Ph.D. THESIS

EXPERIMENTAL AND CLINICAL INVESTIGATIONS IN ULCERATIVE COLITIS

DR. FARKAS KLAUDIA

First Department of Medicine,
University of Szeged
Szeged, Hungary
2010

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

AE: anion exchanger

5-ASA: 5-aminosalicylate

AZA: azathioprine

BBM: brush border membrane

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

CDAI: Crohn's disease activity index

DRA: solute linked carrier 26A3, downregulated in adenoma

ENaC: epithelial Na^+ channel

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

IBD: inflammatory bowel diseases

IFN- γ: interferon gamma

IL-1: interleukin-1

6-MP: 6-mercaptopurine

NHE: Na^+/H^+ exchangers

NHERF: NHE exchanger regulatory factor

NOD2: nucleotide-binding oligomerization domain 2

PAT: putative anion transporter

pH_i: intracellular pH

PPAR-γ: peroxisome proliferators-activated receptor ligand-γ

TNF-α: tumor necrosis factor alpha

UC: ulcerative colitis

LIST OF FULL PAPERS RELATED TO THE SUBJECT OF THE THESIS

I. Farkas K*, Yeruva S*, Ifj. Rakonczay Z, Ludolph L, Molnár T, Nagy F, Szepes Z, Schnúr A, Wittmann T, Hubricht J, Riederer B, Venglovecz V, Lázár Gy, Király M, Zsembery Á, Varga G, Seidler U, Hegyi P. New therapeutical targets in ulcerative colitis: The importance of ion transporters in the human colon. **Both authors equally contributed to this work*

Inflamm Bowel Dis, 2010 (Epub ahead of print) **IF: 4.643**

II. Yeruva S*, **Farkas K***, Hubricht J, Rode K, Riederer B, Bachmann O, Cinar A, Rakonczay Z, Molnar T, Nagy F, Wedemeyer J, Manns M, Raddatz D, Musch MW, Chang EB, Hegyi P, Seidler U. Preserved Na(+)/H(+) exchanger isoform 3 expression and localization, but decreased NHE3 function indicate regulatory sodium transport defect in ulcerative colitis. **Both authors equally contributed to this work*

Inflamm Bowel Dis, 2010 (Epub ahead of print) **IF: 4.643**

III. Molnár T, **Farkas K,** Nagy F, Szepes Z, Wittmann T. Infliximab safety profile and long-term applicability in inflammatory bowel disease: clinical experiences from the eastern side of Europe.

Aliment Pharmacol Ther, 2010; 31: 1152-1153 **IF: 4.357**

IV. Molnar T, **Farkas K,** Nagy F, Wittmann T. Topically administered infliximab can work in ulcerative proctitis despite the ineffectiveness of intravenous induction therapy.

Am J Gastroenterol, 2009; 104: 1857-1858 **IF: 6.012**

V. Molnár T, **Farkas K,** Miheller P, Nyári T, Szepes Z, Herszényi L, Műzes Gy, Nagy F, Tulassay Zs, Wittmann T. Is the efficacy of successful infliximab induction therapy maintained for one year lasting without retreatment in different behaviour types of Crohn's disease?

JCC, 2008; 2: 322-326 **IF: 0.812**

VI. Molnar T, **Farkas K**, Nagy F, Wittmann T. Successful use of infliximab for treating fistulizing pouchitis with severe extraintestinal manifestation - a case report.

Inflamm Bowel Dis, 2008; 14: 1752-1753 **IF: 4.975**

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

I. Molnár T, **Farkas K,** Palkó A, Eszlári E, Bitay M, Nagy E, Pálinkás A, Nagy F, Bogáts G, Wittmann T. Gastric penetration of epicardial pacemaker leads 8 years after cardiac surgery.

Endoscopy, *2010, (accepted - only online available)* **IF: 5.545**

II. Molnár T, **Farkas K**, Nagy F, Lakatos PL, Miheller P, Nyári T, Horváth G, Szepes Z, Marik A, Wittmann T. Pregnancy outcome in patients with inflammatory bowel disease according to the activity of the disease and the medical treatment – a case-control study.

Scandinavian Journal of Gastroenterology, 2010, (epub ahead of print) **IF: 2.084**

III. Farkas K, Papp M, Nyári T, Nagy F, Szepes Z, Wittmann T, Molnár T. Red blood cell distribution width in combination with serological markers can help in the differentiation between Crohn's disease and ulcerative colitis.

The Open Journal of Gastroenterology, 2010 (accepted for publication) **IF: 0**

IV. Szepes Z, Molnár T, **Farkas K**, Horváth G, Nagy F, Nyári T, Wittmann T. Javuló életminőség konzervatív kezelésre nem reagáló colitis ulcerosa sebészeti ellátását követően: milyen áron?

Orvosi Hetilap, 2010; 151: 1264-1269 **IF: 0**

V. Puskás LG, Fehér LZ, Vizler Cs, Ayaydin F, Rásó E, Molnár E, Magyary I, Kanizsai I, Gyuris M, Madácsi R, Fábián G, **Farkas K**, Hegyi P, Baska F, Ózsvári B, Kitajka K. Polyunsaturated fatty acids synergize with lipid droplet binding thalidomide analogs to induce oxidative stress in cancer cells.

Lipids in Health and Disease, 2010; 9: 56 **IF: 2.14**

VI. Farkas K, Nagy F, Szepes Z, Nyári T, Wittmann T, Molnár T. Risk factors for colectomy in ulcerative colitis patients with severe flare-up: some of the few things comparable between the US and Central-Europe.

Inflamm Bowel Dis, 2010 (accepted for publication) **IF: 4.643**

VII. Molnár T, **Farkas K**, Szepes Z, Nagy F, Wittmann T. Autoimmune hemolytic anemia associated with ulcerative colitis: the most important is to induce complete remission.

Am J Gastroenterol, 2009; 105: 1203-1204 **IF: 6.012**

VIII. Molnár T, **Farkas K**, Szepes Z, Nagy F, Wittmann T. Ask the doctor about inflammatory bowel disease on the Internet: experiences after more than one thousand questions.

Inflamm Bowel Dis, 2009 (accepted for publication) **IF: 4.643**

IX. Molnár T, **Farkas K**, Nagy F, Szepes Z, Tiszlavicz L, Németh I, Nyári T, Wittmann T. Does the endoscopic appearance of the ileocecal valve suggest the severity of Crohn's disease in the terminal ileum?

JCC, 2009; 3: 287-290 **IF: 1.729**

X. Papp M, Lakatos PL, Harsfalvi J, Farkas G, Palatka K, Udvardy M, Molnar T, **Farkas K**, Nagy F, Veres G, Lakatos L, Kovacs A, Dinya T, Kocsis AK, Papp J, Hungarian IBD Study Group, Altorjay I. Mannose-binding lectin level and deficiency is not associated with inflammatory bowel diseases, disease phenotype, serology profile, and NOD2/CARD15 genotype in a large Hungarian cohort.

Human Immunology, 2010; 71: 407-413 **IF: 2.55**

XI. Molnár T, **Farkas K**, Nagy F, Iványi B, Wittmann T. Sulfasalazine induced nephrotic syndrome in a patient with ulcerative colitis.

Inflamm Bowel Dis, 2010; 16: 552-553 **IF: 4.643**

XII. Farkas K, Nagy F, Kovács L, Csajbók É, Kovács G, Wittmann T, Molnár T. Ulcerative colitis and primary sclerosing cholangitis as part of autoimmune polyglandular syndrome type III.

Inflamm Bowel Dis, 2010; 16: 10-11 **IF: 4.643**

XIII. Hegyi P, Rakonczay Z Jr., **Farkas K**, Venglovecz V, Ózsvári B, Seidler U, Gray MA, Argent BE. Controversies in the role of SLC26 anion exchangers in pancreatic ductal bicarbonate secretion.

Pancreas, 2008; 37: 232-234 **IF: 2.708**

XIV. Farkas K, Molnár T, Nagy F, Tiszlavicz L, Németh I, Kemény É, Varga E, Wittmann T. Henoch-Schönlein purpura presenting with multiplex gastrointestinal manifestations and massive nephrotic syndrome in adulthood – a case report.
Central European Journal of Medicine, 2008; 3: 521-524 **IF: 0**

XV. Molnár T, **Farkas K**, Szepes Z, Nagy F, Nyari T, Wittmann T. RDW can be a useful additional marker in diagnosing Crohn's disease and ulcerative colitis.
Dig Dis Sci, 2008; 53: 2828-2829 **IF: 1.583**

XVI. Molnár T, **Farkas K**, Miheller P, Nyári T, Szepes Z, Herszényi L, Műzes Gy, Nagy F, Tulassay Zs, Wittmann T. Infliximab indukciós terápia középtávú (egyéves) hatékonysága Crohn betegekben.
Magyar Belorvosi Archívum, 2008; 61: 307-311 **IF: 0**

XVII. Nagy F, Molnár T, Szepes Z, **Farkas K**, Nyári T, Lonovics J. Efficacy of 6 merkaptopurine (6MP) treatment after azathioprine hypersensitivity in IBD.
World J Gastroenterol, *2007; 14: 4342-4346* **IF: 0**

XVII. Nagy F, Molnár T, Szepes Z, **Farkas K**, Nyári T, Lonovics J. 6 merkaptopurin kezelés hatékonysága azathioprin-hiperszenzitivitási reakció esetén ismeretlen eredetű gyulladásos bélbetegségekben.
Magyar Belorvosi Archívum, 2006; 59 (1): 35-39 **IF: 0**

Number of full publications**:**	**24**
Cumulative impact factor:	**63.722**
Number of abstract publications:	**20**

SUMMARY

Background. The exact etiology and the mechanisms of inflammatory bowel disease (IBD) are still unknown. Recent advances in the understanding of the pathophysiological conditions of IBD have provided new therapeutic targets. In the last decades animal studies suggested the role of ion transport mechanisms in intestinal colonic crypts in the pathogenesis of ulcerative colitis (UC). Despite the comprehensive animal studies, there are only scarce available data on the ion transporter activities of the normal and inflamed human colon. The **aim** of our study was to characterize the segmental differences of ion transport mechanisms (namely Na^+/H^+ exchangers [NHE1-3], the epithelial sodium channel [ENaC] and the SLC26A3 Cl^-/HCO_3^- exchanger downregulated in adenoma [DRA]) in human colonic epithelial cells and to examine the activities of these transporters in UC. We also evaluated the efficacy of the infliximab induction therapy in Crohn's disease (CD) and assessed the safety and long-term applicability of infliximab treatment in patients with IBD. **Materials and methods.** 128 healthy controls and 69 patients suffering from active UC were involved in the first part of the study. Primary colonic crypts were isolated from human biopsy and surgical samples. The expressional and functional characteristics of NHE1-3, ENaC and DRA were determined by using fluorescence, patch clamp and real time RT PCR techniques. In the second part of the study one year review was undertaken of all CD patients who achieved remission or fistula closure with 3 infusions of infliximab. We evaluated the clinical response, the estimated CD Activity Index (CDAI), the number of draining fistulae, the dosages of steroid and immunosuppressive drugs at 6 and 12 months after the last infusion, and the needs for hospitalization and surgical intervention during this period. For the evaluation of the long term applicability and safety of infliximab therapy, data of 127 IBD patients were analyzed. A total of 733 infusions were administered, the mean number of infliximab infusions was 5.8/patient. The mean length of follow up was 2.3 years. **Results.** The activities of electroneutral (via NHE3) and the electrogenic Na^+ absorption (via ENaC) are in inverse ratio to each other in the proximal and distal colon. No significant differences were detected in the activity of NHE2 in different segments of the colon. Surface cell Cl^-/HCO_3^- exchange is more active in the distal vs. the proximal part of the colon. Importantly, both sodium and

chloride absorptions are damaged in UC, whereas, NHE1 which has been shown to promote immune response is up-regulated by 6-fold. Infliximab induction therapy without retreatment resulted in a beneficial effect lasting for at least 1 year in 44% of the patients. 57.9% of the patients with luminal disease remained in steroid-free complete remission, while the fistulas persisted closed in only 35.5% of the patients. 12.6% of the patients had 31 episodes of acute, 5.5% patients had 9 episodes of delayed infusion reaction. 68.8% of those with acute reaction were on concomitant immunomodulator and/or corticosteroid treatment. Listeria meningoencephalitis, sepsis, pulmonary tuberculosis and lymphoma in two cases were the most severe infectious complications. The mortality rate was 3.1%. The beneficial effect of infliximab was also confirmed by two unusual cases. Infliximab maintenance therapy led to complete mucosal healing in severe refractory pouchitis and rectal instillation of infliximab was also successful in severe proctitis. **Discussion.** The experimental results of our comprehensive human study demonstrated the differences in the various ion transport mechanisms between the different parts of the colon. We also revealed that both sodium and chloride transport is damaged, whereas NHE1 is up-regulated in UC. With selective inhibition of NHE1 and/or stimulation of NHE3, ENaC and DRA our results may open up new therapeutical targets in UC. Infliximab induction therapy alone may result in sustained remission mainly in patients with luminal CD, while fistulizing disease requires maintenance infliximab therapy. The rate of serious adverse events is less than 5%, confirming infliximab therapy safe in the long-term.

I. INTRODUCTION

1. Pathogenesis of UC

Inflammatory bowel disease (IBD) consisting of Crohn's disease (CD) and ulcerative colitis (UC) is thought to be caused by an abnormal immune response of the intestinal immune system leading to the dysfunction of intestinal mucosal barrier against enteric bacteria in a genetically susceptible host (1). In spite of the latest advances in the knowledge of the pathogenesis of IBD, the exact etiology and the mechanisms of the disease still remain unknown. Ulcerative colitis is a chronic inflammation restricted to the colon which may present with a range of mild to severe symptoms including abdominal pain, diarrhea and hematochezia. The disease may be localized to the rectum or can be more extensive and involve the left side of the colon or the whole large bowel.

Inflammatory bowel diseases occur on the basis of genetic predisposition, although genetic factors have more dominant roles in the development of CD than in UC. Mutations of the nucleotide-binding oligomerization domain (NOD) 2 gene related to CD were shown not to be more frequent in UC, however stronger association exists between genes of the human leukocyte antigen region and UC than for CD (2) supporting that they are related but distinct disorders. Environmental factors are suggested to affect not only the occurrence but also the phenotype of the diseases. Smoking is an independent risk factor for clinical, surgical and endoscopic recurrence of CD, while it has a protective effect in UC (3). Nonsteroidal anti-inflammatory drugs trigger exacerbation of UC or can induce the disease because of the decreased production of mucosal prostanoids and increased adherence and migration of the leukocytes (4). Stress may also lead to flare-up (5). Appendectomy performed before the age of 20 protects against the development of UC, whereas CD is more frequent among appendectomized patients (6).

A large number of cytokine abnormalities have been described including pro-inflammatory and immunoregulatory molecules. In CD, intestinal CD4+ T cells produce interferon (INF)-γ, while mucosal macrophages produce interleukin (IL)-12 and IL-18 (7). In contrast, in UC NK T-cells produce increased amounts of IL-13, and mucosal T-

cells produce more IL-5 (8). Based on these data, it is now generally accepted that the two main forms of IBD are associated with distinct immune profiles which are classified as a fairly typical Th1 response in CD and an atypical Th2 response in UC.

2. Management of UC

Treatment in UC is directed towards inducing and maintaining remission of symptoms and mucosal inflammation. The choice of the therapeutic method depends on both the extent of colonic involvement and the severity of the disease at presentation, which are the key parameters to be assessed for the most appropriate treatment during the whole disease course. For years the therapeutic repertoire for UC included aminosalicylates, corticosteroids and immunomodulators (9). Recent advances in the understanding of the pathophysiological conditions of IBD have provided new immune system modulators as therapeutic tools. Tumour necrosis factor (TNF)-α has been shown to play a central role in the pathogenesis of mucosal inflammation not only in CD, but in UC as well, the blockade of which with the chimeric monoclonal antibody infliximab has shown its efficacy in various published studies (10).

2.1. Sulfasalazine, consisting of 5-aminosalicylate (5-ASA-active component) and sulfapyridine, has shown its efficacy in inducing and maintaining remission in mild to moderate UC since the 1940s (11). Sulfapyridine moiety, which acts as a carrier molecule, is thought to be responsible for most of the side effects of sulfasalazine treatment, including nausea, headache, anorexia or hemolysis, which can be managed by dosage adjustment in the majority of the cases (12). More severe side effects (hepatotoxicity, nephrotoxicity, haemolytic anemia, pancreatitis, and bone marrow depression) rarely occur in the clinical practice. Sulfasalazine and the newer 5-ASA are considered first-line therapy in maintenance of remission. Topical 5-ASA plays a role in distal disease. 5 ASA agents are likely to have multiple anti-inflammatory effects including inhibition of cyclooxygenase, lipoxygenase, B-cells and several inflammatory cytokines. 5-ASA has recently been shown to activate selective peroxisome proliferators-activated receptor ligand-γ (PPAR-γ) that controls cell proliferation and apoptosis (13). It takes typically between 3 and 6 weeks to induce remission in mild to moderately active UC. It has been shown that the rate of remission induction is almost

the same in case of sulfasalazine use than in case of 5-ASA, although sulfasalazine is not as well tolerated as 5-ASA (14).

2.2. Glucocorticoids have their role in the rapid induction of remission in more active cases with extension of the disease and remain the primary therapy for most patients with moderate-to-severe disease since 1950s, although they could not provide maintenance remission. Corticosteroids activate glucocorticoid responsive elements resulting in the inhibition of the recruitment and proliferation of lymphocytes, monocytes and macrophages, migration of neutrophils to sites of inflammation and decreased production of soluble inflammatory mediators including cytokines, leukotrienes and prostaglandins (15). Approximately 10% of patients with unresponsive UC have severe attacks requiring hospitalization and parenteral steroid treatment.

2.3. The toxicities associated with long-term steroid therapy, combined with their ineffectiveness as maintenance medications, have led to increased use of immunomodulators, such as azathioprine (AZA) and its metabolite 6-mercaptopurine (6-MP), for the treatment of steroid-dependent and steroid-resistant IBD. 6-MP is recommended in case of AZA intolerance. There have been 9 controlled trials evaluating the effectiveness of AZA for the treatment of active UC, including 4 randomized double-blind controlled trials (16-20) and 5 randomised trials comparing AZA to other drugs such as mesalazine, sulphasalazine or methotrexate (20-24). It may take up to 3 months for the treatment to become effective. Therefore, it should be started while the patient is undergoing corticosteroid treatment. Long term use of these immunosuppressants is effective for control of inflammation and for prolonging remission. They may have reversible immediate side effects, such as pancreatitis or bone marrow suppression, which disappear upon discontinuation of therapy. Close monitoring of hematologic and biochemical parameters will improve safety.

2.4. Tumour necrosis factor-α has been found to cause inflammation in CD, rheumatoid arthritis and psoriasis and is currently a target of drug treatment in UC due to elevated levels of TNF-α discovered in the blood, colonic tissue and stools (25). In animal models of chronic colitis, treatment with anti TNF-α antibodies was associated with decreased intestinal inflammation (26). Infliximab is an intravenously administered chimeric (75% human and 25% murine sequences) immunoglobulin G1 monoclonal

antibody to TNF-α which binds soluble and the membrane bound TNF-α found on inflammatory cell surfaces, and induce apoptosis and elimination of those inflammatory cells and lymphocytes and monocytes (27-29). Although UC has been considered to be a Th2-type disease with a less prominent role of TNF-α, it has been shown that TNF-α may also play role in its pathogenesis (30). Infliximab is approved in moderate to severe UC not responding to or showing intolerance against conventional therapy. After some small studies of infliximab, the results of two larger randomized placebo-controlled trials, ACT1 and ACT2 provided definitive evidence supporting the efficacy of infliximab therapy in UC (10). According to a recent meta-analysis, the mean short-term response and remission with infliximab therapy was 68% and 40% respectively. Comparing infliximab to placebo short term response was 65% vs. 33%, long term response was 53% with infliximab and 24% with placebo. Long-term remission was achieved in 33% of the patients treated with infliximab and in 14% of those receiving placebo (31). The role of infliximab in the management of acute severe UC as a rescue therapy is still questionable. A recently published paper by Zabana et al. (32) evaluated the long-term safety profile of infliximab in IBD in the last 9 years in the clinical practice and found infliximab therapy safe with a rate of severe adverse events less than 10 %.

3. New therapeutical targets – the role of ion transporters in the management of IBD

Studies focusing on the pathophysiology of IBD which highlight new therapeutic targets are crucially important. In the last decades more and more animal studies have been published concerning the role of ion transport mechanisms in intestinal epithelial cells (colonic crypts) in the pathogenesis of UC (33-35). Although Teleky et al. has investigated the regulation of intracellular pH (pH_i) in freshly isolated human colonocytes (36), a comprehensive functional characterization of ion transporters of human colonic crypts is still missing.

The ion transporters on the apical and basolateral plasma membranes of polarized colonic epithelial cells determine the selectivity of the cells for the vectorial transport of specific ions. Under physiological conditions, the mammalian colon absorbs Na^+, Cl^- and water and secretes K^+ and HCO_3^- (37). Human colonic

electroneutral sodium absorption is probably mediated via parallel activity of Na^+/H^+ exchangers (NHE) 2 and 3 and Cl^-/HCO_3^- exchangers in the proximal and distal colon (37, 38), whereas, electrogenic sodium uptake is mediated by the epithelial sodium channel (ENaC). Animal studies suggested that colonic sodium chloride absorption (including electroneutral Na^+/H^+ exchange, Cl^-/HCO_3^- exchange and the electrogenic sodium uptake) differs significantly in the proximal and distal segments of the large intestine (39-42). Limited number of human studies also showed segmental heterogenity of these transporters in the colon (43, 44).

Concerning the anion exchange mechanisms in the large intestine, recent studies revealed that Cl^- dependent HCO_3^- secretion and the mucosal surface pH is higher in the distal than in the proximal colon in mouse (45). These data also suggest that the pH_i regulation and the functional activities of the acid-base transporters are different in the proximal and distal colon. A recent study in rodent colon demonstrated strong segmental differences in Slc26a3 epxression, a Cl^-/HCO_3^- exchanger expressed in the luminal membrane, towards the distal colon (42).

In UC Cl^- uptake by the colon is significantly reduced (46). It has been described that there is higher level of Cl^- in the colon of patients with UC compared to controls suggesting that the normal luminal to mucosal exchange of Cl^- for bicarbonate is damaged (47). Excess bacterial fatty acids have been implicated as the cause of acidic stools, especially in infant diarrhoea (48). However, it has been shown that the failure of HCO_3^- secretion rather than excessive bacterial fermentation is the cause of lowered luminal pH of the colon with UC (46). Not only anion exchange, but also sodium uptake is altered in UC. NHE3 and NHE exchanger regulatory factor (NHERF) protein expression have been described to be downregulated in IBD patients and in a mouse colitis model (49). Notably, NHE3-deficient mice spontaneously develop colitis restricted to the distal colonic mucosa suggesting a key role of NHE3 in UC (34). In addition, UC is also associated with substantial decreases in the expression of the electrogenic epithelial Na^+ channel (ENaC) beta and gamma subunits (50).

The regulation of NHE1 on the basolateral membrane behaves opposite to the apical NHE3 in UC. NHE1 was shown to be upregulated in mouse colitis (33). Supporting this observation NHE1 is rapidly activated in response to a variety of

inflammatory signals, such as IL-1 (51), TNF-α (52), IFN- γ (53) and lipopolysaccharide (52, 54). Furthermore, functional NHE1 activity is required for both maximal nuclear factor-kappa B activation and IL-8 production in colonic epithelial cells (33). The regulation of intestinal inflammation by NHE is also operational *in vivo*, because NHE inhibition dramatically attenuates disease activity in the mouse dextran sulfate model of IBD (33).

II. AIMS

1. To characterize the segmental differences of ion transport mechanisms (namely NHE1-3, DRA, and ENaC) in human colonic epithelial cells and to examine the activities of these transporters in UC.

2. To retrospectively evaluate the efficacy of the infliximab induction therapy without retreatment in different subgroups of CD and assess whether induction therapy is worth starting in case we know that financing of continuous treatment for at least a year would be restricted. We also assessed the safety and long-term applicability of infliximab treatment in patients with IBD based on our prospectively recorded database.

III. MATERIALS AND METHODS

1. Characterization of the activity and mRNA expression of ion transporters involved in NaCl absorption in human colon

1.1. Humans involved in the study

Humans were divided into 2 groups. 69 IBD patients with active distal UC and 128 age- and sex-matched healthy controls with normal colonoscopic findings were enrolled in the study. An informed consent was obtained prior to endoscopy or surgery. Protocols of the study were approved by the regional ethical committees (both at the University of Szeged, Szeged, Hungary, and Hannover Medical School, Hannover, Germany). Six colonic biopsies were obtained from each patient undergoing colonoscopy at the First Department of Medicine or surgery at the Department of Surgery, University of Szeged, and 2-3 biopsies from each patients in addition to those needed for histological examination at the Department of Gastroenterology, Hannover Medical School.

In control patients neither macroscopic (by endoscopy) nor microscopic (by histology) evaluation showed any signs of inflammation in the colon. Patients with normal endoscopic finding were examined because of colorectal cancer screening or various abdominal complaints. Patients with IBD did not receive any steroid therapy.

All of the UC patients complained of diarrhea with or without hematochezia. The diagnosis of UC was made based on the colonoscopic findings which showed erythema and friability of the mucosa, confluent superficial ulceration and decreased vascular pattern. In every case histological examination was performed and showed inflammatory cell infiltration in the mucosa, cryptitis and/or crypt abscess. Patients having only mild or moderate UC with no steroid therapy were involved in the study. Please note that we also wanted to include patients with severe colitis as well, however, only a small number of strongly damaged colonic crypts could be isolated from the biopsy samples which were unsuitable for functional experiments.

1.2. Materials & solutions for the experiments

General laboratory chemicals were obtained from Sigma-Aldrich (Budapest, Hungary) unless indicated otherwise. Collagenase A was obtained from Roche Diagnostic GmbH (Mannheim, Germany), nigericin from FLUKA Biochemic (Munich, Germany). Nigericin was dissolved in absolute ethanol. HOE-642 (4-isopropyl-3-methylsulphonylbenzoyl-guanidin methanesulphonate) was kindly provided by Sanofi Aventis (Frankfurt, Germany) and was dissolved in dimethyl sulfoxide (DMSO). 2',7'-biscarboxyethyl-5(6)-carboxyfluorescein-acetoxymethylester (BCECF-AM) was obtained from Invitrogen (Eugene, OR, USA); cell and tissue adhesive from Becton Dickinson Bioscience (Cell Tak, Bedford, MA, USA). BCECF-AM was dissolved in DMSO.

1.3. Isolation of colonic crypts

Colonic crypts were isolated from 6 biopsy specimens obtained from colonoscopy or surgical samples of control and IBD patients. Samples were collected from four different segments of the colon (cecum, transverse, or sigmoid colon or rectum). However, only one segment of the colon was investigated in each patient. The tissue samples were placed immediately in ice cold $NaHCO_3$ containing Hanks balanced salt solution (HBSS) and transferred to the laboratory. The samples were washed 3 times with HBSS and cut into small pieces with a razor blade and incubated in 1 mM dithiothreitol (DTT) in HBSS for 15 min followed by 2 times 30 minutes enzymatic digestion with 0.38 mg/ml collagenase A at 37 ^{o}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 viewed under a Nikon stereo microscope (Jencons-PLS, Grinstead, UK). The crypts (200-300 crypts/isolation) were aspirated into a micropipette and transferred to a Petri dish. For intracellular pH (pH_i) measurements the crypts were kept in a storage solution for 3 hours at +4 ^{o}C before the experiments. However, for patch clamp experiments, the crypts were incubated on 24 mm coverslips in culture solution for 24 hours at 37 ^{o}C with 5% CO_2.

The culture solution contained Dulbecco's Modified Eagle's Medium (DMEM), 10 % fetal bovine serum (FBS; Sigma-Aldrich), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg streptomycin.

1.4. pH_i measurements

The compositions of the solutions used are shown in Table 1. HEPES-buffered solutions were gassed with 100% O_2 and their pH was set to 7.4 with NaOH or HCl at 37 ^oC. HCO_3^--buffered solutions were gassed with 95% O_2/5% CO_2 to set the pH to 7.4 at 37 ^oC.

Colonic crypts were attached with Cell Tak to glass 24 mm diameter coverslips 3h after isolation and placed in a perfusion chamber mounted on the stage of an inverted fluorescent microscope linked to a Cell^R imaging system (Olympus, Budapest, Hungary). Colonic crypts were bathed in standard HEPES solution at 37 ^oC and loaded with the pH-sensitive fluorescent dye BCECF-AM for 20-30 minutes. Crypts were continuously perfused with solutions at a rate of 4-5 ml/min. 3 different areas (base, medium, surface) in each crypt were excited with light at wavelengths of 495 nm and 440 nm and the 495/440 fluorescence emission ratio was measured at 535 nm (55, 57). *In situ* calibration of the fluorescence signal was performed using the high K^+-nigericin technique.

	Standard HEPES	**High-K^+ HEPES**	**NH_4^+ in HEPES**	**Na^+-free HEPES**	**Standard HCO_3^-**	**Cl^--free HCO_3^-**
NaCl	**130**	**5**	**110**		**115**	
KCl	**5**	**130**	**5**	**5**	**5**	
$MgCl_2$	**1**	**1**	**1**	**1**	**1**	
$CaCl_2$	**1**	**1**	**1**	**1**	**1**	
Na-HEPES	**10**	**10**	**10**			
Glucose	**10**	**10**	**10**	**10**	**10**	**10**
$NaHCO_3$					**25**	**25**
NH_4Cl			**20**			
HEPES				**10**		
NMDG-Cl				**140**		
Na-gluconate						**115**
Mg-gluconate						**1**
Ca-gluconate						**6**
K_2-sulfate						**2.5**

Table 1: Composition of solutions. Values are concentrations in mM. NMDG: N-methyl-D-glucamine

1.5. Measurement of NHE activities

The crypts were acid loaded by exposure to a 5-min-pulse of 20 mM 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 was present in the solution), the pH_i was set to a stable acidic level. NHE activity was switched on by re-addition of extracellular sodium and the activity of NHE was determined by measuring the initial rate of pH_i recovery over the first 60 sec (60 data points). The activities of the different NHE isoforms were extracted by using the isoform selective NHE inhibitor HOE-642. 1 μM HOE642 inhibits NHE1 whereas 50 μM HOE642 inhibits both NHE1 and 2 but not NHE3 (53, 54). Although there are 9 different NHE isoforms in mammalian digestive tract (59), only NHE1-3 are localized to the membrane of the cells (60). Therefore, the activities (A) of NHE isoforms can be calculated from the recoveries (R) as follows:

$$A_{NHE1} = R_{0\ \mu M\ HOE642} - R_{1\ \mu M\ HOE642}$$
$$A_{NHE2} = R_{1\ \mu M\ HOE642} - R_{50\ \mu M\ HOE642}$$
$$A_{NHE3} = R_{50\ \mu M\ HOE642}$$

1.6. Measurement of Cl^-/HCO_3^- exchange activity

The Cl^-/HCO_3^- exchange activity of the cells was determined using the Cl^- withdrawal technique. Briefly, removing Cl^- from the standard HCO_3^-/CO_2 buffered solution causes alkalization due to the reverse 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 (30 data points).

1.7. Measurement of ENaC activity

Voltage-clamp recordings were performed using the patch clamp technique in whole-cell configuration at room temperature with an Axopatch 200B amplifier (Axon Instruments, Union City, CA, USA). Micropipettes were fabricated by a P-97 Flaming/Brown type micropipette puller (Sutter Instrument, Novato, CA, USA) from GC120F-10 glass capillary tubes (Harvard Apparatus, Holliston, MA, USA) and had resistances of 5–10 MΩ when filled with pipette solution. Capacitative currents were

compensated with analog compensation. Linear leak currents were not compensated. Series resistance was approximately 8–15 MΩ, and series resistance compensation (70–80%) was used in whole-cell recordings if the current exceeded 1 nA. Currents were filtered at 2 kHz (four-pole Bessel filter) and sampled at 5 kHz. Pulse generation, data acquisition, and analysis were performed using the pClamp 6.03 software (Axon Instruments). The pipette solution contained (in mM) 95 K-gluconate, 5 Na-gluconate, 2 Mg-ATP, 10 HEPES, 2 EGTA-Na, 0.2 $MgCl_2$, 40 CsOH, and 20 TEA-OH, with pH adjusted to 7.2 using gluconic acid. Our standard bath solution contained (in mM) 140 Na-gluconate, 5 K-gluconate, 1 $CaCl_2$, 5 $BaCl_2$, 1 $MgCl_2$, 10 HEPES, and 5 glucose, with pH adjusted to 7.4 using Tris, supplemented when appropriate with 50 µg/ml trypsin or 10 µM amiloride (final concentrations). In some experiments, Na^+ was substituted with N-methyl-d-glucamine ($NMDG^+$) in the extracellular solution. Inward currents (downward current deflections) are defined as negative currents, i.e., movement of positive charge from the extracellular side to the cytoplasmic side. Starting from a V_{hold} of -50 mV, voltage step protocols were performed intermittently using consecutive 300 msec step changes to potentials ranging from -100 up to +100 mV in 20 mV increments. The average current values reached during the last 100 msec of the voltage steps were used for the *I-V* plots. The changes in current amplitudes were expressed as changes in current densities (pA/pF).

1.8. RNA isolation and real-time RT-PCR

The biopsy samples were collected in RNA later (Sigma-Aldrich, Steinheim, Germany) solution. RNA was isolated from the biopsies using Nucleo spin kit (Machery and Nagel, Düren, Germany) as recommended by the manufacturer. The RNA of each sample was tested for intactness of RNA, and only such samples were used in which no RNA breakdown was documented. 1 µg of RNA was reverse transcribed using MMLV superscript III reverse transcriptase (Invitrogen, Karlsruhe, Germany). Real-time PCR was performed using SYBR Green PCR mastermix (Applied Biosystems, Darmstadt, Germany) exactly as previously described (60). Primer sequences are given in Table 2.

Gene	Sequence
NHE1 For	5'-TGAGGAGCTGAAGGGCAAAG-3’
NHE1 Rev	5'-CCTTGCTCCGCATCATGAT-3’
NHE2 For	5'- CTTCCACTTCAACCTCCCGAT-3’
NHE2 Rev	5'-GCTGCTATTGCCATCTGCAA-3’
NHE3 For	5'-AGAAGCGGAGAAACAGCAG-3’
NHE3 Rev	5'-TGGTGACACTAGCCAGGAAC-3’
DRA For	5'-AGCACAGGAGGCAAAACACAG-3’
DRA Rev	5'-GACTTTTGTAGAGGCGCCAGG-3’
Villin For	5’-CTATGCCAACACCAAGAGAC- 3’
Villin Rev	5’-CCCAGACATCTAGTAGGAACAC-3’

Table 2: Primer sequences for PCR experiments

1.9. Immunohistochemistry and confocal microscopy

Biopsies were fixed, sectioned and treated as previously described for murine intestine (58), except that the NHE3 antibody was developed against NHE3 in the lab of Dr. Eugene Chang (61). Cover slides were imaged on a Leica DM IRB with a TCS SP2 AOBS scan head equipped with a 405 nm laser for excitation of blue dyes.

1.10. Statistical analysis

Values are means ± SE. Statistical analyses were performed using unpaired Student's t-test or ANOVA as appropriate. $P \leq 0.05$ was accepted as significant. n = 10-15 colonic crypts isolated from 4-6 patients. For the quantitative mRNA results, the number of biopsies used for the experiments is indicated in the figure legends.

2. Assessment of the efficacy, the safety profile and long-term applicability in inflammatory bowel disease

2.1. 50 CD patients (26 females, 24 males; mean age at the diagnosis 29.3 years [range 13-59]), who had reached successful remission (luminal group) or complete fistula closure (fistulizing group) after infliximab induction therapy, and who had been diagnosed for at least 24 months based on the appropriate criteria (62), were enrolled in

the study to determine the efficacy of infliximab induction therapy without retreatment. Indication of infliximab induction therapy was active luminal disease in 19 and presence of draining fistula in 31 patients. According to the application and clinical practice guidelines, infliximab was administered in a dose of 5 mg/kg 3 times, in weeks 0, 2 and 6 for all patients in both groups during induction therapy. Groups formed by disease localizations included 23 colonic, 13 ileal, 13 ileocolonic and 1 duodenal. Eighteen patients received corticosteroids, 43 immunosuppressants and 16 of them combined corticosteroid and immunosuppressive treatment at the time of the induction therapy. Azathioprine was administered to 39, 6-MP to 3 and methotrexate to 1 patient. After the successful induction therapy patients were examined at least once in every three months. Data at month 6 and 12 were selected for statistical analysis. Clinical symptoms, the estimated CD Activity Index (CDAI), the number of draining fistulae, changes in the dosages of steroid and immunosuppressive drugs, the need for repeated (on-demand) infliximab infusion due to exacerbation were evaluated. We also assessed the need for hospital admissions and the number of surgical interventions related to CD during the 12 months after the induction therapy. Clinical remission was defined as an estimated CDAI <150 at both controls of months 6 and 12 in the luminal group, the absence of draining fistula, if the dose of the immunosuppressants was steady and the dose of corticosteroids could be tapered. Relapse was defined as a flare up (CDAI>150) or as a recurrence of draining fistulas within a year after successful induction therapy, if the dose of corticosteroid and immunosuppressive drugs had to be increased or surgical intervention was needed due to the development of fistula, perianal abscess or stenosis and if readministration of infliximab was required. Breslow (Generalized Wilcoxon) test was used for statistical analysis and p values<0.05 were considered statistically significant.

2.2. For the evaluation of the long term applicability and safety of infliximab therapy, data of 127 IBD patients (70 females, 57 males; mean age at the diagnosis 27 years [range 12-67]) infused for refractory or steroid-dependent CD (n=81) or UC (n=42) or pouchitis (n=4) during a 7 year period between January 2003 and December 2009 were analyzed. 26% of the patients received only induction therapy while 62.2% of the patients were on maintenance treatment. A total of 733 infusions were administered, the

mean number of infliximab infusions was 5.8/patient. The mean length of follow up was 2.3 years. 32 patients of the CD group were diagnosed with colonic, 21 with ileal, 21 with ileocolonic and 7 with upper gastrointestinal tract localization. Considering disease behaviour 16 patients had inflammatory, 19 stricturing, 38 penetrating and 8 combined stricturing and penetrating CD. Extension of the UC was pancolitis in 34 and left-sided in 12 patients. The mean disease duration at the time of the first infliximab infusion was 6.6 years in CD and 9.8 years in UC.

IV. RESULTS

1. Characterization of the activity and mRNA expression of ion transporters involved in NaCl absorption in human colon

1.1. Determination of the resting pH_i of human colonic crypts in different parts of the colon

In the first series of experiments, our aim was to determine the resting pH_i of human colonic crypts (Figure 1A-B).

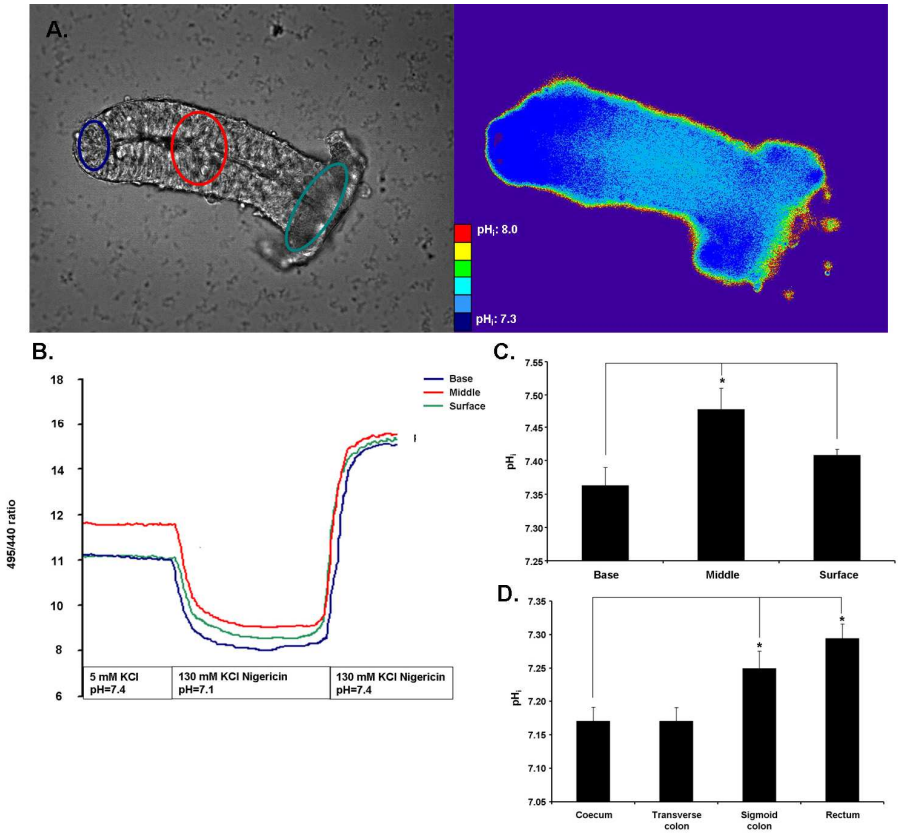

Figure 1. Resting pH_i of colonic epithelial cells. (A) Light and fluorescence microscopy. Isolated colonic crypts were fixed on a cover glass. 3 areas (ROIs) of each crypt were excited with light at wavelengths of 495 nm and 440 nm and the 495/440 fluorescence emission ratio was measured at 535 nm. **(B)** Crypts were exposed to nigericin/high K^+ HEPES solutions of pH 7.1 and 7.4. Resting pH_i was calculated by linear regression analysis. Bar charts show the summary of resting pH_i in different parts of the **(C)** crypts and **(D)** the colon. Significantly higher resting pH_i was measured in the middle part of the crypts vs. the surface and the base and in the distal vs. the proximal part of the normal colon. n = 19 colonic crypts isolated from 7 patients. Data are presented as means ± SEM. * indicates significant difference ($p<0.05$) vs. the respective groups.

We used the classical linear model (55, 56) to determine the resting pHi which proved to be significantly higher in the middle vs. the surface or the base of the crypts (Figure 1C) in all parts of the colon. Importantly, the resting pHi was significantly higher in the distal vs. proximal part of the colon (Figure 1D).

1.2. Activities and expression of NHE isoforms in different parts of the normal colon

Since NHEs play a key role in the regulation of pH_i, we investigated the activity of different NHE isoforms in the normal human colon. Removal of Na^+ from the standard HEPES solution caused rapid decrease in the pH_i, while, adding back Na^+ led to a complete pH_i recovery confirming the presence of a Na^+ dependent H^+ efflux in the epithelial cells of the colonic crypts (data are not shown). The NH_4Cl pulse technique was used to measure the activity of NHE isoforms. Exposure of colonic crypts to 20 mM NH_4Cl induced an immediate rise in pH_i because of the rapid entry of the lipophilic base, NH_3 into the cell. After the removal of NH_4Cl, pH_i rapidly decreased (Figure 2A). 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. After this acidification, pH_i starts to recover due to activation of pH_i regulatory mechanisms. In the absence of extracellular HCO_3^- in the solution and due to the low activity of H^+ efflux mechanisms from the cells, the recovery from acidosis (in the presence of Na^+) reflects the activity of NHE. Cariporide (HOE-642) the well known isoform specific NHE inhibitor (63) was used to test the activities of NHE1, NHE2 and NHE3, as previously validated in murine colonic crypts of NHE knockout mice (57, 58). The activities of NHE1 and 3 isoforms showed a decreasing pattern from the proximal to the distal part of the colon, whereas the activity of NHE2 did not change significantly (Figure 2B-D). The activities of all examined NHE isoforms could be detected in the different parts of the colonic crypts (surface, middle, base). Importantly, in the proximal colon NHE3 activity was significantly higher in the surface of the crypt (Figure 2D).

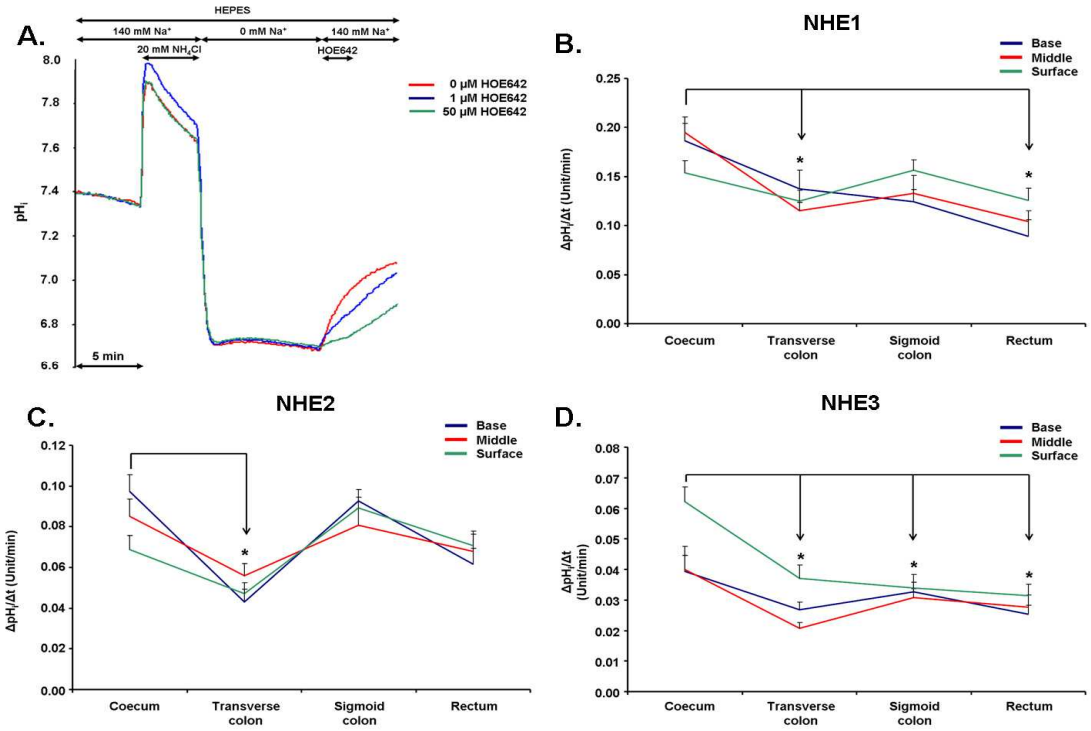

Figure 2: Determination of the Na^+/H^+ exchange activity in colonic epithelial cells. (A) Representative pH_i trace. The crypts were acid loaded by an exposure to a 5-min-pulse of 20 mM NH_4Cl followed by a 10-min-exposure of Na^+-free HEPES solution. The activities of the different NHE isoforms were measured by the isoform selective NHE inhibitor HOE-642. 1 µM HOE-642 inhibits the activity of NHE1 whereas 50 µM HOE-642 inhibits the activity of NHE2 and 3. The line charts **(B-D)** show that the activities of NHE1 and 3 were significantly higher in the proximal vs. the distal part of the colon. NHE3 activity proved to be the highest at the surface of the isolated colonic crypts. n = 26 colonic crypts isolated from 13 patients. Data are presented as means ± SEM. * indicates significant difference ($p<0.05$) vs. the respective groups.

Next, we were interested in whether the alterations of NHE1, NHE2 and NHE3 activities are associated with the changes of mRNA levels of these transporters. RT-PCR analyses revealed that mRNA expression of NHE3 was clearly decreased in the distal part of the colon in relation to villin, an epithelial marker (Figure 3C), β-actin, and β2-microglobulin which are non-epithelial markers (data not shown). However, there were no significant differences between the NHE1 and 2 mRNA levels in the different segments of the colon (Figures 3A-B).

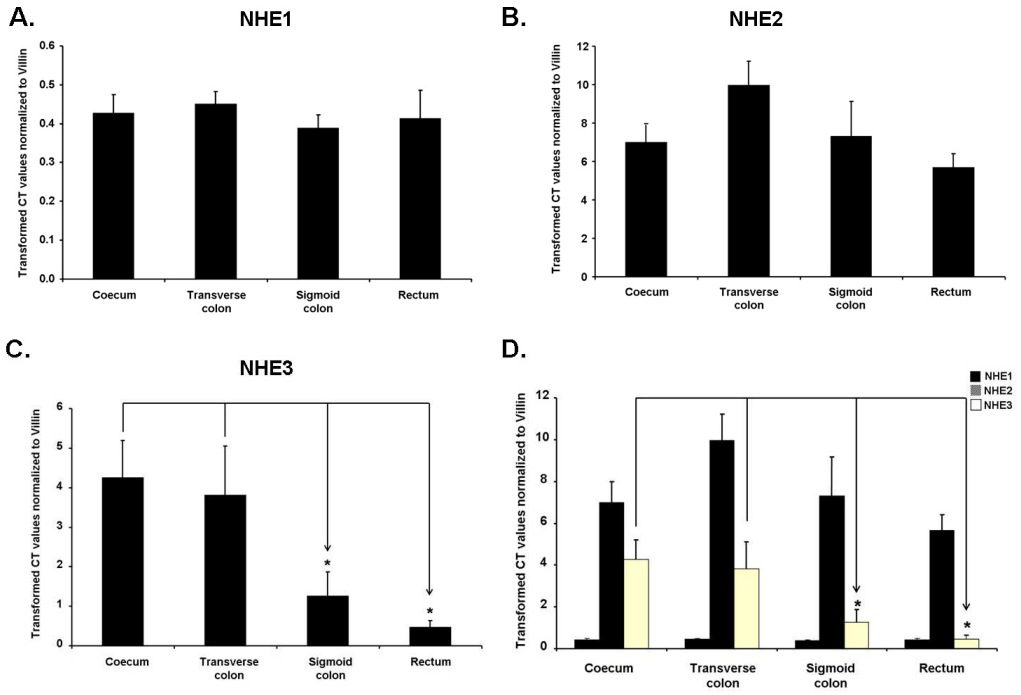

Figure 3. mRNA expression of NHE 1, 2 and 3 in the non-inflamed human biopsies collected from coecum till rectum. RNA was isolated from human biopsies and RT-PCRs were conducted as mentioned in the Materials and Methods. **(A)** NHE1, **(B)** NHE2 and **(C)** NHE3 mRNA expression levels were normalized against villin (an epithelial marker). **(D)** An overview of all the three genes normalized against villin, shows the differential expression of these genes from coecum to rectum. For each region, RNA was isolated from several biopsies of 3-5 controls. Data are presented as means ± SEM. * indicates significant difference ($p<0.05$) between the different parts of the colon.
CT: The cycle number at the threshold level of log-based fluorescence.

1.3. Differences in functional Na^+ channel activity along the large intestine

In order to compare the Na^+ channel activity in different parts of the large intestine, we isolated colonocytes from the coecum, transverse colon, and sigmoid colon/ rectum of individuals without UC. Colonic crypts were seeded on glass coverslips covered by Matrigel, where they preserved the original crypt structures and attached in a perpendicular orientation (Figure 4A). There is recent evidence that proteases contribute to ENaC regulation by cleaving specific sites in the extracellular loops of the α- and γ-subunits but not the β-subunit (64). Several studies indicate that trypsin activates ENaC by interacting with its α and/or γ subunits (65, 66). This interaction could alter the binding of amiloride to ENaC decreasing the potency of the inhibitor (67). Thus, trypsin was chosen to stimulate Na^+ channel activity. Figures 4B and 4C show that inward currents were activated by trypsin (50 µg/ml) in colonocytes isolated from sigmoid colon/rectum. These effects were abolished when extracellular

Na^+ was replaced with NMDG (Figure 4D). On the other hand, trypsin remained ineffective if cells were perfused with $NMDG^+$-rich external solution. Re-administration of Na^+ to the bath solution restored the stimulatory effects of trypsin (data not shown). In our hands amiloride (10 μM) inhibited trypsin-induced activation of the inward currents in only two cells out of ten experiments. In contrast to colonic crypts isolated from sigmoid colon/rectum, trypsin did not cause significant activation of inward currents in cells derived from the coecum or transverse colon (Figure 4E and Table 3). Following trypsin administration the reversal potential shifted towards more positive values in cells isolated from both sigmoid colon/rectum and transverse colon suggesting the activation of inward Na^+ current (Figure 4F).

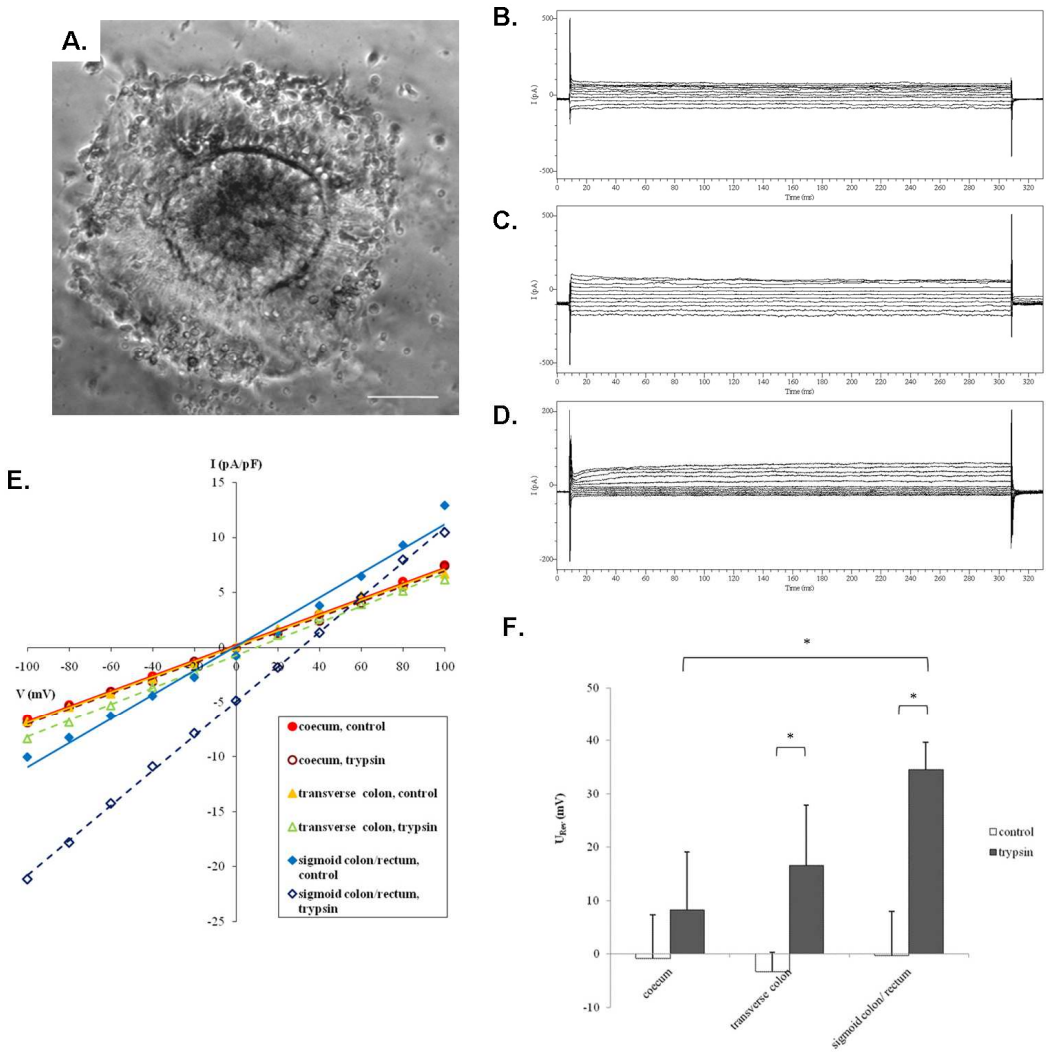

Figure 4. Differences in the functional Na^+ channel activity along the large intestine. (A) Phase contrast image of a control rectum-derived crypt adhered to a Matrigel-coated coverglass. The length of the bar indicates 100 µm. **(B)** Representative whole-cell current recording measured on control rectum-derived colonic crypts. **(C)** Exposure to trypsin caused an increase in inward currents. **(D)** Inward currents were rapidly suppressed when Na^+ was replaced with NMDG in the extracellular solution. **(E)** Average I/V plots obtained in cells derived from coecum (n=12), transverse colon (n=12) and sigmoid colon/rectum (n=13) isolated from 5 control individuals in each location. Trypsin significantly activated the Na^+ channel activity in control sigmoid colon/rectum samples. **(F)** Trypsin caused a significant shift of the reversal potentials towards more positive values in cells derived from transverse and sigmoid colon/rectum samples of control individuals. Note that trypsin-induced changes in reversal potentials were significantly larger in sigmoid colon or rectum derived cells than in coecum. Data represent mean ± SEM (*$p < 0.05$). n = 12-13 colonic crypts isolated from 5 patients.

	coecum (12) basal ENaC activities	coecum (12) trypsin-induced ENaC activities
-100 mV	-6,57 ± 4,44	-6,94 ± 4,14
-80 mV	-5,23 ± 3,70	-5,42 ± 3,22
-60 mV	-3,96 ± 2,93	-4,04 ± 2,33
-40 mV	-2,58 ± 2,17	-3,14 ± 2,19
-20 mV	-1,40 ± 1,46	-1,27 ± 0,37
0 mV	-0,06 ± 0,96	-0,09 ± 0,31
+20 mV	1,28 ± 1,05	1,31 ± 1,13
+40 mV	3,03 ± 1,58	2,37 ± 1,68
+60 mV	4,51 ± 2,32	4,02 ± 2,90
+80 mV	5,96 ± 3,01	5,53 ± 3,66
+100 mV	7,49 ± 3,60	7,40 ± 5,15

	transverse colon (12) basal ENaC activities	transverse colon (12) trypsin-induced ENaC activities
-100 mV	-6,83 ± 5,07	-8,36 ± 5,74
-80 mV	-5,46 ± 3,95	-6,87 ± 4,78
-60 mV	-4,25 ± 3,02	-5,29 ± 3,88
-40 mV	-2,87 ± 2,09	-3,63 ± 2,97
-20 mV	-1,48 ± 1,53	-2,16 ± 2,41
0 mV	0,05 ± 1,52	-0,61 ± 2,09
+20 mV	1,67 ± 2,29	1,10 ± 2,34
+40 mV	3,33 ± 3,20	2,71 ± 2,91
+60 mV	4,73 ± 3,61	3,90 ± 3,78
+80 mV	5,65 ± 4,09	5,12 ± 4,67
+100 mV	6,65 ± 4,70	6,15 ± 5,62

	sigmoid colon/ rectum (13) basal ENaC activities	sigmoid colon/ rectum (13) trypsin-induced ENaC activities
-100 mV	-10,03 ± 6,94	-21,15 ± 13,02*
-80 mV	-8,25 ± 6,16	-17,82 ± 11,11*
-60 mV	-6,26 ± 4,81	-14,26 ± 8,95*
-40 mV	-4,41 ± 3,79	-10,89 ± 6,86*
-20 mV	-2,72 ± 3,08	-7,87 ± 5,10*
0 mV	-0,76 ± 2,36	-4,84 ± 3,39*
+20 mV	1,39 ± 2,74	-1,80 ± 1,76*
+40 mV	3,80 ± 4,37	1,36 ± 1,24
+60 mV	6,46 ± 7,06	4,57 ± 3,01
+80 mV	9,28 ± 10,02	8,00 ± 5,82
+100 mV	12,89 ± 15,09	10,48 ± 7,76

Table 3: Whole cell current recordings in samples isolated from coecum, transverse colon, and sigmoid colon/rectum of control individuals, prior and after trypsin administration. Currents are expressed as current densities (pA/pF), negative values represent inward currents. Data represent mean ± SE (*$p < 0.05$). Number of cells is shown in parentheses.

1.4. Cl^-/HCO_3^- exchange activities in different parts of the normal colon

To test the activity of the Cl^-/HCO_3^- exchange (AE) mechanisms of colonic crypts, we utilized the Cl^- removal technique in the presence of HCO_3^- ions. Cl^- removal from the standard HCO_3^-/CO_2 solution caused a marked alkalization in colonic crypts suggesting active anion exchange. Notably, the alkalization was significantly higher in the surface vs. the middle and the base of the crypts (Figure 5A), suggesting that it may be predominantly caused by Slc26a3 (DRA) located on surface (66). In contrast to the activities of apical NHE3, AE activity was significantly higher in the distal vs. the proximal part of the colon (Figure 5B). Since solute linked carrier 26A3 (DRA) is the dominant anion exchanger on the apical membrane of colonic epithelial cells (42, 68), we also tested the expression level of this transporter. RT-PCR experiments showed no significant difference between the mRNA expressions at the distal and proximal parts of the colon (Figure 5C).

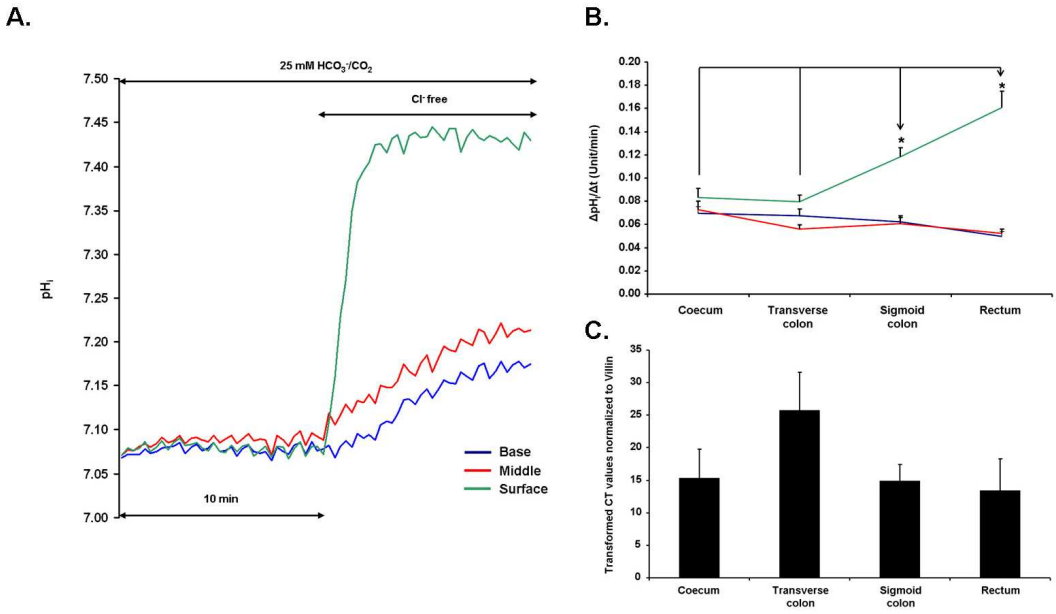

Figure 5. Cl^-/HCO_3^- exchange (AE) activity and DRA expression in control colonic crypts. (A) Representative pH_i trace of colonic crypts isolated from patients with normal endoscopic findings. Removal of Cl^- from the standard HCO_3^-/CO_2 solution resulted in an alkalization of pH_i. Anion exchanger activity was the highest in the surface of the crypts. **(B)** Activity of the Cl^-/HCO_3^- exchanger in different parts of the colon. Significantly higher AE activity was measured in the distal vs. the proximal part of the colon. n = 17-31 colonic crypts isolated from 4-10 patients. **(C)** Real-time PCR analysis of DRA mRNA expression in different parts of colon in relation to villin (an epithelial marker). For each region, RNA was isolated from several biopsies of 3-5 controls. Data are presented as means ± SEM. * indicates significant difference ($p<0.05$) between the different parts of the colon.

1.5. Changes in the activities of NHE1 and NHE3 in colonic crypts of UC patients

After the basic characterisation of ion transport mechanisms of the normal human colon, we examined the changes of these ion transport activities in patients suffering from UC. Since UC always involves the rectum, and in many cases sigmoidoscopy is enough for a control endoscopy, biopsy samples were collected only from the sigma and rectum. The resting pH_i of epithelial cells of the crypts isolated from patients with UC did not differ from the controls (Figure 6A).

The activity of NHE1 in the colonic crypts isolated from the inflamed region of the distal colon was approximately two times higher than in healthy colonic crypts isolated from the same region (Figure 6B). However, not only the activity, but also the expression of NHE1 was highly elevated. NHE1 was strongly upregulated in UC patients vs. controls (Figure 6D).

The activities of NHE3 significantly decreased in the middle and in the surface of the crypts in UC patients vs. controls. As it was predictable, no changes were observed in the base of the colonic crypts (Figure 6C). Quantitative RT-PCR revealed no significant changes in the UC vs. the control group suggesting that the defect of NHE3 activity is functional, rather than due to a downregulation of NHE3 expression (Figure 6E). The activity of NHE2 showed no significant differences in UC vs. controls (data are not shown).

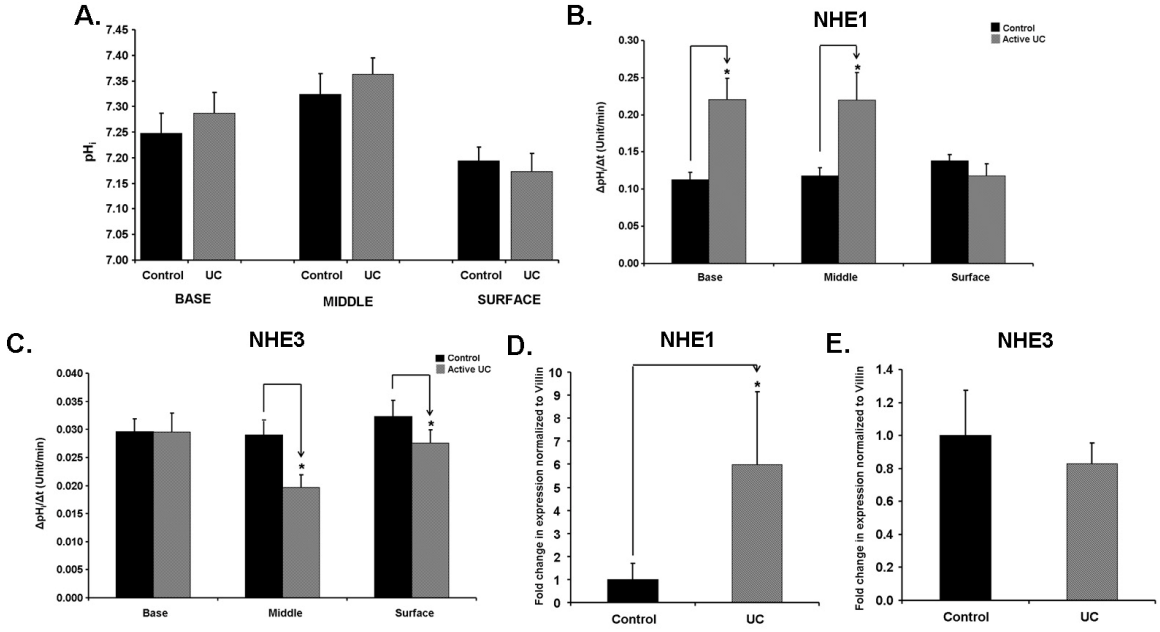

Figure 6. Resting pH_i and changes in the activity and expression of NHE1 and NHE3 in colonic crypts of UC patients. Biopsy samples were collected from the distal part of the colon of UC patients. Resting pH_i was determined in UC in the same way as in case of control colonic crypts. **(A)** No difference was detected in the resting pH_i between active UC and control colonic crypts. n = 10 colonic crypts isolated from 4 patients. **(B)** The activity of NHE 1 isoform was determined both in active UC epithelial cells with the use of 1 and 50 μM HOE642. Significantly higher NHE1 activity was detected in the base and middle parts of the colonic crypts in active UC compared to the controls. **(C)** Activity of NHE3 was significantly decreased in active UC throughout the crypt except for the base. n = 18 colonic crypts isolated from 7 patients.
Real-time PCRs were performed to determine the mRNA expression of **(D)** NHE1 and **(E)** NHE3 in biopsies collected from the sigmoid colon in relation to villin expression. Results showed a significant and strong increase of NHE1 mRNA expression in UC compared to non-inflamed controls, whereas NHE3 mRNA levels were not significantly altered. For RNA isolation, several biopsies from 7 control and 14 UC (mild to moderate inflammation of the sigmoid colon) patients were used. Data are presented as means ± SEM. * indicates significant difference ($p<0.05$) between the different parts of the colon.

1.6. Immunolocalization of NHE3 in the brush border membrane (BBM) of sigmoid colonic biopsies from UC patients with different histological disease states

We localized NHE3 immunohistochemically in relation to F-actin, which is a marker for the subapical cytoskeletal network. Apical as well as subapical NHE3 staining was seen in the surface area as well as the upper parts of the crypts, whereas the crypt base was free of NHE3 staining in both normal and UC sigmoid colon (Figure 7A-D). Subapical staining for NHE3 was seen in both control and UC enterocytes (Figure 7E for control enterocytes). NHE3 was located in the apical membrane in both non-inflamed sigmoid colon and in all stages of colitis (Figure 8A, shown for controls, moderate and severe colitis). Many different areas were assessed with the quantification

software Image-J (NIH, Bethesda, MD, USA, http://rsb.info.nih.gov/ij/) as described (57), and the intensity peak of F-actin was set to 1. Neither NHE3 fluorescence intensity nor its distribution in relation to F-actin was significantly different from non-inflamed controls (Figure 8B). This demonstrates that in inflamed sigmoid colon, NHE3 BBM abundance and localization is normal. We analyzed both steroid-treated and untreated patients' biopsies in the severely inflamed group, but did not find a higher NHE3 abundance in steroid-treated vs. untreated patients.

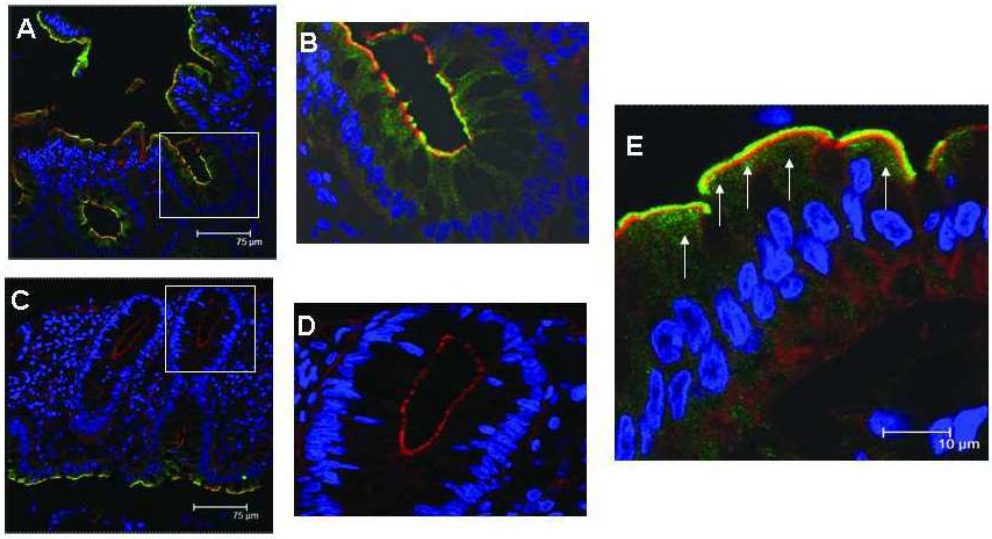

Figure 7: NHE3 immunolocalization in the human colon of control and UC patients. NHE3 staining was evident in the surface region and mid crypt, but not lower crypt area of sigmoid colon from UC patients. A) Lower magnification. NHE3 stains in green, F-actin was stained with phalloidin (red) and the nuclei are blue. Higher magnification of the B) surface region, C) upper-mid crypt region, D) lower crypt region. Some NHE3 is diffusely located in the subapical region. This was seen in UC as well as control mucosa, which is shown in (E).

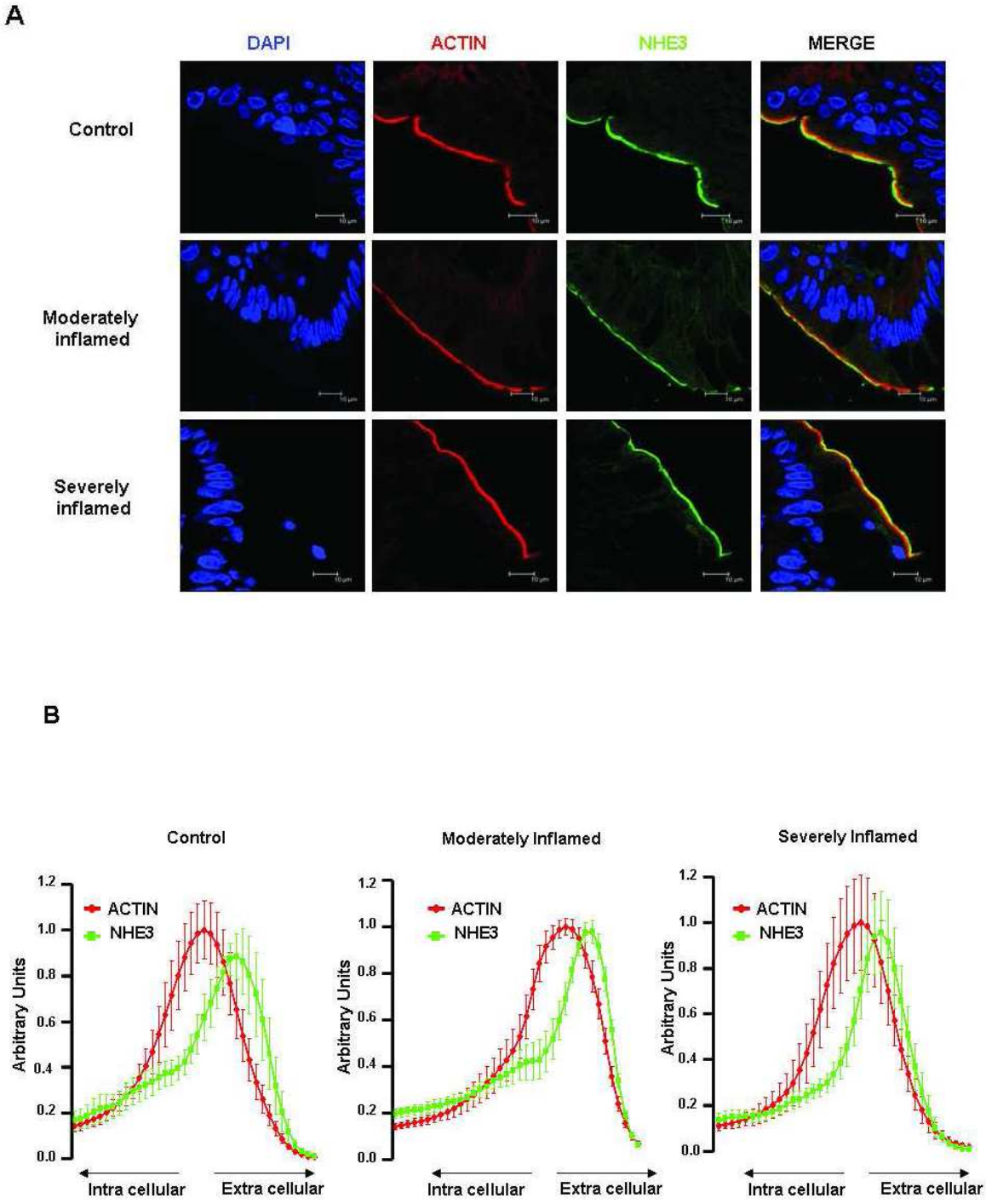

Figure 8: Immunolocalization of NHE3 in the brush border membrane of sigmoid colonic biopsies from UC patients with different histological disease states. A) NHE3 immunolocalization was performed in the biopsies from all disease states (here shown form moderate and severe UC) and B) NHE3 membrane abundance and localization in respect to the actin staining of the subapical network was assessed. Surprisingly, when many areas from at least 3 biopsies of each disease state were studied, no statistically significant difference was detected in the overall membrane abundance of NHE3. Brush border localization in relation to actin was also unchanged in the inflamed mucosa. n = 3-5.

1.7. Impaired activity of ENaC in UC

Since the effects of trypsin on the ENaC activity were more pronounced in colonic crypts isolated from sigmoid colon/rectum biopsies and the disease always involves this region, we decided to compare Na^+ channel activity on this part of the large intestine in subjects with and without UC. In contrary to the control cells, in UC colonocytes trypsin did not elicit significant increase in Na^+ current (Figure 9A and Table 4). Although we detected a moderate positive shift in reversal potential following administration of trypsin, the difference did not reach the level of significance (Figure 9B). These data suggest that besides alterations of other ion transport mechanisms and accumulation of local inflammatory mediators, disorders of Na^+ transport also contribute to development of diarrhea in UC.

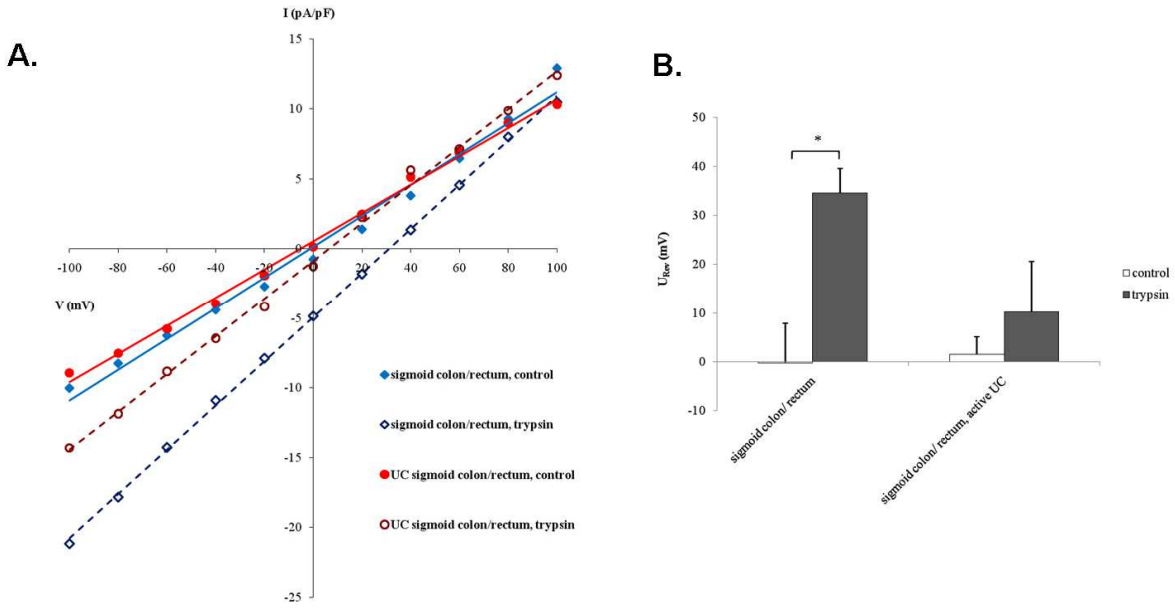

Figure 9. Differences in functional Na^+ channel activity. (A) Average I/V plots obtained in cells derived from sigmoid colon/rectum (n=12) of individuals with and without UC (n=5, respectively). Trypsin did not activate inward currents in colonic crypts isolated from UC patients. **(B)** Trypsin caused a significant shift of the reversal potentials towards more positive values in control but not in UC cells. Data are presented as means ± SEM.
* $P<0.05$ indicates significant differences vs. the controls.

	UC sigmoid colon/ rectum (12) basal ENaC activities	UC sigmoid colon/ rectum (12) trypsin-induced ENaC activities
-100 mV	-8,94 ± 4,35	-14,30 ± 10,44
-80 mV	-7,54 ± 3,87	-11,86 ± 8,67
-60 mV	-5,77 ± 2,98	-8,84 ± 6,79
-40 mV	-3,96 ± 2,20	-6,45 ± 5,30
-20 mV	-1,92 ± 1,07	-4,15 ± 3,24
0 mV	0,15 ± 0,75	-1,26 ± 1,89
+20 mV	2,45 ± 2,07	2,28 ± 3,69
+40 mV	5,15 ± 4,34	5,63 ± 5,16
+60 mV	6,91 ± 6,35	7,12 ± 6,59
+80 mV	9,00 ± 8,20	9,88 ± 8,79
+100 mV	10,31 ± 7,55	12,37 ± 10,42

Table 4: Whole cell current measurements in colonic epithelial cells derived from sigmoid colon/rectum of control individuals and patients with UC, prior and after treatment with trypsin. Currents are shown as current densities (pA/pF). Data represent mean ± SE (*p < 0.05). Number of cells is shown in parentheses.

1.8. Apical chloride transport is diminished in UC patients

In the surface of the crypts significantly lower Cl^-/HCO_3^- exchange activity was observed in UC patients vs. controls (Figure 10A). mRNA levels of DRA were reduced by approximately 50% in UC patients compared to non-inflamed controls (Figure 10B).

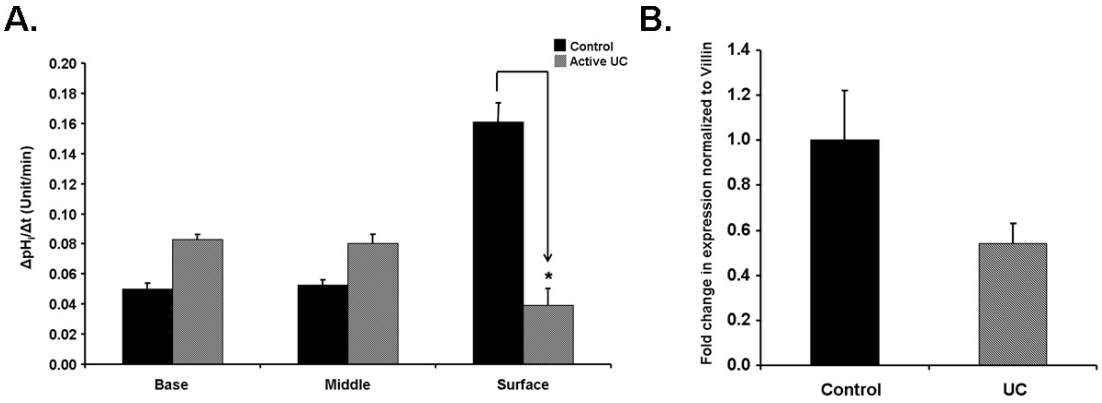

Figure 10. The Cl^-/HCO_3^- exchange activity and DRA expression in UC epithelial cells. (A) The anion exchange activity significantly decreased in the surface of UC colonic crypts. n = 10 colonic crypts isolated from 4 patients. **(B)** Real-time RT-PCRs were performed from the RNA isolated from the biopsies of sigmoid colon for DRA. Results showed a significant decrease of DRA mRNA expression in UC patients compared to controls. * P<0.05 indicates significant differences vs. the controls. For RNA isolation, several biopsies from 7 control and 14 UC (mild to moderate inflammation of sigmoid colon) patients were used. Data are presented as means ± SEM.

2. Assessment of the efficacy, the safety profile and long-term applicability of inflammatory bowel disease

2.1. Efficacy of successful infliximab induction therapy maintained for one year lasting without retreatment in different behavior types of Crohn's disease

At 6th month. Beneficial effect achieved by infliximab induction therapy remained in 28 of the 50 (56%) patients in the first 6 months. 15 of the 19 (78.9%) patients with luminal CD did not have relapse during this period. In all these 15 cases there was no change in the immunosuppressive drug dose, while corticosteroid treatment could be discontinued in 12 patients. Out of the 4 patients in whom exacerbation developed, 2 required infliximab retreatment, 2 needed parenteral steroid therapy and increase in the dose of immunosuppressants during hospital admission in all cases. In the first 6 months drainage from fistulae ceased in 13 of the 31 (41.9%) patients suffering from fistulizing CD besides unchanged type and dose of immunosuppressive treatment. Surgical treatment of complicated fistula was performed in 3 cases, on demand infliximab treatment was required in 8 patients and an increase in the dose of the immunosuppressive drugs was indicated in 7 cases.

At 12th month. 22 (44%) patients remained in complete steroid-free remission at the end of month 12. 11 (57.9%) of the 19 patients with luminal and also 11 (35.5%) of the 31 cases with fistulizing disease did not show clinical activity or fistula draining at the end of the first year (there is a significant difference between the two groups, p=0.014) (Figure 11). Hospital admission was indicated in further 7 cases of all of the patients (4 because of disease activity, 3 due to fistulae treatment).

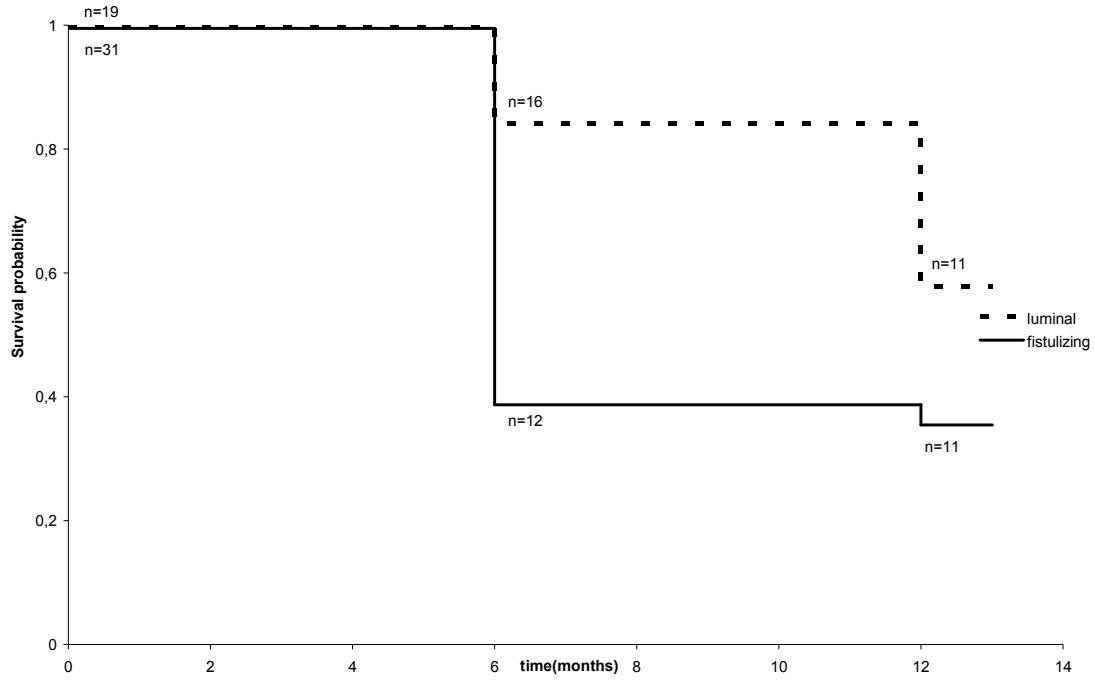

Figure 11. Kaplan-Meier survival curve for patients remaining in remission in luminal and fistulizing group.

Analyzing the patients being in remission at month 6 (15 luminal, 13 fistulizing types) 4 patients in the luminal group had flare up in the second term of the study, and parenteral steroid therapy was applied in all cases. 2 of the 13 patients with active, complicated fistulizing disease needed surgical intervention. Infliximab induction therapy was not repeated in the second term of the investigation. Eight of the patients with colonic, 7 with ileal, 6 with ileocolonic, and 1 with duodenal localization remained in remission at the end of the year. Six of the 19 smoker patients (31.6%) remained in remission at the end of the month 12, vs.16 of the 31 non-smoker (51.6%) patients. Statistical significance was not detected in groups made by localization and smoking between patients having active disease and being in remission.

2.2. Infliximab safety profile and long-term applicability

Sixteen (12.6%) patients (9 CD, 7 UC) had 31 episodes of acute, 7 (5.5%) patients (6 CD, 1 UC) had 9 episodes of delayed infusion reaction. 68.8% of those with acute reaction was on concomitant immunomodulator and/or corticosteroid treatment. Infliximab had to be discontinued in 12 patients because of the allergic reactions.

Listeria meningoencephalitis, sepsis of unknown origin and pulmonary tuberculosis were the most severe infectious complications in three cases. All patients were on concomitant immunosuppressive treatment. One patient with UC on infliximab monotherapy developed primary colonic lymphoma diagnosed after the fourth infusion and an other on concomitant immunosuppressants developed mediastinal lymphoma. The mortality rate was 3.1% (4 patients); 2 patients during and other 2 in the post-infliximab period.

2.3. Experiences with the unusual use of infliximab in two cases

2.3.1. Due to the intractable ulcerative pancolitis of a female patient not responding to the standard treatments, and to the lack of biological agents in that time, rescue operation was performed, which - according to the patient's choice - was subtotal colectomy and ileorectal anastomosis after 4 years disease duration. Six months later severe recurrent inflammation developed in the retained rectum indicating infliximab therapy. Because no response was seen to the 3-infusion induction therapy, systemic infliximab treatment was changed to rectal instillation of 100 mg infliximab daily for 6 days. Significant improvement was observed on clinical, endoscopic and histological examination.

2.3.2. Because of the steroid dependent, azathioprine- and cyclosporine-resistant chronic UC in a 14 year-old girl, ileal pouch anal anastomosis (IPAA) was performed. Due to the severe pouchitis (69) developing after the repeated reconstruction of the bowel continuity, which was accompanied by pyoderma gangraenosum and did not respond to combined antibiotics, topical budesonide, oral corticosteroid and azathioprine therapy, infliximab was initiated. After the induction with infliximab, all of the patient's symptoms and complaints ceased, pyoderma gangraenosum healed, and the endoscopic findings also demonstrated complete regression (Figure 12).

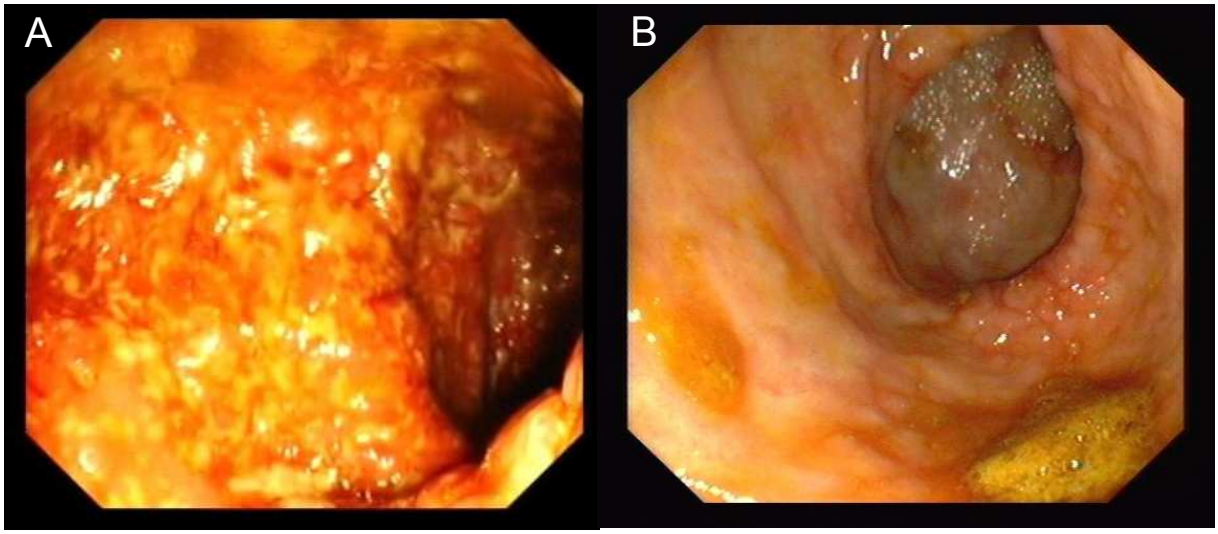

Figure 12. Endoscopic finding of the ileal pouch anal anastomosis before and after infliximab therapy.

V. DISCUSSION

1. Characterization of the activity and mRNA expression of ion transporters involved in NaCl absorption in human colon

The absorption of water and ions (especially Na^+ and Cl^-) is an important function of colonic epithelial cells. When the cells become unable to maintain normal level of absorption, different gastrointestinal symptoms such as diarrhoea or constipation develop, which in severe forms can be life threatening. Therefore, understanding the physiology and pathophysiology of absorption in the colon is crucially important. In healthy humans approximately 1.5 liter of fluid flows into the colon from the small intestine and 90% of this fluid is absorbed (70). The main driving force of this large amount of water reuptake is the Na^+ absorption from the lumen through the colonic crypts (70). In this study we show that the activities of electroneutral (via NHE3) and electrogenic Na^+ absorption (via ENaC) are in inverse ratio to each other in the proximal and distal colon. The segmental differences of electrogenic Na^+ absorption have been suggested by Sandle et al. (44, 71). They found that electrogenic Na^+ transport is higher in the human descending colon vs. the ascending colon *in vitro* (44, 72). In addition, Sellin and De Soignie showed that all segments of the human colon actively absorb Na^+ and Cl^- (72). Although ENaC was

found to be more active in the distal part of the colon, *in vivo* studies revealed that water and Na^+ absorption is greater in the proximal vs. the distal part of the colon (43, 71, 73). These data predict that electroneutral Na^+ absorption must be more dominant in the proximal colon. Consistent with recently published data from rodent colon (42), we show here that both the activity and the expression of NHE3, but not NHE2 are decreasing from the proximal to distal colon in human. In addition, the activity of NHE3 approximately doubled in the coecum vs. the rectum. These results are in accordance with the data obtained in animal models. Aldosterone was found to stimulate intestinal Na^+ absorption and increase NHE3 expression by 3-fold in the proximal colon but not in the distal colon (74). Notably, the expression of NHE2 was unaffected. The prominent role of NHE3 in Na^+ absorption is suggested by mouse models as well. Diarrhoea develops in NHE3-deficient, but not in NHE2 deficient mice (75). Furthermore, the loss of NHE2 transporter has no effect on the diarrhoeal state of NHE3 deficient mice (76).

Although NHE1 is not involved in the absorption of Na^+ (they are located in the basolateral membrane), but they are significantly involved in the pH_i regulation of colonic epithelial cells (41, 77). The importance of NHE1 was connected to inflammatory disorders as well, therefore, we extended our study to investigate both the activity and expression of NHE1 (33). Contrary to NHE3, we could not find segmental differences between the different parts of colon.

Colonic Cl^-/HCO_3^- exchange also plays an important role in water and Na^+ absorption from the large intestine. The two main apical anion exchangers in the gastrointestinal tract are the putative anion transporter 1 (PAT1 or SLC26A6) and the downregulated in adenoma (DRA or SLC26A3) (60, 78). Notably, PAT1 is only expressed in the small intestine, whereas DRA has a colon-predominant expression (77, 78). The prominent role of DRA in the colon is also suggested by the fact that DRA deficient, but not PAT1 deficient mice display dehydration due to severe diarrhoea (79). Importantly, mutations of DRA cause congenital diarrhoea in humans (79). Our experiments demonstrate that Cl^-/HCO_3^- exchange is more active in the distal vs. the proximal colon. The basolateral anion exchanger 2 (AE2) has been identified in the distal colon both in the surface and crypt cells; however, DRA was localized only to the

surface of crypt cells (81, 82). It is noteworthy that the differences between the Cl^-/HCO_3^- exchange activities were detected in the surface of the crypts suggesting alterations of DRA rather than other anion exchangers. This increased Cl^-/HCO_3^- exchange rate in surface colonocytes was not paralleled by higher DRA mRNA expression levels. Since DRA mRNA expression was standardized to villin expression, which is expressed along the whole crypt length (albeit more strongly towards to surface) whereas DRA is expressed in a strongly surface predominant fashion in the human colon (68), and since crypt length increases towards to distal colon, such expression studies may fail to reveal a selective increase in surface cell expression.

Following the first, basic physiological part of the study, we investigated the role of the same ion transport mechanisms in UC in which diarrhea is one of the leading symptoms. We found that the expression of NHE1 is strongly up-regulated in UC which is in accordance with elevation of functional activity. This is a very important finding since experiments in mice revealed that inhibition of NHE1 activity with amiloride strongly decreased the inflammatory processes *in vivo* in a mouse model of IBD (33). In addition, there are other evidences showing that NHE1 may be involved in the development of UC. NHE1 has been shown to be an important regulator in many types of immune response (83). NHE1 promotes TNF-α and IL-8 production (54, 84), neutrophil migration (85) and myeloperoxidase activity and release (86). The 6-fold elevation in the mRNA expression of NHE1 in UC and the above described previous observations strongly suggest that inhibition of NHE1 could be a potential therapeutical target.

Next we investigated the two major Na^+ transport mechanisms (NHE3 and ENaC) in patients suffering from active UC. Our results clearly demonstrate that both mechanisms are functionally damaged. The loss of ENaC function is most probably due to transcriptional alterations. The expression of ENaC β subunit was shown to be downregulated in UC (49). In addition, Amasheh et al. (87) clearly demonstrated that elevated proinflammatory cytokine levels selectively impair β- and γ-ENaC expression in UC. However, the loss of NHE3 function in UC is less clear. Although Sullivan et al. demonstrated that the expression of NHE3 is decreased in colitis based on Western analysis of colonic biopsies (49), we found no such significant differences between

control and UC patients, when the expression levels of NHE3 were compared to those of a structural brush border membrane protein with similar expression pattern than NHE3. In addition, NHE3 immunostaining was found to be normal in the brush border membrane of inflamed colonocytes. Therefore, the molecular mechanism of NHE3 functional damage still remains unsolved, and may be due to changes in the regulatory proteins that ensure NHE3 regulation and membrane retention (88).

Finally, we provided evidence that both the activity of anion exchange and the expression of the dominant anion exchanger, namely DRA, are significantly decreased. Downregulated in adenoma transporter is dominantly expressed in the surface of the crypts, whereas the basolateral anion exchangers are expressed in all parts of the crypts (82). Our experiments on normal crypts showed that the strongest activity of Cl^-/HCO_3^- exchange occur in the surface of the crypts. Importantly, anion exchange activity decreased by approximately 80% in UC indicating that Cl^- absorption via DRA is seriously damaged. It has also been shown that IL-1β reduces DRA mRNA expression *in vitro* by inhibiting gene transcription (89). Furthermore, highly reactive oxygen metabolites which are known to be involved in the pathogenesis of UC (90) inhibit Cl^-/OH^- exchange activity in Caco-2 cells (91). These results suggest that gene transcription by pro-inflammatory cytokines and/or functional changes of Cl^- absorption may play a crucial role in the pathogenesis of diarrhea in colitis.

In conclusion, we have demonstrated in this comprehensive human study that (i) electroneutral (via NHE3) and electrogenic Na^+ absorption (via ENaC) are in inverse ratio to each other in the proximal and distal colon, (ii) the anion exchanger DRA is more active in the distal part of the colon (iii). Both sodium and chloride transport is damaged in UC, whereas, (iv) NHE1 which has been shown to promote immune response is strongly up-regulated. These results open up new therapeutical targets in UC. Drug development and clinical studies are crucially needed to determine whether selective inhibition of NHE1 and/or stimulation of NHE3, ENaC and DRA would be beneficial in UC.

2. Assessment of the efficacy, the safety profile and long-term applicability of inflammatory bowel disease

Infliximab is an accepted treatment for inducing and maintaining remission in active luminal and fistulizing CD not responding to conventional corticosteroid or immunosuppressive therapy or developing intolerance or hypersensitivity to them (92). Beneficial induction therapy is recommended to be followed by maintenance of infliximab infusions every 8 weeks thereafter. However, in clinical practice on demand treatment is often required due to the lack of patient cooperation (missed controls) or financial causes. In such cases patients who relapse receive repeated infliximab induction therapy apart from the elapsed time. ACCENT I and II, which are one of the largest clinical trials dealing with the therapeutic results of CD, confirmed the beneficial effect of the regular maintenance infliximab therapy both in luminal and fistulizing CD. 58% of 573 patients with luminal CD participating in ACCENT I study responded to infliximab induction therapy (only one infusion!). 28 and 38% of them receiving maintenance infliximab therapy 5 and 10 mg/kg every 8 weeks remained in remission at week 54. Corticosteroid treatment was used in 67%, immunosuppressants in 29% of the patients at the beginning of ACCENT I study. Corticosteroid could completely be discontinued in 25% of the patients during the treatment. In addition, a significant decrease was seen in the need for hospitalization and the number of surgical interventions. These study findings suggested that significantly greater proportion of the patients were in remission in case of regular maintenance treatment with infliximab compared to episodic therapy (93). In the ACCENT II trial 36% of 195 patients, who responded to the infliximab induction therapy of the total 306 patients with fistulizing CD, remained in remission with the absence of draining fistula at week 54 (94).

However, an earlier observation by Domenech et al. (95) revealed a surprisingly good 6-month result after successful three-infusion based induction therapy without retreatment in patients with luminal CD, only 5 of the 18 patients relapsed during that time. Since our facilities were not enough to ensure regular 8 weekly maintenance treatment with infliximab for all patients responding to the induction therapy at that time, we retrospectively analyzed which useful conclusions can be made in case of this

application form and in which subgroups of patients with CD is episodic infliximab therapy recommended. Data of those patients were retrospectively analyzed, who were not treated with infliximab regulary after a successful induction with anti-TNF-α antibody. We were interested in which subgroups of patients is infliximab induction therapy worth to be used if maintenance treatment would not probably be available. One year, "medium-term" efficacy was assessed during our study.

22 of 50 patients remained in remission for at least one year without retreatment after successful infliximab induction therapy. The significant majority of the patients being in permanent remission was classified in the luminal patient group. 57.9% of the patients with luminal CD were in remission at the end of the year compared to the remission rate of 35.5% in case of fistulizing patients (p=0.014). Corticosteroid treatment using at the beginning of the infliximab induction therapy could completely be discontinued in patients remaining in remission at the end of month 12.

According to literature data active inflammation with high CRP level, isolated colon localization of CD, absence of intestinal stenosis, co-administration of immunosuppressive drugs and newly diagnosed childhood CD suggest beneficial influence on the outcome of infliximab treatment, while smoking impairs the therapeutic results (96, 97). Contrary to these data, results of Domenech et al. (95) and our findings did not confirm that remission continuity was influenced by the disease location, while among smoker patients – consistent with the literature data - therapeutic failure was more frequent (51.6 vs 31%), although statistical significance could not be verified. Our results support that regular retreatment with infliximab helps to achieve permanent therapeutic effect after successful induction therapy in patients with fistulas. In luminal CD patients, especially in non-smokers, retreatment can be used only if needed. Comparing the data of ACCENT I trial and ours, we observed high remission rate at one year among patients with luminal CD without maintenance biological treatment. This can be explained by the use of immunosuppressive therapy in most of the patients (86% of our patients received immunosuppressive drugs vs. 29% of the patients in the ACCENT I study) and by the fact that only those patients were enrolled in the study who achieved remission or whose fistulas completely closed at the end of induction therapy. Our results represent the medium-term efficacy of infliximab

induction therapy in luminal cases, non-smokers, apart from the disease localization and with continuous use of immunosuppressants. Results of patients with fistulizing CD suggest that infliximab induction therapy might not be worth using in the absence of the possibility of a regular retreatment with infliximab.

Since long-term follow-up safety data coming from clinical practice remain very important we also evaluated the safety profile of infliximab in IBD in a different cohort of IBD patients treated during the last 7 year period. Taken together, our data confirm that the safety profile of infliximab, as demonstrated in controlled trials, is reproducible in a clinical-based IBD cohort. The rate of serious adverse events is less than 5%, considering infliximab therapy safe in the long-term.

The use of infliximab in two unusual cases confirms that infliximab is effective in the management of refractory pouchitis and proctitis. However, further controlled studies are needed to evaluate the long-term outcome and the safety of infliximab in these conditions.

VI. ACKNOWLEDGEMENTS

I would like to thank all of the people who have helped and inspired me during my doctoral study.

I am grateful to **Prof. Dr. Tibor Wittmann**, head of the First Department of Medicine and to **Prof. Dr. András Varró**, the Head of Department of Pharmacology and Pharmacotherapy, who gave me the opportunity to work their Departments.

I would like to thank my supervisors **Dr. Péter Hegyi** and **Dr. Tamás Molnár** for their support and guidance. In addition, a very special thank goes to **Prof. Dr. Ferenc Nagy and Dr. Zoltán Rakonczay Jr.** whose understanding, encouraging and personal guidance have provided a good basis for the present thesis. I appreciate all their contributions of time, ideas, and funding to make my Ph.D. experience productive and stimulating.

I also would like to thank to **the gastroenterologist** at the First Department of Medicine and to **the surgeons** at the Department of Surgery for collecting research samples and to **the gastrointestinal research team** for their help and discussions.

I am deeply grateful to **Prof. Dr. Ursula Seidler** and **her research team** at the Hannover Medical School, Germany and to **Prof. Gábor Varga, Dr. Ákos Zsembery and Marianna Király** at the Semmelweis University, Hungary for their outstanding collaboration.

Lastly, I would like to thank **my family** for all their love, never-ending support, endless patience and encouragement. To them I dedicate this thesis.

VII. REFERENCES

1. Kucharzik T, Maaser C, Lügering A et al. Recent understanding of IBD pathogenesis: Implications for future therapies. *Inflamm Bowel Dis*, 2006; **12:** 1068-1083

2. Satsangi J, Welsh KI, Bunce M. Contribution of genes of the major histocompatibility complex to susceptibility and disease phenotype in inflammatory bowel disease. *Lancet,* 1996; **347:** 1212-1217

3. Cottone M, Rosselli M, Camma C et al. Smoking habits and recurrence in Crohn's disease. *Gastroenterology,* 1994; **106:** 643-648

4. Evans JM, McMahon AD, Murray FE et al. Non-steroidal anti-inflammatory drugs were associated with emergency admission to hospital for colitis due to inflammatory bowel disease. *Gut,* 1997; **40:** 619-622

5. Levenstein S, Prantera C, Varvo V. Stress and exacerbation in ulcerative colitis: a prospective study of patients enrolled in remission. *Am J Gastroenterol*, 2000; **95:** 1213-1220

6. Rutgeerts P, D'Haens G, Hicle M et al. Appendectomy protects against ulcerative colitis. *Gastroenterology*, 1994; **106:** 1251-1253

7. Monteleone G, Biancone L, Marasco R et al. Interleukin 12 is expressed and actively released by Crohn's disease intestinal lamina propria mononuclear cells. *Gastroenterology*, 1997; **112:** 1169-1178

8. Fuss IJ, Heller F, Boirivant M et al. Nonclassical CD1d-restricted NK T cells that produce IL-13 characterize an atypical Th2 response in ulcerative colitis. *J Clin Invest*, 2004; **113:** 1490-1497

9. Hanauer S. Medical therapy for ulcerative colitis. *Gastroenterology*, 2004; **126:** 1582-1592

10. Rutgeerts P, Sandborn WJ, Feagan BG et al. Infliximab for induction and maintenance therapy for ulcerative colitis. *N Engl J Med*, 2005; **353:** 2462-2476

11. Svartz N. Salazopyrin, a new Sulfanilamide preparation. A. Therapeutic results in rheumatic polyarthritis. B. Therapeutic results in ulcerative colitis. C. Toxic manifestations in the treatment with sulphanilamide preparations. *Acta Med Scand*., 1942; **110:** 577-598

12. Peppercorn MA. Sulfasalazine. Pharmacology, clinical use, toxicity and related new drug development. *Ann Intern Med*, 1984; **101:** 377-386

13. Desreumaux P, Ghosh S. Review article: mode of action and delivery of 5-aminosalicylic acid – new evidence. *Aliment Pharmacol Ther*, 2006; **24 suppl 1:** 2-9

14. Sutherland L, Macdonald JK. Oral 5-aminosalicylic acid for induction of remission in ulcerative colitis. *Cochrane Database Syst Rev*, 2006; **2:** CD000543

15. Katz JA. Treatment of inflammatory bowel disease with corticosteroids. *Gastroenterol Clin North Am*, 2004; **33:** 171-189

16. Jewell DP, Truelove SC. Azathioprine in UC: Final report on a controlled therapeutic trial. *BMJ*, 1974; **4:** 627-630

17. Kirk P, Lennard-Jones JE. Controlled trial of azathioprine in chronic ulcerative colitis. *BMJ*, 1982; **284:** 1291-1292

18. Rosenberg JL, Wall AJ, Levin B et al. A controlled trial of azathioprine in the management of chronic ulcerative colitis. *Gastroenterology*, 1975; **69:** 96-99

19. Hawthrone AB, Logan RF, Hawkey CJ et al. Controlled trial of azathioprine withdrawal in ulcerative colitis. *BMJ*, 1992; **305:** 20-22

20. Ardizzone S, Maconi G, Russo A et al. Randomised controlled trial of azathioprine and 5-aminosalicylic for treatment of steroid dependent ulcerative colitis. *Gut*, 2006; **55:** 47-53

21. Sood A, Midha V, Sood N et al. Role of azathioprine in severe ulcerative colitis: one-year, placebo-controlled, randomized trial. *Indian J Gastroenterol*, 2000; **19:** 14-16

22. Caprilli R, Carratu R, Babbini M et al. A double-blind comparison of the effectiveness of azathioprine and sulfasalazine in idiopathic proctocolitis. A preliminary report. *Dig Dis Sci*, 1975; **20:** 115-120

23. Mate-Jimenez J, Hermida C, Cantero-Perona J et al. 6-Mercaptopurine or methotrexate added to prednisone induces and maintains remission in steroid-dependent inflammatory bowel disease. *Eur J Gastroenterol Hepatol*, 2000; **12:** 1227-1233

24. Paoluzi OA, Pica R, Marceggiano A et al. Azathioprine or methotrexate in the treatment of patients with steroid-dependent or steroid resistant ulcerative colitis: results of an open-label study on efficacy and tolerability in inducing and maintaining remission. *Aliment Pharmacol Ther*, 2002; **16:** 1751-1759

25. Thukral C, Cheifetz A, Peppercorn MA. Anti-tumour necrosis factor therapy for ulcerative colitis: Evidence to date. *Drugs,* 2006; **66:** 2059-2065

26. Watkins PE, Warren BF, Stephens S et al. Treatment of ulcerative colitis in the cottontop tamarin using antibody to tumour necrosis factor alpha. *Gut*, 1997; **40:** 628-633

27. Rutgeerts P, van Assche G, Vermeire S. Optimizing anti-TNF treatment in inflammatory bowel disease. *Gastroenterology*, 2004; **126:** 1593-1610

28. Travassos WJ, Cheifetz AS. Infliximab: Use in inflammatory bowel disease. *Curr Treat Options Gastroenterol*, 2005; **8:** 187-196

29. Summers RW. Novel and future medical management of inflammatory bowel disease. *Surg Clin North Am*, 2007; **87:** 727-741

30. Hanauer S. Medical therapy for ulcerative colitis 2004. *Gastroenterology*, 2004; **126:** 1582-1592

31. Gisbert JP, González-Lama Y, Maté J. Systemic review: infliximab therapy in ulcerative colitis. *Aliment Pharmacol Ther*, 2006; **25:** 19-37

32. Zabana Y, Doménech E, Mañosa M et al. Infliximab safety profile and long-term applicability in inflammatory bowel disease: 9-year experience in clinical practice. *Aliment Pharmacol Ther*, 2010; **31:** 553-560

33. Németh HZ, Deitch EA, Szabó C et al.: Na+/H+ exchanger blockade inhibits enterocyte inflammatory response and protects against colitis. *Am J Physiol Gastrointest Liver Physiol*, 2002; **283:** 122-132

34. Laubitz D, Larmonier CB, Bai A et al. Colonic gene expression profile in NHE3-deficient mice: evidence for spontaneous distal colitis. *Am J Physiol Gastrointest Liver Physiol*, 2008; **295:** G63-G77

35. Seidler U, Lenzen H, Cinar A, et al. Molecular mechanisms of disturbed electrolyte transport in intestinal inflammation. *Ann N Y Acad Sci*, 2006; **1072:** 262-275

36. Teleky B, Hamilton G, Cosentini E, et al. Intracellular pH regulation of human colonic crypts cells. *Pflugers Arch*, 1994; **426:** 267-275

37. Kunzelmann K, Mall M: Electrolyte transport in the mammalian colon: mechanism and implications for disease. *Physiol Rev*, 2002; **82:** 245-289

38. Musch M, Arvans DL, WU GD et al. Functional coupling of the downregulated in adenoma Cl-/base exchanger DRA and the apical Na+/H+ exchangers NHE2 and NHE3. *Am J Physiol Gastrointest Liver Physiol*, 2009; **296:** G202-210

39. Clauss W, Schäfer H, Horch I et al. Segmental differences in electrical properties and Na-transport of rabbit coecum, proximal and distal colon in vitro. *Pflugers Arch*, 1985; **403:** 278-282

40. Yau WM, Makhlouf GM. Comparison of transport mechanisms in isolated ascending and descending rat colon. *Am J Physiol*, 1975; **228:** 191-195

41. Ikuma M, Kahgarian M, Binder HJ et al. Differential regulation of NHE isoforms by sodium depletion in proximal and distal segments of rat colon. *Am J Physiol Gastrointest Liver Physiol*, 1999; **276:** G539-G549

42. Talbot C and Lytle C. Segregation of Na/H exchanger-3 and Cl/HCO3 exchanger SLC26A3 (DRA) in rodent cecum and colon. *Am J Physiol*, 2010, epub

43. Levitan R, Fordtran JS, Burrows BA et al. Water and salt absorption in the human colon. *J Clin Invest*, 1962; **41:** 1754-1759

44. Sandle GI, Wills NK, Alles W et al. Electrophysiology of the human colon: evidence of segmental heterogeneity. *Gut*, 1986; **27:** 999-1005

45. Kawamata K, Hayashi H, Suzuki Y. Chloride dependent bicarbonate secretion in the mouse large intestine. *Biomed Res*, 2006; **27:** 15-21

46. Roediger WE, Lawson MJ, Kwok V et al. Colonic bicarbonate output as a test of disease activity in ulcerative colitis. *J Clin Pathol*, 1984; **37:** 704-707

47. Schilli R, Breuer RI, Klein F et al. Comparison of the composition of faecal fluid in Crohn's disease and ulcerative colitis. *Gut*, 1982; **23:** 326-332

48. Weijers HA, Van De Kamer JH. Alteration of intestinal bacterial flora as a cause of diarrhoea. *Nutrition Abstracts and Reviews*, 1965; **35:** 591-604

49. Sullivan S, Alex P, Dassopoulos T et al. Downregulation of sodium transporters and NHERF proteins in IBD patients and mouse colitis models: Potential contributors to IBD-associated diarrhea. *Inflamm Bowel Dis*, 2009; **15:** 261-274

50. Greig ER, Boot-Handford RP, Mani V, Sandle GI: Decreased expression of apical Na+ channels and basolateral Na+-K+-ATPase in ulcerative colitis. *J Pathol*., 2004; **204:** 84-92

51. Civitelli R, Teitelbaum SL, Hruska KA et al. IL-1 activates the Na+/H+ antiport in a murine T cell. *J Immunol*, 1989; **143:** 4000-4008

52. Vairo G, Royston AK, Hamilton JA. Biochemical events accompanying macrophage activation and the inhibition of colony-stimulating factor-1-induced macrophage proliferation by tumor necrosis factor-α, interferon-γ, and lipopolysaccharide. *J Cell Physiol*, 1992; **151:** 630-641

53. Prpic V, Yu SF, Figueiredo F et al. Role of Na+/H+ exchange by interferon-gamma in enhanced expression of JE and I-A beta genes. *Science*, 1989; **244:** 469-471

54. Orlinska U, Newton RC. Modification of tumor necrosis factor-alpha (TNF- α) production by the Na+ dependent HCO3 cotransport in lipopolysaccharide-activated human monocytes, *Immunopharmacology*, 1995; **30:** 41-50

55. Thomas JA, Buchsbaum RN, Zimniak A, Racker E: Intracellular pH-measurements in Ehrlich ascites tumor cells utilizing spectroscopic probes generated in situ. *Biochemistry,* 1979; **18:** 2210-2218

56. Hegyi P, Rakonczay Z Jr., Gray MA, Argent BE: Measurement of intracellular pH in pancreatic duct cells: A new method for calibrating the fluorescence data. *Pancreas,* 2004; **28:** 427-434

57. Bachmann O, Riederer B, Rossmann H et al. The Na+/H+ exchanger isoform 2 is the predominant NHE isoform in murine colonic crypts and its lack causes NHE3 upregulation. *Am J Physiol Gastrointest Liver Physiol,* 2004; **287:** 125-133

58. Cinar A, Chen M, Riederer B et al. NHE3 inhibition by cAMP and Ca2+ is abolished in PDZ-domain protein PDZK1-deficient murine enterocytes. *J Physiol,* 2007; **581:**1235-1246

59. Kiela PR, Ghishan FK. Na+-H+ exchange in mammalian digestive tract. *In: Johnson LR Physiology of the Gastrointestinal Tract.* Elsevier Press; 2006: 1847-1881

60. Zachos NC, Tse M, Donowitz M. Molecular physiology of intestinal Na+/H+ exchange, *Annu Rev Physiol*, 2005; **67:** 411-443

61. Bookstein C, DePaoli AM, Xie Y et al. Na+/H+ exchangers, NHE-1 and NHE-3, of rat intestine. Expression and localization. *J Clin Invest,* 1994; **93:** 106-113.

62. Lennard-Jones JL. Classification of inflammatory bowel disease. *Scand J Gastroenterol*, 1989; **24(suppl):** 2-6.

63. Scholz W, Albus U, Counillon L et al. Protective effects of HOE642, a selective sodium-hydrogen exchange subtype 1 inhibitor, on cardiac ischaemia and reperfusion. *Cardiovasc Res*, 1995; **29:** 260-268

64. Rossier BC. The epithelial sodium channel: activation by membrane-bound serine proteases. *Proc Am Thorac Soc*, 2004; **1:** 4-9

65. Nesterov V, Dahlmann A, Bertog M et al. Trypsin can activate the epithelial sodium channel (ENaC) in microdissected mouse distal nepron. *Am J Physiol Renal Physiol*, 2008; **295:** F1052-F1062

66. Kleyman TR, Carattino MD, Hughey RP. ENaC at the cutting edge: regulation of epithelial sodium channels by proteases. *J Biol Chem*, 2009; **284:** 20447-20451

67. Kellenberger S, Gautschi I, Schild L. Mutations in the epithelial Na+ channel ENaC outer pore disrupt amiloride block by increasing its dissociation rate. *Mol Pharmacol*, 2003; **64:** 848-856.

68. Jacob P, Rossmann H, Lamprecht G et al. Down-regulated in adenoma mediates apical Cl-/HCO3- exchange in rabbit, rat, and human duodenum. *Gastroenterology,* 2002; **22:** 709-724

69. Heuschen UA, Allemeyer EH, Hinz U, Autschbach F, Uehlein T, Herfarth C, Heuschen G: Diagnosing pouchitis – Comparative validation of two scoring systems in routine follow-up. *Dis Colon Rectum*, 2002; **45:** 776-788

70. Binder HJ, Sandle GI. Electrolyte transport in the mammalian colon. In *Physiology of the Gastrointestinal Tract*, L.R. Johnson, Ed.: Raven Press. New York, 1994: 2133-2171

71. Sandle GI Salt and water absorption in the human colon: a modern appraisal. *Gut,* 1998; **43:** 294-299

72. Sellin JH, De Soignie R. Ion transport in human colon in vitro. *Gastroenterology,* 1987; **93:** 441–448

73. Devroede GJ, Phillips SF, Code CF, *et al*. Regional differences in rates of insorption of sodium and water from the human large intestine. *Can J Physiol Pharmacol,* 1971; **49:** 1023–1029

74. Cho JH, Musch MW, Bookstein CM et al. Aldosterone stimulates intestinal Na+ absorption in rats by increasing NHE3 expression of the proximal colon. *Am J Physiol*, 1998; **274:** C586-C594

75. Schultheis PJ, Clarke LL, Meneton P, et al. Renal and intestinal absorptive defects in mice lacking the NHE3 Na+/H+ exchanger. *Nat Genet,* 1998; **19:** 282–285

76. Ledoussal C, Woo Al, Miller Ml et al. Loss of the NHE2 Na+/H+ exchanger has no apparent effect on diarrheal state of NHE3-deficient mice. *Am J Physiol Gastrointest Liver Physiol*, 2001; **281:** G1385-G1396

77. Hasselblatt P, Warth R, Schulz-Baldes A, et al. pH regulation in isolated in vitro perfused rat colonic crypts. *Pflugers Arch*, 2000; **441:** 118-124

78. Wang Z, Wang T, Petrovic S et al. Renal and intestinal transport defect in Slc26a6-null mice. *Am J Physiol Cell Physiol*, 2004; **288:** C957-C965

79. Schweinfest CW, Spyropoulos DD, Henderson KW et al. Slc26a3 (dra)-deficient mice display chloride-losing diarrhea, enhanced colonic proliferation, and distinct up-regulation of ion transporters in the colon. *J Biol Chem*, 2006; **281:** 37962-37971

80. Mäkelä S, Kere J, Holmberg C, et al. SLC26A3 mutations in congenital chloride diarrhea. *Hum Mutat*, 2002; **20:** 425-438

81. Ikuma M, Geibel J, Binder HJ et al. Characterization of Cl-HCO3 exchange in basolateral membrane of rat distal colon. *Am J Physiol Cell Physiol*, 2003; **285:** C912-C921

82. Rajendran VM, Binder HJ. Characterization and molecular localization of anion transporters in colonic epithelial cells. *Ann N Y Acad Sci*, 2000; **915:** 15-29

83. De Vito P. The sodium/hydrogen exchanger: a possible mediator of immunity. *Cell Immunol*, 2006; **240:** 69-85

84. Ohmori Y, Reynolds E, and Hamilton TA. Modulation of Na+/K+ exchange potentiates lipopolysaccharide-induced gene expression in murine peritoneal macrophages. *J Cell Physiol,* 1991; **148:** 96–105

85. Rosengren S, Henson PM, and Worthen GS. Migration associated volume changes in neutrophils facilitate the migratory process in vitro. *Am J Physiol Cell Physiol,* 1994; **267:** C1623–C1632

86. Witko-Sarsat V, Allen RC, Paulais M, et al. Disturbed myeloperoxidase-dependent activity of neutrophils in cystic fibrosis homozygotes and heterozygotes, and its correction by amiloride. *J Immunol,* 1996; **157:** 2728–2735

87. Amasheh S, Barmeyer C, Koch CS et al. Cytokine dependent transcriptional down-regulation of epithelial down-regulation of epithelial sodium channel in ulcerative colitis. *Gastroenterology*. 2004; **126:** 1711-1720

88. He P, Yun CC. Mechanisms of the regulation of the intestinal Na+/H+ exchanger NHE3. *J Biomed Biotechnol*, 2010; epub.

89. Yang H, Jiang W, Furth EE et al. Intestinal inflammation reduces expression of DRA, atransporter responsible for congenital chloride diarrhea. *Am J Physiol*, 1998; **275:** 1445-1453

90. McKenzie SJ, Baker MS, Buffinton GD et al. Evidence of oxidant-induced injury to epithelial cells during inflammatory bowel disease. *J Clin Invest*, 1996; **98:** 136-141

91. Saksena S, Gill RK, Tyagi S et al. Role of Fyn and PI3K in H2O2-induced inhibition of apical Cl-/OH- exchange activity in human intestinal epithelial cells. *Biochem J*, 2008; **416:** 99-108

92. Lichtenstein GR, Abreu MT, Cohen R et al. American Gastroenterological Association Institute Technical Review on Corticosteroids, Immunomodulators, and Infliximab in Inflammatory Bowel Disease. *Gastroenterology,* 2006; **130:** 940-987

93. Hanauer SB, Feagan BG, Lichtenstein GR et al. Maintenance infliximab for Crohn's disease: the ACCENT I randomized trial. *The Lancet*, 2002; **359:** 1541-1549

94. Sands BE, Anderson FH, Bernstein CN et al. Infliximab maintenance therapy for fistulizing Crohn's disease. *N Eng J Med*, 2004; **350:** 934-936

95. Doménech E, Hinojosa J, Nos P et al.: Clinical evolution of luminal and perianal Crohn's disease after inducing remission with infliximab: how long should patients be treated? *Aliment Pharmacol Ther*, 2005; **22:** 1107-1113

96. Chaudhary R, Ghosh S: Prediction of response to infliximab in Crohn's disease. *Digestive and Liver Disease*, 2005; **37:** 559-563

97. Parsi MA, Achkar J, Richardson S, et al. Predictors of response to infliximab in patients with Crohn's disease. *Gastroenterology*, 2002; **123:** 707-713