Collagenous colitis

The influence of inflammation and bile acids on intestinal barrier function

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“Jedes Naturgesetz, das sich dem Beobachter offenbart, lässt auf ein höheres, noch unerkanntes schließen.“

Alexander von Humboldt (1769-1859)

Dedicated to my patients
ABSTRACT

Background and aims
Collagenous colitis (CC) is a diarrheal disorder with an incidence rate of 5-6/100000 inhabitants, affecting mainly middle-aged women. The diagnosis is made by histology of the colonic mucosa. Classical findings are a thickened subepithelial collagenous layer and chronic inflammation in the lamina propria. In inflammatory bowel disease (IBD) the mucosal barrier function is important in pathogenesis. The main purpose of the thesis was therefore to describe the barrier function in CC. The cause of CC is uncertain but the condition seems to be associated with bile acid malabsorption. Increased faecal bile acids are known to induce diarrhea. In functional studies the influence of bile acids on mucosal permeability in biopsies of healthy human individuals and in patients with CC was investigated.

Methods and patients
In the first paper a single patient with intractable CC was examined before surgery, with loop-ileostomy and after bowel reconstruction. For the other studies a total of 25 patients with CC were included (20 women, 5 men, mean age 66 years). There were three groups (14 patients in clinical remission without medical treatment, 11 with active disease, and 8 of these again after 6 weeks of budesonide treatment); 17 individuals with normal histology served as controls. Endoscopic biopsies from the sigmoid colon were mounted in modified Ussing chambers and assessed for short-circuit current (Isc), transepithelial resistance (TER), and transmucosal passage of chemically killed E. coli K12 after addition of chenodeoxycholic acid (CDCA) and deoxycholic acid (DCA). The biopsies were further investigated with confocal microscopy to assess bacterial transepithelial passage.

Results:
Para- and transcellular permeability was increased in active CC, but normalized with histological improvement due to faecal stream diversion. After bowel reconstruction, permeability to CrEDTA and HRP increased again.

In CC, bacterial uptake in colonic biopsies was significantly higher in all groups than in controls. Despite significant alleviation of symptoms, budesonide did not normalize the increased bacterial passage. Histology was unchanged after 6 weeks of budesonide treatment. DCA augmented mucosal permeability to CrEDTA in a dose-dependent manner and even such a low dose as 100 µmol/l DCA increased bacterial uptake significantly. The combination of bile acids and E.coli K12 had additive effects on TER.

100 µmol/l CDCA and DCA increased bacterial uptake in biopsies of CC patients in remission 4-fold, but had no additive effect on biopsies from patients with active disease. Furthermore, patients in clinical remission on budesonide treatment showed no bile acid-induced effects on E.coli K12 passage.

Conclusion:
Collagenous colitis presents with increased para/transcellular permeability and bacterial uptake, irrespective of disease activity or budesonide treatment, signifying an underlying mucosal barrier defect. Faecal stream diversion can normalize the barrier dysfunction, but budesonide does not, despite its beneficial clinical effects which alleviate diarrhea or bowel symptoms. Bile acids in physiological concentrations have the potential to augment bacterial uptake, especially in mucosa from CC patients in remission. Budesonide treatment appears to counteract the bile acid induced mucosal impairment. These detrimental effects of bile acids on mucosal barrier function might facilitate initiation and perpetuation of mucosal inflammation in CC.
LIST OF PAPERS

I. Dynamics of mucosal permeability and inflammation in collagenous colitis before, during and after loop-ileostomy.

II. Dihydroxy bile acids increase mucosal permeability and bacterial uptake in human colonic biopsies.

III. Increased transmucosal uptake of E. coli K12 in collagenous colitis persists after budesonide treatment.

IV. Physiological levels of bile acids increase bacterial uptake in colonic biopsies of collagenous colitis patients in remission.
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ABBREVIATIONS

ASBT  Apical sodium-dependent bile acid transporter
AZA  Azathioprine
BAM  Bile acid malabsorption
CC  Collagenous colitis
CDCA  Chenodeoxycholic acid
CEC  Colonic epithelial cells
CFU  Colonic forming unit
CLSM  Confocal laser scanning microscopy
$^{51}$CrEDTA  $^{51}$ Chromium-ethylene diamine tetra-acetic acid
DCA  Deoxycholic acid
E. coli  Escherichia coli
ECP  Eosinophil cationic protein
EGFR  Epithelial growth factor receptor
FACS  Fluorescence Activated Cell Sorting
FAE  Follicle-associated epithelium
HLA  Human leukocyte antigen
HRP  Horseradish peroxidase
IBD  Inflammatory bowel disease
IFN $\gamma$  Interferon gamma
iNOS  Inducible nitric oxide synthase
Isc  Short circuit current
JAM  Junctional adhesion molecules
LC  Lymphocytic colitis
LCA  Lithocholic acid
MC  Microscopic colitis
MHC  Major histocompatibility complex
MLC  Myosin regulatory light chain
MLCK  Myosin light chain kinase
MMP  Matrix metalloproteinase
6-MP  6-Mercaptopurine
NNT  Number needed to treat
NSAID  Non-steroidal anti-inflammatory drug
PD  Transepithelial potential difference
PEG  Polyethylene glycols
$^{75}$SeHCAT  $^{75}$ Selenium-labelled homocholic acid-taurine
SSRI  Selective serotonin reuptake inhibitors
TER  Transepithelial electrical resistance
TIMP  Tissue inhibitor of metalloproteinases
TJs  Tight junctions
TNF$\alpha$  Tumour necrosis factor alpha
UDCA  Ursodeoxycholic acid
VEGF  Vascular endothelial growth factor
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1. INTRODUCTION

Collagenous colitis (CC) belongs to the disease group called microscopic colitis. They are diarrheal disorders in which the diagnosis has to be established by histology. Although already described in 1976 by Lindström, collagenous colitis has attracted more scientific attention only in the last decade, when firm epidemiological data from Sweden and the USA have identified a rising incidence in the population, most certainly due to a greater awareness of physicians to take biopsies from patients with chronic diarrhea (Wickbom, 2009; Pardi, 2007). The growing numbers of epidemiological studies have shown that CC is a disease affecting mainly middle-aged women, although it can be seen in all ages and also in men. As these patients frequently have concomitant autoimmune diseases such as celiac disease, rheumatoid arthritis, diabetes mellitus or thyroid disorders, it becomes likely that a disturbed immunological response plays a pathogenic role (Nyhlin, 2008). Furthermore, studies showed that diverting luminal content via a loop-ileostomy can resolve intestinal inflammation. Restoration of bowel continuity reinstalls the classical histological signs of CC, making it evident that a luminal agent triggers the inflammatory process (Järnerot, 1995). As CC is associated with bile acid malabsorption (Ung, 2000) it has been hypothesized that an increased content of faecal bile acids might be of pathophysiological importance.

In classical inflammatory bowel disease (IBD) it is believed that an environmental factor triggers an uncontrolled immunological response in the intestine of individuals who are genetically predisposed. Hitherto no susceptibility gene has been detected in CC. In addition to genetic, immunological and environmental factors, recent research has been examining the role of disturbed mucosal barrier function as an important link in the pathogenesis of IBD (Xavier, 2007). The intestinal mucosa as the main interface between the outer and inner environment plays a crucial role in the defence from potentially harmful agents. The human gut content is complex, consisting of more than 400-500 different bacterial species. Animal models have shown that intestinal inflammation does not occur under sterile conditions and the beneficial use of antibiotics in IBD further emphasizes the role that intestinal bacteria might play in causing intestinal inflammation (Sartor, 2008). Especially newer data have described that commensals in our own gut flora seem to have the potential to adhere and invade the mucosa (Rhodes, 2007; Barnich, 2007).
A broader scientific interest in different aspects of CC has emerged, in particular double-blind, randomized trials have been conducted, looking at various medical treatment options. Budesonide has the best documented efficacy for inducing and maintaining remission in CC (Chande, 2009). On the other hand, cessation of budesonide treatment leads to clinical relapse within 3 months in most patients (Miehlke, 2005).

The objective of this thesis has been to describe the mucosal barrier function in CC in different phases of clinical activity and during budesonide therapy. Particular attention has been paid to the passage of \textit{E.coli} bacteria and the effects of bile acids on colonic mucosal functions in biopsies, in Ussing chamber experiments.

**BACKGROUND**

**Collagenous colitis**

**Historical remarks**

The term collagenous colitis (CC) was first introduced by the Swedish pathologist Lindström in 1976. He published the case report “Collagenous colitis with watery diarrhoea- a new entity?” describing a 48-year-old woman with chronic watery diarrhea who showed no abnormal macroscopic signs at rectoscopy, while histological examination of rectal biopsies revealed a remarkable thickened subepithelial collagenous deposition (Lindström, 1976). He furthermore described the clinical characteristics and showed that this condition lacks abnormal laboratory, microbiological and endoscopic findings. He speculated that the thickened collagenous layer formed a barrier to absorption of water and electrolytes, resulting in diarrhea and that this disease is a separate entity of immunological origin. In the same year Freeman et al. published a similar case describing the same condition (Freeman, 1976).

In 1980 the term “microscopic colitis” (MC) was introduced by Read et al. (Read, 1980) which, in 1989 was renamed to “lymphocytic colitis” (LC) when Lazenby et al. showed that the main feature of this diarrheal disease was an increase content of intra-epithelial lymphocytes (IEL) (Lazenby, 1989). As CC and LC share similar clinical findings and were
found to show no endoscopic abnormality, a French and American research team proclaimed in 1993 that these diseases should be combined under the common term “microscopic colitis” (Levison, 1993; Flejou, 1993).

Particularly in the last decade, there has been increasing scientific focus in this field and the number of original papers has increased exponentially reaching more than 900 publications in January 2010. Despite the increasing interest in this field, the cause of MC and the relationship between the two forms are still unknown.

**Epidemiology**
Epidemiological studies on CC have been conducted in different countries, but most of the work with the longest follow-up has been conducted in the USA and Sweden.
In the observation period between 1984 and 2008, epidemiological data have been collected in Olmsted County, USA and in Örebro, Sweden and both places have reported gradually increasing incidence rates. Today it is believed that the incidence rate lies at 5.8 per 100 000 inhabitants (Wickbom, 2009) and the prevalence was reported to be 39 per 100 000 inhabitants for CC in the year 2001 (Pardi, 2007). It is most likely that the rise in incidence is an effect of greater awareness of clinicians and pathologists when diagnosing this condition. In all epidemiological studies, CC seems to affect mainly middle-aged women, though the disease can occur in all ages, even in children (Benchimol, 2007). In the Örebro cohort the mean age at diagnosis was 65 (range 53-74) years and the female: male ratio was 7:1 (Tysk, 2008).

**Diagnosis: Clinical findings**
The predominant symptom of CC is chronic, non-bloody, watery diarrhea (Bohr, 1996). Abdominal pain is common, even during clinical remission (Nyhlin, 2008). Furthermore CC is often associated with weight loss, fatigue, nausea and urgency, leading to faecal incontinence which seriously impairs the health-related quality of life of these patients (Hjortswang, 2005; Madisch, 2005). In one Swedish study, patients with CC were asked to record their bowel movements in diaries and to fill out various health-related quality of life questionnaires, making it possible to define clinical criteria for disease activity. The patients witnessed a deterioration in their quality of life when a mean ≥ 3 stools/day or a mean ≥1
watery stool/day in a one week registration was noted as a sign of disease activity (relapse) (Hjortswang, 2009).

The onset of CC can be a sudden necessitating exclusion of an infectious cause, but in most cases symptoms evolve gradually (Bohr, 1996). The course is usually chronic, intermittent, but spontaneous remissions can occur. The risk of colorectal cancer is not increased (Chan, 1999).

CC patients often have a concomitant autoimmune co-morbidity, most commonly thyroid disorders, celiac disease, diabetes mellitus and rheumatoid arthritis (Bohr, 1996, Kao, 2009, Jobse, 2009). The association with other autoimmune diseases suggests an underlying autoimmune process in CC, but hitherto no specific autoantibody or marker has been detected. Routine blood samples are non-diagnostic and non-invasive screening for patients with CC is not yet possible.

**Endoscopic findings**

Initially, CC was defined as presenting with a normal endoscopic picture but recent reports describe abnormalities in the colon, e.g. mucosal tears as longitudinal lesions (Wickbom, 2006). Greater friability of the colon also seems to give a higher risk of post-endoscopic perforations (Bohr, 2005; Allende, 2008).

**Histology**

As the diagnosis of CC is based on typical histopathological findings, it is essential that patients with chronic diarrhea are assigned to a colonoscopy for biopsy taking. The diagnostic features of CC are a thickening of the subepithelial collagenous layer ≥10µm in well-orientated sections, in contrast to a normal basal membrane of <3 µm and furthermore a chronic mononuclear inflammation in the lamina propria, epithelial cell damage and occasionally an increased number of intra-epithelial lymphocytes, as presented in Fig.1. In uncertain cases the use of tenascin immunostaining has been recommended (Müller, 2001). The distribution of the typical histological findings in CC can be patchy in the colon and are most prominent in the ascending and transverse colon and can be absent in the sigmoid colon or rectum (Tanaka, 1992). Flexible sigmoidoscopy with multiple biopsy specimens from the left colon is not sufficient to exclude CC when based on the presence of a thickened
collagenous band alone. Yantiss et al. have proposed an optimal approach to obtain mucosal biopsies for assessment of IBD of the gastrointestinal tract. To detect CC they recommend taking two or more biopsies each from the right, transverse, descending and sigmoid colon and additional sampling of endoscopically visible abnormalities (Yantiss, 2009).

Figure 1: A: Human colonic biopsy showing normal histology.
B: Human colonic biopsy showing typical findings of collagenous colitis—increased subepithelial collagen layer, inflammation in lamina propria and epithelial cell damage with intra-epithelial lymphocytes. Staining with trichrom.
Source=http://www.flickr.com/photos/euthman/2800899442/

Aetiology

The cause of CC is not known but it is believed that a luminal agent triggers an uncontrolled intestinal inflammation in predisposed individuals. That a luminal agent is a precondition in the pathogenesis of CC is best demonstrated by diversion of intestinal content via a loop-ileostomy, leading to clinical and histopathological remission. After operative rearrangement of the intestinal continuity the clinical symptoms and the classical histological findings of CC resume (Järnerot, 1995). Current knowledge of CC pathogenesis is best divided into mucosal and luminal factors.
Mucosal factors

The mucosal inflammation in the epithelium is characterized by mainly CD8+ T lymphocytes that carry the α/β form of the T-cell receptor. In the lamina propria there are mainly CD 4+ T-lymphocytes (Mosnier, 1996). In a more recent study it could be demonstrated that the increased CD 4+ T and CD8+ T cell infiltration in colonic mucosa displayed a suppressed activation, but on the other hand the increased infiltration of eosinophils were functionally activated in active CC (Wagner, 2009).

The thickened subepithelial collagenous layer stains intensely for collagen types I, III, VI and particularly for tenascin. This histological finding is potentially reversible, but it is believed that the characteristic linear deposition of extracellular matrix relies on a restricted matrix metalloproteinase (MMP-1) RNA and increased tissue inhibitor of metalloproteinases (TIMP) expression, leading to an imbalance of fibrogenesis and fibrolysis in CC (Günther, 1999).

Furthermore it has been suggested that vascular endothelial growth factor (VEGF) might play a role in the accumulation of immature subepithelial matrix (Griga, 2004). By using a colonoscope-based, segmental perfusion technique it could be shown that VEGF was increased in the perfusate and was reduced by steroid therapy, giving rise to the hypothesis that VEGF could be involved in the inflammatory reaction and affect mucosal permeability (Taha, 2004).

CC demonstrates a Th1 mucosal cytokine profile with interferon gamma (IFN γ) as the predominantly upregulated cytokine. Mucosal mRNA levels of interleukin (IL) 15, tumour necrosis factor alpha (TNFα) and inducible nitric oxide synthase (iNOS) were also increased (Tagkalidis, 2007).

Cytokines are known to alter tight junction permeability, especially IFN γ (Sugi, 2001) and TNFα (Schmitz, 1999). Tagkalidis et al. found a reduction of E-cadherin and ZO-1 expression induced by IFNγ in CC as a sign of alteration in epithelial barrier function. That paracellular permeability is impaired in CC is corroborated by a study by Bürgel et al. showing diminished expression of occludin and claudin 4 which are important tight junction proteins and these findings correlated with reduced epithelial resistance, reflecting mucosal barrier dysfunction. Furthermore the same group used Ussing chamber technique to describe the diarrheal
mechanism in CC as being a reduced Na⁺ and Cl⁻ absorption accompanied by a secretory component of active chloride secretion (Bürgel, 2002).

Increased expression of iNOS correlated with luminal nitric oxide (NO) concentrations and clinical activity measured as frequency of daily bowel movements (Olesen, 2003). In colonic biopsies of CC patients, NFκB is activated and recruited to the iNOS promoter in vivo via an IKKβ mediated pathway (Andresen, 2005).

Faecal markers such as eosinophil protein X, myeloperoxidase and tryptase, can be increased in the stool of CC patients (Lettesjö, 2006). The findings on faecal calprotectin as a marker for intestinal inflammation are contradictory and can not be recommended as a diagnostic tool (Wildt, 2007).

Genetics
A familial occurrence of CC has been reported, but the role of genetic factors remains unclear (Abdo, 2001; Järnerot, 2001). Human leukocyte antigen (HLA) studies have demonstrated an association between CC and HLA-DQ2 or DQ1/3 and a higher frequency of HLA-DR3DQ2 haplotype and TNFα polymorphism in CC, compared with controls (Fine, 2000; Koskela, 2008). In contrast to Crohn’s disease, functional polymorphism in the NOD2/CARD15 gene has not been detected (Madisch, 2007) but on the other hand polymorphism of the matrix metalloproteinase-9 gene does appear to be associated with CC (Madisch, 2006).

Luminal factors
Drug-induced CC
Several drugs have been suspected of playing a causal role in inducing microscopic colitis and anecdotal clinical observations have been published since the early 1990s. In a systematic review of the literature, Beauserie and Pardi have listed 8 drugs (acarbose, aspirin, cyclo3Fort, lansoprazole, nonsteroidal anti-inflammatory drugs, ranitidine, sertraline and ticlopidine) as highly likely to cause LC or CC. The median interval between drug intake and
onset of diarrhea is around 4 days and after drug withdrawal diarrhea stopped after about 5 days (median) (Beaugerie, 2005).

In a case control study the risk associated with use of non-steroidal anti-inflammatory drugs (NSAIDs) in CC was confirmed; on the other hand selective serotonin reuptake inhibitors (SSRI) caused diarrhea but not necessarily MC (Fernandez-Banares, 2007). In all patients with chronic diarrhea a thorough drug history should be taken and cessation of suspected drugs should be tried.

**Infection**

As CC can present with a sudden onset an infectious cause has been suspected. The association of CC and *Clostridium difficile* infection has been discussed and presented in case reports. The symptoms persisted after treatment of *C. difficile* and it was suggested that *C. difficile* may be a noxious stimulant that could catalyse a chain of events resulting in CC (Erim, 2003). Furthermore antibodies to Yersinia were more common in CC patients than in controls, which led to the speculation that a previous Yersinia infection could have triggered CC (Bohr, 2002). In most CC cases, despite its sudden onset, stool cultures remain negative.

**Bile acids**

Bile acid malabsorption (BAM) can coexist with CC, leading to more frequent bowel movements and looser stool consistency. By using $^{75}$Selenium-labelled homocholic acid-taurine (SeHCAT), concurrent bile acid malabsorption (BAM) was found in up to 44% of patients with CC. Bile acid binding treatment has been shown to be effective in CC, especially when BAM is concomitant (Ung, 2000). The same research group studied the long term course (mean 4.2 years) in CC, BAM and bile acid sequestrants on histopathology and clinical features and found: 1. BAM seems to be a long-standing finding in a considerable number of patients with CC. 2. Patients on bile acids binders had no significant change in histopathology despite they have good effect on the symptoms. 3. Furthermore, in conclusion BAM and CC seem to be associated although presumably independent diseases.
Treatment

By taking a thorough history, the excessive use of dietary products or concomitant drugs which can lead to chronic diarrhea should be excluded. In patients with mild symptoms, a trial of loperamide or cholestyramine can be tested. In minor uncontrolled studies, bismuth subsalicylate (Fine, 1999), prednisolon (Munck, 2003) and mesalamine (Calabrese, 2007) demonstrated a favourable clinical response but sample sizes were too small to give a general recommendation. On the other hand treatment, with Boswellia serrata extract (Madisch, 2007) and probiotics (Wildt, 2006) failed to show efficacy.

Budesonide has the best documented efficacy in significantly alleviating symptoms and improving quality of life. In a Cochrane meta-analysis, budesonide was described as being effective and well tolerated for inducing and maintaining clinical and histological responses in patients with CC (Chande, 2009). A total of 94 patients were enrolled in three trial studies on budesonide (9 mg daily or in a tapering schedule for 6 to 8 weeks) (Miehlke, 2002; Baert, 2002; Bonderup, 2003). Clinical remission occurred in 81% of patients given budesonide, compared with 17% of patients who received placebo ($p<0.00001$). The pooled odds ratio for clinical remission to treatment with budesonide was $12.32$ (95% CI 5.53 to 27.46), with a “number needed to treat” (NNT) = 2. A statistically significant histological response followed treatment in all three trials studying budesonide therapy. Budesonide induction therapy has also been shown to improve quality of life (Madisch, 2005).

On the other hand, after withholding of short-term budesonide treatment, relapse rates lied around 61% in a follow-up period and the median time until recurrence of symptoms was 2 weeks (range 1-104 weeks) (Miehlke, 2005).

In treatment studies for maintaining remission, 80 patients who had responded to open-label budesonide were enrolled in two trials studying budesonide (6 mg daily for 6 months) (Bonderup, 2009; Miehlke, 2008). Clinical response was maintained in 83% of those given budesonide, compared with 28% of patients given placebo ($p=0.0002$). The pooled odds ratio for maintenance of clinical response to treatment with budesonide was $8.40$ (95% CI 2.73 to 25.81), with a “number needed to treat” (NNT) = 2. Histological response was maintained in
48% of patients given budesonide, compared with 15% given placebo ($p=0.002$) (Chande, 2009).

In severe cases of CC who are steroid dependent or refractory, immunomodulating therapy with azathioprine (AZA) or 6-mercaptopurine (6-MP) can be initiated. In a small group of patients (N=9) AZA or 6-MP gave a response rate of 89% and a steroid sparing effect (Pardi, 2001). In a retrospective study, beneficial effects of oral low-dose methotrexate was observed in CC patients (Riddell, 2007).

Medical therapy in CC has become so effective that surgical treatment is very seldom needed nowadays, but an ileostomy is still an option in patients with severe and therapy resistant illness.

**Intestinal barrier function**

**Structures**
The intestinal tract represents the body’s most important interface between internal and external environment. The intestinal epithelium is a single-cell layer serving as a highly selective barrier. Its role is dual, by permitting absorption of vital nutrients, electrolytes and water on the one hand while on the other, maintaining an effective defence against intraluminal toxins, antigens and enteric flora. The barrier is built up of a complex interaction between several components including the unstirred water layer, mucosal surface hydrophobicity, mucus layer containing immunoglobulins/defensins, the epithelium (cells held together by tight junctions) and immune cells in the lamina propria that all have different barrier-protecting properties (Fig.2).

The magnitude of transepithelial permeation of molecules gives information on mucosal barrier integrity in health and disease. Intestinal permeability is strictly regulated and several factors participate in this process. Not all aspects of barrier function can be discussed in this chapter but the main focus lies on the structural components of the intestinal barrier, especially those that are investigated with endoscopic biopsies in the modified Ussing
chamber. Furthermore, a basic understanding of intestinal barrier dysfunction in IBD will be highlighted.

**Figure 2:** A simplified view of the different structures comprising the intestinal barrier function. In the lumen gastric acid, pancreatic juices, and bile take part in barrier function by degradation of bacteria and antigens; pathogenic bacteria are kept under control by the normal gut flora. The mucus acts as a physical barrier and contains defensins and secreted immunoglobulins, primarily IgA. The epithelium constitutes the principal barrier to permeation, through which molecules can pass either transcellularly (transcytosis) or paracellularly via the junctional complexes, including tight junctions. The lamina propria contains various cells such as myofibroblasts or cells of the innate and acquired immunity which interact with enterocytes. Furthermore the enteric nervous system communicates with immune cells and neural impulses influence the mucosal barrier function. Through the endothelium of the capillaries the mucosa has contact with the circulation.
**Mucus layer**

Goblet cells in the gastrointestinal tract produce a mucous gel coat that serves as a lubricant and provides non-specific protection against chemical digestion and adhesion of bacteria. The hydrophobic character of gastrointestinal mucus relies on a layer of surface active phospholipids that line the top of the mucus covering the epithelium. The phospholipid layer protects against luminal acidity by repelling the diffusing hydrogen ions. Not only in the gastric mucosa is surface hydrophobicity high, but also in the colon. In a study where detergents were applied to remove the phospholipid layer in rat colon, an increased mucosal permeability to macromolecules and toxins was found (Lugea, 2000).

As already studied in the 1960s, mucus seems to envelop particles so that they do not come into contact with epithelial cells (Florey 1962). Furthermore it protects against pathogens by acting as a physical barrier, having binding sites for bacterial adhesins, maintaining high concentrations of secreted immunoglobulins, primarily IgA and defensins, and also acts as a free radical scavanger (Cross, 1984; Forstner, 1994).

Mucus is secreted continuously, nearly 10 litres daily, which is digested and mostly recycled. The rest is shed in faeces. The thickness of the mucus layer (approx. 110-160 µm) is determined by the balance between the rate of secretion and rate of degradation and shedding. In an animal model it was recently demonstrated that the colonic mucus consists of two layers, the inner layer being densely packed and devoid of bacteria. Proteomics revealed that the gel-forming mucin Muc2 was the major structural component (Johansson, 2008).

Toxic and irritating substances can greatly stimulate mucus secretion, increasing the thickness of the mucus layer while efficiently and rapidly moving the irritants away from the epithelium.

**The epithelial layer**

The gastrointestinal epithelium is a single-cell layer that acts as a selectively permeable barrier. It undergoes perpetual self-renewal originating from a limited pool of pluripotent stem cells situated at or near the base of intestinal crypts (Karam, 1999). The epithelium faces the complex task of permitting absorption of nutrients, electrolytes and water, while also protecting the internal environment from potentially toxic products.
There are two major routes for epithelial permeation: paracellular and transcellular (Spring, 1998). The paracellular transit is the key regulator of intestinal permeability and is formed by a complex protein-protein network, also called the junctional complex, that mechanically links adjacent cells and seals the intercellular space. The protein network connecting epithelial cells forms three adhesive complexes: desmosomes, adherens junction and tight junctions (TJs), the latter being most critical for paracellular permeability. Small hydrophilic compounds succeed in passing through the cell via passive diffusion or via aqueous pores, whereas larger molecules tend to pass via the paracellular route. The transcellular pathways are only briefly mentioned as they are not further discussed.

**Paracellular permeability/Tight junctions**

Tight junctions are the apically-most adhesive junctional complexes in mammalian epithelial cells. They form a continuous belt-like ring around epithelial cells at the border between the apical and lateral membrane regions (Farquhar, 1963). They act as a dynamic gateway, able to change in size under various conditions to facilitate or hinder passage of different products. TJs structures can be altered by osmotic load or hypertonic solution reflected by changes in transepithelial resistance and an increased paracellular uptake of macromolecules (Madara, 1983).

TJs are a multiprotein complex build-up of four unique families of transmembrane proteins: occludin, claudin, junctional adhesion molecules (JAM) and tricellulin. Occludin and at least 20 members of the claudin family have different barrier-sealing properties which are variable among cell types in terms of electrical resistance, solute and water flux, and charge selectivity (Mitic, 2000). In the tight junctions, permeation is also regulated by size and charge selectivity, whereby hydrophilic, positively charged molecules and ions pass more easily. Of the junctional adhesion molecule protein family, mainly JAM-1 seems to play a major role in intestinal homeostasis by regulating epithelial permeability, inflammation and proliferation (Laukoetter, 2007).

At points where three cells meet, tricellulin forms a central tube in a tricellular junction, allowing passage of solutes. Tricellulin is expressed in large amounts in epithelium-derived tissue and when tricellulin expression is suppressed, the epithelial barrier function will be compromised (Ikenouchi, 2005).
Furthermore, intracellular proteins such as zonula occludens (ZO) family members and cingulin link these molecules to the actin cytoskeleton, which provides the cell with structural integrity. The cytoskeleton includes three types of proteins filament: actin, microtubules, and intermediate filaments that extend throughout the cytosol and make contact with the cell to cell outer surface. Hence, the cytoskeleton is also essential for the paracellular pathway and a critical structure for maintaining intestinal barrier function (Fig. 3).

The intimate relationship between the tight junctions and the cytoskeleton is also demonstrated by the observation that phosphorylation of the myosin regulatory light chain (MLC) is involved in tight junction regulation. The myosin ATPase-mediated contraction of the perijunctional actomyosin ring subsequently leads to physical tension on the TJs (Turner, 1997). Furthermore it has been shown that proinflammatory cytokines, like interferon gamma and tumour necrosis factor alpha, influence paracellular permeability by either inducing endocytosis of epithelial TJ proteins (Utech, 2005) or by downregulating the expression of the tight junction strand protein occludin (Mankertz, 2000).

**Figure 3: Schematic representation of the basic structural transmembrane components of tight junctions.**

The main transmembrane proteins are occludin, claudin, junctional adhesion molecules (JAM) and tricellulin. ZO-1 or ZO-2 is important for clustering of claudins and occludin, resulting in the formation of tight junctional strands. The ZOs and cingulin can provide a direct link to the actin cytoskeleton.

**Transcellular permeability**

A controlled protein uptake via the transcytotic route is physiological and essential for antigen surveillance in the gastrointestinal tract (Ponda, 2005). The transcellular pathway allows many molecules to enter the cell from the luminal side and exit on the basolateral side and is also important for the regulation of intestinal permeability. There are active mediated uptake mechanisms for sugars, amino acids and vitamins, while larger peptides, proteins and particles are transported through the cell by endocytosis. Endocytosis in epithelial cells can occur along different routes, depending on the nature of the substance. There are highly specific receptor-bound processes via the clathrin-mediated endocytosis (Liu, 2001) or more unselected uptake of luminal antigens via phagocytosis or macropinocytosis (Conner, 2003). Most of what is internalized is recycled to the apical membrane but the remaining proteins are degraded by lysosomal enzymes. This process is believed to play a role in induction of oral tolerance (Zimmer, 2000).

In in-vitro studies, horseradish peroxidise (HRP) is used as a trancellular marker and is known to be taken up in endosomes in human colonocytes (Wallon, 2005). In one animal study it was seen that increased intestinal permeation of HRP was associated with increases in the number and size of the epithelial endosomes (Santos, 2001). Furthermore, epithelium under metabolic stress increases its endocytotic activity which can result in a microtubule-, microfilament-dependent internalization and transcytosis of bacteria (Nazli, 2006).

**Intestinal barrier dysfunction in IBD**

Mucosal barrier function has been extensively examined in ulcerative colitis and Crohn’s disease. These inflammatory bowel diseases (IBD) are of polygenetic origin characterized by an exaggerated inflammatory response to the microbial flora inhabiting the lumen of the gut. Microscopic colitis and IBD are clearly different entities but in rare cases, however, a double diagnosis was made or progression of CC to genuine ulcerative colitis was observed (Geboes, 2008).

Accumulating evidence underscores the important role that the epithelium plays in both pathogenesis and pathophysiology of IBD. Early studies suggested that functional modification in the barrier function (increased permeability) also described with the term
“leaky gut” could be found not only in patients with IBD but also in some first-degree relatives (Hollander, 1999; Söderholm, 1999). In in vivo and in vitro studies abnormal permeability refers to a measurable increase in flux of markers across the intestinal epithelium, whereby several mechanisms contribute to this defect.

The rate of movement is regulated primarily by the functional state of the tight junctions controlling paracellular passage. In IBD, altered tight junction structures have been shown to contribute to impaired epithelial barrier function (Schmitz, 1999). In Crohn’s disease an upregulation of pore-forming claudin 2 and downregulation of sealing claudins 5 and 8 were found (Zeissig, 2007). Furthermore, TJs are influenced by proinflammatory cytokines such as TNF-α and IFN-γ, which increase in the IBD mucosa (Niessner, 1995). Both TNF-α and IFN-γ have been shown to impair epithelial barrier function in cell line experiments and also modify mucosal morphology and TJ protein rearrangement (Amasheh, 2009).

In addition to TJ changes, other changes also play a role in IBD barrier dysfunction, such as increased transcytosis and induction of epithelial cell apoptosis and lesions. There is increasing evidence that antigens can be taken up to a significant extent via the transcellular route by endocytotic uptake and transcytosis. This could be identified by electron microscopy studies in Crohn’s disease, but the transport mechanisms are still not known (Schürmann, 1999; Söderholm, 2004).

In recent years the role of luminal bacteria in the pathogenesis of IBD has attracted increased attention as intestinal bacteria are essential for the development of mucosal inflammation as demonstrated in numerous animal models of IBD (Barnich, 2007). Patients with IBD have greater numbers of mucosa-associated bacteria than control patients (Swidsinski, 2002) and a high prevalence of adherent-invasive Escherichia coli was found in the ileal mucosa in Crohn’s disease (Darfeuille-Michaud, 2004). Crohn’s disease presents initially with small lesions at the specialized follicle-associated epithelium (FAE) that lines the Peyer’s patches in the terminal ileum. One study demonstrated that the barrier dysfunction was localized to the FAE of Crohn’s patients showing increased transcellular uptake of non-pathogenic bacteria (Keita, 2008).

Little is known about mucosal barrier function in CC. In a recent study it was demonstrated that active CC reduced E-cadherin and ZO-1 expression induced by IFNγ, signifying modification of epithelial barrier function (Tagkalidis, 2007). That mucosal barrier function is
impaired in CC was further corroborated in a study by Bürgel et al. showing diminished expression of occludin and claudin 4 which are important tight junction proteins. These findings correlated with decreased epithelial resistance reflecting increased paracellular permeability (Bürgel, 2002).

**Bile acids**

**Biochemistry**

The common bile acids are synthesized from cholesterol in the liver and contain a saturated ring system and a five-carbon side chain terminating in a carboxyl group. In all bile acids the ring system is the same, though, the number and position of hydroxyl groups and the presence or absence of conjugation to amino acids bring about important differences in the structure and consequent physical properties. Subtle changes, such as the addition of one hydroxyl group at position 3, 7 or 12 or the change from α- to β- configuration of the hydroxyl group may give very different crystalline packing, solubility and behaviour in the aqueous systems (Fig. 4 b). The α-hydroxyl groups all lie on one side of the ring and give the molecule amphipathic character with polar and a nonpolar face responsible for its solubilizing properties. The hydroxyl group’s location, orientation and hydrophilic properties are given in Table 1.

| Bile acid | pos. 3 | pos. 7 | pos.12 |  
|-----------|--------|--------|--------|---|
| CA        | α OH   | α OH   | α OH   | hydrophilic |
| UDCA      | α OH   | β OH   | H      |
| CDCA      | α OH   | α OH   | H      |
| DCA       | α OH   | H      | α OH   |
| LCA       | α OH   | H      | H      | lipophilic |

**Table 1:** Common bile acids showing their position of the hydroxy groups, α- to β- configuration and hydrophobicity.
The naturally occurring bile acids in humans are cholic acid (CA), chenodeoxycholic acid (CDCA), deoxycholic acid (DCA), and lithocholic acid (LCA) and in a minor proportion also ursodeoxycholic acid (UDCA) (Fig. 4 a). The primary bile acids CA and CDCA are formed in the liver and before excretion from the hepatocytes they are conjugated with an amino acid, taurine or glycine, by linkage to the carboxyl group of the side chain. Thus, conjugation makes it impermeable for membranes. The secondary bile acids DCA and LCA are formed by bacterial 7α-dehydroxylation from the primary bile acids in the intestine and, furthermore deconjugation takes place resulting in major changes in hydrophobicity (Cabral, 2001).

Figure 4: Biochemistry of the common bile acids. The primary bile acids cholic acid (CA), chenodeoxycholic acid (CDCA) are synthesized from cholesterol and conjugated with an amino acid, taurine or glycine, by linkage to the carboxyl group of the side chain. Furthermore hydroxyl groups are added to positions 3, 7 and 12 (b). In the intestine the secondary bile acids DCA and LCA are formed by bacterial 7α-dehydroxylation and deconjugation (a). Scheme of a micelle formed by phospholipids in aqueous solution (c).
Physiology
Bile acids are hydrophobic derivates of cholesterol that play an important role in the digestion and absorption of fats. They are synthesized in the liver, stored in the gallbladder, and secreted into the intestine as conjugated bile acids linked to glycine and taurine. Bile acids serve many important physiological functions, including cholesterol homeostasis, lipid and vitamin absorption and excretion of drugs (Vlahcevic, 1999). Typically, after secretion into the intestine, bile acids are efficiently reabsorbed via the apical sodium-dependent bile acid transporter (ASBT) in the terminal ileum forming the enterohepatic circulation, although a small percentage (~5%) is known to escape into the colon. In a steady state this faecal loss equates approximately to the daily synthesis. Our knowledge of faecal bile acids is based mainly on qualitative and quantitative analyses using gas-liquid chromatography-mass spectrometry (Setchell, 1988). Quantitative determination of faecal bile acid excretion provides important information about bile acid kinetics, whereas qualitative analysis gives us insight into intraluminal events involving bacteria and bile acid interaction.

In general, total faecal bile acid excretion in healthy adults has been quoted to an average range of 200-300 mg/day, mainly in unconjugated form owing to deconjugation during passage through the small intestine and colon. The inter- and intra-individual range can differ greatly from day to day, measurement mainly reflecting the influence that diet has on faecal bile acid excretion. Numerous analyses have revealed a tremendous complexity and composition of >40 different bile acids found in faeces. Lithocholic and deoxycholic acids are quantitatively the major bile acids, accounting for about 30-55% of all faecal bile acids excreted. The proportions of chenodeoxycholic and cholic acids are generally low in healthy humans. Bile acids are bound to dietary residue and intestinal microorganisms but, in the colon, passive absorption has been demonstrated, contributing significantly to the conservation of the bile acid pool in the healthy state. This is also demonstrated by the presence of numerous unconjugated and secondary bile acids in peripheral blood. Our knowledge of faecal bile acid composition in humans is based on faecal samples that have been excreted and have passed the whole colon. Recently, in an interesting study, Hamilton et al. looked at the concentrations and spectrum of bile acids in the human caecum. They found that 90% of bile acids were unconjugated and dehydroxylation of bile acids was nearly complete in the right colon. The total 3-hydroxyl bile acid concentration was 0.6±0.3mM, thereof deoxycholic 34±16%, lithocholic 26±6%, cholic 6±9% and chenodeoxycholic acid 7±8% (Hamilton, 2007).
Various factors can influence bile acid excretion, the most crucial in the conservation of the bile acid pool being the active transport of bile acids in the terminal ileum. Resection or dysfunction due to inflammation in this region will seriously compromise the integrity of the enterohepatic circulation. At normal intraluminal pH, conjugated bile acids will be present principally in ionized form with high water solubility by virtue of forming micelles. Ionized conjugated bile acids are favoured by active transport processes and a decrease in intraluminal pH can influence bile acid uptake. The intestinal microflora metabolizes bile acids by a number of reactions, mainly hydrolysis of the amide bond of the conjugates and 7α-dehydroxylation. Changes in the microflora of the gut can alter both the quantitative and qualitative patterns of faecal bile acids. Furthermore, conditions with decreased transit time or diarrhea can lead to an excessive spillage of primary bile acids into the colon (Setchell, 1988).

**The influence of bile acids on intestinal barrier function**

The role that bile acids might have in the carcinogenesis of colon cancer has been vigorously investigated but the focus of this chapter is to describe the toxicity of bile acids to the colonic mucosa and effects on barrier function.

In perfusion studies in animals and humans, bile acids induced marked morphological changes in the colonic mucosa, often associated with changes in fluid and electrolyte secretion (Mekhjian, 1971; Chadwick, 1979).

Animal studies showed that bile acids with two hydroxyl groups in the alpha configuration (CDCA and DCA) in concentrations between 1-8 mM gave a dose-related increase in paracellular mucosal permeability and damaged the mucosa (epitheliolysis) as demonstrated by light and electron microscopy (Camilleri, 1980; Goerg, 1982). The potency of several bile acids as inducers of these changes appears to be related to their surface properties as determined by critical micelle concentration and thereby loss of surface epithelium is directly related to their detergent activity (Gullikson, 1977).

In a more recent study, moderate concentrations of bile acids induced increased permeability in vivo in rat colon by mechanisms involving muscarinic and nicotinic receptors as a link between the central nervous system and colonic mucosal barrier function (Sun, 2004). Looking at more physiological concentrations of bile acids Mühlbauer et al. investigated the
molecular mechanism of bile acid-induced gene and cytokine expression in colonic epithelial cells (CECs). They demonstrated that DCA can induce IL-8 gene expression via the NF-κB signal transduction pathway in primary colonic epithelial cells, suggesting that bile acids can trigger a proinflammatory reaction (Mühlbauer, 2004). In a further study it was shown that physiological concentrations of bile acids inhibited recovery of ischaemic-injured porcine ileum, thereby implying that DCA was deleterious to mucosal barrier function due to increased paracellular permeability (Campbell, 2004). Investigations into the effects that more physiological concentrations of bile acids might have on barrier function, especially in human tissue, are lacking. As CC is associated with bile acid malabsorption, presents with diarrhea and is driven by an intestinal inflammation, the question arises to what extent bile acids influence the barrier function in this condition?
3. AIMS OF THE THESIS

As patients with inflammatory bowel disease are described as having a “leaky gut” the major aim of this thesis was to describe barrier function in colonic biopsy material from patients with collagenous colitis (CC), by using the Ussing chamber technique.

Furthermore, CC is associated with bile acid malabsorption, implying higher faecal bile acid concentrations in the colon. We speculated that bile acids might affect barrier function in CC.

The specific aims of the papers are as follows:

I. to describe mucosal permeability and histological features in a single patient with active CC, before and during faecal diversion via loop-ileostomy and after bowel reconstruction;

II. to elucidate the effects of µM concentrations of bile acids on mucosal barrier function in biopsies from healthy individuals with normal histology;

III. to analyse mucosal barrier function in patients with CC in clinical remission, with active disease and during budesonide treatment.

IV. to determinate whether physiological concentrations of bile acids further exacerbate the impaired barrier function in CC.
4. SUBJECTS AND METHODS

Patients
In the first paper we examined a single female patient (age 59 years) with intractable CC who had not responded to various medical treatment options. A loop-ileostomy was performed and she agreed to undergo repeated biopsy taking before, during faecal stream diversion and after bowel reconstruction for functional and histological examinations.

In the second study, patients planned to be examined with endoscopy at the University Hospital in Linköping, Sweden, and in whom we suspected a normal histology in the sigmoid colon agreed to provide us with biopsies for research purposes. Indications for colonoscopy were mainly screening for malignancy because of occult blood in faeces, constipation, or previously radiographically verified polyps outside the sigmoid colon. 17 patients were included: 12 women mean age 62 years (range 38-78) and 5 men mean age 60 years (range 44-73). The patients were divided into two subgroups: Group A: 9 patients for electrophysiological and permeability measurements, group B: 8 patients for analysis of bacterial uptake. A further criterion for inclusion was the absence of NSAID or steroid medication.

In the other two studies a total of 25 patients (20 women, 5 men, mean age 66 years) with CC were included from December 2005 to April 2008. There were three groups: 14 patients in clinical remission without medical treatment, 11 with active disease, and 8 of these were studied again after 6 weeks of budesonide treatment. The subjects of the second study with normal histology served as controls when comparing electrophysiological parameters and bacterial uptake. All patients were asked to register their bowel movements during one week on a diary chart, and thereafter undergo sigmoidoscopy where biopsies were taken from the mid-part of the sigmoid colon. Stools were collected during a 24-hour period prior to endoscopy, to measure stool weight and faecal calprotectin levels. Stool cultures were performed to rule out ongoing Campylobacter, Salmonella, Shigella, Yersinia and Clostridium infection. Routine blood samples (blood count, creatinine, CRP) were taken to detect other possible infectious conditions. From the diary chart the mean stool frequency and quantity of watery stools per day/week was calculated and served as reference to classify the patients into groups of remission or active disease (relapse), according to the score by Hjortswang et al.
Active disease (relapse) was defined as a mean of ≥ 3 stools/day or a mean of ≥ 1 watery stool/day over a one-week registration. The consistency of the stool was classified in arbitrary order (1 = watery, 2 = soft, 3 = normal). 8 patients with active disease were treated with budesonide (Entocort®) 9 mg o.d. for 4 weeks and further 6 mg o.d. for 2 weeks. After 6 weeks of treatment, all patients attained clinical remission, stool collection was repeated and the patients were re-examined with sigmoidoscopy and biopsies taken for histology and Ussing chamber analysis. None of the patients took NSAID or other immunomodulating agents.

All patients gave their informed consent and the studies was approved by the Ethics Committee, Faculty of Health Sciences, Linköping, Sweden.

Ussing chamber

The “Ussing Chamber” is named after its inventor, Hans Ussing, a Danish physiologist (Ussing, 1951). Designed initially to study vectorial ion transport through frog skin, it has emerged to become a widely used instrument within pharmaceutical research for studies of drug absorption (Hillgren, 1994). It has also been increasingly applied to the study of pathophysiological processes in the intestinal mucosa of animals and humans (Stack, 1995; Biljsma, 1995). The initial methodology was rather complicated and the technique has since been modified and simplified (Grass, 1988).

The modified Ussing chamber, which has been extensively used by our group and in these experiments, consists of two half chambers and the endoscopically taken biopsy is mounted between the halves, as shown in Fig. 5/6. The two compartments, one on either side of the tissue, are filled with buffer and continuously oxygenated (95% O₂, 5% CO₂). The gas flow keeps the buffer in motion, reducing the thickness of the unstirred water layer (Karlsson, 1992). A heat block keeps the solution at 37°C. The marker solutions are applied to the mucosal or serosal compartment and withdrawn from either side for analysis. The system is furthermore equipped with a pair of Ag/AgCl- electrodes with agar-salt bridges and a pair of current-giving platinum electrodes to enable monitoring of electrophysiological parameters.
Epithelium displays two features that distinguish it from other tissue: polarity and tightness. Polarity or the transepithelial potential difference (PD) is generated by the sum of ions and proteins that are asymmetrically distributed either to the apical or basolateral membrane. It reflects the electrogenic pump activity (mainly Na+/K+-ATPase) in the membrane but also passive ion flow through channels (Armstrong, 1987).

In order to measure short-circuit current (Isc) the epithelium is short circuited by injecting a current that is adjusted by a feedback amplifier to keep PD = 0 mV. The amount of current needed for this reflects the summation of all active ion pump activity.

Furthermore the integrity of the tissue is determined by the formation and permeability of the tight junction, an assembly of proteins responsible for the “tightness” between epithelial cells. Tightness can be measured electrically by the transepithelial resistance (TER) and represents the passive flow of ions via the paracellular pathway. Resistance is calculated by applying Ohm’s law: TER = PD/I.

**Figure 5: Schematic illustration of the modified Ussing chamber**

The biopsy, taken by endoscopy, is mounted between the two half-chambers and continuously oxygenated. One pair of Ag/AgCl-electrodes is used to measure the potential difference (Pd) and another pair of platinum electrodes supplies current to the system (I) which allows calculation of the transepithelial resistance (TER). Buffer solution is given into both compartments and different markers can be added.
Permeability markers
To study the mucosal barrier function, various markers such as C-mannitol, FITC-dextran and polyethylene glycols (PEG) of varying size have been tested in Ussing chamber experiments. We chose to use $^{51}$Cr-EDTA and the 45 kD protein antigen horseradish peroxidase (HRP) which are widely used for permeability studies.

As a paracellular marker we chose to apply the inert probe $^{51}$Cr-EDTA (MW 384D; Perkin Elmer, Boston, Mass., USA (3.25 µM)). EDTA binds strongly to the radioactive Cr, which ensures that the Cr passage is equal to the passage of EDTA, and no Ca$^{2+}$ can be bind to EDTA to give detergent effects.

As a transcellular marker we applied HRP (Typ VI, 10 µM), which is known to be taken up through the epithelial cell via macropinocytosis (Schürmann, 1999).

HRP and $^{51}$Cr-EDTA were added to the mucosal side and serosal samples were collected at 0, 30, 60, 90 and 120 min after start. An aliquot from each sample was saved for HRP analysis and the remainder was placed in a gamma-counter for $^{51}$Cr-EDTA measurements. For HRP analysis we used the QuantaBlu fluorogenic peroxidase substrate Kit (Pierce, Rockford; Ill.)
USA). Permeability was calculated during the 30-90 min period for both markers. $^{51}$Cr-EDTA permeability was expressed as $P_{\text{app}}$ (apparent permeability coefficient; cm/s x 10^{-6}), and HRP permeability presented as transmucosal flux (pmol/h/cm²).

**E.coli K-12**

As commensals are increasingly known to play a pathogenetic role in IBD we wanted to study the transmucosal passage of non-pathogenic bacteria. In papers II, III and IV all patients were investigated for uptake of chemically killed, fluorescein conjugated *E.coli* K-12 BioParticles (Molecular Probes, Leiden, The Netherlands). These bacteria are killed with paraformaldehyde, which stops their reproduction but retains antigenicity and has previously been used for phagocytosis studies (Wan, 1993). A concentration corresponding to $1.0 \times 10^8$ CFU/ml was added to the mucosal compartment as previously described (Keita, 2006). After 2 hours the whole content of the serosal compartment was analysed at 488 nm in a fluorimeter (Cary Eclipse, Varian) where 1 unit corresponds to $3 \times 10^3$ CFU/ml, assessed by FACS analysis.

**Bile acids**

We chose to apply CDCA and DCA in our experiments because they represent a primary and a secondary bile acid and have been used frequently in many previous studies. Furthermore they are known to be most abundant in the large intestine, mainly in a non-conjugated status. Sodium-chenodeoxycholate (3α, 7α- dihydroxyl-5β-cholan-24-oic acid, ≥97%, Sigma) and sodium-deoxycholic acid (3α, 12α- dihydroxyl-5β-cholan-24-oic acid, ≥99%, Sigma, St Louis, Mo, USA) were diluted with mannitol Krebs to obtain concentrations of 100, 500, 1000 µmol/l. After 40 min equilibration, CDCA and DCA in mannitol Krebs were added to the mucosal compartment.
Histology

All biopsies were examined by the same pathologist (Åke Öst). Two biopsies from the sigmoid colon were taken at each investigation and stained with haematoxylin-eosin (HE) and van Gieson. The degree of surface epithelial cell degeneration was assessed in arbitrary units (0=none, 1=mild, 2=moderate, 3=severe). The thickness of the collagenous band was measured in five different areas and the mean value was determined. Immunohistochemical staining for CD3 was also performed according to routine procedures. The number of intraepithelial lymphocytes (IEL/100 enterocytes; mean value of three counts) was assessed. The infiltration of mononuclear cells (lymphocytes and plasma cells) in the lamina propria was defined in arbitrary units (0=none, 1=mild, 2=moderate, 3=severe) (Geboes, 2000).

Confocal laser scanning microscopy

From 2 patients in the second and fourth paper, six extra biopsies were processed for confocal laser scanning microscopy to study the passage routes. *E.coli* K-12 and 100 or 1000 µmol/l of CDCA or DCA were added to the mucosal side and after 15 min the tissues were rinsed in phosphate-buffered saline (PBS) and then carefully removed to be mounted in OCT Compound (Miles Inc., Ind., USA). The biopsies were stored at –72°C. The tissue blocks were subsequently cryosectioned (6 μm thickness) onto glass slides using a Leica CM3050 microtome (Sollentuna, Sweden). The slides were air-dried overnight, fixed in ice-cold acetone for 30 min, and stored at 4°C until further use. The sections were then incubated for 10 min with Alexa Fluor 581 conjugated phalloidin (Molecular Probes, Leiden, The Netherlands). The slides were thoroughly washed with PBS (5 times). A drop of mounting medium (Dako Cytomation, CA, USA) was added. Prolong Gold with DAPI was used as mounting medium to achieve a parallel nuclear and chromosome stain. In experiments where rhodamine conjugated dextran (10,000 MW) (Invitrogen) was used it was added in the Ussing chambers at the same time as the bacteria. The slides were examined in a Nikon Eclipse E600W confocal laser-scanning microscope (Nikon, NY, USA) using Nikon EZ-C1 software, with a 60x oil-immersion objective. An ion laser permitted simultaneous excitation wavelengths of 488 nm for fluorescein-labelled *E. coli* and 594 nm for Alexa-labelled phalloidin.
Methodological considerations

The taking of human colonic biopsies cannot be standardized; the biopsies may vary in size and thickness. This leads to a scattering of results due to variability of the examined tissue. To reduce inter-individual differences in biopsy taking, this task was performed mainly by one doctor (Magnus Ström), as mounting the biopsies in the Ussing chamber was done by Andreas Münch. To avoid systematic repetition and unconscious mounting of the largest biopsies first, the order of placement of the Ussing chambers in the system was randomly changed. To further reduce biological variability, multiple biopsies were examined. In CC the typical histological findings are patchy throughout the colon but more present in the right side of the colon. In this region the concentration of bile acids is greater, declining on their way through the colon due to passive absorption. In the studies biopsies were taken from the sigmoid colon, due mainly to practical reasons and to reduce discomfort for the patients. To what extent results could differ between the right and sigmoid colon is not known but should be considered.

Furthermore, to extrapolate findings derived from in-vitro experiments into the in-vivo situation should be undertaken with caution. The complexity of the biological circumstance can not be reproduced by the Ussing chamber, which has obvious limitations such as the lack of circulation and nervous control, making viability crucial for the specimens. Nevertheless it was found that colonic biopsies had good viability and could be used to study transmucosal uptake of various molecules for 160 min with stable levels of ATP and lactate (Wallon, 2005). Biopsies that did not fulfil the viability criteria (PD > -0.5 mV) at the beginning of the experiment were excluded.

Statistics

In all papers the data were presented as mean/SEM, median and 25th-75th percentiles. As our results in humans are not normally distributed we used non-parametric methods for the permeability calculations. Comparisons between groups were initially done with Kruskal-Wallis test and further analysed with the Mann-Whitney test. For the comparison of patients before and after budesonide treatment, Wilcoxon’s-matched pairs signed rank test was applied. Spearman’s test was used for correlation between histological findings and bacterial uptake. The two-sided p-value <0.05 was considered significant.
5. RESULTS

Detailed descriptions of the results obtained are given in the respective papers. This section will only highlight the main findings.

Paper 1

In paper I we described a patient with intractable collagenous colitis who was treated with a temporary loop-ileostomy. She was followed clinically, histopathologically, and functionally by measuring mucosal permeability before, with ileostomy, and after bowel reconstruction. The changes in histological findings at different time-points are given in Table 2.

Table 2. Time schedule of sigmoid histology mean (range) of 3-5 counts.

<table>
<thead>
<tr>
<th></th>
<th>Before surgery</th>
<th>2 months with diverting stoma</th>
<th>4 months with diverting stoma</th>
<th>7 months after bowel reconstruction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epithelial cell degeneration (au 0-3)</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Collagenous band thickness (µm)</td>
<td>30 (22-38)</td>
<td>25 (2-50)</td>
<td>&lt;10</td>
<td>&lt;10</td>
</tr>
<tr>
<td>Intraepithelial lymphocytes (number per 100 enterocytes)</td>
<td>17 (13-23)</td>
<td>8 (5—11)</td>
<td>14 (11-17)</td>
<td>12 (9-16)</td>
</tr>
<tr>
<td>Density of inflammatory (mononuclear cells) in lamina propria (au 0-3)</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
</tbody>
</table>

• au = arbitrary units no=0, slight=1, moderate=2 and heavy=3

In the Ussing chamber experiments, Cr-EDTA and HRP permeability was substantially increased before surgery, when the patient had active colitis. Permeability decreased at 2 months and normalized after 4 months when compared with the control group. Seven months after bowel reconstruction, colonic mucosa permeability increased again to a level above the 95th percentile for the controls (Fig.7). Electrophysiological measurements (Pd) were stable,
indicating viability of all specimens. The results indicate that faecal stream diversion leads not only to histological remission but also restores barrier function.

**Figure 7.** Permeability of Cr-EDTA and HRP in the sigmoid colon of 19 healthy controls and at different stages of disease in the patient with CC (3-5 biopsies of the sigmoid colon studied at each time point). Permeability is expressed as the apparent permeability coefficient ($P_{app}$). Bars indicate mean values with SEM. The dashed line indicates 95th percentile of controls.

**Paper 2**

In this paper Ussing chamber experiments were performed in biopsies taken from patients with normal histology. Nine patients were included for electrophysiological measurement (Pd, Isc and TER) and analysis for Cr-EDTA and HRP permeability while adding increasing concentrations of bile acids. Bacterial uptake with addition of CDCA and DCA was investigated separately in 8 patients, in these patients an analysis of bacterial effects on TER was also carried out. A total of 204 biopsies were investigated and 14 were excluded because of Pd above -0.5 mV indicating loss of viability.

Both bile acids caused increased bacterial uptake. Significant differences were present between controls and 500 µmol/l ($p=0.01$) and 1000 µmol/l ($p=0.04$) CDCA. With DCA, bacterial uptake increased significantly already with 100 µmol/l ($p=0.03$) (Fig.8).
The results showed that *E. coli* K12 can per se decrease TER. When combining 100 µmol/l of CDCA or DCA with bacteria, we observed stronger effects on TER and especially CDCA seems to augment this effect, though not reaching significance compared with control biopsies (*p*=0.06) (Fig. 9).

**Figure 8:** Uptake of *E. coli* bacteria during 120 min exposure to 0/100/500/1000 µmol/l CDCA and DCA. Values are given as % passage of mucosal concentration. * = *p*<0.05 compared to controls.

**Figure 9:** Changes in transepithelial resistance (TER) during luminal exposure to *E. coli* (*bac*) and/or 100 µmol/l CDCA (*bile*).
When using confocal microscopy, fluorescent *E.coli* K12 bacteria were detectable in the lamina propria after 15 min when the biopsies had been exposed to 1000 µmol/l CDCA or DCA. *E.coli* bacteria adhered to the epithelium and were found to cross the cell layer mainly via the paracellular route (Figs 10/11).

**Figure 10:** Confocal microscopy of colonic biopsies after exposure to 1000 µmol/l CDCA for 15 min. Overview showing fluorescent *E.coli* bacteria in the epithelium (*fine arrow*) and translocation of bacteria into lamina propria (*thick arrow*).

**Figure 11:** Magnified view showing adhesion and initiated paracellular uptake of fluorescent *E.coli* in colonic epithelium after 15 min exposure to 1000 µmol/l CDCA.
Paper 3

The main finding of the third paper was the significantly increased uptake of *E. coli* bacteria in the Ussing chamber in all groups of CC patients, compared with controls. Active disease also showed significantly increased uptake compared with patients in remission (*p*=0.03). After 6 weeks of budesonide treatment the passage of *E. coli* K12 decreased numerically, though not significantly compared with active disease and the values did not normalize (Fig. 12).

![Figure 12: Uptake of *E. coli* bacteria during 120 min in Ussing chamber. Comparison in controls vs. patients with CC in remission or active disease, and after budesonide treatment. Values are given as units and IQR. One unit denotes to $3 \times 10^3$ CFU/ml.](image)

On commencing the experiments (time 0) TER in the active disease group was significantly increased compared with controls; 47 (38-53) Ωcm$^2$ versus 34 (27-37) Ωcm$^2$ (*p*=0.005). After mucosal exposure to *E. coli* K12, TER decreased significantly more in active disease compared with remission and controls. TER did not change after budesonide treatment (Fig.13).
Figure 13: Change in electrical resistance during 120 min exposure to E. coli K12 in Ussing chamber. Comparison between controls and patients with CC in remission or active disease, and after budesonide treatment.

After addition of E. coli K12 in the mucosal compartment, the change in short-circuit current was significantly altered in active disease, compared with controls. The change in Isc (Δ 0-120min) by E. coli stimulation normalized after budesonide treatment (Fig. 14).

Figure 14: Change in short-circuit current (Isc) during 120 min in Ussing chamber and after adding of E. coli.
Paper 4

In paper 4 the biopsies of CC patients in all groups (remission, active disease and after budesonide treatment) were stimulated with either 100 µmol/l CDCA or 100 µmol/l DCA. The most interesting result is the 4-fold increase in *E. coli* uptake in biopsies of patients in clinical remission, due to addition of bile acids. In patients with active disease, no further increase in bacterial passage was induced by bile acids and this was also the case in individuals undergoing budesonide treatment (Fig. 15).

**Figure 15:** Uptake of *E. coli* bacteria during 120 min in Ussing chamber with or without adding 100 µmol/l CDCA or DCA to the mucosal side of colonic biopsies. Comparison in controls and in groups of patients with CC in remission, with active disease and during budesonide treatment. In remission, addition of bile acids increased bacterial uptake significantly in colonic biopsies (♦ *p*<0.01). In healthy controls 100 µmol/L DCA increased bacterial passage (* p* =<0.05). Values are given as units and range on a logarithmic scale. One unit corresponds to 3x10³ CFU/ml.
6. DISCUSSION

During the last decade, numerous, mainly epidemiological studies have given us more insight into collagenous colitis. We have learned that this diarrheal disorder is not as uncommon as previously predicted and that the incidence rate lies between 5-6/100000 inhabitants making it nearly as common as Crohn’s disease (Wickbom, 2009; Pardi, 2007). Furthermore, it has been shown that patients with CC have poorer quality of life compared with a background population (Hjortswang, 2005). Luckily, good treatment is available and budesonide has the best documented efficacy for inducing and maintaining clinical and histological remission with a “number needed to treat” (NNT) of 2 as published in a Cochrane meta-analysis (Chande, 2009).

Despite the increasing interest in clinical studies on collagenous colitis, little experimental research has been undertaken into the pathogenesis of this disorder.

In classical inflammatory bowel disease the cause of intestinal inflammation is apparently multifactorial (Xavier, 2007; Baumgart, 2007). It is believed that an environmental factor triggers an uncontrolled intestinal inflammatory response in a genetically predisposed individual. Besides genes, disturbed innate immunity and environmental factors, the intestinal mucosa as a barrier between the inner and outer environment plays a crucial role in IBD. The “leaky gut” theory describes a dysfunctional barrier leading to increased mucosal permeability of potential noxious agents (Hollander, 1999; Clayburgh, 2004; Xavier, 2007). The tight junctions (TJs) constitute the rate-limiting components of the paracellular permeability pathway.) TJs structures and function can be modulated by pro-inflammatory cytokines such as IFN-γ and TNF-α, which increase in the mucosa of IBD patients (Bruewer, 2006).

In recent studies the normal gut flora or commensals, especially E.coli species with the ability to adhere and invade the mucosa, have been focused on as being possible initiators of intestinal inflammation (Rhodes, 2007; Barnich, 2007; Sartor, 2008).

Studies on intestinal permeability in humans have previously been carried out mainly with in vivo techniques (Bjarnason, 1995). The Ussing chamber technique for in vitro studies of epithelial function is well established for experiments on intestinal ion transport, drug absorption, and permeability to variously sized marker molecules and antigens, mainly in the gut mucosa of laboratory animals (Holtug, 1996; Albin; 2001). The modified Ussing chamber
which was used in our experiments allows investigation of endoscopic biopsies of humans (Wallon, 2005). The obvious advantages with this technique are the opportunity for detailed studies on epithelial function in samples from healthy individuals or patients before and after treatment of gut disorders.

By this approach we could for the first time describe that CC has an underlying mucosal barrier defect, irrespective of whether the patients were in clinical remission, had active disease or were undergoing budesonide therapy. All patient groups had significantly increased transmucosal passage of \textit{E. coli} K12 compared with controls, and confocal microscopy revealed that the bacteria apparently crossed the epithelium via the paracellular route. Alterations of paracellular permeability was furthermore demonstrated by a significant reduction of TER when adding \textit{E. coli} K12 in the Ussing chamber.

Information on the expression of tight junction proteins in CC is limited. Bürgel et al. found that the decreased epithelial resistance in CC was accompanied by diminished expression of occludin and claudin 4, while claudin -1, claudin-3, and claudin 5 remained unaltered. Despite that the exact physiological role of these integral membrane proteins are still unclear, the authors conclude that these changes might represent the structural correlate for the epithelial barrier defect (Bürgel, 2002).

CC is associated with a T helper cell type 1 mucosal cytokine profile with 60-100 times greater mRNA levels for interferon gamma (IFN $\gamma$) and tumor necrosis factor alpha (TNF$\alpha$) compared to controls (Tagkalidis, 2007). In the same study CC was associated with reduced staining of cell junction protein E-cadherin and even complete loss of ZO-1 staining in active disease. These T$_{H1}$ cytokines are known to have critical effects on barrier function and especially in Crohn’s disease it has been established that TNF$\alpha$ causes an increase in intestinal epithelial TJ permeability (Gibson, 2004; Ma, 2004).

In other experimental studies it has been speculated that eosinophil activation and vascular endothelial growth factor (VEGF), a potent enhancer of vascular permeability, could be mediators of mucosal permeability in CC. Taha et al. used a unique colonoscopy-based perfusion technique and found increased levels of eosinophil cationic protein (ECP) and VEGF in the perfusion fluid in distal segments of the colon. As ECP and VEGF levels correlated with increased concentrations of albumin, which can be interpreted as a sign of mucosal leakage, the authors concluded that activated eosinophils and VEGF could alter
mucosal permeability (Taha, 2001; Taha, 2004). Activation of eosinophils in CC was also confirmed in a recent study (Wagner, 2009). Furthermore, the epithelium of CC patients shows strong immunostaining of VEGF and it has been suggested that VEGF might have an important role in counteracting the local imbalance of fibrogenesis and fibrolysis, leading to an accumulation of immature subepithelial matrix in CC (Griga, 2003). The thickened subepithelial collagenous band has been described as a significant diffusion barrier, making up one third of the resistance of the epithelium, thus reducing absorptive efficiency of ions (Bürgel, 2002).

In our study histology had not changed at all after 6 weeks budesonide treatment whereas other studies have reported a reduction in thickness of the collagenous layer and decrease of inflammation grade (Baert, 2002; Bonderup, 2003; Miehlke, 2002). These differences could have been due to differing treatment duration (6 or 8 weeks) and dose regimes (without tapering). Another study of CC patients found that the degree of inflammation in lamina propria could be used to predict response to medical treatment; the more intense the inflammation, the more likely it was that the patient needed anti-inflammatory therapy (Abdo, 2002). On the other hand yet another prior study failed to show any correlation between the thickness of the collagenous layer and stool frequency (Wang, 1987). In our studies we found no correlation between grade of histological alterations and transmucosal bacterial uptake.

Inspired by the study by Järnerot et al., describing how faecal stream diversion leads to induction of clinical and histopathological remission in CC, we investigated histology and mucosal permeability in a single patient with CC before operation, with loop-ileostomy, and after bowel reconstruction. Our findings corroborated Järnerots findings and in the period with a diverting stoma, repeated biopsies of the sigmoid colon showed that the transcellular and paracellular permeability decreased at the same time as the intestinal inflammation abated and the thickened sub-epithelial collagenous layer and the epithelial degeneration disappeared. After bowel reconstruction, however intestinal permeability increased prior to the appearance of the thickened collagenous layer. These findings suggest that the disturbed mucosal barrier function is triggered by a hitherto unknown noxious luminal factor and the increased mucosal permeability precedes the histological changes in CC.
Studies by Ung et al. found an association between CC and bile acid malabsorption measured with SeHCAT (Ung, 2000). A pathological outcome in the SeHCAT implies greater losses of bile acids via the colon. Faecal concentrations of bile acids in CC patients have not been analysed, but effective treatment with bile acid resins such as cholestyramine suggests that faecal bile acids contribute to patient’s symptoms. Bile acids are known to induce diarrhea by increasing Cl\(^-\) secretion (Potter, 1998). In animal studies it could be shown that bile acids with two hydroxyl groups in the alpha configuration (CDCA and DCA) in concentrations between 1-8 mM gave a dose-related increase of paracellular mucosal permeability and damaged the mucosa as assessed by light and electron microscopy (Chadwick, 1979; Camilleri, 1980; Goerg, 1982). Little is known however, about the effect of \(\mu M\) concentrations of bile acids on mucosal permeability in the human colon. Therefore we first wanted to investigate the functional effect of bile acids on colonic biopsy material from healthy individuals in the Ussing chamber and speculated that they might impair barrier function. Hereby we found that concentrations of CDCA and DCA below 1mM increased paracellular mucosal permeability and enhanced bacterial uptake in normal human colon biopsies. However these results have to be interpreted with caution as the Ussing chamber experiment cannot simulate the complex \textit{in vivo} situation in all aspects. The luminal content with variable pH and calcium concentrations can influence the toxicity of bile acids (Rafter, 1991). Furthermore it is uncertain how mucus production, as a cell surface protection, is affected in biopsies that lack nervous stimulation and blood circulation. However, stable epithelial PD values during all our experiments contradicted cytotoxicity after addition of 100\(\mu\)mol/l CDCA or DCA.

Hamilton et al. (2007) were the first to demonstrate that the total caecal concentrations of 3-hydroxy bile acids in humans were 0.6mM \(\pm\) 0.3mM. As CDCA constitutes 7\(\pm\)8\% and DCA 34\(\pm\)16\% of the total bile acid composition, a concentration of 100 \(\mu\)mol/l CDCA and DCA lies within the physiological range. We therefore continued our Ussing chamber experiments with only 100 \(\mu\)mol/l CDCA and DCA, looking at the effects bile acids might have on barrier function in CC.

The major finding of paper IV is that physiological concentrations of bile acids increase \textit{E.coli} passage in biopsies of patients in clinical remission by 4-fold, which is highly significant. In experimental studies in Caco-2 monolayers it could be demonstrated that tight junction structures are modulated by \(\mu M\) concentrations of bile acids via epithelial growth factor receptor (EGFR) activation (Raimondi, 2008) or generation of reactive oxygen species (Araki, 2005). Furthermore it has been demonstrated that non-pathogenic \textit{E.coli} strains decrease TER
and alter localization of claudin-1 in T84 cells, signifying tight junction modulation (Zareie, 2005). The combination of bile acids and bacteria seems to have additional effects on paracellular permeability (TER), as shown in paper II, and the question arises if bile acids in concentrations found in the colon contribute to the uptake of commensal bacteria through the mucosa in vivo. Taken together, these findings give rise to the hypothesis that faecal bile acids in CC patients may be of pathogenic importance by affecting the mucosal barrier which could initiate intestinal inflammation and lead to a manifest clinical relapse. Bile acid binders are known to ameliorate diarrheal symptoms in CC, but it has not yet been investigated whether this medication can keep patients in remission, reduce histological signs of intestinal inflammation and improve mucosal barrier function in controlled trials. Furthermore, it could be of interest to study the potential effects of bile acids on the mucosal barrier function in classical IBD.

On the other hand, in active disease where bacterial uptake is already significantly increased, no further augmentation arises with bile acid stimulation. This could imply that structures of the mucosal barrier might already be rearranged in active disease in such degree that no further impairment is induced by bile acids. Moreover, patients in clinical remission during budesonide treatment had no increased passage after adding bile acids, suggesting that steroids have protective properties on bile acid induced mucosal impairment and improve barrier function.

Budesonide has the best documented efficacy for inducing and maintaining remission in CC and all studies have in common that budesonide reduces watery stools significantly and this effect can be seen quite rapidly, within days (Chande, 2009). However, the clinical course of CC is mainly chronic relapsing and the successful induction therapy with budesonide is compromised by a high relapse rate in 61% of patients after 2 weeks and in 88% after 3 months (Miehlke, 2005). Relapse risk remains high even after 6 months of maintenance treatment with budesonide (Bonderup, 2009). The mechanisms underlying frequent relapses in CC have not yet been clarified.

In general, diarrhea is driven by osmotic forces including malabsorption, active secretion and altered ion flux. Bürgel et al. showed that the diarrheal mechanism in CC relies predominantly on a reduced net $\text{Na}^+$ and $\text{Cl}^-$ absorption and is accompanied by a minor component of active chloride secretion (Bürgel, 2002). In our study the short-circuit current (Isc) was significantly
reduced in patients with active disease, possibly due to reduced Na\(^+\) absorption, thus corroborating Bürgels findings. Furthermore Sandle et al. previously found that inflamed but structurally intact human colonic tissue exhibits only a moderate degree of electrogenic sodium transport (Sandle, 1990). In inflamed tissue, T cell derived mucosal cytokines mediated inactivation of Na\(^+\)/K\(^+\) ATPase in mice (Musch, 2002) and in CC a T\(_h\)1 cytokine profile is predominant (Tagkalidis, 2007). Steroids are known to stimulate Na\(^+\) and fluid absorption in the colon (Sellin, 1985) and a more recent study showed that a possible mechanism for this could be a steroid-induced up-regulation of epithelial sodium channels (ENaC) (Zeissig, 2007). In our study, budesonide normalized the alterations in Isc, which were induced by *E.coli* K12 in biopsies from active CC patients. These data demonstrate that budesonide exerts a primarily direct and substantial anti-diarrheal effect, while its anti-inflammatory properties, reflected by improvement of the histological picture, appears more gradually.

Concerning effects of steroids on barrier function it has been demonstrated that the induction of remission in Crohn’s disease with corticosteroids is associated with an improvement in intestinal permeability as measured by the lactulose/mannitol ratio *in vivo* (Wild, 2003). Although it is well established that steroids have positive effects on intestinal permeability it is still unclear whether this is a result of their anti-inflammatory properties, including the ability to inhibit the expression of proinflammatory cytokines such as TNF\(\alpha\) (Barnes, 1998) or due to a direct TJ barrier “tightening” effect of glucocorticoids. To address this question, Boivin et al. looked at mechanism of glucocorticoid regulation of the intestinal tight junction barrier and found that steroids inhibit the TNF\(\alpha\) –induced increase in myosin light chain kinase (MLCK) protein expression, a key process mediating tight junction permeability (Boivin, 2007).

Despite favourable results of short-term budesonide treatment our findings suggest that increased bacterial uptake may be a genuine phenomenon in patients with CC that does not normalize after effective clinical treatment with budesonide and one may speculate that this ongoing barrier dysfunction has pathogenetic significance in explaining the development and recurrence of CC.
7. CONCLUSIONS

- Collagenous colitis is associated with increased paracellular and transcellular permeability, which normalizes following faecal stream diversion. Mucosal permeability was altered prior to reappearance of the thickened collagenous layer after restoration of bowel continuity.

- Collagenous colitis is associated with increased mucosal bacterial passage irrespective of disease activity.

- Active CC is associated with altered tight junction reactivity.

- Dihydroxy bile acids alter gut barrier function, causing increased antigen and bacterial uptake in normal human colonic biopsies.

- Physiological concentrations of bile acids exacerbate barrier dysfunction in colonic biopsies from CC patients in clinical remission.

- Budesonide gives short-term clinical relief but does not restore the gut barrier function.

- Budesonide appears to counteract the bile acid-induced mucosal impairment.

- It can be speculated that faecal bile might exacerbate and perpetuate mucosal inflammation in CC.
8. SVENSK SAMMANFATTNING


**Metod och patienter:** I det första arbetet undersöktes en kvinnlig patient med uttalad KK innan operation, efter loop-ileostomi and vid tarm rekonstruktion. I de andra studierna inkluderades sammanlagd 25 patienter med KK (20 kvinnor, 5 män, genomsnittlig ålder 66 år). De delades in i tre grupper: 14 patienter i klinik remission, 11 med aktiv sjukdom (8 av dessa igen med budesonid behandling) och 17 friska kontrollpersoner med normal histologi. Endoskopiska vävnadsprover från sigmoideum monterades i en modifierad Ussing kammare och undersöks med elektrofysiologiska parametrar (kortslutningsström Isc, transepitelial resistans TER) och transmukosal passage av avdödade *E.coli* K12 bakterier efter tillägg av chenodeoxychol syra (CDCA) och deoxychol syra (DCA) i olika koncentrationer. Vävnadsproverna undersöks också med konfokal mikroskopi för att studera passagväg genom slemhinnan.

**Resultat:** I fallbeskrivningen visar aktiv sjukdomen tecken på ökad paracellular och transcellular permeabilitet men detta normalisera vid urkoppling av tarmmen och via stomi. Efter återkoppling återupptas den mucosala permeabiliteten för makromolekyler igen. Vid KK syns ett signifikant ökat upptag av bakterier både i remission och aktivitet samt under budesonid behandling jämfört med kontroller. Trots signifikant förbättrade symtom under budesonid behandling normalisera inte den ökade passagen av bakterier. Histologin är oförändrad efter 6 veckors behandling med budesonid. DCA påverkar den mucosala permeabiliteten dosberoende och redan 100µmol/l DCA ökar det bakteriella upptaget signifikant i biopsierna från friska försökspersoner. Kombinationen av gallsalter och *E.coli* K12 verkar ha additiva effekter på TER. 100µmol/l CDCA och DCA leder till en 4 faldig ökning av bakteriepassagen hos patienter i klinisk remission men har ingen effekt på bakterier från patienter med aktiv sjukdom. Yetterligare visar det sig att gallsaltern inte har någon inverkan på bakteriers upptag i biopsier från patienter som står på budesonid behandling.

**Slutsats:** KK har en ökad permeabilitet för makromolekyler och bakterier oberoende av sjukdomsaktivitet eller budesonid behandling som ett tecken på en underliggande barriärsstörning i slemhinnan. Att koppla ur tarmen via en stomi resulterar i normalisering av barriärfunktionen men så är inte fallet vid budesonid behandling trots dess goda effekt att minska symtomen. Gallsalter i fysiologiska koncentrationer har potentialen att öka bakteriernas upptag framförallt i biopsier från patienter i klinisk remission. Budesonid behandling tycks motverka gallsalternas inverkan på slemhinnan. Dessa negativa effekter av gallsalter och inverkan på den mucosala barriär funktionen kan möjligtvis initiera och upprätthålla den intestinala inflammationen vid KK.
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