



Gastrointestinal dysbiosis and *Escherichia coli* pathobionts in inflammatory bowel diseases

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PREFACE

My interest in the field of gastrointestinal microbiology started early in my medical career with a study of bacterial gastroenteritis that is included in this dissertation. Over time I have steadily become more involved in the search for new areas where microbiology and gastroenterology may be linked. During my PhD research in the exciting field of *Helicobacter pylori* infection, I realized that novelty can often be found in the areas lying between fixed sets of thought.

No one believed the Australian doctors Warren and Marshall when they claimed that a gastric infection was the cause of gastroduodenal ulcers, but in the end their innovation and persistence managed to convince even the most doubtful scientist. It has certainly underscored that specific or consortia of microorganisms may very well cause, or at least play a central role in, many other chronic diseases such as inflammatory bowel diseases (IBD). Today it is not controversial to perform research trying to improve the dysbiosis linked to IBD using fecal microbiota transplantation (FMT), just as FMT has treated the dysbiosis linked to *C. difficile* infection. All of these areas are at the core of this dissertation. Hopefully, our research has been one small additional step in the journey leading to new and microbiome-based treatments of IBD, a disease in which we today can't always find a medical cure, but instead must perform surgical removal of parts of the intestine.

Finally, I would like to express my profound gratitude to my main collaborators: Professor Karen A. Krogfelt, Post Doc Hengameh Chloé Mirsepasi-Lauridsen and Post Doc Sofie Ingdam Halkjær with their manifold talents in networking, lab research and project managing. This gratitude is also directed to all my co-authors and all my colleagues in all professions who have helped and supported me along the way, and to my wonderful and adventurous family, Joe and Noline.

This thesis is based on the following 10 previously published papers, which will be referred to in the text by their Roman numerals:

- I. **Petersen AM**, Nielsen SV, Meyer D, Ganer P, Ladefoged K. Bacterial gastroenteritis among hospitalized patients in a Danish County, 1991–93. *Scand J Gastroenterol.* 1996;31(9):906–11.
- II. **Petersen AM**, Stensvold CR, Mirsepasi H, Engberg J, Friis-Møller A, Porsbo LJ, Hammerum AM, Nordgaard-Lassen I, Nielsen HV, Krogfelt KA. Active ulcerative colitis associated with low prevalence of *Blastocystis* and *Dientamoeba fragilis* infection. *Scand J Gastroenterol.* 2013;48(5):638–9.
- III. Halkjær SI, Christensen AH, Lo BZS, Browne PD, Günther S, Hansen LH, **Petersen AM**. Fecal Microbiota Transplantation alters gut microbiota in Patients with Irritable Bowel Syndrome: Results from a randomized, double-blind placebo-controlled study. *Gut.* 2018. pii: gutjnl-2018-316434.
- IV. **Petersen AM**, Mirsepasi-Lauridsen HC, Vester-Andersen MK, Sørensen N, Krogfelt KA, Bendtsen F. High abundance of Proteobacteria in Ileo-anal pouch anastomosis and increased abundance of Fusobacteria associated with increased pouch inflammation. *Antibiotics (Basel).* 2020;9(5):237.
- V. **Petersen AM**, Nielsen EM, Litrup E, Brynskov J, Mirsepasi H, Krogfelt KA. A phylogenetic group of *Escherichia coli* associated with active left-sided inflammatory bowel disease. *BMC Microbiol.* 2009;9:171.
- VI. **Petersen AM**, Halkjær SI, Gluud LL. Intestinal colonization with phylogenetic group B2 *Escherichia coli* related to inflammatory bowel disease: a systematic review and meta-analysis. *Scand J Gastroenterol.* 2015;50(10):1199–207.
- VII. **Petersen AM**, Schou C, Mirsepasi H, Engberg J, Friis-Møller A, Nordgaard-Lassen I, Wildt S, Krogfelt KA. Seroreactivity to *E. coli* outer membrane protein C antibodies in active inflammatory bowel disease; diagnostic value and correlation with phylogroup B2 *E. coli* infection. *Scand J Gastroenterol.* 2012;47(2):155–61.
- VIII. Kantsø B, Halkjær SI, Thomsen OØ, Belard E, Gottschalck IB, Jørgensen CS, Krogfelt KA, Slotved HC, Ingels H, **Petersen AM**. Immunosuppressive drugs impair antibody response of the polysaccharide and conjugated pneumococcal vaccines in patients with Crohn’s disease. *Vaccine.* 2015;33(41):5464–5469.
- IX. **Petersen AM**, Schjørring S, Gerstrøm SC, Krogfelt KA. Treatment of inflammatory bowel disease-associated *E. coli* with ciprofloxacin and *E. coli* Nissle in the streptomycin-treated mouse intestine. *PLoS One.* 2011;6(8):e22823.
- X. **Petersen AM**, Mirsepasi H, Halkjær SI, Mortensen EM, Nordgaard-Lassen I, Krogfelt KA. Ciprofloxacin and probiotic *Escherichia coli* Nissle add-on treatment in active ulcerative colitis: A double-blind randomized placebo controlled clinical trial. *J Crohns Colitis.* 2014;8(11):1498–505.

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Ulla M. Wewer, Head of Faculty.

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Summary

In this thesis, based on ten previously published papers, the intestinal dysbiosis, links to pathobionts and manipulations of the intestinal microbiome have been examined in inflammatory bowel disease (IBD). IBD has previously been linked to acute and chronic gastrointestinal infections due to overlapping symptoms, Paper I, and sometimes even identical endoscopic presentation. IBD is associated with intestinal low diversity dysbiosis, with probable negative effects on both the availability of intestinal nutrients, the intestinal immune function and the mucosal barrier integrity. Conversely, normobiosis including colonization with apathogenic parasites is linked to IBD in remission, Paper II. The possibility of correcting the intestinal dysbiosis using fecal microbiota transplant (FMT) from healthy donors was proven in patients with irritable bowel syndrome, Paper III, and FMT has been found efficient in inducing clinical response in patients with ulcerative colitis (UC). Some intestinal bacterial phylae, such as Firmicutes and Bacteroidetes, are found in lower abundance during flare-ups of IBD, whereas others, such as Proteobacteria, e.g., *E. coli*, are increased, Paper IV. In addition, different intestinal microorganisms have been claimed as possible pathogenic causes of IBD. Among the most frequently identified candidates are *E. coli* pathobionts. *E. coli* exists in different subsets, most are commensal, but others harbor virulence genes. Among *E. coli* with virulence genes some are considered classic enteropathogenic, while others are considered extraintestinal pathogenic (ExPEC). The association between ExPEC intestinal colonization and flares of IBD was confirmed, Paper V, VI. Specific virulence factors, e.g., adhesins and α -hemolysin, in ExPEC links this *E. coli* to IBD pathogenesis through disruption of the intestinal epithelial cell barrier, a possible invasive potential of some *E. coli* and an ability to survive within macrophages (adherent invasive *E. coli*). Increased levels of antibodies towards *E. coli* antigens in IBD patients, Paper VII, supports a more direct interaction of *E. coli* with the immune system in IBD patients, even though IBD medications will also influence the serologic response, Paper VIII. Colonization experiments in a mouse model, Paper IX, and a probiotic *E. coli* (*E. coli* Nissle) placebo-controlled treatment study in patients with active UC, Paper X, demonstrate that *E. coli* Nissle does not eradicate ExPEC but instead has the ability to act as a symbiont with ExPEC, thereby hindering de-colonization and presumably preventing remission of UC. In conclusion, the presumed microbial pathogenic mechanism behind flares of IBD could be aligned with the pathogenesis of recurrent *C. difficile* infection. The involved pathobiont only results in intestinal inflammation if a general intestinal dysbiosis co-exists. Accepting this hypothesis, remission-inducing treatment options in IBD could in the future be specific antibiotics, anti-adhesives or phage therapy removing the pathobiont, followed by FMT or probiotics in order to maintain remission.

Dansk Resumé

Baseret på ti tidligere publicerede artikler undersøges i denne afhandling betydningen af ubalance i sammensætning af tarmens mikroorganismer for patienter med kronisk inflammatoriske tarmsygdomme (IBD). Endvidere undersøges mulighederne for at genoprette tarmens mikrobiom. Diagnostisk kan debut eller opblussen af kroniske inflammatoriske tarmsygdomme (IBD) være svære at skelne fra akutte og kroniske mave-tarm infektioner, både når det gælder symptomer, artikel I, og de endoskopiske fund. IBD er associeret med specifikke ændringer i tarmens mikrobiom, den såkaldte 'lav diversitets' dysbiose, med sandsynlige negative konsekvenser for tarmens ernæring, tarmens immunfunktion og slimhinde-barrierens integritet. Modsat er normobiose inklusive kolonisering med apatogene parasitter associeret med IBD remission, artikel II. Muligheden for korrektion af den intestinale dysbiose ved brug af fæces transplantation (FMT) fra raske donorer bevises for patienter med irritabel tyktarm, artikel III, og FMT er tidligere fundet at kunne inducere et klinisk respons hos patienter med colitis ulcerosa (UC). Nogle tarmbakterier, såsom Firmicutes og Bacteroidetes, findes i lavere forekomst ved opblussen i IBD, hvorimod andre, såsom Proteobakterier, herunder *Escherichia coli*, findes med øget forekomst, artikel IV. Ydermere er en række forskellige mikroorganismer blevet udråbt som mulige sygdomsfremkaldende årsager til IBD. Blandt de hyppigst beskrevne fund er *E. coli* pathobionter. *E. coli* består af en lang række forskellige undertyper, de fleste er såkaldte kommensale, mens andre indeholder virulens gener. *E. coli* med virulens gener kan deles op i klassiske enteropatogene *E. coli*, og i extraintestinalt patogene (ExPEC). Associationen mellem kolonisering af tarmen med ExPEC og opblussen i IBD kunne bekræftes, artikel V, VI. Visse virulens- faktorer, f.eks. adhæsiner og α -hæmolysin, i ExPEC kobler yderligere disse *E. coli* til IBD-patogenesen ved nedbrydning af tarmens epitelcelle barriere, et invasivt potentiale og en evne til intracellulær overlevelse i makrofager (adherent invasive *E. coli*). En øget forekomst af antistoffer mod et *E. coli* overflade antigen, hos IBD-patienter, artikel VII, støtter ligeledes en mere direkte interaktion mellem *E. coli* og IBD patientens immunsystem. Dog er det også et faktum at den immunmodulerende medicin, der anvendes til IBD behandling også vil kunne influere på det serologisk respons, artikel VIII. Kolonisations eksperimenter i en musemodel, artikel IX, og et probiotisk *E. coli* (*E. coli* Nissle) placebokontrolleret behandlingsstudie, artikel X, blandt patienter med aktiv UC viser, at *E. coli* Nissle ikke eradikerer ExPEC, men derimod fungerer som en symbiont, resulterende i en hindring af de-kolonisering og formodentlig en hindring af UC remission. Som konklusion kan det udledes at en formodet mikrobiel patogenese til IBD kan sammenlignes med patogenesen ved rekurrent *C. difficile* infektion. Den implicerede pathobiont resulterer kun i tarminflammation, hvis der samtidigt eksisterer en tarmdysbiose. Hvis denne hypotese accepteres, er der grundlag for fremtidige remissionsinducerende behandlinger af IBD med specifikke antibiotika, anti-adhæsiner eller fagterapi rettet mod den mistænkte pathobiont fulgt af FMT eller probiotika som remissionsbevarende behandling.

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INTRODUCTION

Inflammatory bowel diseases, definition and pathogenic mechanisms

Inflammatory bowel disease (IBD) is the umbrella term for chronic inflammatory diseases of the intestine often affecting children and young adults [1]. Diagnosis of IBD is based on a combination of history, physical and laboratory examination, esophagogastroduodenoscopy and ileocolonoscopy with histology, imaging of the small bowel and, importantly, absence of enteric infections [2]. Currently, IBD is divided into ulcerative colitis (UC) and Crohn's disease (CD) based on intestinal dissemination of the inflammation, in addition to macroscopic and histological features; UC affects the colon, starting from the anus extending in varying degrees into the colon, in some cases resulting in inflammation of the entire colon, defined as pan-colitis [3]. UC generally affects the superficial layers of the epithelium inducing crypt abscesses and subepithelial gathering of inflammatory cells [4]. In contrast, CD can affect any part of the intestine; however, in the majority of cases, inflammation will be found in the terminal ileum, the colon or both of these [5]. CD is associated with deeper penetrating inflammation, occasionally resulting in fistulizing disease and abscess formation [6]. Both UC and CD can, if medical therapy fail, lead to the need for surgery; among patients with CD, intestinal surgery is required for as many as 80%, and a permanent stoma is required in more than 10%. In patients with UC, the lesions usually remain superficial and extend proximally; colectomy is however still required for 10%–30% of patients [7]. It has been

suggested that earlier introduction of immunosuppressants and biologics may be associated with a lower risk for surgery, even though data also suggest that a decrease in surgery rates had been achieved even before introduction of biologics [8–10]. Furthermore, the need for surgery has also increased in some cohorts even after introduction of anti-TNF and other biological treatments in IBD [11]. This implies that the introduction of new types of medical treatments, such as biologics, has not changed the need to search for new fundamentally different treatment options for IBD patients.

Diagnosis of CD and UC do overlap. Sometimes the diagnosis will shift from UC to CD or from colonic CD to UC over time, and for some patients, the colonic inflammation will remain indeterminate [12]. The anticipation is, however, that IBD in the future will be categorized into additional subtypes taking into account the genetic, the immunological and the intestinal microbiome profile of the individual patient [13]. The pathogenic mechanisms of IBD have been researched intensely and in general, it is believed that genetic, immunological and environmental factors are involved simultaneously. It is, however, remarkable that the intestinal inflammation in IBD has a close macroscopic and microscopic resemblance to infectious diseases of the gut, such as intestinal tuberculosis (TB), cytomegalovirus (CMV) enteritis, *Clostridioides difficile* infection, campylobacteriosis and many more, and the differential diagnosis can sometimes be difficult [14]. What is even more intriguing is the fact that IBD often has a variation in manifestation in the individual patient over time, with shorter or longer periods with quiescent disease and other periods with a full

flare occasionally resulting in the need for surgery [15]. Based on these observations it is of utmost importance also to investigate the causes of IBD flares. One likely event is the occurrence of a gastrointestinal (GI) infection triggering the onset of disease flares. In this respect, it is interesting that the debut of IBD is probably not associated with the most common GI infections such as *Salmonella* and *Campylobacter* infections [16]. Over time, many different bacteria (e.g., *Mycobacterium avium* subspecies *paratuberculosis* and *Escherichia coli* pathotypes), viruses (e.g., measles and CMV) and other microorganisms have been suspected to be the pathogenic cause of IBD, or at least to contribute to IBD flares [17]. It is by now well established that luminal factors in the intestine are involved in the inflammatory process of CD and UC. One often referred example is that the diversion of the continuity of the intestines results in healing of the resting gut, whereas the inflammation will return when continuity is reestablished [18]. Furthermore, animal IBD models have documented the importance of the presence of bacteria in the inflammatory process [19]. Numerous experiments involving animal colitis models, such as IL-10 knock-out mice, have shown that these mice will develop fulminant colitis living under normal conditions, whereas they will be disease-free living in germ-free surroundings [20]. Moreover, it has been demonstrated that probiotics will reduce the inflammatory damage of the intestine in, e.g., IL-10 knock-out mice [21,22]. But without convincing discoveries of the involvement of specific microorganisms in IBD, and acknowledging the fact that immune regulatory medications often have the ability to cure flares of these diseases, CD and UC have for a long time been perceived as immunological diseases with elements of autoimmunity [23]. In this context it is important to underline that the most frequently used medications in IBD are immunomodulators; the medications used to induce remission include 5-aminosalicylic acid products, steroids and tumor necrosis factor (TNF), $\alpha 4\beta 7$ integrin or JAK kinase inhibitors and medications used to maintain remission include 5-aminosalicylic acid products, immunomo-

dulators (Azathioprine, 6-mercaptopurine, methotrexate) and TNF-, $\alpha 4\beta 7$ integrin or JAK kinase inhibitors [24,25]. Genetic studies have, however, revealed that the mutations associated with IBD (currently 163 IBD-specific loci) are frequently linked to the intestinal immunogenic defense against microorganisms [26]. This has caused the pendulum to swing back from the assumption of IBD as mainly autoimmune diseases towards a greater appreciation of a contribution from the intestinal microbiome [27]. Many of the IBD-associated genetic mutations result in inefficient innate immune responses, e.g., malfunctioning defensins, and defective phagocytic processing of bacteria [27]. Importantly, the early finding of a defect in the caspase recruitment domain family, member 15 (*NOD2/CARD15*) gene among CD patients, has reawakened the search for specific involved pathogens [28]. *NOD2/CARD15* is involved in the innate immune system including the production of defensins; therefore, defects in this gene could imply that the host is more susceptible to gastrointestinal infections [29]. It has, furthermore, been shown that the number of viable internalized *Salmonella typhimurium* in Caco-2 cells was higher when the Caco-2 cells were transfected with a variant *CARD15/NOD2* expression plasmid associated with Crohn's disease [30]. Even though genetic mutations affecting the immune system do not seem to account for all IBD cases, these interesting results created a bridge upon which the immunological and the microbiological believers could meet, regarding the major contributing factors to the pathogenicity of IBD. Therefore, current theories describe IBD as a result of an unfortunate interplay between immunity (based on possible faults in the immunological maturation and in genetics) and specific perturbations in the composition of the intestinal microbiome, the so-called intestinal dysbiosis [31]. This will in the near future, probably lead to new definitions of IBD, with subtypes being primarily based on genetics, subtypes based primarily on immunological dysfunctions, and the possibility that certain subtypes will be explained to a large degree by imbalances in the intestinal microbiome, as illustrated in Figure 1.

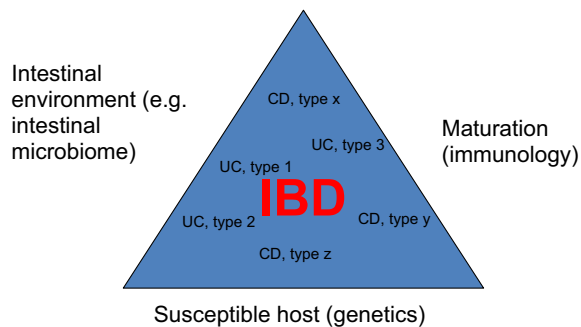


Fig. 1. An Illustration of the perception of IBD and IBD subtype pathogenesis being based on varying contributions from genetics, immunology and the intestinal microbiome.

Aim of the present thesis

Intestinal dysbiosis, defined as increased levels of harmful bacteria and decreased levels of beneficial bacteria, is associated with IBD. Furthermore, infections with specific microorganisms have been claimed as a possible cause of IBD or IBD flares. Colonization with specific *E. coli* pathotypes has frequently been reported to be associated with both UC and CD. Based on these observations I have, in this thesis, focused on examinations of:

1. Acute gastrointestinal infectious diseases as a differential diagnosis to flares of IBD, and the intestinal dysbiosis related to IBD (I, II, III, IV);
2. The phylogenetic and virulence profile of *E. coli* intestinal isolates, *E. coli* serology and serological responses in IBD (V, VI, VII, VIII), and;
3. Treatment trials designed to eliminate *E. coli* pathotypes and improve clinical outcomes in active IBD (IX, X).

INFLAMMATORY BOWEL DISEASES AND INFECTIOUS GASTROENTERITIS

Diagnostically it is occasionally difficult to separate acute infectious gastroenteritis (AG) from a debut or a flare of IBD [32]. An initial flare of IBD will often be misdiagnosed as a case of AG, and a more prolonged episode of AG could easily be mistaken for IBD [14]. The core symptoms are similar; frequent stools,

occasionally blood in stools, abdominal pain, fever, weight loss and sometimes extraintestinal complications such as reactive/inflammatory arthritis. Even endoscopic appearances are sometimes indistinguishable, with hyperemia, edema, submucosal bleeding and ulcers. Some gastrointestinal infections, such as *C. difficile* infection, intestinal tuberculosis, *Giardia*, CMV colitis, can furthermore be chronic, or the symptomatology can be prolonged by post-infectious irritable bowel syndrome after an episode of AG [33]. *C. difficile* has, furthermore, frequently been found in stools of patients with IBD even during quiescent disease [34]. In some countries, e.g., India, intestinal tuberculosis is a very relevant alternative diagnosis to IBD in patients with intestinal inflammation and chronic intestinal ulcers [35]. Therefore, stool sampling and intestinal biopsies with culture/PCR diagnostics for enteropathogens will often be necessary during both initial diagnosis and continuous follow-up of IBD patients. Based on an increase in the number of zoonotic *Salmonella* infections in Denmark in the 1990s, from a previous level of 500–1000 cases a year to more than 4000 cases a year, we reviewed, retrospectively, the outcome and the symptomatology in patients admitted with more common bacterial gastrointestinal infections such as Salmonellosis, Campylobacteriosis and Yersiniosis, Paper I. Overall *Salmonella* infection was associated with the highest rate of admission of patients (44%) compared with the other bacterial causes of gastroenteritis, suggesting a more severe disease course during infection with *Salmonella* spp. However, blood in stools was most frequent in patients infected with *Campylobacter*, Table 1, Paper I. In general, both *Campylobacter* and *Salmonella* infections have been linked to IBD. A search on ‘*Salmonella*’ and ‘Inflammatory Bowel Disease’ resulted in 406 articles, and a search on ‘*Campylobacter*’ and ‘Inflammatory Bowel Disease’ resulted in 283 articles, date 17-08-2020. Increased risk of IBD has been described after clinical gastroenteritis especially caused by *Salmonella* species, *Campylobacter* species and *Clostridioides difficile* without any particular difference in odds ratio (OR) for IBD between these microorganisms [36,37]. However, it has been found in an epidemiological study that also a negative

Table 1. Paper I. Symptoms due to infection with enteropathogenic bacteria among hospitalized patients in Roskilde county, Denmark, 1991–93

	All, n	Diarrhea %	Abdominal pain%	Vomiting%	Blood in stools%	Fever > 38 C,%
<i>Salmonella typhimurium</i>	40	98	68	28	38	81
<i>Salmonella enteritidis</i>	48	92	65	40	19	85
Other zoonotic <i>Salmonella</i>	39	90	56	57	26	78
<i>Campylobacter</i>	40	98	78	35	40	63
<i>Yersinia enterocolitica</i>	27	93	63	43	22	71

stool sample for enteropathogens was associated with a subsequent diagnosis of IBD. This indicates that a patient with IBD with a concomitant *Salmonella*, *Campylobacter* or *C. difficile* infection probably has a higher risk of a positive stool sample simply because of more frequent submission of stool samples from IBD patients compared to patients with acute gastroenteritis alone [16]. Historically, it seemed necessary to make at least three stool cultures to secure a bacteriologic diagnosis based on the culture of fecal samples in patients with longer disease duration, Paper I. This could presumably have affected the ability to associate IBD with gastrointestinal microorganisms. Currently, diagnosis of GI infection has in many clinical microbiology departments shifted to PCR-based methods with new possibilities for the diagnosis of GI infections, with higher sensitivity and specificity. These diagnostic changes might again affect the frequencies of how often a link will be found between acute GI infections and flares of IBD.

Another link between AG and IBD is reactive arthritis linked to AG and inflammatory arthropathy linked to IBD. Reactive arthritis occurred in 4.8% of our cohort of patients with AG, Paper I, compared with a frequency of up to 30% of patients with IBD having some form of inflammatory arthropathy [38]. However, the frequencies in IBD are naturally reported for a much longer disease period compared to patients with AG. It is possible that a common pathogenetic mechanism exists in reactive arthritis and inflammatory arthropathy giving rise to further difficulties when trying to separate AG and IBD diagnostically.

Gastrointestinal infections, other than *Salmonella*, *Campylobacter* or *Yersinia*, are, however, even more likely to be a possible

differential diagnosis to IBD or to complicate the course of IBD. In many parts of the world where tuberculosis is common, it will be crucial to exclude that a suspected case of IBD is not in fact a case of intestinal tuberculosis. To exclude tuberculosis among IBD patients has now become increasingly important in all parts of the world, since treatment of IBD with TNF- α inhibitors will increase the risk of reactivating a concomitant tuberculosis infection [39]. IBD and intestinal tuberculosis are very similar in symptoms and findings such as weight loss, malabsorption, diarrhea, blood in stools, abdominal pain and even in macroscopic appearance during endoscopy, with CD and intestinal tuberculosis both causing chronic intestinal inflammation with the risk of stenotic behavior and deep ulcerations in the intestinal wall [35]. Furthermore, *C. difficile* infection can complicate IBD and raise doubts about the right treatment approach; should gastroenterologists in these cases choose metronidazole, vancomycin and/or increased doses of anti-inflammatory medications [40]? In clinical practice, a case of *C. difficile* infection during a flare of IBD will often result in both antibiotic and anti-inflammatory treatment. Especially diagnostically difficult are cases of CMV colitis, which are often directly caused by the immunosuppressive treatment given to IBD patients [41]. This makes it important to look for and treat a possible CMV infection when initial beneficial treatment of IBD such as high-dose prednisolone suddenly fails. An additional problem is emerging infections; e.g., Shiga toxin-producing *E. coli* infection (STEC) can cause hemorrhagic colitis, which has been confused with flares of IBD [42]. Bacterial gastroenteritis requiring hospitalization affects mainly children < 5 years [43]; likewise IBD affecting

young children has been associated with a more severe disease course [44]. So even without evidence for a direct link between intestinal dysbiosis or specific enteropathogens in IBD pathogenesis, intestinal infectious complications to IBD are an ever-present consideration for clinicians. So, thorough initial microbiological examinations are diagnostically important, as are frequent re-evaluations during IBD treatment courses. This all rests on the diagnostic approach in the clinical and supporting para-clinical departments, and the following question must always be considered when anti-inflammatory treatments fail: Is the current anti-inflammatory treatment failing, requiring treatment replacement with other more intensive anti-inflammatory treatments or surgical intervention, or is a more intensive search for complicating gastrointestinal infectious diseases necessary?

INFLAMMATORY BOWEL DISEASES AND THE INTESTINAL MICROBIOME

The intestinal microbiome, which refers to the microorganisms (gut microbiota), their genomes and the local environment in the human intestine, contains approximately 100 times as many genes as the human genome [45]. Even though an imbalance in the intestinal microbial communities, also referred to as intestinal dysbiosis, is associated with flares of IBD, the exact nature of the involvement of the dysbiotic microbiome in IBD pathogenesis is not fully understood. Furthermore, the IBD-associated intestinal dysbiosis could be driven by the disease through a substrate effect of an inflamed and possibly bleeding mucosa or through effects of IBD medications on the

microbiome. In a recent systematic review, a notable impact was found of non-antibiotic prescription drugs on the overall architecture of the intestinal microbiome, e.g., Proton Pump Inhibitors, metformin, NSAIDs, opioids and antipsychotics were associated with increases in members of Proteobacteria (including *E. coli*) or members of *Enterococcaceae* [46]. In addition, it has been shown that nitrate generated by the intestinal inflammatory response conferred a growth advantage to *E. coli* possibly contributing to the dysbiosis associated with IBD [47]. It has, however, also been demonstrated that introduction of IBD dysbiotic communities can stimulate intestinal inflammation in mouse models, even though, it is still not verified whether a healthy intestinal microbiome can be sufficient to prevent the induction of IBD flares [48]. A direct effect of the IBD-associated microbiome on IBD pathogenesis could be due to one of the three following mechanisms: (1) the diseased intestine with epithelial damages and ulcers will be a natural transmission zone for the intestinal microbiome at random, resulting in possible bacteremia, and micro or macroabscess formation; (2) a dysbiotic microbiome could represent microorganisms, which are involved in epithelial destruction (through induction of barrier defects in mucus and/or epithelial cells) and damages to the immune system in the genetically susceptible individual; (3) or, specific emerging pathogens such as e. g. *Mycobacteria avium* subspecies *paratuberculosis* and *E. coli* pathotypes could be the underlying cause of IBD, even in non-genetically susceptible individuals, Table 2. That microorganisms could be involved in IBD pathogenesis, is supported by several meta-analyses of the effect of antibiotics in IBD, which all concluded that

Table 2. Three possible mechanisms by which the intestinal microbiome could be involved in IBD pathogenesis

Excessive translocation of intestinal bacteria (leaky gut)	Inflammation results from an abnormal mucosal permeability/abnormal mucosal immune response
Altered gut microbiome (dysbiosis)	Inflammation results from loss of the protective barrier provided by normal bacterial populations and negative effects on the epithelial cells and/or immune cells from microorganisms representing the dysbiotic microbiome
Persistent pathogen	Inflammation results from the persistence of a not yet identified bacterial, parasitic or viral pathogen

there was a positive effect of antibiotic treatment during flares of IBD [49–51].

It has been documented that IBD and flares of IBD are linked to reproducible changes in the gut microbiome based on gut bacteria classification studies [52]. The IBD-associated dysbiosis (not unlike changes found in other diseases such as *C. difficile* infection, irritable bowel syndrome (IBS), cancer, liver diseases and metabolic syndrome) is characterized by a low diversity microbiome with reduced presence of anaerobes, often found in high numbers in healthy individuals, and an increase in facultative anaerobes [53]. These data provide evidence of a shift in the balance between the bacterial phylae, including depletion of Firmicutes subtypes (such as *Faecalibacterium prausnitzii*) and Bacteroidetes [54–56]. Furthermore, the increased presence of Proteobacteria (including the family *Enterobacteriaceae*) has frequently been described [56–58]. In addition, specific changes in bacteria, viruses and parasites at the species level have been documented, which further have added evidence to the assumption that IBD is linked to a changed/dysbiotic intestinal microbiome. Among the many studied microorganisms, a decrease in the bacterial populations *Clostridium leptum* group (IV), *Roseburia hominis* and *F. prausnitzii* [59,60] and *Lachnospiraceae* [61] have been described in IBD patients compared with controls, illustrating the many microbiological studies that are available, Table 3. These shifts in microbial communities, e.g., lower levels of *Roseburia hominis* and *F. prausnitzii*, have also been associated with low levels of the short-chain fatty acid (SCFA) butyrate, which is mainly produced from complex sugars by anaerobic bacteria in the colon. Butyrate is an important energy source for the maintenance of a normal colonic epithelium [63], and butyrate has more direct anti-inflammatory properties [64]. Both consequences of the

shortage of butyrate could be central in IBD pathogenesis. In addition, the consumption of butyrate by colonic epithelial cells provides a hypoxic environment in the colon [65], which is related to a disadvantage of colonization with facultative anaerobic bacteria such as *Salmonella* [66]. Interestingly, the sustained response of pediatric CD patients to TNF- α inhibitors was associated with abundance of SCFA-producing bacteria [67]. As another example of dysbiosis-related consequences for the intestinal inflammation seen in IBD, *Akkermansia* has been found to be decreased in UC, and this could be especially interesting due to an anti-inflammatory effect of *Akkermansia*-derived vesicles [68]. Parts of the phyla Actinobacteria have been found to convert bile acids, related to a reduced inflammatory response in the colon [69]. This may also play a role in IBD, since these strains have also been found to be decreased in IBD compared with controls [70]. Many of the studies of changes in microbiome structure have so far focused largely on bacterial species; however, microbiome changes in IBD do include other enteric microorganisms. Findings from our own study regarding the presence of the intestinal parasites *Blastocystis hominis* and *Dientamoeba fragilis*, likewise, found a change of presence, in this case, a decrease of these microorganisms among patients with IBD compared with controls, Paper II, Table 4.

In addition, a significant difference in *D. fragilis* colonization was found between inactive and active UC, 33% and 5%, respectively, ($p < 0.05$). Likewise, *Blastocystis* was found primarily in inactive UC, ($p < 0.01$), Table 4, Paper II. Similar results have been found in a study from 2010, where *Blastocystis* was detected in 33% (2/6) of IBD patients compared with 76% (16/21) of IBS patients [71].

These findings could be in accordance with the hygiene theory, which proposes that the

Table 3. Examples of the many proposed protective and aggressive microorganisms in patients with IBD [62]

Increased	Reduced
Proteobacteria, e.g., <i>E. coli</i>	Firmicutes, e.g., <i>Clostridium</i> groups IV, XIVa (<i>Faecalibacterium prausnitzii</i>)
<i>Ruminococcus gnavus</i>	Bifidobacteria
<i>Candida</i>	<i>Saccharomyces cerevisiae</i>
<i>Veillonellaceae</i>	Bacteroidetes

Table 4. Paper II. Gender, age and parasitological findings in different IBD groups

Diagnose N	CDA 22	CDI 20	UCA 20	UCI 21	PA 9	PI 8	HC ^M 32	HC ^R 64
Blastocystis-positive (%)	1 (5)	0	0	4 (19)	0	0	10 (31)	8 (13)
Dientamoeba-positive (%)	2 (9)	3 (15)	1 (5)	7 (33)	0	1 (13)	5 (16)	9 (14)
Gender (% M)	32	45	55	43	56	63	31	91
Age, median	39,5	49,5	38	49	45	43	33,5	20
Range	23–76	23–73	28–72	23–87	25–84	26–60	19–73	19–30

Active Crohn's disease (CDA), inactive Crohn's disease (CDI), active ulcerative colitis (UCA), inactive ulcerative colitis (UCI), active pouchitis (PA), inactive pouchitis (PI), healthy controls, medical and laboratory staff (HC^M) and recruits (HC^R). Male (M).

microbial challenges to the intestinal immune system are limited in the western parts of the world, and that a continuous microbial challenge is necessary to remain healthy [72]. In this context, it has been speculated that the increased prevalence of IBD could be associated with the decreased prevalence of intestinal helminths, when the distribution of these diseases is compared on a global scale [73]. Furthermore, ingestion of helminths, such as *Trichuris suis* ova, has been evaluated as treatment against flares of IBD, although no final conclusion can be made regarding the efficiency of this treatment [74,75]. These data underline the extremely complex nature of the human intestinal microbiome and the possible links to health and disease of a general intestinal low diversity dysbiosis. An obvious strategy is to demonstrate that restoring a healthy high diversity microbiome can prevent the induction of or cure intestinal disorders such as IBD. As demonstrated in our recently published study, Paper III, it is not evident that simply restoring a reduced diversity microbiome with a high diversity microbiome will cure all intestinal dysbiosis-related illnesses. In our placebo-controlled study of fecal microbiota transplant (FMT) treatment of patients with IBS, we found that FMT capsules based on a mixture of fecal donor material from 4 healthy donors increased the diversity of the gut microbiome significantly in IBS patients treated with FMT compared to IBS patients treated with placebo, Figure 2. Furthermore, it was shown that bacteria originating from the donors were established in the recipients for at least six months. But surprisingly, patients in the placebo group experienced greater symptom relief compared with the FMT group after 3 months.

In this context, it is interesting that low-grade inflammation is described both in IBS and in quiescent IBD [76], and that paracellular permeability was significantly increased in both quiescent IBD with IBS-like symptoms and IBS compared with quiescent IBD without IBS-like symptoms [77]. Proteobacteria and Bacteroides have been shown to be increased in patients with IBS compared with controls, whereas uncultured Clostridiales, *Faecalibacterium prausnitzii* and *Bifidobacterium* were decreased in patients with IBS [78]. Similar microbiome changes are also seen when comparing IBD patients to healthy controls as already described. It is, however, important to note that FMT has shown promise as a possible treatment of active UC. In a meta-analysis by Costello et al. [79] including 4 placebo-controlled trials of FMT for UC, it was reported that clinical remission was achieved in 39 of 140 (28%) patients in the FMT groups compared with 13 of 137 (9%) patients in the placebo groups. In FMT-treated UC patients, microbial diversity increased with and persisted after FMT [80], similarly to what we have shown in FMT-treated IBS patients, Paper III. Interestingly, stool of donors with a high bacterial richness and a high relative abundance of *Akkermansia muciniphila*, unclassified *Ruminococcaceae* and *Ruminococcus* spp. have been found to be more likely to induce remission in UC [81]. Importantly, it is evident that in the future the use of more advanced sequencing techniques will provide further knowledge regarding the involvement and function of the intestinal microbiome in IBD pathogenesis. 16 S rRNA sequencing mainly provides us with knowledge of involved bacteria. Unquestionably more data on metagenomic, metabolomic and proteomic profiles of IBD patients (with and without flares) and

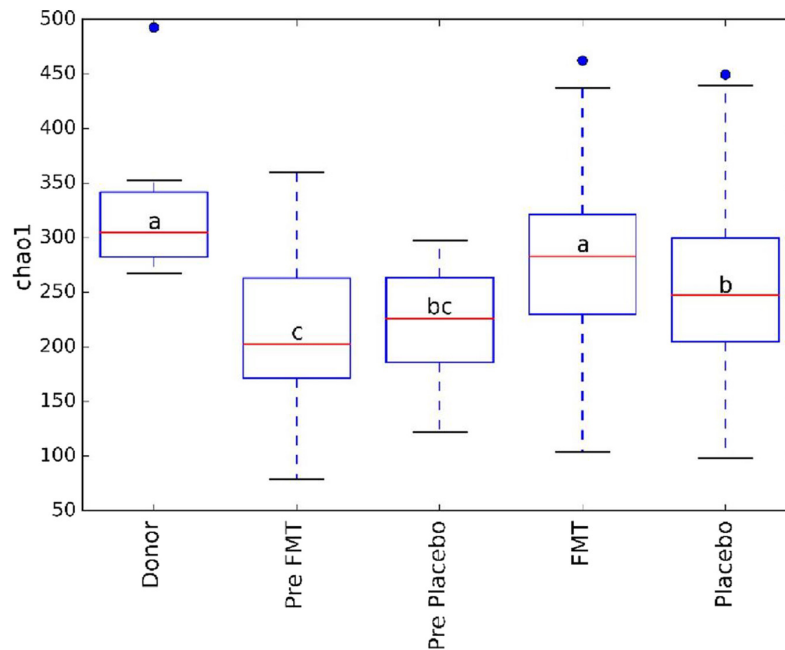


Fig. 2. Paper III. α -Diversities of donors, IBS patients before FMT treatment at inclusion (Pre FMT), placebo IBS patients before placebo treatment at inclusion (pre-placebo) and FMT-treated IBS patients (FMT) and placebo-treated IBS patients (Placebo).

controls will be able to further elucidate IBD pathogenesis, both regarding the involvement of other microorganisms than bacteria and the immunological regulation performed by the IBD-related dysbiosis, but these data are limited [62].

In this light, it does seem reasonable that individual microorganisms with a possible pathogenic potential have been subject to an increased number of studies within the field of IBD research. Specifically, the low diversity dysbiosis linked to IBD includes an expansion of facultative anaerobes of the Enterobacteriaceae family (Proteobacteria) [82], increased Proteobacteria abundance has been found associated with both UC patients with an ileal-pouch anal anastomosis after a colectomy and a history of pouchitis, Paper IV, Figure 3 and Crohn's disease patients with an aggressive disease course, [83]. Increased abundance of Proteobacteria was in our study not associated with acute pouch inflammation in patients with a history of pouchitis, Paper IV, whereas the abundance of Fusobacteria was. In a recent paper, fecal metagenomics showed an increased abundance of Proteobacteria and

Fusobacteria to be linked to future relapse in patients with IBD [84]. *Escherichia coli* has been found in increased numbers in fecal and mucosal samples in patients with both UC and CD suggesting a possible involvement of *E. coli* in both diseases [85]. In a recent review of gut microbiome differences between IBD patients and controls, Proteobacteria was, at the phylum level, highlighted as possibly associated with both UC and CD. Interestingly, of all potentially harmful bacteria associated with IBD, increased *E. coli* was found to be the most consistent finding [86]. IBD patients had compared with controls enrichment of bacterial virulence factors linked to *E. coli* [87] and the treatment naïve microbiome in newly onset IBD is especially enriched in *E. coli* [88]. A recent paper using metagenomics and studies of co-abundance of species, again confirmed the association of a high level of *E. coli* with IBD [89]. Most studies do not examine the microbiome differences at the strain level, so there is still a lack of studies thoroughly determining if the increased presence of *E. coli* in IBD patients is due to colonization with especially virulent or commensal strains. These

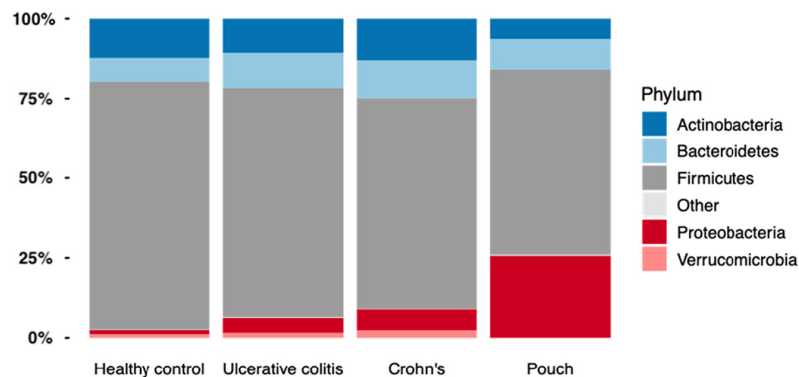


Fig. 3. Paper IV. The relative abundance of bacterial phyla found in inflammatory bowel disease (Crohn's disease, $N = 58$ and Ulcerative colitis, $N = 82$), controls, $N = 30$, and in patients with an ileo-anal pouch anastomosis after colectomy for UC and a history of pouchitis, $N = 20$.

factors highlight that the search for a possible link between emerging enteropathogenic *Escherichia coli* (pathobionts) and IBD, as described in the following, is of major importance.

PATHOGENIC *E. COLI* IN INTESTINAL AND EXTRAINTestinal DISORDERS

Diarrheagenic: acute gastroenteritis and chronic diarrhea

Even though *E. coli* does not constitute the major part of the intestinal microbiome, *E. coli* has the capacity to be both a peaceful commensal and a pathogen with links to a wide range of human infectious diseases. All depend on the virulence genes associated with the colonizing *E. coli* strain. *E. coli* is part of the intestinal microbiome in over 90% of humans, and even though *E. coli* strains are outnumbered by anaerobic bacteria, they do constitute the predominant aerobic microorganism in the human intestine. Furthermore, *E. coli* strains are some of the earliest colonizers of a child's intestine just after birth [90]. Most frequently these *E. coli* are commensal, harmless symbionts [91], natural inhabitants of the intestine not causing any diseases. The commensal *E. coli* strains are often found in specific phylogenetic groups (phylogroup A and B1) [92], and they are furthermore free of virulence genes normally associated with intestinal and extraintestinal disease [92]. In addition, different commercially available

E. coli strains have been used as probiotics [93]. Early on it was, however, discovered that certain subtypes of *E. coli* are linked to infectious diarrhea: this group of *E. coli* has steadily increased in the number of pathotypes, and now includes enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), verotoxin producing *E. coli* (STEC), enteroinvasive *E. coli* (EIEC), diffusely adherent *E. coli* (DAEC) and enteroaggregative *E. coli* (EAEC) [94,95]. Many of these pathotypes are defined by specific genetic characteristics that are now easily found by PCR tests. EPEC is most often linked to childhood diarrhea, ETEC and EAEC to tourist diarrhea, EIEC to more severe disease often represented by bloody diarrhea, STEC to bloody diarrhea and frequently complicated by hemolytic uremic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP), and the emerging pathogens DAEC and EAEC have been linked to chronic diarrhea and gastrointestinal disease in the developing world [94]. The different *E. coli* pathotypes elicit their pathogenicity through adhesion, invasion and/or toxin production, e.g., EPEC infection diarrhea results from increased ion secretion, increased intestinal permeability, intestinal inflammation and loss of absorptive surface area resulting from microvillus effacement [96]. ETEC strains produce a heat-stable enterotoxin (ST) and a heat-labile (LT) cholera toxin-like enterotoxin, both toxins cause increased secretion of Cl^- from secretory crypt cells, which culminates in diarrhea [95]. EIEC shares pathogenic

mechanisms with *Shigella*, and pathogenesis initially involves epithelial cell penetration, followed by lysis of the endocytic vacuole, intracellular multiplication, directional movement through the cytoplasm, extension into adjacent epithelial cells and finally the induction of apoptosis in infected macrophages and the release of IL-1 β [97]. The pathogenic mechanisms of STEC are linked to both adherence and toxin production, STEC adheres to gastrointestinal epithelial cells via the bacterial outer membrane protein, intimin [98], similar to the adherence mechanisms of EPEC. Then, the STEC toxin is transported into the kidney via blood or by transmigration of neutrophils (PMN) [99]. EAEC pathogenic mechanisms initially involve bacterial adherence to the intestinal mucosa via aggregative adherence fimbriae. EAEC surface structures along with its release of flagellin and toxins cause inflammation by inducing the release of IL-8, stimulating neutrophil transmigration across the epithelium and as a result tissue damage [100]. This implies that the complexity of the diarrheagenic *E. coli* is ever-changing, with new emerging infectious *E. coli*, identification of new virulence factors and new mixtures of genes linked to diarrhea. The outbreak in 2011 of a HUS-associated *E. coli* in Germany is a frightening example [101]. This specific *E. coli* turned out to be an EAEC incorporating virulence factors from STEC resulting in more serious disease outcomes, including an increased death rate, in comparison to the previously known STEC strains.

Extraintestinal pathogenic *E. coli*

Escherichia coli out of their natural intestinal habitat, are linked to urinary tract infection, to septicemia and to neonatal meningitis [102]. *Escherichia coli* strains found outside the intestine and linked to infectious diseases are defined as Extraintestinal Pathogenic *E. coli* (ExPEC). The definition of an ExPEC prototype is, however, still obscure; in some sense, the definition, as mentioned, includes any *E. coli* found outside the intestinal tract and linked to disease. *E. coli* strains can, however, be classified into four phylogenetic groups; A,

B1, B2 and D. Groups A and B1 are, as already mentioned, usually commensal strains and carry few virulence genes, whereas the more pathogenic groups B2 and D often possess virulence genes which are associated with persistence, adhesion and extraintestinal infection [92,103]. *Escherichia coli* strains with the ability to cause infection outside the intestine are most often found in the phylogenetic group B2 and, to a lesser degree, in phylogroup D [103, 104]. B2 *E. coli* has been found to differ considerably from other phylogroups based on MLST typing, and to show the lowest intergroup recombination frequency compared with the other phylogroups [105,106]. ExPEC is furthermore often found to have specific genetic characteristics, the so-called ExPEC genes, such as *papA* (P fimbriae), *papC* (pilus assembly), *afa* (afimbrial adhesion), *sfa/foc* (S fimbriae/F1C fimbriae), *iut* (aerobactin system) and *kpsM* (capsular synthesis) [107,108]. Some of these traits are also colonization factors (e.g., P fimbriae, siderophore systems, toxins) and not as such classical virulence factors [104]. These specific abilities would give ExPEC isolates an advantage as colonizers of the intestinal tract, which perhaps makes it more likely, that these specific *E. coli* can cause disease extraintestinally in patients with an unbalanced intestinal microbiome due to underlying illness, diarrhea or antibiotic treatment. Even though ExPEC does frequently belong to specific lineages, and are often carrying specific colonization/virulence genes, they are probably best defined as facultative pathogens. Increased typing, including whole-genome sequencing of ExPEC isolates from various sites compared with commensals, might in the future give a more precise definition of ExPEC. Presumably, commensals, of non-common phylogenetic lineages and without specific virulence traits, will probably still be found as extraintestinal pathogens in subgroups of especially immune impaired patients. However, if the genetic characteristics of the classical ExPEC could also be directly linked to the pathogenesis of intestinal disease is uncertain; the link of intestinal colonization with ExPEC to IBD will be described in the following.

***ESCHERICHIA COLI* PATHOTYPES IN ULCERATIVE COLITIS AND CROHN'S DISEASE**

Involvement of specific *E. coli* pathotypes (e.g., ExPEC) in UC

It was observed as early as in the 1970s that *E. coli* isolated from patients with active UC, were of specific serotypes, most often serotypes linked to urinary tract infections [109]. In addition, these *E. coli* were found to be hemolytic [110]. However, the changes in the dominance of specific *E. coli* subtypes between UC in remission and UC during flares were attributed to possible substrate effects, which would imply that their presence in the colon was assisted by the inflammation but did not cause it. Subtypes of *E. coli* strains have developed a greater ability to acquire iron (heme receptor and numerous siderophores) [111], which in an inflamed, ulcerated and bleeding intestine will be an advantage for these *E. coli*. Diarrheagenic *E. coli* subtypes have been isolated from fecal samples from IBD patients, most often described as acute infections masking or initiating a flare of the disease, where antibiotics have been described as efficient in controlling the disease in these specific cases [42,112]. Recently, through metagenomics in a pediatric population comparing IBD patients with their healthy siblings, a strong correlation to IBD has been found with the abundance of bacterial virulence genes, enriched specifically in the UC microbiome with *E. coli* abundance, suggesting that *E. coli* is a central driver in UC pathogenesis [113]. Furthermore, after culture, specific phylogenetic groups of *E. coli* were found to be more frequent among patients with CD and UC [114]. In addition, an increased inflammatory response to *E. coli* has been found in patients with UC [115]. Finally, it has been shown that *E. coli* are very predominant in inflamed mucosa of patients with UC, and that these strains based on 16S rRNA PCR are 'active' and overrepresented in comparison with the microbiome of healthy controls, which generally had much higher biodiversity [116].

Subcharacterization and phylogenetic typing of *E. coli* isolated from IBD patients have revealed that they are not only of very distinct

serotypes, but they also frequently belong to the phylogenetic group B2, which is often linked to ExPEC [114,117], Paper V, Figure 4. Phylogenetic groups can be determined by multilocus ribotyping but can also be determined by a simple and rapid phylogenetic grouping technique based on triplex PCR, based on a combination of two genes (*chuA* and *yjaA*) and an anonymous DNA fragment [103], resulting in the previously mentioned phylogroups, A, B1, B2 and D. When comparing the number of B2 *E. coli* with at least one positive ExPEC gene, 6 of 7 were found positive among IBD patients with active disease, 1 of 8 among IBD patients in remission ($p < 0.05$) and 1 of 9 among healthy controls ($p < 0.05$), Paper V. This further lends support to the observation that these IBD-associated *E. coli* are comparable to uropathogenic *E. coli* (UPEC), which by definition, is part of ExPEC and which often, as previously mentioned, are of the phylogenetic group B2. In a meta-analysis, Paper VI, five studies reported the risk of *E. coli* phylogenetic group B2 colonization among patients with UC compared with controls, Figure 5, Random-effects meta-analysis showed that patients with UC had an increased risk of being infected/colonized with these *E. coli* (OR 3.364; 1.456 to 7.775; I² 0%). After excluding patients without active disease, patients with active UC were even more likely to be infected than were controls (OR 4.417; 1.067 to 18.289; I² 40%).

UC patients with an ileal-pouch anal anastomosis after a colectomy frequently develop pouchitis. This condition could be speculated to be an interesting model of UC microbiome-related pathogenesis, due to the established effect of antibiotics, e.g., with metronidazole or ciprofloxacin in pouchitis, where traditional treatment of UC, in the form of immunomodulators, seems less efficient [118]. In this context, it was surprising that no link to any phylogenetic *E. coli* was found when comparing active to inactive pouchitis, Paper IV, instead we found *Fusobacteria* abundance linked to current pouch inflammation. After surgical treatment of UC, the target organ for the possible link between Proteobacteria abundance and, specifically, phylogroup B2 *E. coli* and IBD are no longer present. It might, therefore, not be surprising that a similar link

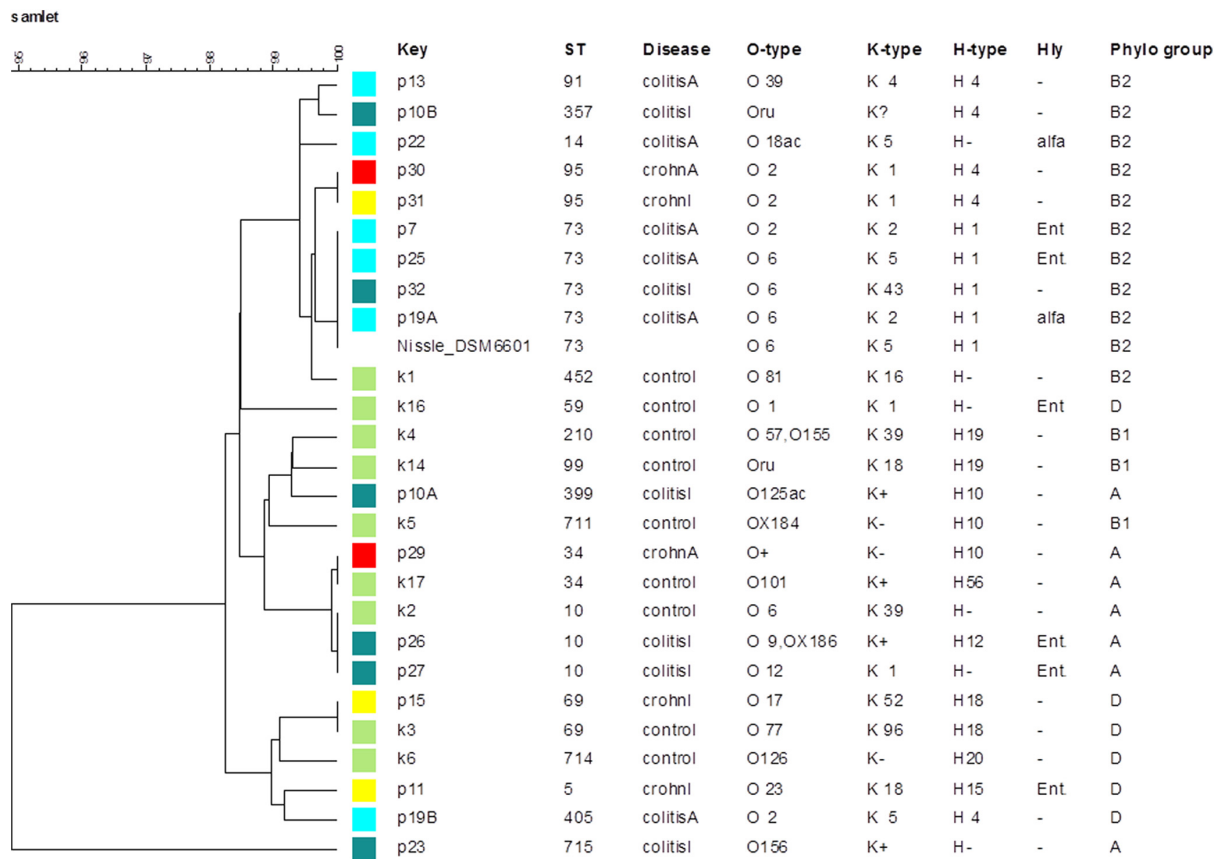


Fig. 4. Paper V. Phylogenetic tree based on multilocus sequence typing of *Escherichia coli* isolated from fecal samples from patients with active and inactive IBD, all with past or present involvement of the left side of the colon, and from controls. Also presented are serotype and phylogenetic groups A, B1, B2 or D (based on a simple triplex PCR). ColitisI, inactive ulcerative colitis; colitisA, active ulcerative colitis, crohnI, inactive Crohn's disease; crohnA, active Crohn's disease; ST, sequence type. The probiotic strain *E. coli* Nissle has been added to the phylogenetic tree for comparison.

between inflammation and *E. coli* B2 colonization cannot be found in pouchitis.

At present IBD-associated B2 *E. coli* cannot be differentiated from ExPEC strains isolated from non-intestinal samples, even when testing multiple genes [117]. Others have found the IBD-associated *E. coli* to possess specific adherence factors, identifying these *E. coli* as diffusely adherent *E. coli* (DAEC) [119]. However, ExPEC has certain interesting virulence profiles, such as the production of α -hemolysin [120], and in animal studies, α -hemolysin-producing *E. coli* has been found to be associated with increased intestinal inflammation [121]. Furthermore, α -hemolysin producing *E. coli* has in our research groups in *in vitro* studies using epithelial cell lines been found to cause

disruption of tight junctions [122,123]. *Escherichia coli* α -hemolysin has also been demonstrated to cause focal leaks in epithelial cells in a mouse model and increased levels of α -hemolysin in inflamed intestinal tissue from patients with UC [124]. In our own study, hemolytic *E. coli* were isolated more frequently from patients with IBD, 7 of 15, compared with healthy controls, 1 of 9; this difference did, however, not reach statistical significance ($p = 0.18$), Paper V. The clonal nature of *E. coli* isolated from IBD patients contradicts the possible assumption that IBD through an impaired immune system allows an overgrowth of *E. coli* at random. It remains to be determined, whether the demonstrated relationship with ExPEC, is a marker of inflammation,

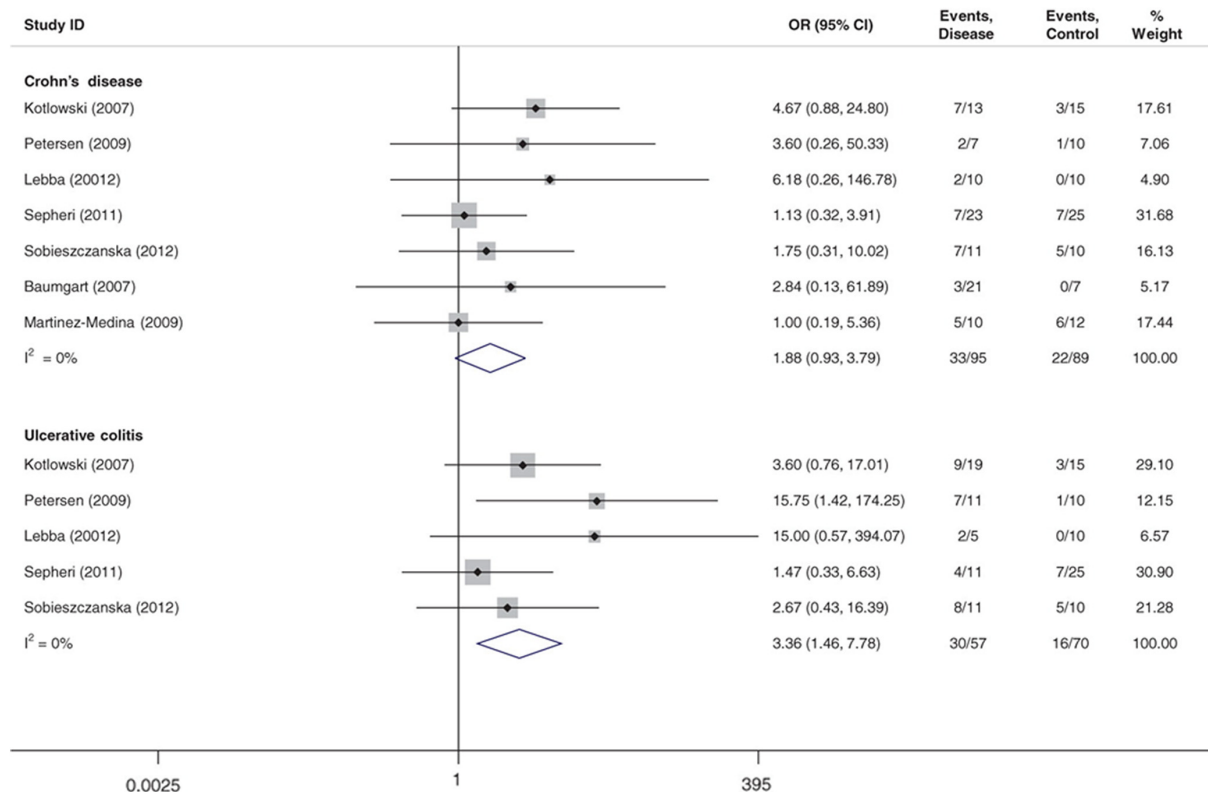


Fig. 5. Paper VI. Odds of phylogenetic group B2 *E. coli* colonization in ulcerative colitis patients and Crohn's disease patients compared with controls. Random-effects meta-analysis showed that patients with UC had an increased risk of being colonized with B2 *E. coli* (OR: 3.364; 95% CI: 1.456–7.775; I^2 0%). There was no difference between patients with CD and controls (OR: 1.882; 95% CI: 0.934–3.794; I^2 0%). Analysis comparing phylogenetic group B2 colonization and the odds of IBD for patients with CD or UC shows no difference between subgroups ($p = 0.25$).

possibly based on a substrate effect as described, or if ExPEC could serve as a trigger of flares of IBD.

Involvement of specific *E. coli* pathotypes (e.g., AIEC) in CD

Among patients with CD *E. coli* has also been found in increased numbers in fecal samples, and furthermore, within the intestinal mucosa and within granulomas and macrophages [125]. In 1978, Keighley observed a modification of luminal bacteria concentrations in CD patients with a significant increase in *E. coli* [126]. Additionally in 1988, a significantly higher index of adhesion in *E. coli* present in active CD patients was found in comparison to controls [127]. *Escherichia coli* strains isolated from the ileal mucosa of patients with Crohn's disease

were found to adhere to intestinal cell lines at a high degree compared with controls, and furthermore, Boudeau et al. [128] found that *E. coli* isolated from the terminal ileum of patients with CD were especially invasive upon attachment to epithelial cells. This led to the definition of a new subtype of *E. coli*, given the name adherent invasive *E. coli* (AIEC) [129]. Especially interesting is the fact that AIEC has been found to be present at first diagnosis of IBD, suggesting that they may have a role in the early stages of disease onset, even before IBD treatments may have influenced the microbiome [130]. The diagnosis of these *E. coli* is, however, only based on phenotypic tests such as adherent-invasive assays, and not on PCR tests for specific virulence genes, such as it is possible for the other well-defined intestinal pathogenic *E. coli*.

Interestingly, the prototype AIEC strain LF82 is of the phylogenetic group B2, even though it is unknown whether all AIEC are B2 *E. coli* [131]. In our meta-analysis, Paper VI, six studies reported the risk of *E. coli* phylogenetic group B2 colonization among patients with CD compared with controls, Figure 5. There was no difference between patients and controls when including all patients (1.882; 0.934 to 3.794 I2 0%) or when including only patients with active CD (1.554; 0.671 to 3.601 I2 0%). The possibility remains that UC *E. coli* and CD *E. coli* are truly two different entities, each with specific pathogenic features.

ESCHERICHIA COLI PATHOTYPES IN IBD; THE CIRCUMSTANTIAL EVIDENCES

Serology

IBD is, as described previously, primarily diagnosed based on endoscopic, histological and/or radiological and, not least, clinical criteria. However, several serological markers have been linked to IBD; including pANCA: Perinuclear anti-neutrophil cytoplasmic antibody; Anti-pancreas antibody; ASCA: Anti-*Saccharomyces cerevisiae* antibody; ACCA: Anti-chitobioside carbohydrate antibody; ALCA: Anti-laminaribioside carbohydrate antibody; AMCA: Anti-mannobioside carbohydrate antibody; OmpC: Outer-membrane porin C: Anit-Cbir1 antibody (a Clostridial flagellin protein); Anti-I2 antibody (a *Pseudomonas*

fluorescens-associated protein). The two first mentioned are auto-antibodies, but the last seven are microbial antibodies [132]. Only pANCA and ASCA will reach reasonably high positive rates in UC and CD, respectively, positive pANCA is found in approximately 50% of patients with UC [133] and positive ASCA is found in approximately 40% of patients with CD [134]. Among the microbial antibodies linked to IBD is anti-Omp-C, which interestingly are antibodies against an Outer Membrane Protein found in *E. coli* [135]. The seroprevalence for anti-OmpC is reported to be higher in CD patients than in UC patients [136]. We did, however, in our study find a similar level of positivity in CD and UC patients, Paper VII, Table 5. CD patients have been reported to be at a higher risk for progression of disease, fibrostenosing/perforating disease and small bowel surgery, if they were positive for OmpC antibodies [137]. Furthermore, patients with 2 or more resections were more likely to be anti-OmpC positive, and after an operation for stenosing or structuring Crohn's disease, recurrence of disease was more frequent in patients positive for anti-OmpC at the time of operation [138]. The presence of ANCA has not correlated with activity in UC [139], and similarly, the presence of ASCA has not correlated with activity in CD [140]. Furthermore, the seropositive/seronegative antibody status remains relatively stable over time in both UC and CD [141]. However, since significantly more patients with active IBD were found to be colonized with ExPEC strains

Table 5. Paper VII. Prevalence of serological markers in patients with active and inactive inflammatory bowel disease and in controls

Diagnosis	ANCA-positive (%)	ASCA-positive (%)	OmpC-positive (%)
UCA	48	0	48
UCI	50	0	46
CDA	25	5	15
CDI	23	13	52
PA	63	0	50
PI	36	0	45
K	0	0	11
KD	0	0	9
AG	15	3	3

Active Crohn's disease (CDA), inactive Crohn's disease (CDI), active ulcerative colitis (UCA), inactive ulcerative colitis (UCI), active pouchitis (PA), inactive pouchitis (PI), control (K), chronic diarrhea from non-IBD causes (KD) and a group of patients tested positive for *Yersinia*, *Campylobacter* or *Salmonella* (AG).

compared to IBD patients with inactive disease, Paper V, theoretically these more invasive *E. coli* could result in an increase in OmpC antibody levels during active disease. Among IBD patients with a culture of phylogroup B2 *E. coli*, we did in fact find a higher number of patients, 65%, being positive for anti-OmpC, compared with 33% being positive among patients with the culture of non-B2 *E. coli*, $p < 0.05$, Paper VII. A future perspective of the finding of a more frequent anti-OmpC positivity among patients with B2 *E. coli* could be a study comparing the effect of antibiotics in IBD patients based on both their anti-OmpC level and the presence of B2 *E. coli* in fecal samples. Interestingly, in CD patients with a positive anti-OmpC test, it has previously been shown that their clinical response to corticosteroids plus antibiotics is better than to corticosteroids alone, whereas patients with a negative anti-OmpC test responded better to corticosteroids alone [142].

In the study of OmpC antibodies, we did not find any association with immunomodulating therapies and the antibody level of the serological markers examined, Paper VII. However, immunosuppressant treatments do influence serological responses as has been seen in other studies including our own study, Paper VIII. When comparing the antibody response after pneumococcal vaccination

between 3 treatment groups (no treatment, thiopurines alone or thiopurines with TNF- α), it was shown that patients receiving immunosuppressing treatment with thiopurines and TNF- α have a lower antibody response to pneumococcal vaccination than both patients treated with thiopurines and untreated patients, Paper VIII, Table 6. Furthermore, patients with IBD also have an impaired immune response to influenza vaccination among those on immunosuppressive therapies [143], and the same has been concluded regarding Hepatitis B vaccination [144]. When testing for exposure to tuberculosis among patients with IBD, it was found that prednisolone treatment significantly impaired interferon-gamma response to mitogen stimulation compared with patients not receiving corticosteroids. Furthermore, prednisolone treatment was strongly associated with a negative tuberculin skin test. Single use of azathioprine, methotrexate or 5-aminosalicylate (5-ASA) did, however, not affect these test results [145]. This underlines that both microbiome associations and serological tests in IBD should always be evaluated in the context of current medical treatment.

Mouse models

Several IBD mouse models exist examining IBD pathogenesis and treatment, such as

Table 6. Paper VIII. Antibody response as geometric mean concentrations (in $\mu\text{g/ml}$). (post-vaccination concentration – pre-vaccination concentration) in patients with CD after pneumococcal vaccination with the 23-valent pneumococcal polysaccharide vaccine or the 13-valent pneumococcal conjugated vaccine. Patients were divided according to treatment with thiopurines alone (IS), treatment with thiopurines and TNF- α inhibitors (IS + TNF), or no current treatment (Untreated). Colored according to value

	IS		IS + TNF		Untreated		Color scale
	PPV23	PCV13	PPV23	PCV13	PPV23	PCV13	
Serotype 1	0.27	0.27	0.12	0.11	0.21	0.39	<0.5
Serotype 3	0.39	0.29	0.07	0.08	0.32	0.40	<1
Serotype 4	0.42	0.44	0.28	0.30	0.29	1.23	<2
Serotype 5	0.51	0.27	0.20	0.31	0.78	1.10	<4
Serotype 6B	0.90	0.88	0.37	0.92	1.09	2.17	<6
Serotype 7F	1.12	1.48	0.54	0.49	1.21	2.26	<8
Serotype 9V	0.36	0.88	0.17	0.35	0.56	2.54	<10
Serotype 14	2.51	4.29	2.57	0.90	4.43	9.95	<12
Serotype 18C	1.25	2.53	0.37	1.12	2.38	7.64	
Serotype 19A	0.36	0.54	0.24	0.30	0.82	1.06	
Serotype 19F	1.23	1.02	0.62	0.41	1.79	3.72	
Serotype 23F	1.01	2.38	0.98	4.41	2.48	10.29	

IS, immunosuppressive drugs.

chemically induced colitis models, genetic models (the major group), adoptive transfer models and spontaneous models [146]. *Escherichia coli* infection has been tested frequently in some of these IBD mouse models, signifying the possible role of *E. coli* in IBD. Colonization with specific *E. coli* leads to increased inflammation in mice with disrupted T-cell homeostasis (*Rag1*^{−/−} mice), whereas LPS mutants of these *E. coli* prevented inflammation [147]. Hemolysin-producing *E. coli* induces focal leaks in the intestinal epithelium in mouse models, including both wildtype and Il-10 deficient mice, thus increasing intestinal inflammation in susceptible mouse models [124]. AIEC has proved especially efficient in inducing inflammation in several IBD mouse models [148–150].

Furthermore, the probiotic *E. coli* strain Nissle 1917 (EcN) was reported to maintain remission of ulcerative colitis and pouchitis and to prevent colitis in dextran sodium sulfate (DSS) induced colitis murine models [151–155]. Part of EcN's probiotic abilities is presumably linked to its ability to prevent the colonization of the gut with pathogenic microorganisms. The proposed mechanism behind this ability is the production of both a strong biofilm and of two microcins by EcN [156,157]. It has been shown in the streptomycin-treated mouse model that EcN can limit the growth of pathogenic *E. coli* O157 when

administrated as treatment in pre-colonized mice [158]. These facts made it plausible that EcN or a combination of an antimicrobial and EcN could be efficient in eradicating *E. coli* pathotypes associated with IBD, and thereby possibly abolishing a trigger mechanism involved in IBD disease flares. Previously, the streptomycin-treated mouse model was developed under the assumption that streptomycin treatment would eliminate the gram-negative microbiota from the gut, while leaving intact the Gram-positive and anaerobic microbiota making this model relevant when testing the ability of Gram-negatives to colonize the gut [159]. We tested the ability of ciprofloxacin and/or EcN to eradicate phylogroup B2 *E. coli* strains isolated from patients with active IBD in the streptomycin-treated mouse intestine, Paper IX. However, introduction of EcN did not result in any notable changes in the colonization capacity of tested B2 IBD-associated *E. coli* strains; instead, co-colonization was obtained. Pretreatment of inoculated mice with ciprofloxacin before introduction of EcN revealed an apparent efficient eradication of the tested strains, although one of the IBD-associated *E. coli* strains was surprisingly able to reappear 4 days after ciprofloxacin treatment, Figure 6. Several mechanisms could be speculated, first that a subset of tested *E. coli* became ciprofloxacin-resistant, possibly surviving at a dormant level. Their reappearance is

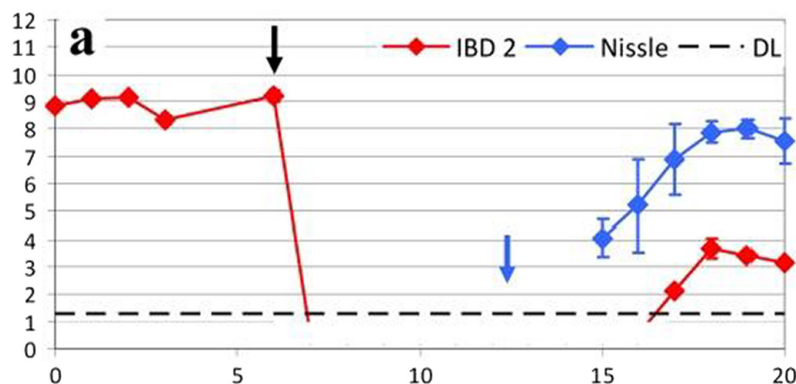


Fig. 6. Paper IX. Three days treatment with ciprofloxacin and a subsequent treatment with *E. coli* Nissle daily in mice pre-colonized with IBD-associated *E. coli*. Sets of three mice were used in each experiment. CFU counts of the inoculated strains from fecal samples of mice. CFU of the inoculation suspension is shown at day 0 ($\sim 10^9$ CFU/mouse). Black arrow indicates the initiation of ciprofloxacin treatment from day 6–9 (0.2 mg/mouse) every 6h. Blue arrow indicates the initiation of inoculation with *E. coli* Nissle strain at high levels ($\sim 10^9$ CFU/mouse) every day throughout the experiment. Each graph represents the CFU counts of three mice, and bars represent SEM; detection limit (DL) at 20 CFU/g feces.

perhaps effectuated by EcN, since EcN has a marked ability to form biofilm thereby delivering the perfect microenvironment for other *E. coli* [157].

It must, however, be emphasized that colonization is only the first step in infection and that the streptomycin-treated mouse intestine is only a model for *E. coli* colonization and not pathogenesis. Furthermore, it might have been more appropriate to use one of the colitis mouse models also in this line of colonization experiments. Even though we did not find any effect in our mouse model regarding colonization with IBD-associated phylogroup B2 *E. coli*, this does not rule out that EcN in the human intestine interacts with possible harmful *E. coli* by blocking their attachment to epithelial cells, perhaps even in a more profound way in the inflamed intestine. This theory could be in concordance with a study showing EcN's ability to block the adherence of AIEC in experiments with a human intestinal cell line [160]. As previously mentioned, it has been demonstrated that different *E. coli* strains, including experiments with EcN, can co-exist based on the utilization of different nutrients [161]. However, it was also shown that infection with three different non-pathogenic *E. coli* including EcN was able to prevent recolonization with a pathogenic (enterohemorrhagic) *E. coli* [158]. Therefore, it cannot be ruled out that EcN with the 'assistance' of other commensal Gram-negative bacteria would be successful in preventing recolonization with *E. coli* pathotypes in the non-inflamed human intestine.

Epithelial cell models

Escherichia coli pathotypes associated with IBD have been tested in immortalized intestinal epithelial cell models and in models of infection-related cells such as macrophages. AIEC has been found to adhere to intestinal epithelial cells, to invade intestinal epithelial cells via a macropinocytosis-like process and to survive and replicate intracellularly in intestinal epithelial cells (Caco-2 cells) and macrophages [128,162,163]. However, we have also tested hemolysin-producing B2 *E. coli* (with ExPEC characteristics) from patients with active UC in Caco-2 cells, demonstrating

the ability to interrupt the epithelial cell barrier through degradation of tight junctions [122,123]. This phenotype was lost in a mutant with knock-out of both hemolysin loci ($p < 0.001$). In another set of experiments, we tested viable IBD-associated *E. coli* cultured with human monocyte-derived dendritic cells (moDCs) and intestinal epithelial cells (Caco-2 cells), followed by analysis of secreted cytokines and cellular death. Two *E. coli* isolates (phylogroup B2) isolated from patients with ulcerative colitis induced enhanced killing of moDCs and IECs, coupled with elevated IL-18 [164]. The cytopathic nature of these two IBD *E. coli* isolates, in contrast to other tested *E. coli* subtypes, suggests that colonization with specific non-diarrheagenic B2 *E. coli* could induce intestinal barrier defects and considerable intestinal inflammation. One important limitation of results from these immortalized epithelial cell lines is the lack of a mucus layer, e.g., an adherent mucus layer on epithelial cells has been shown to attenuate the production of colibactin. Colibactin, which is often found in phylogroup B2 *E. coli*, induces DNA double-strand breaks (DSBs) in eukaryotic cells [165]. Removal of the adherent mucus layer restored the occurrence of DNA-DSBs. If something similar could be found regarding hemolysin production in phylogroup B2 *E. coli* is unknown.

Antibiotic treatment

Antibiotics have long been known to have a place in the treatment of IBD, especially in fistulizing Crohn's disease and in pouchitis. Further, during flares of both CD and UC, antibiotics are often used in clinical practice, due to a suspicion of bacterial translocation. In a review and meta-analysis, it was concluded that antibiotics might also play a role in the maintenance of remission in post-operative CD [166]. In a meta-analysis of placebo-controlled trials, it was concluded that antimicrobials have some effect in the treatment of both CD [167] and UC [50,51], but the effect of antibiotics is believed to be short-lived.

Many different regimes have been used. Antibiotics directed against Gram-negative bacteria, such as *E. coli* has proven especially efficient, e.g., in pouchitis and when treating

fistulizing CD [167,168], but long-term effects have not been demonstrated. Data for ulcerative colitis consists of small prospective trials evaluating ciprofloxacin, metronidazole and rifaximin, and most trials did not show a benefit for the treatment of active ulcerative colitis with antibiotics, even though the previously mentioned meta-analyses concluded that antibiotic therapy is associated with a modest improvement in clinical symptoms [169]. However, papers are very diverse regarding both antibiotic type and length of treatment and in no cases has a cure for IBD been proposed. It could, therefore, be speculated that the beneficial effect is primarily linked to the effect on superinfections imposed on patients in relation to the immune-modulatory treatment that these patients have often received, e.g., effect of Metronidazole on IBD, where the effect on a common occurrence in IBD patients, such as an underlying *C. difficile* infection, could explain the positive results. In our own study, Paper X, antibiotic treatment (Ciprofloxacin) did not benefit patients with moderately active UC, although, it did not seem to worsen their outcome either.

Escherichia coli Nissle

Interestingly, it has been found that the probiotic *E. coli* Nissle (EcN) has an equivalent effect to mesalazine (5-ASA) in preventing disease flares in patients with UC [170]. Furthermore, the ability of EcN to induce remission in patients with active disease has also been found comparable to 5-ASA [171]. It has been shown that 5-ASA does not have an antimicrobial effect on EcN [172], and therefore, a possible additive effect of EcN to the standard of care in UC may be possible. Our study, Paper X, is the first randomized double-blinded study to evaluate the efficacy of orally administered EcN as an add-on treatment to conventional medical therapies in relapsing UC. Our hypothesis was, that treatment with EcN would result in more patients in remission at the end of our study, and secondarily, that patients treated with EcN would reach remission faster, without differences in withdrawal rates. Surprisingly, we observed that significantly fewer patients treated with EcN reached remission and that significantly more patients treated with EcN withdrew

from the study compared with the placebo-treated patients. A cautious interpretation is reasonable regarding the study power, since our patients treated with 7 weeks of EcN or placebo, were also randomized to initial treatment with 1 week of Ciprofloxacin or placebo. However, this approach revealed that patients treated with EcN as add-on treatment without an initial antibiotic cure, did significantly worse also in the per-protocol analysis, than did patients receiving only placebo-placebo treatment alongside the standard of care medication given to all patients participating in this study, Figure 7. EcN is phylogenetically surprisingly similar to the *E. coli* strains we found in active UC, and also of the phylogenetic group B2, Figure 4, thus presumably sharing many traits with the IBD-associated *E. coli* pathotypes [173]. This might make EcN especially suited as a replacement for the IBD-associated *E. coli* in their specific niche, especially since EcN lacks i.e., alpha-hemolysin, P-fimbrial adhesins and the semi-rough lipopolysaccharide phenotype and expresses fitness factors such as microcins, different iron uptake systems, adhesins and proteases [156]. If *E. coli* pathotypes do contribute to IBD pathogenesis, a possible explanation for the negative effect of the placebo-EcN treatment could be that EcN in a symbiotic manner supports the IBD-associated B2 *E. coli* still present in the group of patients, who were not treated with Ciprofloxacin initially.

In the study by Rembacken et al, EcN was found to be non-inferior ($P=0.0508$) to mesalazine in the induction of remission among patients with active UC. However, all their patients did receive a one-week induction with gentamicin [171]. Furthermore, all patients received standard corticosteroid therapy, depending on the severity of involvement (topical or systemic), thus making it impossible to truly evaluate the effect of EcN in active UC and impossible to compare this study to our study. In a double-blinded placebo-controlled study it was found that EcN enemas did improve remission rates in patients with left-sided UC in a per-protocol analysis. However, it was found that no differences could be seen in an intention-to-treat analysis, since more patients in the groups treated with EcN were withdrawn due to adverse events [174]. Furthermore, many patients in the study by

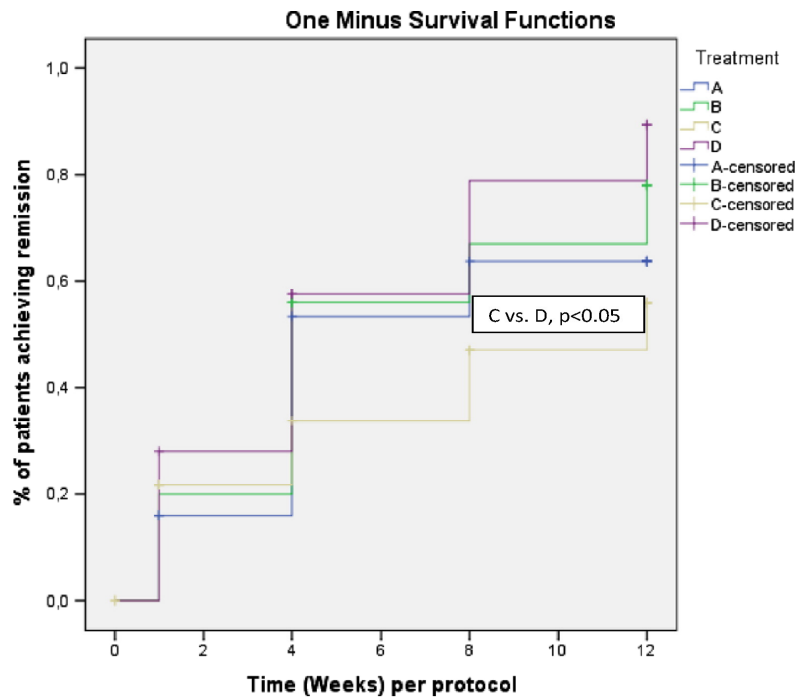


Fig. 7. Paper X. Kaplan–Meier curves were used to compare groups, censored when reaching remission. Per-protocol analysis during 12 weeks of follow-up in patients treated with Cipro/EcN (A), with Cipro/placebo (B) or with placebo/EcN (C) as add-on to conventional treatment compared to patients (D) treated with placebo/placebo as add-on to conventional treatment.

Matthes et al. [174] were excluded due to intake of non-permissible concomitant medication, again making it difficult to compare these results with ours.

Under the assumption that B2 *E. coli* is a part of UC pathogenicity, our study could be interpreted as if we simply did not find the right cure for this *E. coli*. On the other hand, our data indicate that B2 *E. coli* do in fact play a role in UC pathogenesis, since EcN (also a B2 *E. coli*) treatment left fewer patients in remission at the end of the study compared with placebo-treated patients. We have used the CAI score, which has proven efficient in determining flares of UC [175], and if this symptom score occasionally will include patients without endoscopically active disease, this risk would have been the same in all four treatment groups in our study. In addition, our follow-up study using fecal calprotectin as a marker of disease activity, [122], does support our findings, and, furthermore, confirms the observation that B2 *E. coli* colonization is

linked to increased levels of inflammation compared to colonization with other *E. coli* phylogroups. Our study provides an important lesson regarding the use of probiotics in general. Probiotics are not subjected to the comprehensive safety evaluation that pharmaceuticals receive, but before the use of probiotics can be recommended, randomized placebo-controlled studies of sufficient power are required.

In conclusion, our data do confirm the link between B2 *E. coli* and increased intestinal inflammation in IBD. Our data, however, do not support the use of EcN as an add-on therapy to conventional medication in acute flares of UC.

DISCUSSION AND PERSPECTIVES

IBD pathogenesis is, as described in this thesis, influenced by both: (1) gastrointestinal infections; (2) intestinal low diversity dysbiosis and

(3) presumably by specific emerging *E. coli* pathobionts. IBD has previously been linked to acute and chronic gastrointestinal infections due to overlapping symptoms, Paper 1. In daily clinical practice, the constant awareness of concomitant infections mimicking IBD flares is important. Both in order to avoid unnecessary endoscopic examinations but also to avoid potentially harmful use of immunosuppressants. IBD is associated with intestinal low diversity dysbiosis, with probable negative effects on both the availability of intestinal nutrients, the intestinal immune function and the mucosal barrier integrity. Conversely, normobiosis including colonization with apathogenic parasites are linked to IBD in remission, Paper 2. The possibility of correcting intestinal dysbiosis using FMT from healthy donors has been proven, Paper 3. The new perspectives provided by FMT, including the possibility to obtain long-term effects on the intestinal microbiome diversity and composition in patients with IBD and IBS, should, however, be followed closely; so far, long-term effects and the long-term risks of FMT are still basically unknown. Some intestinal bacterial phyla, such as Firmicutes and Bacteroidetes, are found in lower abundance during flare-ups of IBD, whereas others, such as Proteobacteria, e.g., *E. coli*, are increased, Paper IV. In this context, an ongoing search for key microorganisms (both beneficial and harmful) in IBD and IBS pathogenesis, and targeted treatment trials using well-defined and controlled probiotic microorganisms are sound approaches. Among key microorganism *E. coli* pathobionts are particularly often described in current IBD microbiome literature as associated with flares of IBD. Even though a substrate effect in the inflamed colonic mucosa could enhance the survival and growth of *E. coli*, current evidence does support *E. coli* pathobionts colonization as central in IBD pathogenesis. The association between B2 *E. coli* intestinal colonization and flares of IBD was confirmed in this thesis, Paper V, VI. Increased levels of antibodies towards *E. coli* antigens in IBD patients further support a more direct interaction of *E. coli* with the immune system in IBD patients, Paper VII, even though IBD medications will also

influence the serologic response, Paper VIII. Colonization experiments in a mouse model, Paper IX, and a probiotic *E. coli* (*E. coli* Nissle) placebo-controlled treatment study, Paper X, in patients with UC, demonstrate that *E. coli* Nissle does not eradicate IBD-associated *E. coli* but instead has the ability to act as a symbiont, and, thereby, presumably prevent remission of UC.

As an inspiration for future research in IBD-associated *E. coli* pathobionts, other emerging IBD-associated pathogens and the associated intestinal dysbiosis the following questions need elucidation;

Is only one *E. coli* pathobiont involved in UC and CD?

Both AIEC from CD patients and UC-associated *E. coli* have been linked to phylogenetic group B2 *E. coli* [176]. However, if differences exist in virulence or colonization factors between AIEC and UC-associated B2 *E. coli* are so far unknown. Some find no differences [177] while others find, e.g., less invasive potential in UC-associated *E. coli* strains compared with AIEC [178]. It has therefore not been excluded that different subsets of IBD-associated *E. coli* are linked to the inflammation in CD and in UC, each subset of *E. coli* with distinct pathogenic mechanisms. From both disease groups, *E. coli* are often of the phylogenetic group B2, with characteristics indistinguishably from ExPEC [117,176], even though it has been reported that the invasive potential of AIEC is only shared by a few percent of ExPEC isolates in general [179]. Unfortunately, AIEC is still mainly defined by its phenotypic behavior, making it difficult to determine the distribution of AIEC among different disease groups. In conclusion, no definite evidence separates B2 *E. coli* from patients with CD or UC from ExPEC, and it would be a reasonable assumption, that the IBD-associated *E. coli* are in fact identical to ExPEC. Theoretically, ExPEC is perhaps under the right circumstances of impaired immune function and general intestinal dysbiosis as seen in IBD patients, also an Intra-Intestinal Pathogenic *E. coli* capable of initiating inflammatory relapses in IBD patients.

What specific *E. coli* virulence mechanisms could be involved in the inflammatory process in UC vs CD?

Overrepresentation of B2 *E. coli* in the intestinal microbiome might forego inflammation and be directly linked to flares of UC. B2 *E. coli* binds to epithelial cells and hemolysin-positive B2 *E. coli* has the ability to induce degradation of epithelial cell tight junctions [123,124], possibly resulting in inflammation and in crypt abscess formation. Other experiments have revealed that UC-associated *E. coli* are able to mediate a cytokine response with high IL-18 and low IL-12p70, possibly linked to an expansion of Th2 cells rather than Th1, the latter requiring IL-12p70 [164]. Since the discovery of AIEC, several studies have been performed both regarding the prevalence of these *E. coli* among CD patients and controls and regarding the possible pathogenic mechanisms. AIEC pathogenesis has been linked to binding to carcinoembryonic antigen-related cell adhesion molecules 6, CAECAM6 receptors, which are overrepresented in the ileal part of the intestines in response to inflammation, especially in patients with CD [180]. AIEC have an invasive potential in epithelial cells [181]. Furthermore, AIEC has been located in macrophages and has been found to survive efficiently inside these cells, exposing a possible link to the inabilities of the innate immune system in patients with IBD [182]. AIEC also replicates within the phagocytes without inducing cell death and AIEC are able to induce secretion of large amounts of IL8 and TNF- α and finally also to promote a granulomatous inflammatory response, further linking AIEC to CD [163,178].

What could explain the effect of immunomodulators if pathobiont colonization and intestinal dysbiosis are the causes of IBD flares?

Immunomodulators are the drugs of choice in IBD, which could be a contradiction to the involvement of intestinal dysbiosis or of specific microbial pathogens in IBD pathogenesis. However, the effects of at least some immunomodulators on the microbiome have been demonstrated previously, such as changes seen in the microbiome, including reduction in Enterobacteriaceae during treatment with

Salazopyrin [183]. In addition, 5-ASA have recently been shown to inhibit *E. coli* growth in a dose-dependent manner and downregulated the expression of bacterial virulence genes associated with IBD and reduced *E. coli* survival in macrophages and TNF- α secretion by infected macrophages [184]. In azathioprine-treated patients, the suppressed migration of leukocytes was accompanied by a 28-fold higher concentration of mucosal bacteria when compared with the 5-ASA group or a 1000-fold increase when compared with healthy controls [185]. So different mechanisms could be speculated: Both a direct effect of immune-modulating drugs on *E. coli* and an effect through the interruption of the immune system, which could make IBD-associated *E. coli* lose the battle against other coexisting intestinal microorganisms. Furthermore, IBD-associated *E. coli* might only become pathogenic with a simultaneous stimulus from the immune system, and without that stimulus, they will not adhere, proliferate and/or activate the production of important toxins or other virulence factors. In addition, immunomodulators are believed to have an effect, at least on mortality, in other infectious diseases such as TB [186] and meningitis [187], even though the immunomodulators do not have any direct effect on the infectious cause of these diseases. IBD pathogenesis is most likely, as previously described, a mixture of immunology, genetics and environmental factors. The specific *E. coli* might be the trigger of inflammation, but only in susceptible individuals. If the immune response upholds a normal function, B2 *E. coli* will not act as a sole driver of inflammation; therefore, intestinal B2 *E. coli* are not true intestinal pathogens, but instead so-called pathobionts, as defined previously. In other words, B2 *E. coli*/ExPEC are commensal intestinal bacteria with virulent abilities, that are only expressed under very specific conditions such as an intestinal dysbiosis in patients with simultaneous defects in the inflammatory response. But if so, how should these pathobionts then be eradicated?

Treatment trials?

No specific trial has to date been performed, in which a diagnostic test for intestinal ExPEC or

AIEC has been performed followed by antibiotic treatment according to susceptibility pattern and, furthermore, followed by relevant probiotics to avoid recolonization. In IBD, antibiotics have been evaluated as described previously and as concluded in a recent review: Treatment of abscesses and fistulas in CD, includes antibiotic therapy, most often ciprofloxacin and/or metronidazole. Antibiotics might also play a role in the maintenance of remission even in post-operative recurrence of CD. Also in active ulcerative colitis antibiotics, e.g., ciprofloxacin, with a possible effect on *E. coli*, has proven to be moderately efficient [169]. However, antibiotic treatment of specific IBD-associated pathobionts will indisputably also worsen the general intestinal dysbiosis linked to IBD, in this context other possible microbiome-sparing therapies such as strain-specific phage therapy or anti-adhesive therapies should be considered. Supporting the hypothesis that flares of IBD are dysbiosis driven is the number of papers demonstrating a positive effect of probiotics such as, e.g., *Saccharomyces boulardii*, Bifidobacteria and Lactobacilli containing yogurts, *Lactobacillus* GG and VSL#3 (a probiotic mixture of 8 strains) [188]. However, restoring the total fecal microbiome with a fecal donation from a healthy person is the most ultimate form of a 'probiotic cure'. FMT as a treatment option in IBD has been described in case series and smaller uncontrolled trials, indicating a success rate of around 36% [189]. So far four randomized, placebo-controlled trials regarding the use of FMT to UC have been published, [79,80,190,191]. Three of these showed a moderately positive effect, while the last could not reveal any differences when compared to placebo treatment. No similar placebo-controlled trials exist regarding Crohn's disease. But many FMT-IBD studies are currently being performed according to recent registrations in Clinicaltrials.gov making it likely that further clarification regarding FMT's use in IBD will soon arrive. If FMT is in fact efficient during flares of IBD, an interesting link to *C. difficile* infection has been established. Recurrent *C. difficile* infections are based on both the presence of an entero-pathogen (*C. difficile*), an underlying intestinal dysbiosis and an impaired immune system, where a combination of vancomycin and FMT is found to be the

most efficient cure [192,193]. In IBD, ExPEC or AIEC could be the equivalent to *C. difficile* in a similar pathogenetic triangle also involving intestinal dysbiosis and the host's impaired immune system.

However, the ultimate question remains unanswered:

Are pathobionts and intestinal dysbiosis the cause, a culprit or just a marker of IBD?

To answer this more prospective longitudinal follow-up studies of *E. coli* colonization and the intestinal dysbiosis in IBD patients are still necessary, including associations to medical treatments, newly onset disease and disease course, and including studies of both children and adult patients using up-to-date metagenomics. Most importantly, more intervention studies are needed to be directed specifically at eradicating IBD-associated pathobionts and restoring normobiosis in specified subgroups of IBD patients. If possible, these interventions should be supported by studies in mouse models and in vitro assays.

In conclusion; in order to reach clarification regarding the influence of the intestinal microbiome on IBD pathogenesis and possibly obtain efficient microbiome-based cures of IBD, future microbiome correction trials in IBD should possibly focus on three levels of treatment: (1) removing the trigger, e.g., *E. coli* pathotypes or other hypothesized pathobionts, through antibiotics, anti-adhesive or phage therapy; (2) correction of the underlying dysbiosis through probiotics or FMT, together with (3) a treatment targeting the IBD-associated immune system defects.

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