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Low Levels of Bile Acids Increase Bacterial Uptake in Colonic biopsies from Collagenous Colitis Patients in Remission

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Running title: Bile acids in collagenous colitis

Keywords: microscopic colitis, permeability, intestinal mucosa, diffusion chamber, bile acids

Abbreviations: CC = collagenous colitis, IBD = inflammatory bowel disease; Isc = short-circuit-current; PD = transepithelial potential difference; TER = transepithelial resistance; CDCA = chenodeoxycholic acid, DCA = deoxycholic acid

Abstract

Background: Patients with collagenous colitis (CC) have an impaired mucosal barrier.

Moreover, CC is associated with bile acid malabsorption. Bile acids can increase bacterial mucosal uptake in humans. Mucosal barrier function was investigated by exposing colonic biopsies to chenodeoxycholic acid (CDCA) or deoxycholic acid (DCA) in Ussing chamber experiments.

Methods: The study comprised 33 individuals; 25 with CC (14 in clinical remission without treatment, 11 with active disease, and 10 examined in clinical remission resulting from treatment with 6 mg budesonide); 8 healthy individuals undergoing screening colonoscopy served as controls. Endoscopic biopsies from the sigmoid colon were mounted in modified Ussing chambers and assessed for short circuit current (I_{sc}), potential difference (PD), transepithelial resistance (TER), and transmucosal passage of *E. coli K12* after adding 100 $\mu\text{mol/l}$ CDCA or DCA.

Results: When adding 100 $\mu\text{mol/l}$ CDCA or DCA, bacterial uptake increased 4-fold in biopsies of patients in remission; CDCA 6.5 units [2.5-9.8] and DCA 6.2 units [2.1-22] (median [IQR]), compared with uptake in biopsies without added bile acids 1.6 units [1.1-3]; ($p=0.004$ and $p=0.01$, respectively). In active disease and in patients in remission due to budesonide treatment, bile acids did not affect bacterial uptake. Confocal microscopy revealed transepithelial passage of *E.coli K12* within 30 min.

Conclusions: Low concentrations of dihydroxy-bile acids exacerbate mucosal barrier dysfunction in colonic biopsies of patients with CC in remission. This allows a substantially increased bacterial uptake, which may contribute to recurrence of inflammation.

Introduction

Collagenous colitis (CC) is an inflammatory bowel disease of unknown origin having an incidence rate of 5-6/100000 inhabitants (1-3). It affects mainly elderly women (mean age 65) but also men and can occur at any age (1). CC patients present clinically with frequent, non-bloody diarrhea, abdominal pain and weight loss. The diagnosis can be established by histology only as colonic biopsies must show a thickened subepithelial collagenous layer and signs of inflammation in lamina propria. Many pathophysiological mechanisms have been suggested, such as autoimmunity^(4,5), infection⁽⁶⁾, drugs⁽⁷⁾ and bile acid malabsorption (8). Faecal stream diversion by surgery leads to histological remission in CC patients. When bowel continuity is restored, recurrence of mucosal inflammation often ensues, making it likely that an unknown luminal agent has triggered the inflammatory process (9).

Inflammatory bowel diseases, e.g. Crohn's disease and ulcerative colitis, are believed to have a leaky epithelial barrier, leading to increased uptake of proteins and antigens which induce an immunological response in the mucosa (10). In a previous study we showed that biopsies from patients with CC had a disturbed colonic barrier function even when they were in clinical remission (11). Bile acids can stimulate secretion in the colon and in many animal studies chenodeoxycholic acid (CDCA) and deoxycholic acid (DCA) in concentrations between 1-8 mmol/L have produced a dose-related increase in paracellular mucosal permeability and also damaged the mucosa (epitheliolysis), as verified by light- and electron-microscopy (12). Recently we have also found that dihydroxy-bile acids in concentrations as low as 100 $\mu\text{mol/l}$ of can increase mucosal bacterial uptake in human colonic biopsy tissue (13).

In view of these findings we speculate that even low concentrations of bile acids can further increase *E.coli* uptake in colonic biopsies from CC patients, thus testing the hypothesis that

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3 bile acids may affect mucosal barrier function in CC. As budesonide has the best documented
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5 efficacy for treating CC, we also investigated the effect of bile acids on mucosal bacterial
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7 uptake in patients undergoing budesonide treatment.
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16 From December 2005 to February 2009, patients with known collagenous colitis were asked
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18 to register their bowel movements over one week in a diary before undergoing
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20 sigmoidoscopy with biopsy sampling from the mid-part of the sigmoid colon. Mean stool
21
22 frequency and the number of watery stools per day/week were calculated in order to
23
24 categorize the patients as: in remission or with active disease (relapse), as scored *ad modam*
25
26 Hjortswang et al. (14). Active disease (relapse) was defined as producing a mean of ≥ 3
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28 stools/day, of which a mean of at least one watery stool/day over a one-week registration.
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35 The results are based on 25 cases of CC (21 women and 4 men, mean age 66 years), 14 in
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37 clinical remission without treatment, 11 with active disease, and 10 undergoing budesonide
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39 treatment. Of the 11 patients with active disease, 10 were treated with budesonide
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41 (Entocort®) for at least 6 weeks. At re-examination (sigmoidoscopy) all patients were in
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43 clinical remission, taking 6 mg budesonide daily. In all patients drug-induced CC was ruled
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45 out and in patients with active disease stool, cultures were negative. Two patients had
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47 concomitant celiac disease but had normalized histology due to strict glutenfree diet. One
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49 patient in remission and two in the group “active disease” had concomitant bile acid
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51 malabsorption measured with SeHCAT (cutoff $< 10\%$). None of these patients had had any
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53 benefit of bile acids resins and no patient received cholestyramine during the study.
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3 Eight patients (6 women and 2 men, mean age 62 years) who underwent colonoscopy for
4 malignancy screening and who had normal histology were used as controls. Patients taking a
5 NSAID or an immunosuppressive agent were excluded. All patients gave their informed
6 consent and the study was approved by the Ethics Committee, Faculty of Health Sciences,
7 Linköping, Sweden.
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17 **Endoscopy**

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19 Bowel preparation required 3-4 litres of polyethylene glycol (Laxabon®). Sixteen biopsy
20 samples were taken with biopsy forceps (CBF 2.5-230, Cook Sweden AB, Askim, Sweden)
21 from the mid-part of the sigmoid colon: 2 for histology, 12 for the Ussing chamber studies
22 with the following distribution; (2 controls, 2 for *E.coli*, 2 *E.coli*+CDCA, 2 *E.coli*+DCA and 4
23 for confocal microscopy (different time intervals) and 2 to be held in reserve.
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33 **Ussing chamber experiments**

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35 The biopsies were taken in ice-cold oxygenated modified Krebs-Ringer bicarbonate buffer
36 (KRB) to the laboratory within 15 min. They were mounted in modified 1.5 ml Ussing
37 chambers (Grass&Sweetana, Harvard Apparatus Inc., Holliston, Mass, USA) with an exposed
38 tissue area of 1.76 mm² using the technique previously described by Wallon et al. (15).
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44
45 Oxygenation was continuous; pH was kept at 7.4 and temperature maintained at 37°C. The
46 mucosal compartment was filled with 1.5 ml 10 mM mannitol in Krebs buffer and the serosal
47 compartment was filled with 1.5 ml 10 mM glucose in Krebs buffer. Experiments started with
48 a 40-min equilibration period to establish a steady state. Transepithelial potential difference
49 (PD), short-circuit current (I_{sc}) and transepithelial resistance (TER) were studied for 120 min
50 in all specimens, using one pair of Ag/AgCl electrodes with agar-salt bridges and one pair of
51 current-giving platinum electrodes. Only three biopsies had to be excluded because of an
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3 initial PD > -0.5 mV which implies uncertain viability or leakage (15), but all other biopsies
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5 had a stable PD value (< -0.5 mV) for the duration of the experiment (data not shown).
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9 10 **Passage of chemically-killed E.coli K-12**

11 All biopsies were investigated for uptake of chemically killed, fluorescein conjugated *E.coli*
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13 K-12 BioParticles (Molecular Probes, Leiden, The Netherlands). These bacteria are killed
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15 with paraformaldehyde, which stops their reproduction but retains antigenicity. A
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17 concentration corresponding to 1.0×10^8 CFU/ml was added to the mucosal compartment as
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19 previously described by Keita et al. (16). After 2 hours the entire content of the serosal
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21 compartment was analysed at 488 nm in a fluorimeter (Cary Eclipse, Varian) where 1 unit
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23 corresponds to 3×10^3 CFU/ml.
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31 **Mucosal exposure to bile acids**

32 Sodium-chenodeoxycholate ($3\alpha, 7\alpha$ - dihydroxyl- 5β -cholan-24-oic acid, > 97%, Sigma) and
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34 sodium-deoxycholic acid ($3\alpha, 12\alpha$ - dihydroxyl- 5β -cholan-24-oic acid, SigmaUltra >99%,
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36 Saint Louis, Mo., USA) were diluted with mannitol Krebs to obtain concentrations of 100
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38 $\mu\text{mol/l}$. We chose this concentration as it reflects levels present in the colon and showed
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40 effects on barrier function in our previous study (13). After a 40-min equilibration, mannitol
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42 Krebs containing CDCA or DCA was added to the mucosal compartment of two biopsy
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44 samples.
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52 **Histology**

53 All biopsies were examined by one and the same pathologist (ÅÖ, blinded); two from the
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55 sigmoid colon were taken at each investigation and were stained with haematoxylin-eosin
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57 (HE) and van Gieson. The degree of surface epithelial cell degeneration was assessed in
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59 arbitrary units (0=none, 1=mild, 2=moderate, 3=severe). The thickness of the collagenous
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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 intra-epithelial lymphocytes (IEL)/100 enterocytes (mean value of three counts) was determined. The infiltration of mononuclear cells (lymphocytes and plasma cells) in to the lamina propria was defined in arbitrary units (0=none, 1=mild, 2=moderate, 3=severe).

Confocal microscopy studies

For studies on bacterial transport in colonic tissue, samples were fixed in 4% formalin after 30 and 60 min and mounted in Cryomount. Cryosections (10 μ m). They were cut using a microtome (Leica, Wetzlar, Germany) and dried overnight at room temperature on polysine glass slides. After cooling to room temperature, the sections were rinsed three times in PBS, then blocked in PBS containing 5% BSA. Prolong Gold with DAPI was used as mounting medium to achieve concurrent nuclear and chromosome staining. Cryosections were examined using confocal laser scanning microscopy (BioRad Radiance 2000 MP with LaserSharp 2000 software).

Statistics

Data are presented as median and 25th-75th percentiles. Comparisons between the groups were initially done with Kruskal-Wallis and further analysed with Mann-Whitney tests. To compare patients before vs. after budesonide treatment, the Wilcoxon`s matched-pairs signed-rank test was used. A two-sided p -value < 0.05 was considered significant.

Results

Histology

The histology in patients in clinical remission and findings before and after short-term budesonide treatment in patients with active CC are reported in table 1. No significant change was evident before vs. after budesonide treatment. The inflammation in lamina propria was significantly milder in patients in remission than in patients in remission due to budesonide treatment (1 [0-2] vs 2 [1-2]) ($p<0.01$).

Table 1: Histology in patients with clinical remission and findings before and after six weeks budesonide treatment in patients with active collagenous colitis. The groups “remission without budesonide” and “active disease” differed ($*=p<0.05$) but there were no significant changes before vs. after six weeks budesonide treatment. The inflammation in lamina propria was significantly more severe in patients in remission with budesonide treatment than in patients in remission without budesonide ($\# = p<0.01$). The data is presented as median (min-max) and was previously published (11).

E.coli passage

As shown in our previous study (11), uptake of *E.coli* through the colonic epithelium was increased in all CC groups compared with controls ($p=<0.05$).

By adding 100 $\mu\text{mol/l}$ of CDCA or DCA to biopsies from patients with CC in remission, the already increased bacterial uptake compared with controls was further augmented by factor 4 (Fig. 1). In patients with active disease, no further increase in bacterial passage was induced by bile acids, as was also the case when the individuals were receiving budesonide treatment. There were no significant differences between the groups of patients with CC when bile acids were added.

Electrophysiological measurements (TER, I_{sc}, PD)

The increased total wall resistance (TER) at the beginning of the study in CC patients with active disease compared with controls [47 (38-53) vs 34 (27-37) Ohmcm², $p < 0.05$] is a consequence of the thickened subepithelial collagenous layer and an inflammatory cell infiltration leading to submucosal edema, as discussed in our earlier study (11). Otherwise there were no significant differences in TER between the groups at baseline (0 min). Addition of 100 μmol/l CDCA or DCA to biopsies from patients in clinical remission, with active disease, or receiving budesonide treatment did not cause in any significant changes (delta values) in TER during the 120 min experiment period. This indicates that the tight junctions were not affected by this concentration.

Concerning the short-circuit current (I_{sc}), no significant changes were seen in any of the groups when 100 μmol/l CDCA or DCA was added and the potential difference (PD) in all biopsies was stable during the 120 min experiment (data not shown).

Confocal microscopy

Fig.2 shows that *E.coli* K12 crossed the epithelium within 30 min of adding bile acids and was found on the basolateral side of the epithelial cells. Imaging of the transepithelial passage of *E.coli* did not reveal which route (para-/transcellular) was predominant.

Discussion

We demonstrated recently that CC is associated with increased transmural passage of *E.coli* K12 bacteria and that the impaired barrier function is present in biopsies from patients in clinical remission as well as with active disease (11). As up to 44% of CC patients have an

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3 accommodating bile acid malabsorption (8), we wanted to test the hypothesis that bile acids
4 may influence barrier function in CC. The novel findings in the present study were that
5 dihydroxy-bile acids, in concentrations normally found in the colon, exacerbated the already
6 impaired mucosal barrier function by increasing bacterial uptake 4-fold in patients in clinical
7 remission. In contrast, biopsies from patients with active disease or in clinical remission
8 following budesonide treatment were not affected by bile acids and no increased bacterial
9 uptake was evident. Speculatively, this vulnerability to bile acids may offer an explanation for
10 the rapid clinical recurrence often seen after withdrawing budesonide treatment of CC.
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24 Hamilton et al. (17) reported that the total caecal concentration of 3-hydroxy bile acids in
25 humans was $600\mu\text{mol/l} \pm 300\mu\text{mol/l}$. As CDCA constitutes $7\pm 8\%$ (mean \pm SD) and DCA
26 $34\pm 16\%$ of the total bile acid composition, a concentration of $100\mu\text{mol/l}$ CDCA and DCA
27 lies within the physiological range in the caecum. Furthermore, stable epithelial PD values
28 during our experiments contradicted cytotoxicity. In these concentrations, bile acids do not act
29 as detergents (18), but have more specific, receptor-mediated effects. Bile acids can interact
30 with the intestinal mucosa in many ways and it has been demonstrated that they modulate
31 tight junction structures and barrier function in Caco-2 monolayers by activating the epithelial
32 growth factor receptor (EGFR) (19) or generating reactive oxygen species (20). Non-
33 pathogenic *E.coli* strains have been shown to ameliorate epithelial barrier function and
34 mediate tight junction protein rearrangement (21,22). In an earlier study (13) we showed that
35 the combination of *E.coli* K12 and $100\mu\text{mol/l}$ dihydroxy bile acids enhanced bacterial
36 passage and reduced TER in colonic biopsies from healthy subjects, which suggested effects
37 on tight junctions. Moreover, confocal microscopy revealed that the bacteria passed the
38 mucosa within 30 min.
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3 Mucosal barrier dysfunction is believed to play a major role in the pathogenesis of IBD (23),
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5 but to our knowledge no study has been conducted to consider the possible action that bile
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7 acids might have on mucosal integrity in humans affected by IBD. Interestingly, our study
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9 suggests that the already impaired barrier in active disease is not exacerbated by adding bile
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11 acid. This raises the question which structures or mechanisms are involved in biopsies from
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13 patients in clinical remission that make them more vulnerable or hyperreactive to bile acids.
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15 Similar observations were made in our previous study on ileal biopsies from Crohn`s patients,
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17 where stimulation with sodium caprate, a tight junction modulator, induced a more
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19 pronounced increase in flux of ⁵¹Cr-EDTA, a paracellular marker, and reduced TER more in
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21 non-inflamed than in inflamed tissue (24).
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29 Faecal bile acid concentrations in CC patients have not been analysed previously, but a
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31 pathological SeHCAT in 44% of CC patients (8) does reflect higher bile acid loss via the
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33 colon. CC presents with increased numbers of intra-epithelial lymphocytes also in the
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35 terminal ileum (27), indirectly suggesting functional alterations. It can therefore be speculated
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37 that bile acid reabsorption via the active sodium bile acid transporter (ASBT) is affected.
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39 Moreover increased intestinal motility in diarrheal disorders can per se lead to higher
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41 “spillover” of primary bile acids in the right colon. Holmquist et al. (28) reported that patients
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43 with ulcerative colitis who had bile acid malabsorption showed a high degree of mucosal
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45 inflammation in the right colon at colonoscopy. The right colon is also the main site of the
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47 classical histological findings of CC (29). Furthermore Ejderhamn et al. found that children
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49 with IBD in clinical remission had significantly increased bile acid levels in faeces and faecal
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51 water (30). In Crohn`s disease, faecal bile acids are also increased with an altered bile acid
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53 pattern showing a predominance of primary bile acids (31). Considering these observations
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60 together, it could be speculated that luminal bile acids, in susceptible hosts, could be

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3 promoters of colonic inflammation. The possible pathogenic role of bile acids in CC is further
4 corroborated by the symptomatic effects of bile acid-binding resins, such as cholestyramine,
5 that are clinically effective and ameliorate diarrheal symptoms in CC (8).
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10 With consideration that bile acid concentrations and intestinal inflammation are higher in the
11 right colon, it would have been favourable to study biopsies of the ascending colon. This was
12 difficult of practical reasons and some patients would have refused repeated colonoscopies.
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15 One the other hand we have not found any correlation between the degree of histological
16 manifestations and bacterial uptake in our prior study (11).
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24 Budesonide has a well-documented efficacy for inducing and maintaining remission in CC
25 (32). Steroids have a well known immunosuppressive effect and they bind to the
26 glucocorticoid complex suppressing myosin light chain kinase (MLCK) activity which
27 mediates intestinal tight junction permeability (33). Boivin et al. demonstrated that
28 glucocorticoids can inhibit a TNF- α induced TJ barrier defect. Furthermore, budesonide
29 increases ileal bile acid transport in CC and thereby decreases bile acid load on the colon as
30 shown by Bajor et al. (34). These findings lead to the speculation that the good clinical
31 efficacy of budesonide might also partially rely on modulatory effects on bile acid induced
32 diarrhoea or mucosal damage in CC.
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46 In the present study on the other hand, we found no effect of low concentrations of bile acids
47 on biopsies from patients with budesonide treatment. As these biopsies had a higher
48 background level of inflammation and bacterial uptake, this might have overshadowed the
49 experimental deleterious effect of bile acids.
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57 CC runs a mainly chronic, intermittent course. It is still not known what causes the intestinal
58 inflammation or triggers a recurrence. Our data suggest that asymptomatic patients have an
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3 underlying barrier defect and increased mucosal vulnerability to bile acids, leading to
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5 increased bacterial uptake through the colonic epithelium. These detrimental effects on
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7 mucosal barrier function could facilitate initiation and perpetuation of mucosal inflammation
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9 in CC. Our findings may also offer an explanation for the rapid clinical relapse that is often
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11 seen after ending the immunosuppressive and barrier protecting therapy with budesonide (35).
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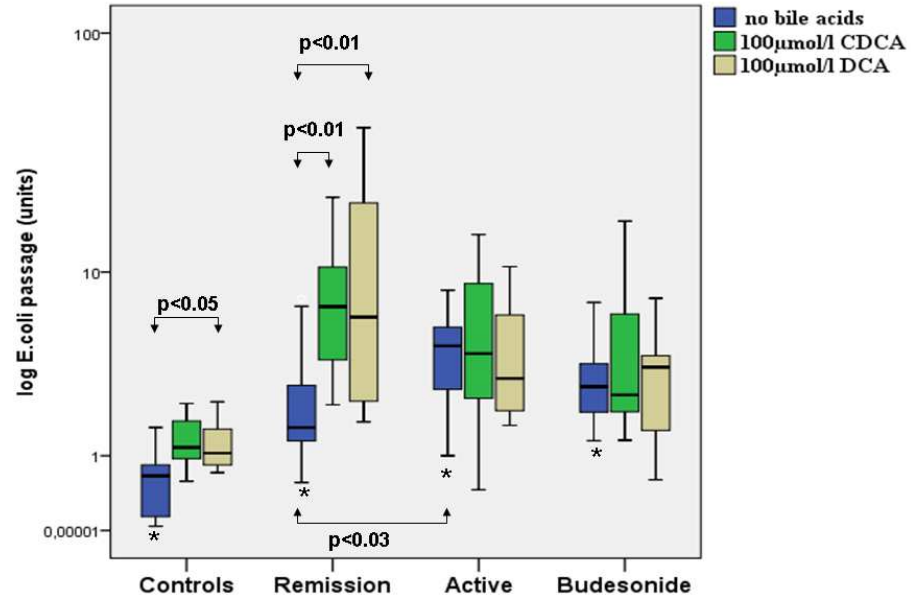
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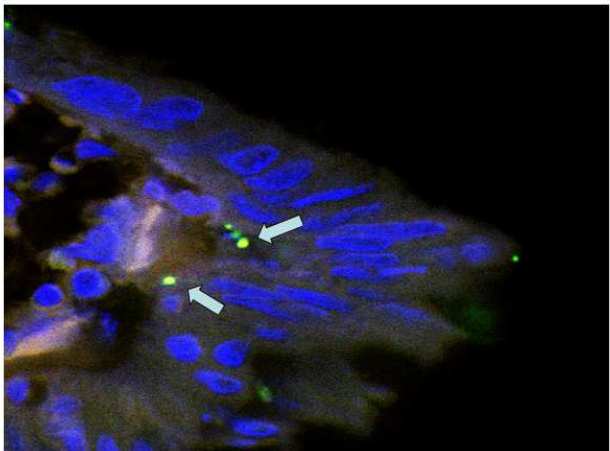


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Fig. 2



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Review

Table 1.

Histology	Active disease before budesonide (n=11)	Remission after six weeks on budesonide (n=10)	p-value	Remission without budesonide (n=14)	p-value
Degeneration	1 (0-3)*	0 (0-3)	ns	0 (0-2)*	<0.05
Collagenous band (µm)	15 (4-55)*	17 (7-25)	ns	8 (4-22)*	<0.05
IEL	30 (4-52)*	27 (6-49)	ns	12 (3-30)*	<0.05
Lamina propria	2 (0-3)*	2 (1-2) #	ns	1 (0-2) # *	<0.05

For Peer Review

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6 **Figure 1** Uptake of *E.coli* bacteria during 120 min in Ussing chambers with or without
7 addition of 100 $\mu\text{mol/L}$ CDCA or DCA to the mucosal side of colonic biopsies. Comparison
8 in controls vs. groups of patients with collagenous colitis (CC) (i) in remission, (ii) with active
9 disease and (iii) during budesonide treatment. Values are expressed as boxplot (line=median,
10 box=25-75 percentile and whiskers 5-95 percentile). One unit corresponds to 3×10^3 CFU/ml.
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14 **Figure 2** Visualization of *E. coli* K12 on the basolateral side of the human intestinal
15 epithelium (*arrow*). White = Phalloidin, F-actin, Green = *E.coli* K12, Blue = DAPI nuclear
16 stain. (x63 objective, x2 zoom).
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21 **Table 1: Histology in patients with clinical remission and findings before and after six**
22 **weeks budesonide treatment in patients with active collagenous colitis. The groups**
23 **“remission without budesonide” and “active disease” differed ($*=p<0.05$) but there were**
24 **no significant changes before vs. after six weeks budesonide treatment. The**
25 **inflammation in lamina propria was significantly more severe in patients in remission**
26 **with budesonide treatment than in patients in remission without budesonide ($\# =$**
27 **$p<0.01$). The data is presented as median (min-max) and was previously published (11).**
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