly with steroids ($P = 0.038$).

Patients	Serum IFX level (μg/ml)	ATI level (μg/ml)
1	2.75	3194.90
2	2.68	258.55
3	2.67	1056.25
4	2.66	3055.04
5	2.93	3712.82
6	2.26	3343.07
7	2.66	129.54
8	2.49	4540.33
9	12.4	58.92
10	2.66	3679.21
11	2.65	536.57
12	1.90	555.53
13	1.71	810.87
14	4.67	46.34

Table 3. Serum IFX and ATI levels in case of ATI positivity

2.2. The impact of influenza-vaccination on the cellular immune response of IBD-patients treated with anti-TNF-α and/or immunosuppressive therapy.

209 IBD patients (127 with CD, 82 with UC) were eligible and enrolled in the study. 156 patients received influenza vaccination, whereas 53 patients (control group) refused the

vaccine – the acceptance rate of vaccination was 66.3%. Whole virion vaccine was given to 57; split virion vaccine was given to 99 patients. The mean age of the vaccinated patients was 27.9 years; 84 were women, 72 were men. In the control group, the mean age was 30.7 years, 29 were women, 24 were men. Out of the 156 vaccinated patients, 98 had CD, 58 had UC. Median disease duration was 9 years for CD (IQR 5-13), and 9 years for UC (IQR 4-15.8). Of the control subjects, 29 had CD and 24 had UC. Median disease duration was 7 years for both CD (IQR 5-14) and UC (IQR 4.5-12).

Out of the 156 vaccinated patients, 115 received immunosuppressive therapy. The non immunosuppressive group of vaccinated subjects was composed of 41 patients. Out of the 53 control subjects, 32 received immunosuppressive therapy. Twenty-one patients were free of immunosuppressive therapy. 8.3% of the patients were regularly vaccinated against seasonal influenza virus. 39 patients (21.5%) had received the last vaccination within one year, 25 patients (13.8%) within 3 years and 3 patients (1.7%) within 5 years. 63% of the patients had received the last vaccination more than 5 years earlier. Demographic and clinical characteristics, disease activity at the time of the vaccination and treatment types are summarised in Table 4.

Leukocyte and lymphocyte levels varied between 2.78-17.6 G/L and 0.38-20.9 G/L before and between 2.41-20.54 G/L and 7.9-43.8 G/L after the vaccination. Leukocyte and lymphocyte levels did not differ significantly after vaccination. The level of INF-γ varied between 9.9 and 39.1 pg/ml before and between 11.7 and 39.1 pg/ml after vaccination. IL-2 levels varied between 14.7 and 152.6 pg/ml before and between 13.8 and 152.3 pg/ml after the vaccination. The levels of TNF-α varied as: 11.6-360.4 pg/ml before and 8.5-216.9 pg/ml after the administration of the vaccine. Neither TNF-α, nor INF-γ levels changed significantly after influenza vaccination; however, a significant decrease was observed in the level of IL-2 after vaccination with split vs. whole virion vaccine (p=0.004).

	All patients (n = 209)	Fluval AB vaccinated (n = 57)	IDFlu9 vaccinated (n = 99)	Controls (n = 53)
Gender				
Male	96	30	42	24
Female	113	27	57	29
Mean age at diagnosis (years)	28.6	27.2	28.3	30.7
Median disease duration (years)	9	9	9	7
Crohn's disease	127	46	52	29
Ulcerative colitis	82	11	47	24
Age of diagnosis				
A1	22	4	11	7
A2	158	47	74	37
A3	29	6	14	9
Disease location				
L1	25	10	10	5
L2	46	14	20	12
L3	54	21	22	11
L4	2	1	0	1
Disease behavior				
B1	41	14	19	8
B2	24	9	12	3
B3	62	23	21	18
Extent of UC				
E1	21	3	11	7
E2	28	5	16	7
E3	33	3	20	10
Therapy				
Aminosalycilates	21	5	6	10
Thiopurines	27	7	14	6
Anti-TNF-α	26	10	12	4
Combined thiopurines and anti-TNF-α	62	20	20	22
Mean CDAI	120.3	108.4	133.2	137.5
Mean pMayo score	1.8	1.7	1.6	2.1

Table 4. Demographic, clinical characteristics, disease activities and treatment types of patients, enrolled in the study.

V. DISCUSSION

1. The role of human colonic epithelial ion transporters in bile-acid induced diarrhoea

One of the main non-motor functions of the colon is to absorb about 90% of the fluid (~1.5 to 1.9 liters), arriving daily from the small intestine [62]. This great absorptive capacity of the large intestine is mediated by the polarised epithelial cell layer which maintains the balance between absorption and secretion. These polarised epithelial cells are equipped with numerous ion channels, pumps and carriers located either on the luminal or basolateral membrane, allowing highly efficient transport of large amount of water and salts, especially Na^+ and Cl^- [63]. The electroneutral NaCl absorption is probably mediated via the coupled activity of Na^+/H^+ exchangers and Cl^-/HCO_3^- exchangers [2, 3]. The fact that impaired activities of these transporters were observed in diarrhoea-associated diseases, such as ulcerative colitis and secretory diarrhoea [12, 13, 64], underlies their importance in colonic electrolyte and water absorption.

BAs are amphipathic molecules aiding fat solubilisation and lipid digestion in the intestine. Besides the physiological functions, BAs are also known to induce diarrhoea, a common feature of BA malabsorption. BAM often develops after small bowel resection or post-cholecystectomy [14-17]. Increased colonic motility [65], stimulated defecation, mucosal damage, increased epithelial permeability and stimulated mucus secretion [66] are the major responses to the elevated amount of BAs in the colon . In addition, the influence of BAs on the secretory function of colonic epithelium is supposed to be critical in the development of bile-induced diarrhoea. Confirming this, the prosecretory action of BAs on colonic epithelial cells was demonstrated [21-23, 67, 68]. Although bile-induced diarrhoea is a frequent complication affecting high number of patients, its pathogenesis is not yet completely understood.

Because BAs can induce diarrhoea when they are present in the colon at high concentration, in our first series of experiments, we wished to clarify whether the decreased activities of acid/base transporters are involved in this process. Thus, we isolated colonic crypts from patients, whose colon is probably continuously exposed to high concentration of BAs. Ileum-resected or cholecystectomised patients were involved in the study and were divided into two groups depending on having (Diarrhoea - D) or not having diarrhoea (Non-Diarrhoea – NON-D) after the surgical intervention. In the D-group, the activities of all

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examined NHE isoforms were markedly lower than in control patients. Next, we tested whether the activity of the CBE is also influenced by chronic BA exposure. Similarly to NHEs, the activity of the CBE was significantly diminished in D-patients compared to controls. It is important to note that the functions of the examined acid/base transporters were unaltered in colonic epithelial cells isolated from biopsy samples of NON-D patients. These data suggest that the reduced absorptive function of the colon is probably due to the continuous presence of non-physiological concentration of BAs. This is in agreement with the observation that CDC reverses the absorption of water and Na^+ into secretion in rat colon [69], moreover, Freel et al. showed inhibited Na^+ absorption in rabbit colon induced by BAs [70]. In a more previous study Binder et al. also reported that excess BAs in rat colon decrease Na^+ and Cl^- absorption [71].

Following our first fundamental observation regarding the long-term influence of BAs, we aimed to characterise their basic effects on human colonic epithelial cells. Thus, colonic crypts isolated from control patients were treated with the non-conjugated BA CDC and the conjugated GCDC. We used conjugated and non-conjugated BAs as well, because due to the conjugation, the natures of the BAs are changing. Being a weak acid, CDC can traverse cell membrane by passive diffusion [72]. On the contrary, conjugated BAs are water soluble and require active transport mechanisms for cellular uptake [73]. Indeed, the presence of the apical sodium dependent BA transporter ASBT was found to be expressed in human biopsies from different segments of the large intestine [74]. In this study, three concentrations of the BAs were applied: 0.1 mM as a low, while 0.3 and 1 mM served as high concentrations, in order to imitate physiological and non-physiological circumstances. This was based on the data of Hamilton et al., that the physiological concentration of total BAs – including conjugated and non-conjugated forms - in the proximal colon (cecum) reaches 1 mM [75]. Moreover, in the large intestine, BAs are metabolized, mainly deconjugated, by resident bacteria [76-78], leading to the elevated formation of the more lipohil non-conjugated BAs. In our experiments, administration of BAs caused an immediate, dose-dependent and reversible decrease of the pH_i, which acidosis was more prominent in case of the CDC, due to the typical characteristics of the non-conjugated BAs.

Because decreased activities of NHEs and CBE were observed in patients suffering from diarrhoea, whose colon is probably continuously exposed to high concentration of BAs, next we investigated the short-term effects of BAs on the functions of ion transporters of colonic epithelial cells. Colonic crypts, isolated from control patients, were treated with 0.1 or

0.3 mM CDC or GCDC. Administration of CDC in a relative high concentration (0.3 mM) resulted in a significant inhibition of NHEs and CBE of human colonic epithelial cells, suggesting the possible toxic effects of high doses of non-conjugated BAs. In order to identify the exact NHE isoform, which is inhibited by 0.3 mM CDC, the isoform-specific NHE inhibitor HOE-642 was applied. Our experiments showed that the functions of all examined NHE isoforms were reduced in response to 0.3 mM CDC. Although the secretion of Cl^- and Na^+ in response to BAs was not investigated in this study, it is already well established that BAs stimulate intestinal electrolyte and fluid secretion [24] and the decreased absorption and elevated secretion of ions may account for the diarrhoea associated with BAM. Surprisingly, 0.3 mM GCDC markedly stimulated the activities of NHEs. Physiologically, most of the BAs are present in conjugated form, which protects the tissues from the strongly detergent non-conjugated BAs.

Having seen that both acute and chronic exposure of the colonic epithelial cells to BAs resulted in reduced electrolyte absorptive processes, the question raised how BAs are able to explain this inhibitory effect and what is the molecular background of this inhibition. First, we investigated the effects of BAs on the morphology of intracellular organelles, as a potent candidate for the target of Bas. Basing this idea on previous studies, demonstrating that BAs can perturb intracellular organelles and induce Golgi fragmentation, ER-stress and disruption of the mitochondria not only in cultured colonic epithelial cells but also in pancreatic ductal cells [56-58]. In our experiments, 10-minute exposure of the colonic epithelial cells to 0.3 mM CDC or 1 mM GCDC did not induce any visible alteration in the morphology of the cell compartments. Nevertheless, 1 mM CDC caused a severe damage in all of the mitochondria. The mitochondria swelled up and the structure of the inner membranes was lost, whereas other intracellular organelles seemed to remain intact. For positive control experiments, the mitochondrial uncoupling toxin CCCP (100 μM) was used, which resulted in the same mitochondrial injury as it was observed after treatment with 1 mM CDC.

Although 0.3 mM CDC inhibited the activities of acid/base transporters, but it did not induce alteration in the structure of intracellular organelles, next we investigated whether ATP_i is affected by 0.3 mM CDC. Administration of 0.3 mM CDC significantly but reversibly depleted the ATP_i of isolated human colonic epithelial cells. In addition, 1 mM CDC caused a more prominent reduction in ATP_i level. Exposure of the isolated colonic epithelial cells to the glycolysis inhibitors 10 mM DOG + 5 mM IAA together with the mitochondrial uncoupler toxin CCCP mimicked the effect of 1 mM CDC. These observations

indicate that non-conjugated BAs in high concentration inhibit both the oxidative and the glycolytic metabolism of the colonic epithelial cells.

In order to more profoundly examine the influence of CDC on the processes concerning the mitochondrial metabolism of the isolated human colonic epithelial cells, we decided to investigate mitochondrial transmembrane potential $(\Delta\psi)_m$, being the essential driving force for ATP synthesis. 0.1 mM and 0.3 mM CDC induced a marked reduction of TMRM fluorescence, which indicates the loss of $\Delta\psi_m$. This effect was reversible, $\Delta\psi_m$ returned to basal level following removal of the BA. The significant and irreversible reduction of $\Delta\psi_m$ caused by 1 mM CDC was more prominent than it was observed after the administration of the mitochondrial uncoupler toxin CCCP. Taken together, these data clearly demonstrate that 0.3 mM CDC is not enough to cause structural damage of the cell compartments but it is still able to deplete ATP_i and diminish $\Delta\psi_m$ by which perturbs the energy homeostasis of human colonic epithelial cells. These processes may play a role in the impaired function of ion absorption.

Next, we investigated another potential intracellular target of BAs, the Ca^{2+} signalisation, a well-known mediator of numerous cellular processes. It was shown previously that the Cl^-/HCO_3^- exchanger DRA and NHE3 are inhibited by the pathological increase of intracellular Ca^{2+} [50, 79]. In our experiments, CDC dose-dependently induced an increase of $[Ca^{2+}]_i$, which was a sustained non-physiological elevation with a plateau-characteristic. The removal of Ca^{2+} from the extracellular solution mostly prevented, while pretreatment of the colonic crypts with BAPTA-AM (40 μM), a chelator of Ca^{2+}_i almost completely abolished the effect of 0.3 mM CDC. When elevation of $[Ca^{2+}]_i$ occurs, the source of Ca^{2+} could be the ER or the extracellular space. From the ER, Ca^{2+} enters into the cytoplasm through IP_3R and/or RyR. In order to identify the orignin of elevated Ca^{2+} during administration of 0.3 mM CDC, the IP_3R antagonist caffeine, the RyR-blocker RR and the plasma membrane Ca^{2+} channel inhibitor gadolinium (Gd^{3+}) were applied. In our experiments, caffeine, but not RR or Gd^{3+}, reduced the $[Ca^{2+}]_i$ elevation, induced by 0.3 mM CDC. The fact that neither RR, nor Gd^{3+} alone were able to prevent the toxic Ca^{2+} signal, suggests that CDC mobilizes stored Ca^{2+} from the ER via IP_3R. However, since the inhibition of IP_3R did not completely abolished the effect of CDC, proposes that extracellular Ca^{2+} influx must be a key player as well. This process is most probably mediated by Gd^{3+} insensitive Ca^{2+} channels or Na^+/Ca^{2+} exchangers (NCXs). Moreover, the non-specific cation channels can also not be excluded. These ion channels have large Na^+, Ca^{2+} and/or K^+ conductance but since specific inhibitors are lacking,

it is difficult to distinguish their functions. Beside, the SERCA inhibitor thapsigargin induced a further elevation of the $[Ca^{2+}]_i$ after or during CDC administration suggests that CDC does not completely empty the ER Ca^{2+} store. These observations lead us to the hypothesis that CDC mobilizes stored Ca^{2+} from the ER and promotes the influx of external Ca^{2+}. The increase in $[Ca^{2+}]_i$ in response to CDC is probably not due to the detergent property of the BA, because this effect is reversible. Nevertheless, the involvement of other minor intracellular Ca^{2+} stores (mitochondria, Golgi, peroxysomes) must also be considered as a potential target of CDC in the colonic epithelial cells.

Finally, we examined whether there is a conjunction between the inhibitory effect of 0.3 mM CDC on the activities of acid/base transporters and its intracellular actions on ATP_i or $[Ca^{2+}]_i$. Therefore, we measured again the ion transporter activities of human colonic crypts, isolated from patients with healthy colon. Depletion of ATP_i with the glycolysis inhibitors DOG+IAA resulted in a similar decrease of the activities of NHEs as it was perceptible following administration of 0.3 mM CDC. In contrast, pretreatment of the colonic epithelial cells with the Ca^{2+}_i chelator BAPTA-AM did not prevent the toxic effect of 0.3 mM CDC on the activities of NHEs. These results indicate that 0.3 mM CDC inhibits the functions of NHEs via depleting ATP_i. Elevation of $[Ca^{2+}]_i$ may play a minor role in this process. This is in agreement with previous observations that NHEs are secondary-active transporters and their function is ATP-dependent [59-61]. Still, the consequences of ATP_i depletion and the role of $[Ca^{2+}]_i$ increase cannot be completely distinguished from each other, since CDC affects them simultaneously. Beside the Na^+ transport, the Cl^- absorptive capacity via the CBE of the colonic epithelial cells was also tested again. ATP_i depletion caused by the glycolysis inhibitors did not have any influence on the activity of CBE. However, preincubation of the colonic crypts with the Ca^{2+}_i chelator BAPTA-AM, which almost completely abolished the sustained elevation of $[Ca^{2+}]_i$ induced by 0.3 mM CDC, restored the decreased activity of CBE due to 0.3 mM CDC. This observation further supports the hypothesis that CBE is inhibited by the non-physiological elevation of $[Ca^{2+}]_i$ [79].

In conclusion, in this study we provided evidence that BAs impair the ion transport mechanisms of human colonic epithelial cells which could play an important role in the development of BAM associated diarrhoea. We demonstrated that chronic exposure of the colon to high concentration of bile acids results in decreased activities of acid/base transporters, responsible for NaCl absorption. In addition, the non-conjugated BA chenodeoxycholate inhibits the activities of NHEs and CBE of isolated human colonic

epithelial cells by depleting ATP_i and inducing toxic sustained intracellular Ca^{2+}-elevation. These processes may reduce fluid and electrolyte absorption in the colon and generate diarrhoea. Thus, our results might contribute to the development of new therapeutical approaches in the treatment of bile-induced diarrhoea.

2. Biological therapy with the anti-TNF-α IFX in the management of IBD

In the past decades, biological therapy revolutionized the treatment of IBD. At the beginning, it was introduced into the medication of those patients, who have failed conventional therapies. Unfortunately, a significant proportion of patients loses response to these agents or develops adverse effects, such as infusion- or hypersensitive reaction during the course of the treatment. Nowadays, clinicians are willing to start biological therapy sooner, parallel with steroids and/or immunomodulators, in order to avoid unfavourable outcomes. The prevention and the management of therapeutic failure with IFX is a significant challenge for clinicians in the field of IBD. Loss of response occurs mostly due to the phenomenon of immunogenicity, the production of neutralizing anti-drug antibodies, that accelerates drug clearance leading to subtherapeutic drug concentrations [80]. Immunogenicity induced by IFX can be determined by monitoring the serum concentrations of ATI, TNF-α and IFX [81]. It has been repeatedly demonstrated, that the formation of ATIs results in a decreased level of serum IFX, increased risk of infusion reactions and diminished clinical response [82, 83]. Maintenance vs. episodical IFX therapy or concomitant immunomodulators are proven to be benefical therapeutical strategies that reduce ATI development and the risk of infusion reactions [84, 85]. Interestingly, antibodies against IFX Fab fragment are detectable in patients never received IFX therapy, they may be present before IFX treatment in patients naive to biological agents, and may predict long-term clinical efficacy and safety of IFX in CD and UC. Measurement of these antibodies before initiation of IFX treatment might help clinicians to select the best type of therapeutic TNF-blocker in individual patients [86].

In our study both increased TNF-α and decreased IFX levels correlated with the presence of ATI, although neither ATI nor serum IFX influenced the outcome of the therapy. A meta-analysis also concluded that the presence of ATIs is associated with a significantly higher risk of loss of clinical response to IFX and lower serum IFX levels in patients with IBD [87]. Although these statements and consequences are logical, the results of the clinical

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practice are confusing. In a prospective cohort study Steenholdt et al. found that improved clinical outcome was associated with a higher increase in IFX levels [88]. Moreover, in a systematic review Chaparro published that there is a close relationship between trough levels of anti-TNF-α and maintenance of response [39]. Although higher serum IFX level proved to predict longer duration of response and clinical remission by some studies both in CD and UC [85, 87, 89, 90], a Japanese study showed that the median trough levels of IFX did not differ significantly in patients who maintained and who lost response to IFX [91]. In the study of Bortlik et al. the median trough levels of IFX were significantly higher and antibody titers were significantly lower in patients with concomitant thiopurines [92]. In our study, previous biological therapy had more significant effect on the outcome of IFX therapy than the concomitant use of thiopurines. According to the study of Afif et al., dose escalation was associated with a high clinical response in patients with subtherapeutical IFX levels and negative ATI, and better clinical outcome was achieved in ATI positive patients switching to another anti-TNF-α-drug [93]. On the basis of previous studies, concomitant corticosteroid therapy is suggested to decrease the effect of TNF-α blocker confirmed by our results regarding the higher TNF-α level in patients receiving steroids [94, 95].

Because of these controversial data, the usefulness of monitoring the trough levels and ATI concentrations in the therapeutic decisions may be questionable. Our results do not confirm the clinical utility of trough level and antibody measurement in the differentiation of "problematic' patients with loss of response of adverse reactions vs. those who respond appropriately to the biological therapy. The reason of the conflicting outcomes of the studies could be due to the differences in patient selection, type of analysis, the large interindividual dissimilarities in the reactions for the treatment or the lack of uniform reporting, which makes difficult to understand the results and to compare the individual studies. Anyway, therapeutic drug monitoring could be a key device in the optimised management of IBD patients, however, the methods should be correctly standardised. Steenholdt et al. underlines the importance of individualised therapy, which provides cost-effectiveness as an extra advantage, without any apparent negative effect on clinical efficacy [96]. The same goals has the Trough level Adapted infliXImab Treatment (TAXIT) trial, which was performed in order to investigate the value of individualised treatment with IFX based on therapeutic drug monitoring. Furthermore, Vande Casteele et al. revealed, that IFX-dosing based on therapeutic drug monitoring was associated with fewer flares during the course of treatment, in comparison with clinically based drug-dosing [97].

In conclusion, in our prospective observational study we found significant association between serum TNF-α level and the presence of ATI; and also between ATI positivity and low trough levels of IFX. However, antibody positivity and lower serum IFX levels did not correlate with loss of response, side-effects and hypersensitivity. Previous use of IFX correlated with the development of ATI. When previous studies determined only ATI positivity or negativity, detectable IFX serum concentration suggested many fals-negative results. This factor was decreased by the quantification of ATI titers in our study. On the basis of the present work, we suggest that further prospective studies are needed to determine whether the simultaneous measurement of serum TNF-α level, serum anti TNF-α concentration and antibodies against anti TNF-α may help to optimize the therapy in the critical situations.

Patients on immunosuppression or biological therapy are supposed to be at increased risk of influenza, since immunotherapy is known to predominantly impair cellular immunity, leaving the humoral immune response more or less intact [98]. Thus, annual influenza vaccination is recommended for all patients with IBD on immunomodulators. In the second part of our study, we examined whether influenza vaccination has an effect on the cell-mediated immune response by measuring the pre- and post-immunisation levels of INF-γ, IL-2 and TNF-α. Interestingly, only the level of IL-2 decreased significantly after vaccination. The study by Holvast et al. assessed cell-mediated responses to influenza vaccination in patients with SLE. They found that the frequencies of CD4+ T cells producing TNF and IL-2 were lower in patients after vaccination compared with healthy control subjects. They also found that this diminished cell-mediated response may reflect the effects of concomitant use of immunosuppressive drugs [99]. The study of Long et al. also found a diminished humoral and cell-mediated immune response to monovalent 2009 pandemic influenza A (H1N1/2009) and seasonal trivalent influenza vaccines in subjects with SLE but not with sickle cell disease or asthma, presumably due to the different immunocompromised status of these children [100]. Our results suggest that IBD patients on immunosuppressive therapy are recommended to be immunised against influenza, but larger and more detailed studies are needed to examine the cell-mediated response and to determine the efficacy of influenza vaccination in immunocompromised IBD-patients.

VI. ACKNOWLEDGEMENTS

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