Studies of barrier function in patients with ulcerative colitis and pouchitis

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ABSTRACT

Background and aim: The cause of ulcerative colitis (UC) is largely unknown. However, there is a presumed genetic component to susceptibility and altered intestinal barrier function has been implicated in the pathophysiology of ulcerative colitis. There is evidence that the increased intestinal permeability in IBD is partly controlled by delicate intercellular circuits in the colonic tissue linked to the enteric nervous system. Little is, however, known about how this is regulated in detail. Ileal pouch-anal anastomosis (IPAA) is a good surgical reconstructive option in UC patients after proctocolectomy. However 10-15% of IPAA patients develop a severe and recurrent inflammation in the constructed pouch. The standard treatment for pouchitis is long and/or frequent use of antibiotics. Probiotics have been shown to reduce the risk of recurrence of pouchitis after induction treatment with antibiotics.

The aim was to characterize macromolecular permeability in non inflamed colon of UC and elucidate the role of cholinergic signaling, mast cells and eosinophils in the regulation of the human colonic permeability. Furthermore, we examine the mucosal barrier function in relation to pouchitis, before and after treatment with probiotics.

Material and methods: In the first study 23 UC patients in remission and 53 healthy volunteers were included. Biopsies from the sigmoid colon were assed for macromolecular permeability (horseradish peroxidase (HRP) and ^51CrEDTA) and electrophysiology during challenge with carbachol. Experiments were repeated with CRF receptor antagonists, carbachol receptor antagonists and mast cell stabilizers in Ussing chambers. Further, pouch biopsies from 16 IPAA patients with pouchitis and 13 IPAA controls were assed in Ussing chambers for macromolecular permeability and electrophysiology as above. In addition E. coli K12 were used to assess the barrier to bacteria. Biopsies were taken on three occasions; before treatment, after antibiotics and after probiotics. Pouchitis Disease Activity Index (PDAI) was used in all subjects.

Results: Colonic tissues from UC patients had significant increase in permeability to protein antigens compared with controls. Permeability was normalized by atropine, α-helical CRF(9-41) and lodoxamide. Eosinophils were increased in number in UC tissues, expressed M2 and M3 muscarinic receptors and showed immunoreactivity to CRF. In pouchitis patients, PDAI was significantly improved after treatment with antibiotics and probiotics. There was a significantly enhanced passage of E. coli K12 and HRP in patients with active pouchitis, which was unchanged during treatment with antibiotics, but significantly normalized by probiotics.

Conclusions and discussion: We identified a neuroimmune intercellular circuit (from cholinergic nerves, via eosinophils to mast cells) that mediates colonic mucosal barrier dysfunction in UC patients. Furthermore we found that probiotics restored the increased permeation to E. coli and HRP in patients with pouchitis. Pouchitis, resembling symptoms in active UC, may well constitute a good model to study acquired intestinal barrier dysfunction in IBD.
LIST OF PAPERS

This thesis is based on following papers:

I. Eosinophils express muscarinic receptors and corticotrophin-releasing factor to disrupt the mucosal barrier in ulcerative colitis.

II. Effects of probiotics (Ecologic 825) on barrier function during maintenance treatment for severe pouchitis.
ABBREVIATIONS:

Ach  Acetylcholine  
Atr  Atropin  
Cch  Carbachol  
CD  Crohn’s disease  
CRF  Corticotropin-releasing Factor  
CFU  Colony-forming unit  
E. coli  Escherichia coli  
FAP  Familial adenomatous polyposis  
GALT  Gut-associated lymphoid tissue  
GWA  Genome-wide association  
Hex  Hexamethonium  
HIT  Human intestinal tract  
HRP  Horseradish peroxidase  
H₂S  Hydrogen sulphide  
Hsps  heat shock proteins  
IBD  Inflammatory bowel disease  
IBS  Irritable Bowel Syndrome  
IL  Interleukin  
IPAA  Ileal pouch-anal anastomosis  
Isc  Short circuit current  
KRB  Krebs-Ringer bicarbonate buffer  
LPS  Lipopolysaccharide  
Lod  Lodoxamide tromethamine  
MLCK  Myosin Light-Chain Kinase  
PD  Potential difference  
PDAI  Pouchitis disease Activity Index  
PPI  Prepouch ileitis  
RA  Rheumatoid arthritis  
SCFA  Short chain fatty acids  
SRB  Sulphate Reducing bacteria  
TER  Transepithelial electrical resistance  
TJ  Tight junction  
TLR  Toll-like Receptors  
TNF-α  tumor necrosis factor alpha  
TTX  Tetrodotoxin  
UC  Ulcerative colitis  
VE  Vehicle  
WHO  World Health Organization  
4-DAMP  4-diphenylacetoxy-N-methylpiperidine methiodide  
⁵¹CrEDTA  Chromium 51 ethylene diamine tetra-acetic acid
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1. BACKGROUND

Ulcerative colitis

Epidemiology and symptoms

Ulcerative colitis (UC), one of the two main conditions of inflammatory bowel disease (IBD) has been known since ancient times but was first described as a formal medical entity in the late 19th century by Wilks and Moxon (1). With its symptoms of bloody diarrhea, fever and abdominal pain in relapse UC affects 10-15/100,000 inhabitants yearly. Even though a second peak incidence is seen in the sixties the most common onset occurs between the ages of 15 and 25. The condition affects females slightly more with a factor of 1.2:1 (2).

Aetiology

The aetiology of UC is not fully elucidated but most likely multifactorial. Epidemiological studies show that a positive family history is the largest independent risk factor for the disease (3). Ethnic background is also important. For instance, UC occurs more commonly among Ashkenazi Jewish people than non-Jewish people. Moreover there is a geographic correlation to UC where the highest incidence is seen in Scandinavia, the United Kingdom, The United States and Canada (4-5). Genetic studies confirm that UC involves polygenetic disorders. Recently, hypothesis-free genome-wide association (GWA) studies have revolutionized the field of complex disease genetics. In addition to previously known mutations on chromosome 6, which encodes the major histo-compatibility complex, over 20 susceptibility loci, specific to UC have been discovered. Most interestingly, genes implicated in mucosal barrier function (ECM1, CDH1, HNF4α and laminin B1) confer risk of UC. Furthermore, impaired IL-10 signaling due to IL-10 receptor mutations has reemerged as a key pathways in intestinal inflammation. IL-10 is a well known anti-inflammatory cytokine that limits the release of proinflammatory cytokines like TNF-α and IL-12 in response to pathogens (6).

Under normal conditions there is a delicate balance in the colonic immune system. In UC numerous studies has established an imbalance where luminal antigens, including the commensal microbiota, gain access to the underlying mucosal tissue via a leaky intestinal barrier. The innate and adaptive subepithelial immune cells respond to the invading antigens and trigger an abnormal inflammatory response. This leads to differentiation and activation of different immune cells, such as T cells, macrophages and mast cells. Furthermore, secretion of different substances of inflammation is seen where the interleukin 13 (IL-13) secreted by natural killer T cells also seem to be a key effector cytokine of UC. IL-13 stimulates epithelial cell apoptosis and increase epithelial permeability due to changes in function of tight junctions which may, in part explain increased uptake of antigens (7-8). Other secreted pro-inflammatory cytokines stimulate macrophages to secrete large amounts of tumor necrosis factor α (TNF-α), IL-1 and IL-6 which are known to increase epithelial permeability to antigens (9-11).

Various environmental factors have been shown to affect the course of UC. While observational studies have shown beneficial effect of cigarette smoking with less frequent relapse of UC (12), psychological stress increases the risk of exacerbation of the disease (13).
In conclusion, a growing bulk of evidence suggests that IBD and consequently UC arises in genetically predisposed individuals, who are exposed to precipitating environmental factors, where the composition of our intestinal microbiota seem to be important in the maintenance of the chronic inflammation.

**Treatment**

**Acute colitis**

Since Truelove and Witt published their work on the effects of cortisone in UC, corticosteroids have served as the cornerstone of treatment in the acute phase of ulcerative colitis (14-15). The common standard treatment in severe acute UC is intravenously administrated steroids, e.g. Betametason during five days (16). Approximately half of these patients will attain full remission. However, about 30% of the patients do not respond to steroids and will need rescue medical treatment or surgery i.e. colectomy (17).

**Maintenance treatment**

**5-aminosalicylic acid (5-ASA)**: In the 1930’s in Sweden the first female professor of medicine, Nanna Swartz developed sulfasalazine, a drug initially aimed for rheumatoid arthritis. Later on sulfasalazine also proved to be effective on intestinal symptoms in patients with ulcerative colitis (18). Today, the active substance of sulfasalazine, 5-ASA is the first line medication for maintenance of remission. It can also be used in patients with mild to moderate relapse of UC (19).

**Immunosuppressants**: As a product of transplantation medicine immunosuppressant drugs were developed in the 1960’s in order to prevent organ rejection. In addition to mentioned features mercaptopurine (20) and azathioprine also proved to be effective in UC. Today both substances are used in UC patients after severe relapse, in cortisone dependant disease or in patients with frequent relapses in spite of 5-ASA-treatment. In 1984 Gupta et al proved that the immunosuppressant agent, cyclosporine was valuable in the treatment of UC (21). The substance has gained popularity internationally but has its limitations due to severe and sometimes lethal side-effects. Furthermore high recurrence rates have been reported (16).

**Biological therapy**: The term is defined as any form of treatment that uses the body’s natural abilities that constitute the immune system to fight infection and disease. In recent years tremendous efforts have been made to put forward monoclonal antibodies, mainly to be used in the fight against malignant disease. In 1994 Elliot et al tested infliximab, a monoclonal antibody against TNF-α, in patients with RA. The antibodies were further tested for UC (22). Today, Infliximab is predominantly used as rescue treatment in patients with severe relapsing disease, resistant to standard therapy (23).

**Probiotics**: Have shown to be effective in inducing and maintaining remission of UC (24-25). Moreover, probiotics reduce the risk of recurrence of pouchitis after induction treatment with antibiotics (26) and work as a prophylactic treatment against pouchitis in patients with newly constructed reservoirs (27). Probiotics will be further discussed in a separate chapter.

**Surgery**

After primary colectomy with the creation of an ileostomy, there are different restorative surgical options. Nils Kock at Sahlgrenska Sjukhuset in Sweden constructed a continent pouch ileostomy in 1969 (28). The internal reservoir is continent due to a valve mechanism and is emptied by intubation through a stoma regularly. In 1978 Parks et al introduced a surgical procedure whereby the distal
part of ileum was formed into a pouch and then was anastomosed to the anal canal after complementary proctectomy. The procedure is called ileal pouch anal anastomosis (IPAA) and has become the most widely accepted procedure of choice for the surgical treatment of patients with ulcerative colitis. Patient satisfaction is good and IPAA provide a near-normal life. More recently, due to more effective and potent anti-inflammatory medical therapies the procedure of ileorectal anastomosis has gained in popularity. The advantages are less surgical trauma and reduced risk of infertility. However there is limited knowledge concerning the risk of developing malignant disease in the remaining rectum over time.

**Intestinal barrier function**

**Large intestine**

The human large intestine is about 1.5 meters long and is divided into colon and rectum. The main function is to absorb water. Colon and rectum houses 300-500 different bacterial species and constitute the important commensal microbiota. Bacteria make up most of the content in colon and represent about 60% of the fecal mass. In addition to the delicate interaction between the microbiota and host, the bacteria degrade indigestible carbohydrates for epithelial nourishment and produce vitamins for colonic absorption into the blood. The wall of the large intestine is composed of three different layers; the mucosa (consisting of the epithelium, the lamina propria and muscularis mucosae) the submucosa and the muscle layer.

The colonic epithelium consists of a single-cell polarized layer. Colonocytes constitutes together with goblet cells, which secrete mucin, about 95% of the cells of the mucosa. The adjacent colonocytes are interconnected by junctional complexes e.g. tight junctions. Amongst other cells of the colonic mucosa, Plasma cells have previously been described as an important source of β-defensins (29). The beta defensins are antimicrobial peptides implicated in the resistance of epithelial surfaces to microbial colonization.

**Tight junctions**

This intercellular junction is located at the most apical part of the lateral cell membrane forming linking strands between adjacent epithelial cells. Tight junctions consist of three transmembrane proteins, claudins, occluding and JAM (30). These proteins are anchored by different cytoplasmic components where zona ocludens protein 1-3 (ZO-1-3), Cingulin and F-actin play a crucial role. In addition to holding cells together tight junctions constitute a gate-like, paracellular barrier for passage of ions and molecules. This passage is rate limited where there is a size and charge selectivity. For instance, positively charged molecules and ions diffuse more easily. Furthermore, tight junctions have the ability to separate lipids and protein components of the apical and basolateral cell membrane. These features are essential to maintain barrier function. The passage of positively charged ions help to maintain the polarity of cells ensuring the specialized functions of each surface to be preserved i.e receptor-mediated endocytosis at the apical part and exocytosis at the basolateral surface. Certain molecules and ions are prevented to pass through the space between the cells; hence the tight junctions force these components to actually enter the cells in order to pass through the tissue. In UC, increased production of interleukin 13 elevates the claudin protein expression in TJ. TNF-α is known to activate certain proteins, myosin light-chain kinase...
(MLCK) involved in the contraction ability of TJ (31). Altogether, this results in increased paracellular permeability by reducing the number of intercellular strand molecules in TJ (32).

**Barrier layers**
While allowing absorption of water and nutrients, the intestinal barrier has the complex task of serving as a selective barrier against harmful food components, bacteria and other antigens. The normal intestinal barrier allows small amounts of antigens to cross the mucosa to interact with the innate and adaptive immune system – immunosampling. The different layers of the barrier consist of:

- **Lumen:** The growth of bacteria and other pathogens are inhibited by the effects and competition of the commensal bacterial flora. The milieu consisting of bile, gastric and pancreatic juice and the motility of the gut also have an inhibitory effect.

- **Microclimate:** Adjacent to the apical surface of the epithelium a layer of mucus produced by the goblet cells is found. In the mucus there is immunoglobulin i.e. secretory IgA which play an important role in the barrier defense. Above the layer of mucus there is an unstirred water layer.

- **Epithelium:** A polarized single layer epithelium connected by junctional complexes such as tight junctions with passive and active transport mechanisms.

- **Lamina propria:** Underneath the epithelium cells of innate and acquired immunity are found. They constitute key components in the defense against pathogens. The enteric nervous system is present as well as endothelium of capillaries and lymphatics.

**Components of the intestinal barrier:**
- **Lumen:** degradation of bacteria and antigens by gastric acid, pancreas and biliary juices (3). Microclimate: the unstirred water layer (2), the mucus layer and glycocalyx (3) Immunoglobulins, primarily IgA (4).
- **Epithelium:** the enterocytes, interconnected by junctional complexes (5), chloride secretion (6) and the basal lamina (7). Lamina propria: immunoglobulins (8), cells of acquired immunity (9) and innate immunity (10). The enteric nervous system and hormones (11). The endothelium and capillaries (12).
Probiotics

**The commensal intestinal microbiota**
The bacterial colonization of the gut occurs the moment the child passes through the birth canal during birth (33). This process is further refined as the child starts to breastfeed (34). The initial colonization is of great importance for the permanent composition of the intestinal microbiota through life since pioneer bacteria can modulate and create a favourable environment for themselves and inhibit the growth of other bacteria introduced later into the ecosystem (35). The bacterial distribution alters throughout the course of the intestine. Because of the exposure to HCl, bile and pancreatic secretion together with enhanced gut motility the bacterial count as well as the amount of different species is low in the upper part of the gut. The bacterial concentration and diversity gradually increases along the jejunum and ileum (36). In colon the bacterial load constitutes approximately 60% of the fecal content (37). An adult individual accommodates 300-500 different bacterial species of which the majority has never been cultivated and many are yet to be identified. About 30-40 of these species represents 99% of the total intestinal bacterial population (38). Most of the bacterial species of the gut are anaerobic and the dominant groups in adults are *Bacteriodes*, *Bifidobacterium*, *Eubacterium*, *Clostridium*, *Lactobacillus*, *Fusobacterium* and different gram positive cocci whereas aerobes such as *Enterococcus* and *Enterobacteriaceae* are amongst the subdominant bacterial strains (38).

**Function of intestinal microbiota**
Under normal conditions, commensal microbes and their hosts enjoy a symbiotic relationship. The host provides a hospitable and nutrient rich environment while the diverse intestinal microbiota exerts numerous beneficial effects on the host’s health. The microbes of the gut can control the proliferation and differentiation of the epithelial cells of the mucosa (39). Furthermore the intestinal bacteria play an essential role in the development of the immune system, in terms of Gut-associated lymphoid tissue (GALT) development (40), mucosal immunity (41) and oral tolerance (42). The metabolic function of the gut microbiota consists of fermentation of non-digestible dietary residue and endogenous mucus (38). The resident bacteria in colon work as an anaerobic bioreactor taking part of the breakdown of non-digested and otherwise indigestible polysaccharides (43), the synthesis of micronutrients including vitamins (44), short chain fatty acids (45) and polyamines (46), and the absorption of trace elements (45). The microflora can inhibit the colonization of pathogenic species in various ways. The commensal flora produces and secretes antimicrobial agents such as bacteriocines to kill competitor strains. Further they have the ability modify luminal pH and compete for nutrients required for the growth of pathogens (47-49). Cross-talk between the microbiota and host epithelial cells further ensures that the host provides an adequate milieu in terms of nutrients required by the commensal microbes (38). Through specific receptors, Toll-like Receptors (TLR) on the surface of the enterocytes there is an ongoing communication between the commensal flora and host to distinguish between “good” or “bad” (50).

The normal microbiota of the gut can easily be disturbed. The most common example is diarrhea because of overgrowth by fungi and *Clostridium difficile* after the use of antibiotics (51). Dysbiosis, defined as a state in which the microbiota exerts adverse effects on the host can also be seen after radiation therapy, change in dietary intake, physical and psychological stress, IBD and IBS (52).
**Probiotics – History and definition**

The positive effects of fermented milk products have been known since ancient times but it was first in 1907 when the Nobel Prize laureate Metchnikoff proved that Lactobacillus bulgaricus contained in yogurt is able to displace pathological intestinal flora (53). In 1917, Alfred Nissle isolated a specific *E coli* strain from a World War 1 soldier who seemed to be immune to the diarrheal diseases commonly ravaged in the trenches. The *E coli* strain that later on was named after Nissle, was given to a young woman with ulcerative colitis. Astonishingly a remission was seen after five weeks of treatment (54). This is an early example of how bacteria can be used as therapy but it was not until the sixties the term probiotics started to be used. Today, the definition of probiotics by WHO is “Live microorganisms which when administrated in adequate amounts confer a health benefit on the host” (55). The market for probiotics has grown considerably in recent years together with an increasing number of probiotic-containing commercial products claiming specific health benefits (56). There is a variety of different probiotic strains but the most common type is included in the group of Lactic Acid Bacteria (LAB). Bacteria in this group are functionally related by their ability to produce lactic acid during fermentation. The most commonly used LAB-members are *Bifidobacteria* and *lactobacilli*. Other frequently used probiotic species apart from LAB are *Clostridium butyricum* and the tropical strain of yeast, *Saccharomyces boulardii* (57).

**Mechanisms of probiotic actions**

The mechanisms by which probiotics exert their effect are highly complex and largely unknown, but several of these are directly or indirectly related to intestinal barrier function. Like the commensal microbiota, probiotics have the ability to compete for essential nutrients and modify ecological conditions such as pH. In addition, probiotics exert luminal effects through the ability to reduce the adhesion of pathogens to the intestinal mucosa by enhancing the mucin secretion and compete for adhesion sites in the intestine (58-59). The probiotic strain *E. coli Nissle 1917*, but not other *E. coli* strains, strongly induces expression of antimicrobial peptide human beta-defensin-2 in Caco-2 intestinal epithelial cells. Activation of defensins can inhibit microbial growth of potential pathogens in the gastrointestinal tract (60). Furthermore, specific heat shock proteins (hsp) known for their ability to maintain cytoskeletal integrity were produced in intestinal epithelial cells exposed to VSL#3 conditioned medium (61). *S. boulardii* also strengthen intestinal barrier by protection of the tight junction complex through ensuring zona occludens (ZO)-1 distribution. The tropical yeast strain also prevents chloride secretion induced by *E coli* toxins (62). In animal studies, the probiotic combinations VSL#3 (63) and Ecologic 641 (64) have been shown to prevent increased intestinal permeability to macromolecules in colitis and acute pancreatitis, respectively, by mechanisms involving stabilisation of the cytoskeleton and tight junction proteins. Moreover, the epithelial cells of the intestinal mucosa can further be strengthen by probiotic strains *L. Rhamnosus GG* and *C. Butyricum* as they seem to enhance epithelial cell proliferation rates and by antiapoptotic effects (65-66).

Probiotics capability to interact with the immune system has been studied extensively. The NF-κB signalling pathway plays a central role in the host’s immune response and is known to be influenced by host-microbe interactions. For instance, it is via this pathway *L. Rhamnosus GG* exert its effect by reducing TNF-α-induced IL-8 production in Caco-2 intestinal cells (67). In addition, some probiotics are thought to stimulate the production of secretory IgA (68). This immunoglobulin constitutes an important immunological defence barrier to intraluminal toxins and pathogens. Like the intestinal commensal flora, probiotics communication with the host via Toll-like Receptors (TLR) on the surface...
of the enterocytes. In an animal study, *E. coli* Nissle 1917 were able to ameliorate dextran sodium sulfate-induced colitis in mice via TLR-2 and TLR-4 dependent pathways (69). There is a growing bulk of evidence that probiotics affect the enteric nervous system. In IBS studies certain LAB strain has proved to reduce intestinal pain perception via cannabinoid and µ-opioid receptors (70). Moreover probiotics seem to influence gut motility by shorten the colonic transit time (71).

**Probiotic use in medical conditions**

Hence, probiotic compounds have been used extensively for both the prevention and treatment of various inflammatory and infectious intestinal disorders. For instance, probiotics have shown to be effective against infectious diarrhea in children (72-73). Furthermore probiotics have shown positive results in treatment of antibiotic associated diarrhea (74-75) and recurrent *Clostridium difficile* induced infections (76). There are also a number of studies showing that probiotics can prevent secondary infections in patients with surgical trauma or surgical infections (77-79). In clinical studies there is indirect evidence of probiotics counteracting the consequences of stress. In a study of IBS patients the probiotic strain *Bifidobacterium infantis* have been shown to significantly reduce abdominal pain as well as bloating and bowel dysfunction (80). In IBD probiotic strains have shown to be effective in inducing and maintaining remission of UC (24-25). In 2000 Gionchetti et al. showed that probiotics reduce the risk of recurrence of pouchitis after induction treatment with antibiotics (26) and that probiotics also work as a prophylactic treatment against pouchitis in patients with newly constructed reservoirs (27).

Based on a solid number of studies it is obvious that probiotics have potential positive effects on several medical conditions such as irritable bowel syndrome and inflammatory disease. Probiotics is now a recommended treatment in new guidelines from European Crohn’s and colitis organization. We are however far from being able to choose the precise combination of probiotic strains for each clinical setting. Therefore, more emphasis is needed on clinical probiotic studies.
Pouchitis

Since its introduction by Parks et al in 1978 (81) the ileal pouch anal anastomosis (IPAA) has become the most widely accepted procedure of choice for the surgical treatment of patients with ulcerative colitis or familial adenomatous polyposis (FAP). Patient satisfaction is good and IPAA provide a near-normal life. The most serious long-term complication is an inflammation of the ileo-anal pouch – pouchitis.

**Symptoms and epidemiology**

The main symptoms of pouchitis are; increase in stool frequency, rectal bleeding, urgency, abdominal cramping and fever. However, the definition of pouchitis, has been incoherent over time. Some believe that the clinically relevant definition of pouchitis is the patient’s perception of symptoms while others state that the definition should involve symptoms together with endoscopic and/or histological findings. Sandborn et al in 1994 introduced a pouchitis disease activity index (PDAI) which is based on the later three described parameters (82). This scoring system provides simple and objective criteria for pouchitis classification and is for now the most commonly used tool. The reported incidence of pouchitis varies considerably due to the definition of pouchitis but studies have shown that as many as 46% of patients with UC develop at least one episode of pouchitis within 5 years after surgery (83-84). Approximately 10-15% of patients with an ileoanal pouch develop a severe, chronic form of pouchitis that necessitates long and/or frequent use of antibiotics and in rare cases even pouch excision (85).

**Aetiology**

**Fecal stasis**

The etiology of pouchitis is still unknown. Pouchitis may result from several causes and different theories have been presented. In 1986 O’Connell et al believed that the inflammation was due to fecal stasis with bacterial overgrowth (86). The mechanisms remain unclear but recent animal studies have confirmed that fecal stasis of the pouch predispose to mucosal inflammation (87).

**Prepouch ileitis**

It has been implicated that pouchitis is caused by persistence of diseased ileal or rectal mucosa. Prepouch ileitis (PPI), is known to occur in some IPAA patients with UC (compared with backwash ileitis in UC) (88-90). The pathogenesis of PPI has not been studied further.

**Reduced metabolic substrates and bile acid toxicity**

After restorative proctocolectomy all IPAA patients will naturally experience an increase in stool frequency. It has been suggested that this increase will reduce metabolic substrate hence lead to pouchitis. It has been shown that stool concentrations of short chain fatty acids (SCFA) were significantly less in patients with pouchitis compared to controls and that resolution of pouchitis was associated with a significant increase in SCFA (91). Bile acid toxicity has been proposed as a cause of pouchitis but studies have shown variable results (92-93).

**Pouch ischemia**

It has been hypothesized that pouch ischemia might be a significant cofactor in the development of pouchitis. Transient mucosal ischemia may cause oxygen-derived free radical production by xanthine...
oxidase. A study using a xanthine oxidase inhibitor, allopurinol in the treatment of pouchitis has shown promising results (94-95). Simchuck et al 2000 could not verify that patients with diversion of one or more major mesenteric vessels during pouch construction had a significant higher incidence of pouchitis (96).

Defensins
Throughout the gut mucosa different antimicrobial peptides is secreted in response to stimulation of the innate immune system by pathogens. Defensins are small cysteine-rich cationic proteins that are effective against bacteria, fungi and many viruses. Defensins are regarded as natural antibiotics of the mucosa (97). Beside chemotactic properties, they exert their effect on pathogens mainly by forming pore-like membrane defects, allowing efflux of essential ions and nutrients (97). Paneth cells are the major source of antimicrobial peptides and in the small intestine where they exclusively secrete α-defensins HD5 and HD6. In a study published by Wehkamp et al. showed that patients with manifestations of CD restricted to the ileum had a specific decrease in these α-defensins compared to patients with Crohn’s colitis or UC. Wehkamp hypothesized that a deficiency in the innate immune defense, provided by defensins might allow bacteria to adhere to the CD mucosa and trigger an inflammatory response (98).

A decrease of α-defensins HD5 and HD6 have not been seen in patients with pouchitis (98) but transient low levels have been verified in patients with a newly constructed pouch. Some argue that this initial decrease in antimicrobial defense might initiate the development of pouchitis (99).

Sulphate Reducing bacteria (SRB)
Recently Coffey et al presented a hypothesis concerning the pathogenesis of pouchitis. The presence of feces in the ileal pouch results in a morphological transformation of the mucosa into a colonic metaplasia. This metaplasia is due to the presence of sulphomucin, synthesized by specialized goblet cells. The colonic metaplasia is by far more prominent in patients with UC than in FAP. Sulphomucin provides a metabolic substrate for Sulphate-reducing bacteria. This type of bacteria, hence the difference in sulphmucin, exclusively colonizes UC pouches. SRB produce H$_2$S. Hydrogen sulfide exposure may lead to colonocyte apoptosis, villous atrophy, mucosal attenuation and a reactionary crypt cell hyperplasia (100). Furthermore H$_2$S stimulate mucosal release of IL-8 (101). Disease severity in pouchitis closely correlates with fecal levels of hydrogen sulfide (102).

Antibiotic treatment in pouchitis is associated with a reduction in H$_2$S and normalization of the mucosal architecture (102-103). Futhermore, probiotic regimens reduce the rate of relapse correlated to pronounced reductions in IL-8 levels (101).

The above described unifying hypothesis concerning the pathogenesis of pouchitis may well explain two main issues of the condition. Familial adenomatous polyposis (FAP) is an inherited condition in which numerous polyps form mainly in the epithelium of the large intestine. These polyps will eventually transform into colon cancer if not treated. The surgical treatment off choice in these patients is protocolecotmy with formation of IPAA as in UC patients. It is well known that patients with FAP rarely develop inflammation in their reservoirs (104). Hence, pouchitis occurs almost exclusively in patients with UC. The reason for this may be that colonic metaplasia rarely occurs in FAP pouches which are the premise for colonization of SRB, production of H$_2$S that eventually lead to pouchitis. Why the colonic metaplasia occurs in UC pouches and not FAP need to be studied further. The answer may lie in that patients with FAP have a more rapid epithelial turnover rate, thus less
susceptible to the environmental effects of fecal exposure (100). Furthermore, Coffey et al present theories behind the rationale of treatment of pouchitis by antibiotics and probiotics.

**Treatment**

A lot of effort has been made to indentify different bacterial strains as cause of pouchitis without success. As described earlier SRB with production of H2S seem to be more prominent in UC pouches. In some studies the composition between aerobes and anaerobes has been shown to be altered in patients with pouchitis (105) Bacterial diversity appears to be important in maintaining normal gut homeostasis (106-107). This diversity is clearly reduced in patients with ileal pouches and even more so in patients with pouchitis (108).

The first-line treatment of pouchitis is ciprofloxacin and metronidazol for 14 days. If the patient does not respond or relapse shortly after treatment, it is recommended with prolonged treatment with ciprofloxacin and metronidazol for 4 weeks (109). Patients with chronic pouchitis who achieve remission following antibiotic therapy but relapse more than three times yearly should be put on maintenance therapy (110-111). Earlier, the alternative was to use ciprofloxacin continuously. In 2000 Gionchetti et al. showed that probiotics reduce the risk of recurrence of pouchitis after induction treatment with antibiotics. Later probiotics also proved to be effective as pouchitis prophylaxis in patients with newly constructed ileal pouches (26-27). In rare cases, maintenance therapy fails to control symptoms of pouchitis. In these patients definitive diversion with an ileostomy should be considered. Pouch excision rarely needs to be executed after diversion (108).
2. INTRODUCTION TO THE STUDY

Big efforts in research have been made to reveal the enigma of IBD, still the cause of ulcerative colitis is to a large extent unknown. However there is a presumed genetic component to susceptibility and altered intestinal barrier function has been implicated in the pathophysiology of ulcerative colitis in genetic, functional and epidemiological studies (1).

There are evidence that the well-established increased intestinal permeability in IBD patients is partly controlled by delicate intercellular circuits in the mucosa and submucosa of the intestine linked to the central nervous system. For example cholinergic pathways seem to mediate disturbances in epithelial water and salt absorption in the human jejunum in response to acute psychological stress (2). Though, little is known about how the human colonic barrier is regulated in detail. Therefore, further research is needed to understand mechanisms and find new therapeutic targets in ulcerative colitis.

The treatment of UC constitutes mainly of pharmacological regimens. However 15% of UC patients will experience at least one severe episode of relapse during the course of the disease. 30% of these patients will be forced to undergo acute surgical treatment with colectomy and creation of an ileostomy when the medical treatment fails to reverse the course of inflammation. The formation of a pouch by the terminal part of ileum which then is anastomosed to the anal canal, ileal pouch-anal anastomosis (IPAA) is a good surgical reconstructive option in colectomized UC patients. With bowel continuity IPAA offers a return to a near-normal life style with acceptable long-term morbidity. However 10-15% of patients with IPAA develop a severe form of inflammation in the constructed pouch. Pouchitis is characterized by increased stool frequency, bleeding, abdominal cramping and fever, resembling symptoms seen in active UC. The standard treatment for pouchitis is long and/or frequent use of antibiotics. Probiotics have been shown to reduce the risk of recurrence of pouchitis after induction treatment with antibiotics (3). However, little is known about the barrier function in patients with pouchitis and how probiotics in detail exert their effect.
3. AIMS OF THE THESIS

I. To characterize macromolecular permeability in non inflamed colon of UC and to elucidate the role of cholinergic signaling, mast cells and eosinophils in the regulation of the human colonic permeability.

II. To examine the mucosal barrier function in relation to pouchitis, before and after treatment with probiotics.
4. SUBJECTS AND METHODOLOGY

Subjects and Ethics:

UC patients (paper I)
Twenty-six UC patients (16M/10F, median age 47 years, range 19-78) who were scheduled for annual surveillance colonoscopy were enrolled in the study. Inclusion criteria were ulcerative colitis diagnosis based on reviewed PAD specimen and present patient data registration in the Swedish inflammatory bowel disease registry (SWIBREG). Only patients in remission of UC and a history of no further severe illness were included. Exclusion criteria were colitis of other origin than UC, ie. undetermined colitis or Crohn’s colitis (one patient was excluded due to histopathology). The research biopsies were taken during planned surveillance colonoscopy.

Healthy volunteers (paper I)
Fifty-three healthy volunteers were included as controls. Inclusion criteria were: no medication, no smoking, no history of IBS or other bowel symptoms and no family history of IBD, and normal clinical assessment of sigmoid colon biopsies. For direct comparison with the UC patients, colonic biopsies from 15 volunteers (7M/8F, median age 23 years, range 21-28) were subjected to flexible endoscopy. For the assessment of the mechanism of carbachol’s effect and immunofluorescence, biopsies from another 32 healthy volunteers (16 M/16F, median age 23 years, range 20-38) were taken. (In addition, in paper I, biopsies from 10 volunteers were used for FACS analysis and comparison with Crohn disease, which is not presented in this thesis.)

IPAA patients with pouchitis (paper II)
16 UC patients (9 men and 7 women, median age 48 years, range 32-71) with an ileal pouch anal anastomosis (IPAA), with a history of severe chronic pouchitis, defined as needing continuous antibiotic treatment or having had at least three relapses per year, were included in the study group. The study group subjects underwent endoscopy with biopsies of the pouch on three occasions: Once during active pouchitis, second after 4 weeks of treatment with antibiotics (ciprofloxacin 500mg x 2 + metronidazole 500mg x 3) until clinical remission and third after eight weeks of oral treatment of probiotica (Ecologic® 825, Winclove Bio Industries B.V., Amsterdam, The Netherlands) as described below. Two of the patients in the study group were on continuous treatment of antibiotics and entered the study first at the second endoscopic occasion.

IPAA controls (paper II)
13 patients (12 men and 1 woman, median age 50 years, range 35-63) having had an IPAA for at least two years with no clinical signs of pouchitis served as the control group. These patients underwent endoscopy with biopsies of the pouch on one occasion.
The study was approved by the regional ethics committee, Linköping and informed consent was obtained from all patients included in the study.

**Endoscopy**

**UC patients (paper I)** Bowel preparation was performed the day before endoscopy with the orally given macrogol laxative (Laxabon®, BioPhausia AB, Stockholm, Sweden). After laxative treatment only clear drinks were allowed until the examination. Colonoscopy was performed in an outpatient ward. Biopsies were taken during the first part of the exam according to established lab protocol at median level of 30 cm (range 20-50 cm) from anal verge.

**Healthy volunteers (paper I)** Preparation was carried out using the sodium-docusate-sorbitol laxative enema (Klyx®, Ferring, Copenhagen, Denmark) at 12 and 2 hours before exam or with Laxabon as above, with no differences in results between the groups (data not shown). Only clear drinks were allowed after the first enema until exam. Flexible sigmoidoscopy was performed and biopsies were taken at 20-30 cm level in the sigmoid colon.

**IPAA patients with pouchitis and IPAA controls (paper II)** Bowel preparation were conducted the day before with one liter of the orally given laxative Laxabon® (BioPhausia AB, Stockholm, Sweden). After laxative treatment only clear drinks were allowed until exam. Endoscopy of the pouch was conducted in an outpatient unit at the University Hospital, Linköping, Sweden. 8-10 biopsies for research purposes were taken at the level of the pouch corpus. One of the tissue samples were sent for routine clinical histological assessment and later used for histological scoring according to PDAI.

All biopsies were placed into 4°C modified Krebs-Ringer bicarbonate buffer (KRB) and transported to the laboratory within 20 minutes.
Pouchitis Disease Activity Index (PDAI)

First presented by Sandborn et al, Mayo Clinic, USA (1), PDAI is a 18-point diagnostic instrument consisting of three principal component scores: symptom, endoscopy, and histology. Patients with PDAI scores of seven or more were diagnosed as having pouchitis.

<table>
<thead>
<tr>
<th>The Pouchitis Disease Activity Index</th>
</tr>
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<tbody>
<tr>
<td></td>
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<tr>
<td>Criteria</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td><strong>Clinical</strong></td>
</tr>
<tr>
<td>Stool frequency</td>
</tr>
<tr>
<td>Usual postoperative stool frequency</td>
</tr>
<tr>
<td>0</td>
</tr>
<tr>
<td>1–2 stools/day, postoperative usual</td>
</tr>
<tr>
<td>3 or more stools/day, postoperative usual</td>
</tr>
<tr>
<td>Rectal bleeding</td>
</tr>
<tr>
<td>None or rare</td>
</tr>
<tr>
<td>Present daily</td>
</tr>
<tr>
<td>Fecal urgency or abdominal cramps</td>
</tr>
<tr>
<td>None</td>
</tr>
<tr>
<td>Occasional</td>
</tr>
<tr>
<td>Usual</td>
</tr>
<tr>
<td>Fever (temperature &gt; 37.8° C)</td>
</tr>
<tr>
<td>Absent</td>
</tr>
<tr>
<td>Present</td>
</tr>
<tr>
<td>Endoscopic inflammation</td>
</tr>
<tr>
<td>Edema</td>
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<tr>
<td>Granularity</td>
</tr>
<tr>
<td>Friability</td>
</tr>
<tr>
<td>Loss of vascular pattern</td>
</tr>
<tr>
<td>Mucous exudates</td>
</tr>
<tr>
<td>Ulceration</td>
</tr>
<tr>
<td>Acute histologic inflammation</td>
</tr>
<tr>
<td>Polymorphic nuclear leukocyte infiltration</td>
</tr>
<tr>
<td>Mild</td>
</tr>
<tr>
<td>Moderate, crypt abscess</td>
</tr>
<tr>
<td>Severe, crypt abscess</td>
</tr>
<tr>
<td>Ulceration per low-power field (mean)</td>
</tr>
<tr>
<td>&gt;25%</td>
</tr>
<tr>
<td>25–50%</td>
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<tr>
<td>&gt;50%</td>
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</tbody>
</table>

Ecologic 825

Consists of ten different viable, freeze-dried probiotic strains; *Lactobacillus casei, Lactobacillus paracasei, Lactobacillus plantarum, Lactobacillus salivarius, Lactococcus lactis, Bifidobacterium bifidum, Bifidobacterium infantis, Bifidobacterium lactis, Bifidobacterium longum, Lactobacillus acidophilus*. One table spoon of the probiotic compound was mixed in a glass of water and ingested twice daily.
Ussing chamber experiments:

Ussing chambers were invented by the Danish physiologists Ussing, H.H and Zerahn K. who showed that active transport of sodium was the source of electrical current in short-circuited, isolated frog skin (2). The Ussing chambers was further modified by Grass and Sweetana in 1988 (3) and consists of two half chambers with the tissue sample mounted as a semi-permeable membrane between the halves. Marker solutions and modulators can be applied on either side of the tissue sample and samples can be drawn on both sides in order to measure marker permeability (4).

Biopsies were mounted in these modified Ussing chambers (exposed tissue area 1.76 mm²; Harvard Apparatus, Holliston, MA, USA) as previously described and validated (4-5). Mucosal compartments were filled with 1.5 ml, 10 mM mannitol in KRB and the serosal compartment with 10 mM glucose in KRB (5). The KRB was pH adjusted to 7.4 at 37°C, continuously oxygenated with O2/CO2 (95/5%) and stirred by gas flow in the chambers.

After 40 minutes equilibration to achieve steady state conditions, biopsies with a transepithelial potential difference (PD) less negative than -0.5 mV were excluded due to poor viability or leakage. In the present study 22% of the biopsies were excluded due to malfunction, which is in line with our earlier findings (19).

Experiments were performed in open-circuit conditions with assessment of potential difference (PD), transmucosal electrical resistance (TER) and short circuit current (Isc) were noted at the start of the experiment and at 2 min intervals thereafter, using a four-electrode system: Ag/Ag-electrodes (Ref 201, Radiometer, Copenhagen, Denmark) with 3M NaCl/2% agar bridges for PD, and platinum electrodes for current as previously described (6). Samples of 0.3 ml from the serosal side were collected after 0, 30, 60, and 90 minutes (paper I) and 0, 10, 30, 60, 90 and 120 minutes (paper II).
Macromolecular and bacterial passage

Paracellular probes

$^{51}$Cr-EDTA (3.25 μM), (Perkin Elmer, Boston, MA, USA) was added on the mucosal side and permeation measured by appearance of radioactivity in the 0.3 ml serosal samples (counted for 10 min. in a 1282 CompuGamma reader (LKB, Bromma Sweden)).

Flourescein isothiocyanate-dextran-4000 (FD-4), (25 μM, Sigma Chemical Co. St. Louis, MO, USA) was added on the mucosal side and samples of 0.3 ml were then collected from serosal side for permeation measurements. Fluorescence was measured in a spectrofluorimeter with $\lambda_{ex}$/ $\lambda_{em}$ of 480/520 nm, respectively.

Transcellular probe

Horseradish Peroxidase type VI (HRP), (mw 45kDa, 10 μM, Sigma Chemical Co. St. Louis, MO, USA) was added on the mucosal side. Serosal samples were analyzed using the QuantaBlu® Fluorgenic Peroxidase Substrate Kit (Pierce, Rockford, USA) as previously described (7).

In addition we used HRP-specific antibodies (GenWay, San Diego, USA) to further refine our analysis. Dark microtitre plates (Fluoronunc MaxiSorp) were pre-coated with anti-HRP solution overnight. The plates were then washed with phosphate buffered saline (PBS)-Tween. Bovine serum albumin (BSA;200 μl of 5%) was then added as a blocking agent and incubated in a shaker at 300 rpm for 60 min, and then washed again with PBS-Tween. Samples collected from the serosal side of the Ussing chambers were diluted in Krebs buffer with 0.02% BSA. Fifty μl of sample solution was added to each well, incubated in a shaker at 300 rpm for 60 min and then washed as above. Krebs buffer and BSA (50 μl) were added to each well and analyzed with the QuantaBlu® Fluorgenic Peroxidase Substrate Kit. By combining both the techniques we ensure that we measure transmucosal uptake of HRP that is both enzymatically active and detectable as HRP antigen.

Bacterial probe

E. coli K-12, a chemically killed flourescein conjugated strain of Escherichia coli (molecular Probes, Leiden, The Netherlands) were added to the mucosal side of the Ussing reservoirs at a final concentration of 1 x 108 CFU/ml. At start, after 60 minutes and after 120 minutes, the entire volume of the serosal compartments were collected and analyzed at 488 nm in a flourimeter. (Cary Eclipse, Varian, Victoria, Australia)

Electrophysiology

Potential difference (PD): over a transporting polar epithelium is the reflection of all electrogenic pump activities in the epithelial membrane. These consists mainly of Na+/K+-ATP:ases and the passive permeation of ions through channels apically and baso-laterally and ion flux over the tight junctions (8).
Short circuit current (Isc): denotes a current where PD = 0 and reflects the sum of all ion pump activity.

Transepithelial resistance (TER): reflects the resistance against ions passing the epithelial and subepithelial routes. TER is built up by the apical and baso-lateral cell membrane, the resistance in tight junctions and the subepithelial resistance.

Modulators

Carbachol (Cch), \(10^{-7} \text{ M}\) Muscarinic agonist, added on the serosal side of the biopsy. Used concentration according to dose-response experiments as presented in paper I.

Atropine (Atr), \(10^{-6} \text{ M}\) Muscarinic receptor antagonist, added on the serosal side of the biopsy.

Hexamethonium (Hex), \(10^{-6} \text{ M}\) Nicotinic receptor antagonist, added on the serosal side of the biopsies.

Tetrodotoxin (TTX), \(10^{-6} \text{ M}\) Axonal Na+ channel antagonist, added on the serosal side of the biopsies.

4-diphenylacetoxy-N-methylpiperidine methiodide (4-DAMP), \(10^{-7} \text{ M}\) Muscarinic receptor 3 antagonist, added on the serosal side of the biopsies.

\(\alpha\)-helical Corticotrophin-releasing factor (CRF 9-41), \(10^{-5} \text{ M}\) CRF receptor antagonist, added on the serosal side of the biopsy.

Lodoxamide tromethamine (Lod), \(10^{-5} \text{ M}\) - Purchased from Alcon Laboratories R&D, Fort Worth, TX, USA. Mast cell stabilizer, added on the serosal side.

All modulators above were obtained from Sigma Chemical Co. St. Louis, MO, USA if not noted otherwise. The used concentrations of the reagents were based on previous studies at our laboratory and in the literature (12;20), except for Cch. All antagonist agents were added 20 minutes before agonists or vehicle.

Immunohistochemistry

In paper I immunohistochemistry was used to identify muscarinic receptors, mast cells, eosinophils and CRF. Control sections were obtained from consecutive sections present on the same slide as the samples, which ensured a negative control for background and unspecific binding. The control sections were exposed to secondary, but not primary antibodies. Biopsies were immediately immersed in 4% paraformaldehyde and in PBS for 2 hours and then cryoprotected overnight in 30% sucrose before sectioned at 4–10 \(\mu\)m in a cryostat. The sections were incubated with different primary antibody layers (for specification see below) and followed by incubation with secondary antisera conjugated to fluorescent markers. The immunohistochemical procedure was performed according to figure below.
Antibodies used in immunohistochemical analysis of colonic biopsies:

- Mouse anti human mast cell tryptase (Novocastra Laboratories Ltd, UK) detecting mast cells
- Mouse anti human eosinophil peroxidase (EPO) (Diagnostics Development, Sweden) detecting eosinophils
- Rabbit anti M1 (Acris Antibodies GmbH, Germany) detecting muscarinic receptor M1
- Rabbit anti M2 (Acris Antibodies GmbH, Germany) detecting muscarinic receptor M2
- Goat anti M3 (SantaCruz Biotechnology Inc. USA) detecting muscarinic receptor M3
- Goat anti CRF (SantaCruz Biotechnology Inc. USA) detecting CRF

Microscopy

**Routine pathology review**: Routine clinical histology assessments were conducted in all subjects. Briefly, biopsies were taken during endoscopy at the level of sigmoid colon (paper I) or in the pouch (paper II) and then fixed in paraformaldehyde. The biopsies were processed and reviewed according to local hospital routines. Colonic biopsies were normal in all healthy volunteers. For UC patients, see paper I and for IPAA patients and controls see paper II.

**Light microscopy in immunochemistry (paper I)**: Biopsies from healthy volunteers and from UC patients were collected at endoscopy. Samples were processed for visualization of immunostaining as described above. The light microscopic analysis was performed in a Nikon Eclipse E800 light microscope equipped with a VFM EPI-fluorescence attachment (Vector laboratories Inc. USA)
Statistics

**Paper I:** Data are presented as mean ± standard error of mean. Comparisons between treatment groups were done using analysis of variance with post-hoc tests and Student’s t test as applicable; comparisons of paired data were assessed with paired t test.

**Paper II:** Values are given as median (25-75th interquartile range) if not otherwise indicated. In Ussing chamber experiments, the n value represents the number of patients in study group and control group, with a mean value for each person calculated from 2-4 biopsies for each treatment. Comparisons between treatment groups were done using one-way ANOVA (Kruskal-Wallis) with Dunn’s Multiple Comparison post-hoc test.

P <0.05 was considered significant.
5. RESULTS

Paper I

In the first part of this study our aim was to evaluate if there was a difference in mucosal permeability in UC patients in remission compared to healthy controls. Endoscopic biopsies were mounted in Ussing chambers and barrier function was assessed with the transcellular permeability marker, HRP and the paracellular probes, $^{51}$CrEDTA and fluorescein isothiocyanate–dextran-4000. Permeability to HRP was increased in macroscopically noninflamed colon of UC patients compared to mucosa from controls. In contrast, there was no difference in $^{51}$CrEDTA or fluorescein isothiocyanate–dextran-4000 permeation between the groups. UC patients showed small but statistically significant increases in TER compared to controls (Figure 1D). The Isc at start was higher in UC patients (Figure 1E), suggesting a higher secretory activity of the mucosa consistent with previous studies (1).

Previous studies have indicated that cholinergic pathways mediate disturbances in the intestinal barrier function in humans and in animals (2-5). The aim of the second part of the study was to examine the role of cholinergic signaling and mast cells in the regulation of human colonic mucosal permeability and ion secretion. Biopsies from healthy human subjects were tested with the stable Ach analogue, carbachol (Cch). Cch increased both Isc and HRP transport in a dose-response manner, with significantly increased Isc and flux compared to controls at a Cch concentration of $10^{-5}$M. The Cch-induced permeation of HRP was abolished by muscarinic receptor antagonists, atropine and 4-diphenylacetoxy-N-methylpiperidine methiodide (4-DAMP), whereas the nicotinic receptor blocker hexamethonium had no effect. This suggested muscarinic receptor mediated effects of HRP uptake. Pre-treatment with mast cell stabilizer, lodoxamide showed an inhibition of HRP permeation, suggesting mast cell involvement in the transcellular transport of macromolecules. Pre-treatment with the axonal fast Na$^+$ channel blocker tetrodotoxin (TTX), which blocks neural transmission, did not alter the effect of Cch (Figure 2).
Based on present Cch data and previous findings on corticotropin-releasing factor (CRF) and mast cells (6), our further aim was to assess the role of these mediators in UC. The increased permeation of the macroscopically non-inflamed UC biopsies was normalized to control levels by pre-treatment with the muscarinic receptor antagonist atropine, the CRF antagonist α-helical CRF (9-41) and the mast cell stabilizer lodoxamide, respectively (Figure 3).

Figure 2. (A) Augmented permeation of HRP was abolished by muscarinic receptor antagonists atropine and 4-DAMP, but not by the nicotinic receptor antagonist hexamethonium. (B) HRP permeation was further inhibited by the mast cell stabilizer, lodoxamide but not with the axonal fast Na⁺ channel blocker TTX.

In previous studies of human colon specimens, CRF receptor 1 and 2 have been detected by immunohistochemistry and found to be expressed exclusively by mast cells of the lamina propria (6). To verify muscarinic involvement we aimed to elucidate the mucosal distribution of muscarinic receptors (M₁-M₃) by immunoflourscence. M₁ was expressed on a subset of the epithelial cells in the crypts (Figure 4A–B), whereas M₂ and M₃ receptor expression was restricted to subepithelial cells, which were identified as eosinophils by the coexpression of eosinophil peroxidase. Mast cells (defined by tryptase expression) did not express muscarinic receptors, but were often situated in close proximity to M₂ and M₃ receptor expressing eosinophils (Figure 4D–F). Importantly, the absolute number of eosinophils was increased in colonic biopsies from UC patients compared to healthy volunteers (Figure 5).
In the context of the presence of CRF receptors on submucosal mast cells our concluding aim was to examine the mucosal source of CRF, again using the technique of immunofluorescence. We showed that CRF immunoreactivity was expressed only in mucosal inflammatory cells and which were found almost exclusively to be eosinophils both in UC specimens (92% ± 8% of CRF-positive cells) and controls (93% ± 3%) (Fig. 6). Moreover, the absolute number of CRF-positive eosinophils was significantly increased in the colonic mucosa of inactive UC (27 ± 4 vs controls 11 ± 2 cells/0.1 mm$^2$, $P < .05$, Student’s t test).

In paper II our aim was to examine the mucosal barrier function in relation to pouchitis, before and after treatment with probiotics, mainly using the Ussing chamber technique. To elucidate the degree
of inflammation of the pouch, before and after treatment we used Pouchitis Disease Activity Index (PDAI). Treatment with antibiotics significantly reduced PDAI-scores, to control levels, and PDAI scores stayed at a low level during treatment with probiotics (Table 1).

### Table 1: PDAI score in various treatment groups:

<table>
<thead>
<tr>
<th>Group</th>
<th>PDAI score:</th>
<th>n:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre antibiotic treatment:</td>
<td>10.0 (7.3-11.0)</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre probiotic treatment:</td>
<td>3.0 (2.0-5.5)</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Post probiotic treatment:</td>
<td>2.0 (1.0-5.0)</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controls:</td>
<td>1.0 (0-2.0)</td>
<td>13</td>
</tr>
</tbody>
</table>

Values are presented as median (25-75th interquartile range) and numbers of patients in each group (n). Comparisons between groups were done with Kruskal Wallis test, significant differences as above.

To explore the effects of mucosal inflammation on permeability we used the transcellular probe, HRP and the paracellular probe, $^{51}$CrEDTA. For assessment of bacterial passage fluorescence-labelled chemically killed *E. coli* K12 were used. We showed that active pouchitis is associated with a significantly increased permeation of *E. coli* K12. Common treatment with antibiotics was not sufficient to normalize this barrier disturbance. However, a significant difference was seen after the patients had been treated with probiotics. HRP flux covaried with the permeation of *E. coli* K12 and the increased transcellular permeability was restored only after the use of probiotics (Figure 7). No difference in permeation of the paracellular probe $^{51}$CrEDTA, transepithelial resistance (TER) or short-circuit current (Isc) was observed.

![Figure 7](image)

**Figure 7.**

(A) Permeation of chemically killed *E. coli* K-12 during 120 min exposure in Ussing chambers. 1 unit = 1.5 x 10^3 CFU/ml. (B) HRP flux during 30-90 min time interval in Ussing chambers (pmol/cm^2/h). **Pre ab** = before treatment with antibiotics, **pre proB** = before treatment with probiotics and **Post proB** = After treatment with probiotics. Values are presented as median (25-75th interquartile range) and comparisons between groups were done with Kruskal Wallis test.
6. DISCUSSION

The hypothesis of the “leaky gut” asserts that an increased exposure to antigens due to intestinal barrier dysfunction initiates an immune mediated inflammatory response. In the first set of studies we present evidence of involvement of cholinergic mediators, eosinophils and mast cells in the regulation of macromolecular uptake in human colonic mucosa and hence give a novel explanation of increased permeability to antigen-sized molecules in patients with ulcerative colitis. In our second set of studies we show evidence of increased permeation of \textit{E. coli} and increased transcellular flux of the protein antigen HRP with intact paracellular permeability in IPAA patients with pouchitis. This mimics the same dysfunctional profile of the intestinal barrier as in UC in remission. Furthermore we show that, although antibiotic treatment restores the symptom load and the appearance of inflammation macroscopically, it is treatment with probiotics that holds the ability to restore the intestinal barrier of the pouch. Unveiling the mechanisms of how probiotics exert their effect may constitute the key to understanding not only the pathogenesis of pouchitis but also give us an insight in the mechanisms behind barrier dysfunction in IBD.

In paper I, we demonstrated that Cch increased the transcellular uptake of HRP in human colonic mucosa. The increased permeation was abolished by the muscarinic receptor antagonists, atropine and 4-DAMP, respectively, suggesting that increased uptake of protein antigens due to Cch challenge was mediated by muscarinic receptor stimulation. The present findings suggest that acetylcholine, in addition to controlling ion secretion in the normal human colon, is involved in regulating macromolecular permeability. The present results are supported by earlier studies in animals showing increased transcellular transport of large protein antigens by cholinergic challenge(1-2). Cameron et al. (1) proved the presence of cholinergic receptors on jejunal enterocytes in mice, and the muscarinic receptor M\textsubscript{3} was found to be the subtype that regulates the macromolecular uptake. These results are well in line with our present findings as the increased HRP permeation due to Cch challenge were abolished by pre-treatment with 4-DAMP, a specific M\textsubscript{3} receptor antagonist. However, the affinity of different muscarinic antagonists is rather nonspecific and the function of different receptor subtypes must be interpreted with caution (3).

In biopsies of patients with ulcerative colitis we found a more than doubled permeability to the protein antigen HRP compared to healthy controls. This finding confirms earlier reports of increased permeability to macromolecules in patients with UC in remission (4-5). Interestingly, the increased permeability to HRP in patients with UC was lowered to levels of healthy controls when the biopsies were pre-treated with the muscarinic antagonist, atropine, and the CRF antagonist, \(\alpha\) CRF(9-41), respectively.

Eosinophils have been reported to be localized close to cholinergic neurons and mediate nerve remodelling and induce release of acetylcholine from cholinergic nerve cells (6-7). We show for the first time that eosinophils in human colonic mucosa express cholinergic receptors (M\textsubscript{2} and M\textsubscript{3}). Moreover, we present evidence, using immunohistochemistry, that CRF is expressed almost exclusively by eosinophils in colonic mucosa. (Later confirmed with expression of mRNA of CRF in the human eosinophil cell line 15HL-60 and findings of CRF in supernatant of human blood eosinophils – See paper I.)
In UC biopsies there was a fivefold increase of eosinophils compared to healthy controls. The CRF producing subepithelial eosinophils were located in close proximity to nerves and mast cells. In several studies CRF induced responses that were shown to be mediated via mast cells (8-10). This suggests the possibility for CRF as a signalling compound between eosinophils and mast cells in the pathogenesis of UC. Recently, we have shown that colonic biopsies from healthy volunteers challenged with CRF increased HRP uptake due to mast cell activation via CRF receptors (8). Furthermore, mast cells mediate ion transport in the human gut which is altered in UC patients (11). Mast cells have been found in increased number (12-13) and degranulation state (14) in UC patients. In stress studies of rodents, mast cells have been shown to regulate mucin, PGE2 release (15) and intestinal permeability to macromolecules (16-17). In the present study, the mast cell stabilizer lodoxamide was used, and the increased HRP permeability in the UC patients group was normalized.

In summary, the signaling pathway, proposed by our first set of studies begins with a cholinergic signal to muscarinic (M2 and M3) receptors on CRF-expressing eosinophils. The next step includes eosinophil-induced activation of CRF receptors on mast cells which leads to granulation and release of mast cell factors that generate increased macromolecular uptake by the epithelium (8).

![Model of signaling events inducing barrier dysfunction in UC](image-url)

From the present and previous data the following model can be created: Some event (e.g. stress) activates cholinergic nerves (or possibly non-neuronal from lymphocytes) to release Ach. Ach can act directly on enterocytes and goblet cells to evoke secretion (left cell). More importantly, Ach may bind to M2/M3 receptors on CRF-producing eosinophils in the lamina propria (1). This results in activation of CRF-R1/R2 receptors on juxtaposed mast cells (2), which thereby modulates their behaviour to degranulation and release of pre-formed mediators like TNF or tryptase (3). This leads to increased epithelial macromolecular permeability and activation of a transcytosis route (4). This order of signaling would explain the phenotype with increased HRP flux that we found in patients with inactive UC.
In paper II, we studied IPAA patients with active pouchitis, and with a history of frequent recurrence. Gionchetti proved beneficial effects of probiotic treatment in this group of patients (18-19). However, the mechanisms by which probiotics exert their effect are highly complex, largely unknown and permeation studies of pouch mucosa are scarce. Through Ussing chamber experiments we have shown that active pouchitis is associated with an increased permeation of *E. coli* and increased transcellular flux of HRP, but unaltered permeability to the paracellular probe, ^51^CrEDTA. This is well in line with earlier studies (20). Interestingly similar barrier dysfunction is seen in inactive UC. It has been proposed that increased paracellular permeability seen in active UC is secondary to inflammation whereas the increased transcellular permeability is likely to be important in the early phases of recurrent inflammation (21).

The common use of different antibiotic regimens has occasionally proved to be insufficient in patients with chronic relapsing pouchitis. In paper II we showed that antibiotic treatment do significantly lower the PDAI score by reducing the symptom load and the endoscopic appearance of the inflammation but does not restore the transcellular leakage of *E. coli* and HRP. A normalization of the transcellular permeability is first seen after maintenance treatment of probiotics. Various mechanisms may explain the differences in effects on barrier function by antibiotics versus probiotics. Recently a study presented evidence that metronidazole, an antibiotic compound commonly used in the treatment of pouchitis may exert adverse effects in the mucosa of rodents. By alter goblet cell function, the mucus layer becomes thinner enhancing the attachment of pathogens to the intestinal epithelium (22).

There is evidence that the colon metaplasia seen in the ileal pouch is associated with increased production of sulphomucin (23). The presence of sulphomucin promotes the colonization of certain sulphate-reducing bacteria (SRB). SRB produce hydrogen sulphide that induces epithelial apoptosis, inflammation and barrier dysfunction. Antibiotic treatment decreases the levels of SRB, hence H$_2$S enough to let the mucosal inflammation subside. The probiotic treatment however prevents the colonization and dominance of SRB species and consequently disease relapse (24).

It has been proposed that some of the IPAA patients and especially those with UC have a “leaky ileum” even before surgery (25-26) that would predispose to future development of pouchitis. However Keita et al showed no increase in transcellular or paracellular permeability in human ileal samples from UC patients (27). This supports the notion that the increased permeability is acquired as a consequence of ileal pouch transformation. It has further been suggested that the pouch transformation could transiently alter defensin secretion which may lead to an increased adherence of bacteria to the mucosa, hence increased bacterial passage. Studies have indicated such decrease in patients with newly constructed ileal pouches compared to ileal samples from controls. This could contribute to the development of pouchitis (28-29).

The difference in the incidence of pouchitis might be found in the composition of the microbiota in the pouch in different individuals. Interestingly previous studies have shown a tenfold increased risk of developing pouchitis in patients with UC compared with FAP patients. In one study, Duffy et al showed that 80% of pouch samples from patients with UC contained Sulphate-Reducing Bacteria (SRB) compared to none of the FAP samples (23).
Probiotics is known to influence and strengthen the mucosal barrier in various ways. For instance, *L. acidophilus*, one of the component bacterial strains in Ecologic 825 is known to inhibit adhesion of bacterial pathogens to the mucosa (30-31) and to stimulate mucin production (32). Combinations of different strains seem more effective than single strain preparations (33-35), however, little is known about optimal mixture and in what phase of the condition the probiotic compounds should be used. Studies of probiotics used in acute inflammatory disorders in humans and animals have shown ambiguous results (36-37). In this study we used probiotics as prophylaxis and maintenance rather than as a tool to attain remission.

One can argue that treatment with antibiotics for a longer period of time than in this study might restore the barrier function just as successfully as probiotics. We have performed initial complementary studies with extended treatment of antibiotics instead of probiotics without being able to strengthen this argument (data not shown).

In our study, the use of the PDAI score proved to be a good instrument for quantifying symptoms in pouchitis. However we found no paired correlations between PDAI score, histological appearance and *E. coli* passage. It is thus, important to note that the PDAI is a fairly blunt instrument in identifying subtle histological, inflammatory changes.

Previously, we reported that human intestinal biopsies have good viability in Ussing chambers and are a validated technique to study transcellular uptake of protein antigens and paracellular permeability to marker molecules (8, 38). However, *in vitro* techniques, like Ussing chambers have limitations such as lack of circulation and nervous control. However it has become an essential way to study mechanisms involved in human intestinal function.

In paper I the UC patients were on average older (median 51 years than the healthy controls (median 23 years). However, there is no evidence for altered permeability in adults due to advancing chronological age (39). There was no difference seen in HRP flux correlated to the different medical treatments given to the UC patients (data not shown). In paper II, in our opinion, our study group reflects a common demographic distribution of patients with pouchitis. Unfortunately not the same is seen in the control group where male gender is significantly dominant. However there is nothing overt that would indicate a gender difference in barrier function in IPAA patients.

In conclusion, we demonstrate a chain of events whereby cholinergic signaling stimulates eosinophils via muscarinic receptors to release CRF, which by a paracrine manner activates mast cells to release factors that generate increased macromolecular uptake by the epithelium. This may explain, at least in part the increased transcellular permeability in patients with UC in remission. Furthermore, in UC patients with IPAA and pouchitis, showing similar dysfunctional barrier properties as UC in remission, treatment with probiotics after induction treatment with antibiotics restored the increased permeation to *E. coli* and protein antigen HRP. This could be an important factor behind the prevention of recurrence for this inflammatory condition.
7. CONCLUSIONS

- Colonic permeability to HRP is increased in UC colonic biopsies and is normalized by blocking of muscarinic receptors, CRF receptors and mast cells respectively.

- Muscarinic receptor staining in colonic mucosa reveals $M_2$ and $M_3$ positive eosinophils. Eosinophils are important mucosal sources of CRF. Mast cells are activated by CRF to induce barrier dysfunction.

- Increased numbers of colonic subepithelial eosinophils are seen in UC in remission compared to healthy controls.

- Pouchitis disease activity index (PDAI) is improved after treatment with antibiotics and probiotics.

- Active pouchitis is associated with an increased transcellular permeation of *E. coli* K12 and increased flux of HRP, which is normalized by probiotics.
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Tarmslemhinnan är en av kroppens viktigaste barriärer mot yttre miljöpåverkan. Tarmepitelet ska selektivt ta upp viktiga näringsämnen och samtidigt utesluta potentiellt farliga substanser och mikrober från upptag. För patienter med den inflammatoriska tarmsjukdomen ulcerös colit (UC) har man sett en ökad genomsläpplighet i tjocktarmsslemhinnan vid aktiv inflammation, men det är fortfarande oklart huruvida denna ökade genomsläpplighet i tarmbarriären föregår inflammation och sjukdom. tidigare studier har visat att tarmens barriärfunction regleras av lokala signalsystem som involverar nerver, hormoner och immunceller i tarmen. Vi har dock bristfällig kunskap om hur dessa signalsystem i detalj styrs.

10. REFERENCES


