PROGNOSIS OF IBD IN CHILDREN AND ADOLESCENTS

Assessment of outcome, based on clinical, serological and microbial markers at diagnosis

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2 Abbreviations

ASCA anti–Saccharomyces cerevisiae antibodies

anti-OmpC Antibodies against the outer membrane porin of Escherichia coli

anti-I2 antibodies against a Pseudomonas fluorescence associated sequence

anti-CBir1 antibodies against flagellin expressed by Clostridial phylum

5-ASA 5-aminosalicylic acid

AUC area under the curve

CARD15 caspase recruitment domain-containing protein 15

CD Crohn's disease

CI Confidence interval

CRP C-reactive protein

DNA deoxyribonucleic acid

Epstein-Barr virus (EBV)

EEN exclusive enteral nutrition

EGD esophagogastroduodenoscopy

ELISA enzyme-linked immunosorbent assay

EOIBD early onset IBD

ESPGHAN

ESR Erythrocyte sedimentation rate

FC Fecal calprotectin

FSS fluorescence signal strength

GI gastrointestinal

IBD inflammatory bowel disease

IBS irritable bowel syndrome

Ig immunoglobulin

IL interleukin

MRE magnetic resonance enterography

MRI magnetic resonance imaging

NOD2 nucleotide oligomerization domain 2

OR Odds ratio

p-ANCA perinuclear anti-neutrophil cytoplasmic antibodies

PCR polymerase chain reaction

PIBD pediatric inflammatory bowel disease

PSC primary sclerosing cholangitis

RNA ribonucleic acid

ROC receiver operating characteristic

RR Relative risk

rRNA ribosomal ribonucleic acid

SCFA short-chain fatty acid

SES-CD Simple Endoscopic Score for Crohn's Disease

SNP single nucleotide polymorphism

SPSS Statistical Package for

TNF tumor necrosis factor

TPMT thiopurine methyltransferase

UC ulcerative colitis

VEOIBD very early onset IBD

WGS whole genome sequencing

3 List of publications

This thesis is based on the following papers, which are referred to by Roman numerals:

Paper I

Olbjørn C, Nakstad B, Småstuen MC, Thiis-Evensen E, Vatn MH, Perminow G. Early anti-TNF treatment in pediatric Crohn's disease. Predictors of clinical outcome in a population-based cohort of newly diagnosed patients. Scandinavian journal of gastroenterology. 2014;49(12):1425.
DOI: 10.3109/00365521.2014.966316

Paper II

Olbjorn C, Cvancarova Smastuen M, Thiis-Evensen E, Nakstad B, Vatn MH, Perminow G. Serological markers in diagnosis of pediatric inflammatory bowel disease and as predictors for early tumor necrosis factor blocker therapy. Scandinavian journal of gastroenterology. 2017;52(4):414-9.
DOI: 10.1080/00365521.2016.1259653

Paper III

Olbjorn C, Cvancarova Smastuen M, Thiis-Evensen E, Nakstad B, Vatn MH, Perminow G. Fecal microbiota profiles in treatment-naïve pediatric inflammatory bowel disease- associations with disease phenotype, treatment and outcome. (Accepted manuscript). Clinical and experimental gastroenterology.

4 Abstract

Background and aims

Pediatric inflammatory bowel disease (PIBD) patients often present with an extensive disease distribution and an aggressive disease course. The individual disease course is unpredictable. There is a short window of opportunity to diagnose and induce remission in order to avoid stunting, pubertal delay and irreversible complications. This highlights the importance of early diagnosis and effective therapy. In order to improve the knowledge of etiological, diagnostic and prognostic factors we evaluated clinical factors, inflammatory biomarkers, serological markers and the composition of the gut microbiota in treatment naïve PIBD.

Materials and methods

We prospectively followed newly diagnosed patients under 18 years of age with IBD and non-IBD symptomatic controls. Clinical data, demographic data, inflammatory markers (C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), fecal calprotectin (FC)), serological markers (perinuclear anti- neutrophil cytoplasmic antibody (pANCA), anti–Saccharomyces cerevisiae antibodies (ASCA), antibodies against the outer membrane porin of Escherichia coli (anti-OmpC), antibodies against a Pseudomonas fluorescence associated sequence (anti-I2), and flagellin expressed by Clostridial phylum (anti-CBir1)), fecal gut microbiota and histology from all segments of the gastrointestinal tract were obtained. In the first cohort (IBSEN II) there was a follow-up with repeated sampling within 2 years of therapy.

Results

We confirmed that biologic therapy in pediatric Crohn's disease (CD) is safe and effective for induction and maintenance of remission. Half of our CD patients in the IBSEN II cohort received biologic therapy with tumor necrosis factor (TNF) blockers within the first year after diagnosis. TNF blocker therapy was associated with higher inflammatory markers, widespread disease with more upper

gastrointestinal (GI) involvement. ASCA and pANCA status was useful in differentiating UC and CD patients. Positive ASCA and/or negative pANCA was associated with early initiation of TNF blocker therapy in CD patients. ASCA serology was stable, regardless of treatments received, pANCA status declined after therapy in UC.

Fecal microbiota profiles could distinguish healthy children from symptomatic patients, but the microbiota profiles (dysbiosis) in IBD and non-IBD symptomatic patients were similar. Severe dysbiosis in IBD was associated with extensive disease, stricturing and penetrating CD, TNF blocker therapy, and non-mucosal healing. The dysbiosis persisted after therapy, regardless of treatments and remission status.

Conclusions

A combined panel of clinical, serological and microbial markers can increase the diagnostic precision, the choice and timing of treatments in pediatric patients with IBD. Extensive disease distribution, high CRP, ESR, fecal calprotectin, positive serology against ASCA/negative pANCA in CD, and severe dysbiosis are indicators of a severe phenotype that warrants early aggressive therapy.

5 Introduction

Inflammatory bowel diseases (IBD); Crohn's disease (CD), ulcerative colitis (UC), and IBD unclassified (IBDU) are chronic, lifelong diseases in the gastrointestinal (GI) tract with a relapsing and remitting course. These disorders have distinct pathologic and clinical characteristics, but the etiology of IBD is only partly understood. The pathogenesis involves immunological, genetic and microbiological factors. IBD encompasses multiple disease phenotypes and the treatments need to be individualized on the basis of disease severity and prognosis. Without adequate treatment the patients may face deleterious consequences with irreversible complications. In adult CD, variables predictive of a poor prognosis include young age under 40 years at diagnosis, extensive disease, perianal disease, smoking and the use of corticosteroids (1). Similarly, in adult UC, age below 40 at diagnosis, presence of primary sclerosing cholangitis (PSC), lack of mucosal healing after induction treatment, high titers of perinuclear antineutrophil cytoplasmic antibodies (pANCA) as well as deep ulcerations in the colonic mucosa are all variables predictive of an aggressive disease course (2, 3). However, none of these variables are highly predictive when used alone and are not suitable for pediatric onset of IBD.

5.1.1 Pediatric IBD (PIBD)

There is a rising incidence of IBD, especially in the pediatric population (4-8) and up to 25 % of cases are diagnosed during childhood and adolescence. Although the clinical presentation and the disease phenotypes of PIBD can be highly variable, disease distribution as well as disease progression and response to therapy are age dependent (9). Age- specific characteristics led to a reclassification of PIBD (10, 11). Pediatric patients who develop IBD before the age of 10 years are classified as early onset IBD (EOIBD) in contrast to those developing the disease at 10 years or older. Very early onset IBD (VEOIBD) represents children diagnosed before six years of age, infantile onset IBD those

diagnosed younger than 2 years, and neonatal IBD represents those diagnosed within the first 28 days of age (Table 1).

Table 1.

Definition	Age range in years
Pediatric onset IBD	Younger than 17
EOIBD	Younger than 10
VEOIBD	Younger than 6
Infantile IBD	Younger than 2
Neonatal IBD	First 28 days after birth

VEOIBD patients have their own phenotype (12), and monogenetic diseases are more common in this age group, highlighting the importance of ruling out immunodeficiency and IBD mimicking disease in these patients, including interleukin (IL) 10 receptor deficiency and chronic granulomatous disease (11). In children younger than 6 years of age, UC is the predominant form of PIBD, while CD is predominant in older children and adolescents. Gender is associated with age at onset; being female is associated with a lower risk for CD until the age of 10-14 years, with a higher risk in early adulthood, whereas being male is associated with UC in the age group 5-9 years (13).

At onset or during episodes of relapse, the patients experience symptoms due to the GI inflammation; altered bowel movements, blood or mucus in the stool, abdominal pain, general malaise and fatigue. PIBD has its peak incidence in the prepubertal age group, around age 12 years, affecting the time of hormonal changes to obtain puberty and growth spurt. Hence, growth failure and pubertal delay are common findings, especially in pediatric CD, but it also occurs in UC (14, 15).

Diagnosing IBD in pediatric patients can be challenging, as symptoms often are non-specific and similar to those seen in functional GI diseases such as irritable bowel disease (IBS) as well as allergic and infectious gastroenteritis. The

diagnosis warrants upper and lower endoscopies, which in children are performed in general anesthesia. This makes the diagnostic workup invasive and resource intensive, and diagnostic delays are common (16).

The individual disease course of IBD is unpredictable, and patients have different features in terms of severity, distribution, localization, endoscopic and histopathological findings and extra-intestinal manifestations. Patients at high risk for complicated pediatric CD include those with perianal disease, severe growth retardation, deep ulcerations on endoscopy, extensive disease with upper GI and proximal small bowel involvement and the need for corticosteroids at diagnosis (17). In pediatric UC, diagnostic delays and a positive family history of IBD have been found to be associated with disease extension. Extensive disease as well as the presence of extra-intestinal manifestations at diagnosis increases the risk for colectomy (18).

PIBD patients often present with an extensive disease distribution, an aggressive disease course and are more frequently in need of immunosuppressive and biologic therapy compared to IBD in adults (19- 21). There is a short window of opportunity to diagnose and induce remission in order to avoid stunting, irreversible complications as well as school absenteeism, highlighting the import-ance of early diagnosis and effective therapy (16). Biological agents such as the tumor necrosis factor (TNF) blockers infliximab and adalimumab represent a potent treatment option but are hampered with potentially serious side effects, high cost and are not needed by all patients.

CD

Characteristics of CD are transmural and granulomatous inflammation which can involve any site of the GI tract, from the oral cavity to the anus, often with a patchy distribution. The inflammation may involve any layer of the intestinal wall which can cause complications such as fistulas, abscesses or strictures. Granulomas are present in 24-61 % of CD cases (22), more frequent in children than in adults, and are associated with worse outcome (23). In adults, CD most commonly affects the terminal ileum, while this phenotype is rare in younger

children, in which isolated colonic CD is the dominant disease distribution. Older children and adolescents with CD have typically a pan-enteric phenotype, with ileocolitis being the most common disease distribution in CD. Approximately two thirds of pediatric CD patients have ileocolonic affection at debut, about one third have isolated colonic disease, whereas isolated terminal ileitis becomes more common in older children and adolescents (24). Most pediatric CD patients present with an inflammatory phenotype, but one third of patients will progress to complicated disease; stricturing and or penetrating disease behavior within 10 years of disease duration (25). Perianal disease is more common in children than in adults, more so in boys, and is present at time of diagnosis in up to one third of patients. Additionally, 10-20 % will develop perianal complications with time (26).

UC

The inflammation in UC is limited to the colonic mucosa and submucosa, typically starting in the rectum, extending proximally in a continuous fashion. Pediatric UC is often more extensive and severe than adult- onset UC and 60-90% of children present with pancolitis compared to approximately 30% in adult-onset UC (19, 27-29). Disease progression over time is common in both adults and children, but occurs more rapid in childhood-onset UC. Risk factors for progression to extensive disease from isolated left sided disease are long diagnostic delays, positive family history of IBD, extra-intestinal manifestations and a high pediatric ulcerative colitis activity index (PUCAI) score at diagnosis (30). Pediatric UC patients are more often being treated with systemic steroids and immunomodulators than adult patients, and have a higher risk of steroid dependency (27).

IBDU

In approximately 10% of children, differentiation between CD and UC is not possible in spite of a complete diagnostic workup, and a diagnosis of IBDU is made (10). More patients are diagnosed as IBDU in children younger than 10 years, reflecting the difficulties in the initial classification of PIBD. VEOIBD is

related to extensive disease with mostly colonic involvement and later reclassification from IBDU to CD or UC often occurs (31).

5.1.2 PIBD phenotypes, disease distribution, Paris classification

The Paris classification (32) is a pediatric-specific modification of the Montreal classification of IBD (33) which highlights the phenotypic characteristics that are more common in pediatric-onset than in than adult-onset IBD. These include a subdivision of age according to age below or above 10 years at diagnosis, the addition and location of upper GI involvement in CD, as well as disease extension and severity in UC. The Paris classification added a new category of growth delay in CD patients, enabled the classification of both stricturing and penetrating disease in the same patient and further defined specific characteristics of each phenotype. The presence of perianal fistula, abscess or anal canal ulcers are consistent with perianal disease in the Paris classification. Upper GI manifestations are frequent, especially in CD, and atypical presentations of the disease are not uncommon (24, 34).

Table 2. Pediatric modification of the Montreal classification for CD: The Paris classification from Levine et al (32).

	Montreal	Paris
Age at Diagnosis	A1: below 17 y	A1a: 0–<10y
	A2: 17-40 y	A1b: 10–<17 y
	A3: Above 40 y	A2: 17–40 y
		A3: >40 y
Location	L1:terminal ileal± limited cecal disease	L1: distal 1/3 ileum ± limited cecal disease
	L2: colonic	L2: colonic
	L3: ileocolonic	L3: ileocolonic
	L4: Isolated upper disease*	L4a: upper disease proximal to Ligament of Treitz*
		L4b: upper disease distal to ligament of Treitz and proximal to distal 1/3 ileum*
Behavior	B1: non-stricturing non-penetrating	B1: nonstricturing nonpenetrating
	B2: stricturing	B2: stricturing
	B3: penetrating	B3: penetrating
	p: perianal disease modifier	B2B3: both penetrating and and stricturing disease, either at the same or different times
		p: perianal disease modifier
Growth	n/a	G_0: No evidence of growth delay
		G_1: Growth delay

Table 3. Pediatric modification of the Montreal classification for UC: The Paris classification, from Levine et al (32).

	Montreal	Paris
Extent	E1: ulcerative proctitis	E1: ulcerative proctitis
	E2: left-sided UC (distal to splenic flexure)	E2: Left-sided UC (distal to splenic flexure)
	E3: extensive (proximal to splenic flexure)	E3: Extensive (hepatic flexure distally)
		E4: Pancolitis (proximal to hepatic flexure)
Severity	S0: clinical remission	S0: never severe*
	S1: mild UC	S1: ever severe*
	S2: moderate UC	
	S3: severe UC	

*Severe defined by Pediatric Ulcerative Colitis Activity Index (PUCAI) ≥ 65.[58]

5.1.3 Symptoms and signs

Abdominal pain and altered bowel habits are common in children and adolescents, and differentiating IBD from functional GI disorders can be challenging. Both CD and UC commonly present with abdominal pain and diarrhea. Rectal bleeding and urge occurs more often with UC whereas CD can have an insidious onset, with the patient experiencing fatigue, weight loss or perianal symptoms. Physical signs with pallor, delayed onset of puberty, blood or mucus in the stools, aphtous ulcers, abdominal tenderness and perianal skin tags and/or fissures are signs warranting further investigation. More than 20 % of pediatric IBD patients exhibit a non- classical presentation at diagnosis. In 4-5 %

of cases isolated growth failure or perianal disease are the only initial feature of IBD (35).

5.1.4 Extra-intestinal manifestations

More than one fourth of pediatric IBD patients have extra-intestinal manifestations (36, 37), and in one third of patients an extra-intestinal manifestation is the predominant symptom (35). Extra-intestinal manifestations are more common at onset of PIBD compared to adult IBD, more so in CD than in UC and associated with disease severity (37). The most common extra-intestinal manifestations in children are musculoskeletal symptoms such as arthritis, followed by aphtous stomatitis. Ophthalmological problems (episcleritis and uveitis), are other extra-intestinal manifestations (37). Elevated liver enzymes can be a sign of PSC or autoimmune hepatitis. Up to 80% of PSC patients have concomitant IBD, most often diagnosed as UC (38).

Orofacial granulomatosis with lip swelling, angular cheilitis and aphtous ulcers is more common among pediatric CD patients and can manifest before other IBD signs and symptoms (39, 40). Since many children will present with an extra-intestinal manifestation before developing intestinal symptoms, IBD should be suspected in children and adolescents who display an extra-intestinal manifestation and they should be worked up accordingly.

5.1.5 Disease activity indices (PCDAI and PUCAI)

Disease activity and severity is based on history, clinical and laboratory evaluations summarized in scores such as the Pediatric Crohn's Disease Activity Index (PCDAI) for CD and PUCAI for UC (41, 42).

PCDAI

The PCDAI includes laboratory markers; erythrocyte sedimentation rate (ESR), hematocrit and albumin, symptoms; abdominal pain severity, general well-being and activity, number of stools per day, as well as findings of abdominal tenderness on examination, perirectal disease, weight status and extra-intestinal

manifestations. In the abbreviated version, the laboratory markers have been omitted, and 70 points is the maximum score. The PCDAI score as well as its abbreviated version correlates with biomarkers of inflammation; fecal calprotectin (FC), C-reactive protein (CRP) and ESR, but have only a fair correlation with endoscopic inflammation, measured by the Simple Endoscopy Score for CD (SES-CD) (43). A change in 12.5 points in the PCDAI indicates a response. Remission is defined by a score< 10 points (44).

Table 4. PCDAI score, abbreviated version.

From Shepanski MA et al. JPGN 2004; 39:68-72

History (Recall, 1 week)			
Abdominal Pain			**Score**
	0 = None	5 = Mild: Brief, does not interfere with activities	10 = Moderate/ Severe: Daily, longer lasting, affects activities, nocturnal	
Patient Functioning, General Well-Being			**Score**
	0 = No limitation of activities, well	5 = Occasional difficulty in maintaining age appropriate activities, below par	10 = Frequent limitation of activity, very poor	
Stools (per day)			**Score**
	0 = 0-1 liquid stools, no blood	5 = Up to 2 semiformed with small blood, or 2-5 liquid	10 = Gross bleeding, ≥ 6 liquid, or nocturnal diarrhea	
Examination			
Abdomen			**Score**
	0 = No tenderness, no mass	5 = Tenderness, or mass without tenderness	10 = Tenderness, involuntary guarding, definite mass	
Perirectal Disease			**Score**
	0 = None, asymptomatic tags	5 = 1-2 indolent fistula, scant drainage, no tenderness	10 = Active fistula, drainage, tenderness, or abscess	
Weight			**Score**
	0 = Weight gain or voluntary weight stable/loss	5 = Involuntary weight stable, weight loss 1%-9%	10 = Weight loss ≥ 10%	
Extraintestinal Manifestations			**Score**
(Fever ≥ 38.5°C for 3 days over past week, definite arthritis, uveitis, *E. nodosum*, *P. gangrenosum*)			
0 = None	**5** = 1	**10** = ≥ 2	
		Total Score:	

PUCAI

The PUCAI score is a clinical index that incorporates variables such as the number of daily stools, nocturnal stools, amount of blood, as well as abdominal pain and limitation of activity in UC patients. The PUCAI score is normally higher in children with extensive colitis compared to patients with limited left-sided disease (45). A high PUCAI score at diagnosis is associated with disease progression from limited left sided disease to pancolitis (46). It is responsive to change and following the PUCAI score closely in patients with acute severe colitis is a valuable tool in tailoring and deciding treatments (47- 49). Clinical remission is defined as PUCAI$\leq$ 10 points, mild disease as 10-34 points, moderate disease 35-64 points and severe disease $\geq$ 65 points. A PUCAI change of 20 points defines a clinically significant response (50). Early response to therapy measured by a normalized PUCAI score at 12 weeks is associated with a better prognosis (3). The PUCAI score after 8 weeks of infliximab treatment performed better than mucosal healing and CRP levels in predicting steroid-free remission after 1 year of treatment (48). In contrast to the PCDAI, the PUCAI score correlates well with endoscopic appearance of the colonic mucosa and with the Mayo score (49, 51).

Table 5. PUCAI score, from Turner et al (45).

Item	Points
Abdominal pain	
No pain	0
Pain can be ignored	5
Pain cannot be ignored	10
Rectal bleeding	
None	0
Small amount only, in <50% of stools	10
Small amount with most stools	20
Large amount (>50% of the stool content)	30
Stool consistency of most stools	
Formed	0
Partially formed	5
Completely unformed	10
Number of stools per 24 hours	
0 to 2	0
3 to 5	5
6 to 8	10
>8	15
Nocturnal stools (any episode causing wakening)	
No	0
Yes	10
Activity level	
No limitation of activity	0
Occasional limitation of activity	5
Severe restricted activity	10
Sum of PUCAI (0 to 85)	

5.1.6 Etiology and pathogenesis

The pathogenesis of IBD involves both innate (nonspecific system of defense against microorganism) and adaptive (antigen-specific responses mediated by T and B cells) parts of the immune system. The chronic inflammation in IBD is due to a complex interaction of genetic determinants, disruption of mucosal barriers, aberrant inflammatory signals, loss of tolerance, and environmental triggers (52). Genetic studies have revealed defective processing of intracellular bacteria, autophagy and innate immunity to be of particular importance in in CD, while in

UC the focus is on the barrier function of the intestinal epithelium (9). The interaction between the host genome and the gut microbiota is thought to contribute to the pathogenesis of IBD and other immune mediated disorders (53). A recent study showed that genetic risk variants associated with IBD might influence the gut microbiota in healthy individuals with a shift towards pro-inflammatory gut microbial changes, possibly preceding the onset of IBD (54). There is a general increase of IBD and immune mediated inflammatory disorders such as rheumatoid arthritis, psoriasis and multiple sclerosis. Changes in diet and environment as well as the influence of gut microbiota are increasingly recognized to impact on the disease pathogenesis (55- 57).

Genetic markers may have prognostic impact (58). Examples of genes associated with IBD that are involved in bacterial handling are nucleotide oligomerization domain 2 (NOD2), caspase recruitment domain-containing protein 15 (CARD15) and ATG16L. Mutations in the NOD2/CARD15 gene are predictive of small bowel stenosis and the need for early surgery in CD, while mutations in the ATG16L1 gene, a gene that encodes for a protein important in autophagy, are associated with stricturing and perianal disease.

5.1.7 Gut microbiota, an overview

The gut microbiota consists of bacteria, viruses, yeasts and parasites, among which bacteria are the most abundant, with approximately 10^{14} bacterial cells present in the human gut. More than 99% of the gut microbiota belongs to four phylogenetic groups: Firmicutes, Bacteroidetes, Actinobacteria and Proteobacteria. This was discovered using molecular methods as the majority of anaerobic bacteria in the gut cannot be grown. The gut microbiota may be studied using bacterial deoxyribonucleic acid (DNA) in combination with new sequencing techniques, often referred to as next generation or high- throughput sequencing.

16S ribosomal ribonucleic acid (rRNA) sequencing:

Ribosomes are responsible for the protein production in all living cells. The ribosomes of all bacteria have a small subunit that contains one RNA molecule,

the 16S ribosomal RNA (16S rRNA) (59). The gene coding for 16S rRNA contains several variable regions which can be used to identify the bacterial group from which the gene originated, almost like a fingerprint. These hypervariable regions are flanked by other regions that are highly conserved between bacteria (60). By designing genetic primers targeting the conserved regions of this gene, "universal primers", a polymerase chain reaction (PCR) is performed on extracted DNA, amplifying the hypervariable region next to the "universal primers". The amplified hypervariable region is then sequenced, and one can use databases to identify from which bacterial group the sequence originated, generating a compositional overview of the microbiota (60).

Whole genome sequencing (WGS)

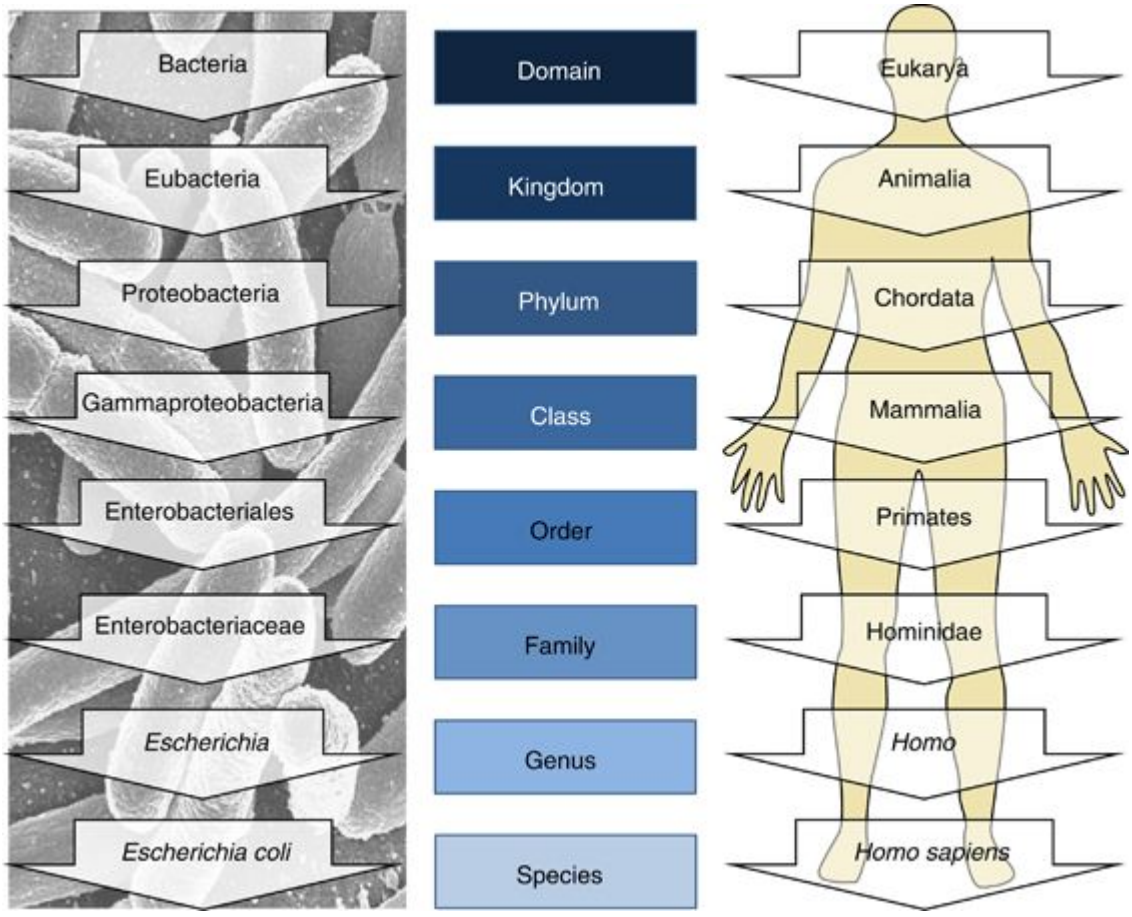
WGS, or shotgun sequencing, is used to sequence all DNA fragments present in a sample (61). The method is still expensive, laborious and requires considerable computational recourses. The advantages are the possible identification of species level taxonomy, estimation of metabolic pathways and thus the ability to predict not only the presence, but also the functional contents of the bacteria in the samples.

Dominant, subdominant and transient microbiota

The strict anaerobic bacteria (Firmicutes, Bacteroidetes and Actinobacteria) are the dominant microbiota in the gut. The subdominant microbiota is made up of facultative anaerobic bacteria, such as *Escherichia coli*, member of the Proteobacteria phylum. The transient microbiota varies over time, dependent on what microorganisms are ingested through the diet, disease and the use of antibiotics. There is a high biodiversity in the human gut microbiota, with more than 1000 species of bacteria. Each individual hosts more than 200 species, with a unique combination. Microbial colonization begins at birth, and it takes time before the gut microbiota becomes stable. Mode of delivery, breastfeeding versus formula feeding, diet and use of medication are all factors that influence the composition of the microbiota in early childhood (62).

Usually the host and microbiota act symbiotic, mutually beneficial, with gut bacteria being involved in many metabolic processes, such as helping to digest nutrients, preventing colonization of the host by pathogenic organisms and contributing to develop both the immune system and intestinal epithelium.

Figure 1. Taxonomic levels used for classification of different organisms. The bacteria domain is on the left hand side, for comparison the taxonomic classification of humans is given on the right. Pictures used in the figure are listed under the Creative <commons Zero license. The figure is from Tyler et al (60).



5.1.8 Gut microbiota in IBD

Disturbances in gut microbiota are thought to be a major factor in the development of IBD (63, 64). A leading hypothesis is that the intestinal inflammation is caused by an inappropriate immune response to commensal bacteria in genetically susceptible individuals (65). Studies of the gut microbiota

in IBD patients have shown an imbalance, dysbiosis, with compositional changes, including decreased bacterial diversity and abundance (64). The shift in the gut microbiota seems to be associated with a depletion of beneficial versus a relative increase of pro-inflammatory bacteria (66, 67). It is recognized that the microbial content of the gut influence disease progress, but whether changes in microbial content in the gut are causative is not known (68, 69).

The intestinal microbiota is unbalanced in various diseases and has the potential to be biomarkers and diagnostic tools (56, 70). Dysbiosis is, however, also a feature in patients with IBS (71), and dysbiosis as well as elevated FC levels are linked to a broad spectrum of GI conditions besides IBD (56, 72). The gut microbiota in adult IBD patients differ from the gut microbiota in patients with IBS and healthy subjects (73, 74) and microbial studies have found characteristic changes in the composition of the gut microbiota in PIBD patients (75, 76). A pediatric study using 16S rRNA amplicon sequencing could distinguish patients with CD from those with UC and patients with IBD from patients with similar symptoms but no inflammatory disease, both in feces and colonic biopsies (77). This last study did not include treatment naïve patients, but children and adolescents with established IBD on treatment, in contrast to this thesis. A large multicenter study in the US analyzing the gut microbiome in treatment naïve CD patients, using both colonic mucosal samples and feces as study material (76), found that stool samples reflected the mucosal dysbiosis less well. They concluded that the mucosal microbiota had a better predictive value compared to fecal samples. However, the different studies have shown conflicting results, and the correlation between the fecal and mucosal associated microbiota and the diagnostic precision of the different samples have yet to be determined.

5.1.9 Diagnosing IBD, the Porto criteria

The Porto IBD Working Group of the European Society of Pediatric Gastroenterology, Hepatology, and Nutrition (ESPGHAN), constructed consensus-based clinical guidelines for the diagnosis of PIBD, which were revised in 2014

(10, 78). In the revised Porto classification, 2 categories of UC are defined, typical and atypical (10). Atypical UC phenotypes include findings of macroscopic rectal sparing, isolated nonserpiginous gastric ulcers, normal crypt architecture, absence of chronicity in biopsies, or a cecal patch, and should be treated as UC. Patients with acute severe colitis may have transmural inflammation.

The PIBD diagnosis is based on a combination of history, physical and laboratory examinations, esophagogastroduodenoscopy (EGD) and ileocolonoscopy with multiple biopsies and histology, as well as visualization of the small bowel with magnetic resonance imaging (MRI) or other imaging techniques.

It is important to exclude enteric infections, and to differentiate PIBD from infectious disease, allergic disease or primary immunodeficiency disorders which can have similar presentations. The treatment for immunodeficiency disorders and monogenetic forms of IBD is different. Especially in younger children and in VEOIBD the diagnostic workup should include immunoglobulin (Ig) measurements, flow cytometry analysis of lymphocyte subsets (CD3, CD4, CD19/CD20, Natural killer cells) as well as analysis of oxidative burst by neutrophils. Specialized tests and genetic workup may be indicated, particularly in the infantile onset of IBD, presence of severe perianal disease or additional features of autoimmunity (11). 50 genes have so far been identified in VEOIBD and primary immunodeficiency (79).

5.1.10 Laboratory examinations and biomarkers

Laboratory examinations are helpful in IBD for diagnosing and assessing disease activity, prognosis and risk of complications, prediction of relapse and for monitoring the effect of therapy. Decreased hemoglobin, elevated total white cell count and platelet count, low serum albumin and elevated inflammatory markers such as CRP, and ESR can be found in active IBD (80). It is however not uncommon for PIBD patients to have normal blood laboratory tests, in UC even more so than in CD (81). Other inflammatory markers such as IL-1 beta, IL-17 and

interferon gamma have been studied, and may be promising, but are not yet fully validated (82).

CRP

CRP is produced by hepatocytes in response to inflammation and is one of the most commonly used acute-phase proteins in humans. The half life of CRP is short, (19 hours) and CRP rises early after the onset of inflammation and decreases rapidly after its resolution. It is elevated in a variety of conditions, such as infections, autoimmune and inflammatory conditions as well as in malignancy, thereby being a non-specific marker of inflammation. CRP elevations may vary from person to person, dependent on their immune status. There is heterogeneity in the CRP response between between CD and UC. CRP may correlate well with disease activity in CD, while UC patients may display only a modest to absent CRP response (83), (84). CRP has prognostic potential; increased baseline levels are associated with better response rates to biologicals in clinical trials (85).

ESR

ESR is the rate at which erythrocytes migrate through the plasma and will depend on the plasma concentration, the number and size of the erythrocytes, as well as on the age of the patient. Conditions such as anemia, polycythemia, and thalassemia thus affect ESR. Compared with CRP, ESR will peak less rapidly and may take several days to decrease. Like CRP, ESR is a non-specific marker of inflammation, and performs less well than CRP (86).

FC

FC is superior to blood markers as a diagnostic marker for intestinal inflammation (87, 88). Calprotectin is a heterodimeric protein which belongs to the calcium-binding protein S100 family. It is released by leukocytes at the site of inflammation and can be detected in the feces where it remains stable for about seven days. Increased FC levels are found in several inflammatory conditions, and cannot distinguish between different causes of intestinal inflammation, type of IBD (CD vs UC), or location of the disease (small versus large bowel), and may occur in apparently healthy infants and toddlers (89, 90). However, elevated FC

levels are helpful in discriminating IBD from intestinal non-inflammatory conditions, nonspecific symptoms (abdominal pain or non– bloody diarrhea) or signs (anemia, elevated CRP or ESR) to favor or avoid endoscopic workup (87, 91, 92). Repeated, normal FC values make the diagnosis of IBD unlikely (93). Further, increasing evidence support the use of FC not only for diagnosis, but also for follow-up and evaluation of response to therapy in PIBD (94). FC correlates well with endoscopic and histological activity in IBD and has emerged as a biomarker for mucosal healing (95, 96). A low FC concentration predicts persistence of clinical remission especially in pediatric non-symptomatic UC and Crohn's colitis and is a useful surrogate marker of postoperative recurrence in both adult and pediatric CD patients (97, 98). FC is of special value in the pediatric population as a non- invasive marker. Disease location in CD has not been shown to affect its performance, and repeated, normal levels of FC may reduce the need for repeated endoscopies in patients with an established IBD diagnosis (99).

Stool culture

In children with suspected IBD, enteric infections should be excluded as cause of the symptoms, before the child is submitted to endoscopy. The search for bacterial infections should include a stool culture to exclude Salmonella, Shigella, Yersinia, Campylobacter, and Clostridium difficile toxins, as well as testing for Giardia lamblia in high-risk populations. However, identification of a pathogen does not necessarily exclude a diagnosis of IBD, since a first episode of IBD may be triggered by an enteric infection.

5.1.11 Serological markers

Several immune-mediated antibodies against microbial antigens have been described in patients with IBD (100). Among autoantibodies and antibodies against microbial antigens in CD and UC patients, perinuclear anti- neutrophil cytoplasmic antibody (pANCA) and anti–Saccharomyces cerevisiae antibodies (ASCA) are the most studied. Antibodies against the outer membrane porin of Escherichia coli (anti-OmpC), antibodies against a Pseudomonas fluorescence

associated sequence (anti-I2), and flagellin expressed by Clostridial phylum (anti-CBir1), have more recently been investigated in IBD patients (100, 101). Evidence from adult studies suggests that serological markers can help establish a diagnosis of IBD, differentiate CD from UC, and that different patterns and levels of antimicrobial antibodies correlate with disease prognosis, risk of complications, extra-intestinal manifestations, need for surgery, and response to treatment (102-106). Other serological markers such as the antiglycan antibodies anti-chitobioside carbohydrate IgA antibody (ACCA), anti-laminaribioside carbohydrate IgG antibody (ALCA), anti-manobioside carbohydrate IgG antibody (AMCA), anti-laminarin IgA antibody (anti-L) and anti-chitin IgA antibody (anti-C) can further aid in the diagnosis and classification of IBD but are not routinely assessed (86). Pediatric studies have indicated that serological markers can predict aggressive CD behavior in children and help in determining diagnosis and guide therapy in IBD patients (107, 108).

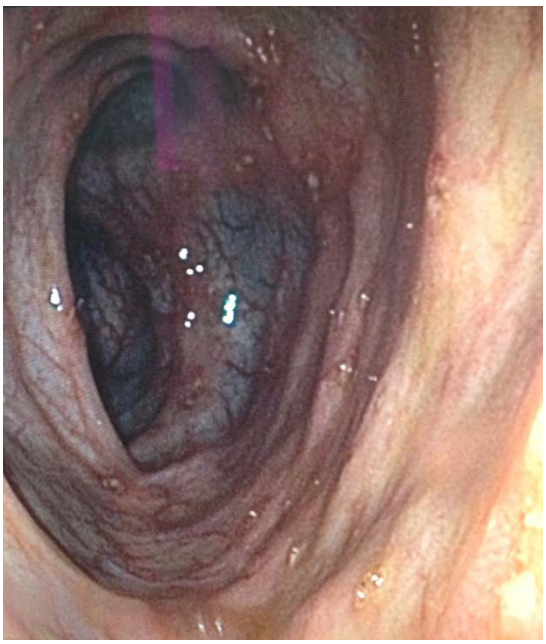
5.1.12 Endoscopy and imaging

According to the Porto criteria, children suspected of having IBD should undergo EGD, ileocolonoscopy, and adequate imaging of the small bowel (109). The significance of a complete endoscopic workup has been well demonstrated. Besides the diagnostic implications of EGD, knowledge about involvement of the esophagus, stomach, and duodenum may have therapeutic consequences (110). For example, it has been shown that in pediatric patients with esophageal CD, there is a high probability for early need of azathioprine (111). The intubation of the terminal ileum increases the diagnostic yield by 13%, and EGD confirms the diagnosis in 7.5% of patients with CD (112). Histology is paramount in distinguishing UC and CD as well as having prognostic potential, presence of granulomas might warrant early aggressive therapy (23).

Currently, magnetic resonance enterography (MRE) is the recommended diagnostic imaging modality for small bowel visualization. It can detect typical IBD-associated features, estimate intestinal inflammation and the degree of

damage, improving the ability to detect mucosal lesions in the small bowel and characterization of PIBD subtypes. In patients who cannot undergo MRE, wireless capsule endoscopy is an alternative. In the ImageKids study, which is a prospective cohort study of pediatric CD, the aim is to develop a magnetic resonance –imaging activity scoring system and a pediatric damage score (113).

Figure 2. Endoscopy image of aphtous lesions in the colon of a CD patient.



SES-CD

Healing of mucosal lesions appears to offer significant benefit and is an important end point in clinical trials of treatment for CD (114). The Crohn's Disease Endoscopic Index of Severity score (115) is complicated and time consuming. The simpler endoscopic score of disease activity, SES-CD is easier to use, and contains scores for ulcer size, ulcerated surface, affected surface, and luminal narrowing (116).

Table 6. SES-CD, from Daperno et al (116)

Variable	0	1	2	3
Size of ulcers (cm)	None	Aphthous ulcers (diameter 0.1-0.5)	Large ulcers (diameter 0.5-2)	Very large ulcers (diameter > 2)
Ulcerated surface	None	< 10%	10-30%	> 30%
Affected surface	Unaffected segment	< 50%	50-75%	> 75%
Presence of narrowings	None	Single, can be passed	Multiple, can be passed	Cannot be passed

SES-CD = sum of all variables for the 5 bowel segments.
Values are given to each variable for every examined bowel segment

Mayo

In UC, the Mayo endoscopic score has been widely used in clinical trials (51) and may be used in pediatric UC. A score of 0 means inactive disease, a score of 0-1 has been considered endoscopic remission, although it may involve mild disease (erythema, decreased vascular pattern and/or mild friability). A score of 2 indicates moderate disease with marked erythema, absent vascular pattern, friability and erosions, while 3 is the maximum score and reflects severe disease with spontaneous bleeding and ulcerations.

Table 7. Mayo UC Endoscopic Score

Score	**0**	**1**	**2**	**3**
Disease severity	Normal	Mild disease	Moderate disease	Severe disease
Mucosal appearance		erythema, decreased vascular pattern, mild friability	marked erythema, absent vascular pattern, friability, erosions	spontaneous bleeding, ulceration

5.1.13 Treatment of PIBD

The goal of treatment of PIBD is not only clinical remission, but "treat to target" with mucosal healing, normalization of biomarkers, histological healing and healing on abdominal imaging (117). Other treatment goals include optimized growth, gaining puberty and psychosocial health with the ability to attend school, activities and to socialize. Treatment strategies are aimed at reducing and preventing complications of the disease and avoiding complications and side effects of medication (118).

Treat to target and tight drug monitoring are important in order to reduce the patient's risks and to avoid inappropriate or over- treatment (119). Also, about one third of patients experience secondary loss of response to TNF blockers and will need treatment optimization (120). Tight drug monitoring may be beneficial in avoiding side effects while maintaining effective drug levels, thus reducing antidrug antibody formation and maintaining response (119). Given the scarce treatment alternatives in PIBD when loss of response occurs, it is highly desirable to optimize both initial response and long-term continuation of therapy. At present, in 2018, the biologic agents approved for PIBD are the TNF blockers infliximab and adalimumab. For adult IBD, newer agents have been approved, such as golimumab, another TNF blocker, as well as other classes of biologics, such as the alpha4beta7 integrin antibody vedolizumab, and ustekinumab, an agent targeting IL-12 and IL -23, bringing future hope for their use in PIBD (121).

Pre-treatment considerations

Screening for infections is crucial, as well as obtaining varicella zoster virus, hepatitis B and C, human deficiency virus, Epstein-Barr virus (EBV) and cytomegalovirus serology and ruling out tuberculosis (122). Optimizing immunizations is part of the treatment algorithm as most PIBD patients will be treated for a long time with immunosuppressive drugs. There are guidelines on how to prevent, diagnose and manage opportunistic infections in IBD (122, 123).

Live vaccines (varicella, measles, mumps, rubella) should, if possible, be delivered prior to immune suppression and immunodulating therapy, as subsequent

immunization with live vaccines only can be administered 3-6 months after cessation of all immunosuppressive drugs. In addition to the routine vaccinations, PIBD patients should be offered varicella zoster vaccination if serology is negative, vaccination against human papilloma virus, inactivated vaccine against influenza once a year, hepatitis B vaccine and pneumococcal vaccine (at diagnosis and after 5 years of disease).

Testing for mutations in the enzyme thiopurine methyltransferase may be indicated prior to commencing treatments with azathioprine, as mutations are associated with severe bone marrow depression with standard doses of azathioprine or 6-mercaptopurine (124-127). Due to a higher risk of lymphoma and hepatosplenic T cell lymphoma in EBV negative young males treated with azathioprine, these patients should receive other treatment options (128, 129). Previously, the notion was that the risk of lymphoma primarily was elevated in young males. Recent publications indicate that young females also have an elevated risk for these malignancies if treated with azathioprine (130), whereas infliximab exposure does not seem to elevate the risk of lymphoma, but instead adds a risk for infections (131).

Age at diagnosis, pubertal and growth development, disease severity, and immune status are some of the factors influencing treatment decisions; conventional versus early aggressive/ top down strategy, monotherapy versus combination therapy, and the use of systemic or locally active corticosteroids (132). The treatments need to be individualized and tailored, often escalated or modified as many patients experience primary or secondary non-response (133).

European Crohn's and Colitis Organization (ECCO) and ESPGHAN have published consensus papers on the medical (17, 134, 135) and surgical (136) management of PIBD. Treatments include nutrition, anti-inflammatory agents, immunosuppressive agents, immunomodulators and biologic therapy targeting inflammatory cytokines.

Nutrition

Nutrition plays a key role in the management (137), for luminal CD also as induction therapy in the form of exclusive enteral nutrition (EEN). It is considered gold standard to start induction treatment with EEN in CD, meaning the exclusive use of a liquid diet in the form of medical formulas for 6-8 weeks, without exposure to other food. The anti-inflammatory effect does not depend on the type of formula (protein source) but on the exclusion of ordinary table food (57). EEN is as efficient in inducing remission as corticosteroids in luminal CD, with an overall remission rate of 73%, and is superior when it comes to mucosal healing (138). The diet has no side effects, but for the patients it is a challenging treatment as no solid food is allowed, since partial enteral nutrition is ineffective for induction of remission (139). Diets are developed with a similar composition as EEN, allowing some table foods, such as the CD exclusion diet (CDED), with promising results (140).

Aminosalicylates

In CD, there is lack of evidence that 5-aminosalicylates (5-ASAs) are effective, whereas the recommendation in UC is to use oral and/or topical 5-ASAs in order to induce and maintain remission. As induction therapy in UC, high doses are required, 60-80 mg/kg/day, and optimal 5-ASA therapy includes using topical and local treatment in addition to or instead of oral medication, including suppositories and enemas. 5-ASAs are indicated for maintenance of remission, in lower dosage, and studies have shown that 5-ASA co-medication result in higher levels of the metabolically active compound in azathioprine; 6- thioguanine nucleotides (141).

Antibiotics

In perianal fistulizing disease antibiotic therapy with metronidazole and/or ciprofloxacin may be beneficial, as they have an effect in reducing fistula drainage (142). Pediatric studies have shown promising results with azithromycin and rifaximin for induction of remission in luminal inflammatory CD (143, 144). In moderate-severe refractory UC and IBDU in children a combination of 3-4 antibiotics including metronidazole, amoxicillin, doxycycline, and vancomycin have yielded good results

(145). However, long-term consequences with development of bacterial resistance to antibiotics and the risk of Clostridium difficile infections raise concerns and limit their practice.

Corticosteroids

It is essential to reduce, and when possible, to avoid the use of corticosteroids in PIBD as these agents can lead to further growth impairment, reduced bone health, adrenal suppression as well as emotional and behavioral side effects. EEN is not efficacious in the induction and maintenance of remission of pediatric UC, and corticosteroids are still part of the routine medical management in UC patients who do not gain remission with 5-ASA therapy. Corticosteroids should only be used in order to induce remission; they have no part in the maintenance therapy of PIBD. High doses over long periods of time should be avoided, and topical corticosteroids such as budesonide with less side effects are preferable when possible (146, 147).

Thiopurines

Maintenance therapy and steroid sparing treatments are essential in PIBD. Thiopurines, (azathioprine and 6-mercaptopurine) are used in patients with aggressive disease, and as adjunct therapy in order to reduce the formation of antibodies to TNF blocker agents. In the SONIC trial, patients on combination therapy with azathioprine and the TNF blocker infliximab, had a better outcome than those on monotherapy with azathioprine or infliximab (148). However, as discussed earlier, thiopurines are not without risks. In addition to a higher risk of lymphoma they increase the risk for non-melanoma skin cancer, also years after cessation. Additionally, they can induce hypersensitivity reactions such as pancreatitis and have a narrow therapeutic window. Measurements of 6-thioguanine nucleotides, the active metabolite of azathioprine, may guide and optimize dosing (149), and complete blood count and liver enzymes need to be closely monitored due to risk of myelotoxicity and hepatotoxicity. It takes time for thiopurines to work, hence they are not suitable as induction therapy.

Methotrexate

Methotrexate can be used as maintenance treatment for children and adolescents with CD at risk for an aggressive disease course, either as the primary maintenance agent or in thiopurine intolerance or failure (17). For UC, the data are not as robust, and methotrexate is usually only considered when other alternatives such as therapy with thiopurines have failed (135, 150). Methotrexate should preferably be given parenteral once weekly, together with antiemetic drugs, and folic acid should be supplemented.

Biologic therapy

In PIBD the TNF blockers infliximab and adalimumab are approved both for induction and maintenance of remission (151) and biological therapy have become part of routine care in the management of moderate to severe PIBD (17, 152). TNF blockers are monoclonal antibodies against TNF alpha, a cytokine that is up regulated in IBD, and these agents have revolutionized the IBD treatment. There is a higher response rate to TNF blockers in pediatric patients compared to adults and these compounds are well tolerated. High costs and the fact that they are not needed by all IBD patients highlights the importance of selecting patients that benefit most from biologic therapy. The effectiveness is better the earlier in the disease course they are given, they may delay the progression to stricturing and penetrating complications in CD, and studies suggest that early TNF blocker therapy may alter the natural disease history of IBD (153, 154).

TNF blockers are associated with a risk of antibody formation, and studies have shown that combination therapy with immunomodulators is associated with higher TNF blocker trough levels and decreased antibody formation. Combination therapy may reduce the risk of stricturing disease, surgery and colon cancer but increases the risk of toxicity and infections (130, 153, 155). The risks and benefits of combination treatment must be weighed against each other and decided individually in agreement with the patients and their families.

Biosimilars are biological agents that are very similar to a reference drug already authorized for use, and the quality, safety and efficacy should be equal (156, 157).

Recently, biosimilars were authorized for use in PIBD. Post marketing surveillance is mandatory and ESPGHAN recommends caution, as uncertainty still exist regarding drug efficacy, immunogenicity and pharmacology of biosimilars (158). Reports on use of the biosimilar infliximab in PIBD indicate that they are as effective as the originator and are associated with significant cost savings (157, 159).

Surgery

Even though the biologic agents have revolutionized the treatment of IBD, there are still indications for surgery such as severe medically refractory disease, steroid- dependent disease in UC, or stenosis, abscesses or fistulas in CD (134, 136, 160). A recent study showed that the 5-year risk of surgery in pediatric CD has not changed from 2002 through 2014, remaining around 13,5% and that early TNF blocker therapy failed to prevent early surgery with most surgeries occurring within 3 years from diagnosis (153). In PIBD the timing of surgery is essential in order to promote growth and puberty, and surgery should be an integrated part of the treatment algorithms and reflections.

5.1.14 Clinical significance of prognostic considerations

Although it is established that the PIBD phenotype is more aggressive, it remains difficult to estimate the individual prognosis and risk of complicated disease. With growth restriction and puberty often co-occurring with disease onset, it is essential with effective and aggressive intervention in patients with phenotypes at risk for an aggressive disease course. In CD, disease activity, rate of recurrence and complications over time, the need for surgery and the disease impact on quality of life are all factors to be considered when determining treatment strategy (161). In UC, disease extent and severity at diagnosis are clinical characteristics associated with a higher rate of colectomy (2). In the era prior to the use of biologic therapy, studies of the natural history of CD confirmed the disease to be a chronic, progressive disease, which also was confirmed in pediatric studies, with half of patients initially presenting with uncomplicated

disease progressing to complicated disease with time (20, 162). Immunosuppression and biologic therapy may reduce the risk of progression to stricturing and penetrating disease and surgery (163), but are associated with malignancies, hemophagocytic lymphohistiocytosis and opportunistic infections. On the other hand, longstanding and uncontrolled inflammation is a risk factor for developing colorectal adenocarcinoma (164). A recent paper from the pediatric IBD Porto group of ESPGHAN found that malignancies and infections were the major cause of mortality in PIBD. This multinational study also showed that death in PIBD patients more often is due to disease related adenocarcinomas than to lymphomas (164).

The challenge is to identify patients who should get early aggressive therapy/ top down treatment, and those who can be treated with cheaper and potentially safer options. Conventional therapies for both UC and CD include 5-ASAs, corticosteroids, and the immunomodulators azathioprine and methotrexate. These agents are part of the "step up" strategy, starting with cheaper but less efficacious medications. "Aggressive" or "top down" treatment involves early biological therapy. More knowledge of etiological and prognostic factors is needed to increase the diagnostic precision, the efficacy and timing of treatments in order to improve the disease course and quality of life of patients.

5.1.15 Known prognostic markers prior to the start of this thesis

In population based cohorts, ileal disease, upper GI disease, complicated behavior, age less than 40 years at diagnosis and perianal disease were identified as clinical risk factors for complicated CD, while in UC, extensive disease and young age at diagnosis were risk factors for worse outcomes (165).

In pediatric CD, deep colonic ulcerations on endoscopy, persistent severe disease despite adequate induction therapy, extensive disease, marked growth retardation, severe osteoporosis, stricturing and penetrating disease as well as severe perianal disease at diagnosis are associated with a poor outcome (17). Poor nutrition, smoke exposure, and genotype have been found to have

prognostic value as well (1, 166, 167). Presence of extra-intestinal manifestations, male gender, young age and growth retardation at diagnosis in pediatric CD was in a population based cohort shown to be associated with diminished target height in adulthood (167). In pediatric UC, younger age, a positive family history of IBD and extensive disease have been associated with worse outcome, as well as the presence of extra-intestinal manifestations (18, 19, 168). On endoscopy, severe ulcerations and extensive findings are indicative of a less favorable disease course, and adult UC patients who reach mucosal healing after one year of therapy are less likely to need a colectomy during 5 years of follow-up (114).

Inflammatory and serological markers

The prognostic value of biomarkers such as CRP, ESR and FC has been studied, and patients with higher levels are more prone to relapse (84, 169, 170). High titers of ASCA, anti-OmpC, and anti-CBir1 are variables at diagnosis associated with aggressive CD (2, 171-173). In adult UC patients, the presence of pANCA is associated with an aggressive disease course, higher relapse rates, resistance to therapy as well as more frequent need for early surgery (104, 174, 175). In pediatric patients, both positivity and high titers of serological antibodies have been found to be associated with worse outcomes (107).

Gut microbiota

The Human Microbiome Project Consortium has generated new knowledge about the microbiota in humans (176), and the number of reports about how the microbiota is altered in different diseases is expanding rapidly. Studies in IBD have shown an overall decrease in diversity, with a decrease in Firmicutes, and an increase in Proteobacteria abundance (177, 178).

A study of established PIBD patients on treatment could distinguish between CD and UC and between IBD and non-IBD, both in feces and colonic biopsies (77). A large multicenter study in the US analyzing the gut microbiome in treatment naïve CD patients, found that the mucosal microbiota showed an overall decrease in species richness and shifts in abundance of several taxa but that stool samples

reflected the mucosal dysbiosis less well (76). In a study of pediatric patients with severe UC, the fecal microbial diversity and evenness was reduced compared to healthy controls, and even mores so in patients not responding to steroids, indicating that gut microbiota composition may have prognostic potential (179).

5.1.16 Prognosis, unanswered questions

Despite progress in unravelling the pathogenesis in IBD, moving towards precision medicine, there are only a few biomarkers that are widely used in clinical practice, such as FC and CRP. These markers have a good negative predictive value regarding inflammation, but how they can aid in stratifying patients at diagnosis is less studied. Reliable and validated predictors of poor prognosis that could aid in deciding treatment early in the disease course are still needed. Although several studies have shown that serological markers are associated with disease phenotypes and disease course in IBD, the prevalence of the serological markers pANCA, ASCA, OmpC, I2 and CBir1 in unselected Norwegian children and adolescents with PIBD is unknown. Additionally, the significance of these markers from the disease onset, their variation over time and their ability to predict treatment response and outcome are not fully explored in pediatric patients.

Microbiome- based biomarkers are yet in their infancy. The different studies have shown conflicting results, and the correlation between the fecal and mucosa associated microbiota and the diagnostic precision of the different samples have yet to be determined. The significance of fecal microbiota profiles as a predictive tool in PIBD is largely unknown.

6 Aims of the study

Main aims

- To evaluate clinical factors and biomarkers as predictors of PIBD outcome and response to treatment.

Secondary aims

- To study whether clinical, endoscopic and biochemical markers at diagnosis are associated with the need for early initiation of treatment with TNF blockers in pediatric CD patients.
- To evaluate if early intervention with biologic treatments is safe and efficient in inducing and maintaining remission within the first 1-2 years after diagnosis in children and adolescents with CD.
- To study whether profiles of serological markers at the time of diagnosis can aid in distinguishing patients with IBD from patients with abdominal symptoms without GI inflammation.
- To study the changes in prevalence and titers of serological markers before and after treatment.
- To study whether the fecal microbiota differentiates pediatric patients with treatment-naïve IBD from non-IBD patients with gastrointestinal symptoms and healthy children.
- To study whether fecal microbiota profiles can identify different IBD phenotypes.
- To study fecal microbiota profiles before and after therapy.

7 Summary of results

7.1.1 Paper I

We wanted to study if clinical, endoscopic and biochemical markers at diagnosis could predict the need for early initiation of treatment with TNF blockers in pediatric CD patients and whether early intervention with biologic treatment would be safe and efficient in inducing and maintaining remission within the first 1-2 years after diagnosis.

In paper I we prospectively followed a cohort of 36 pediatric CD patients. Fifty % received biologic therapy with TNF blockers within the first year after diagnosis. These patients had significantly shorter disease duration, higher levels of FC, CRP and ESR at diagnosis, as well as more extensive disease distribution with more upper GI involvement compared to CD patients who gained remission with conventional treatments (EEN, 5-ASAs, corticosteroids, immunomodulators). The rate of clinical remission and ileomucosal healing was high and comparable for both treatment groups (TNF blocker treated and conventional treated) with no serious side effects observed. A substantial proportion of patients had unspecific lesions in the upper GI tract at follow-up regardless of treatment and despite mucosal healing in the ileocolon. We confirmed that biologic therapy in pediatric CD is associated with higher inflammatory markers and widespread disease at baseline. Biologic therapy seems safe and effective for induction and maintenance of remission, but is not necessary for all patients, highlighting the importance of individualizing treatments in PIBD.

7.1.2 Paper II

In paper II we describe the prevalence of serological markers in 55 treatment-naïve PIBD patients at the time of diagnosis and their utility in differentiating CD, UC and symptomatic non-IBD patients. We assessed the association between presence of autoantibodies and initiation of early TNF blocker therapy. Moreover,

we compared the clinical relevance of serological markers to the biomarkers CRP, ESR and FC.

The prevalence of antibodies against ASCA, pANCA, and I2 were significantly higher in an unselected Norwegian cohort of PIBD patients compared to a group of symptomatic non-IBD controls, 36% vs 8%, 47% vs 27 %, and 38% vs 11%, respectively. ASCA and pANCA status was useful in differentiating UC and CD patients, and positive ASCA and/or negative pANCA was associated with early initiation of TNF blocker therapy in CD patients. In multivariate analyses we found pANCA and/or ASCA status but not CRP, ESR and fecal calprotectin levels to be associated with early TNF blocker therapy.

ASCA serology was stable, regardless of treatment modality, and might be a prognostic tool at any time in the disease course, whereas there was a decline in pANCA positivity after treatment.

7.1.3 Paper III

We explored the fecal microbiota abundance in pediatric patients with treatment naïve IBD, non-IBD patients with GI symptoms, and healthy children with a 16SRNA based test with the GA-map™ technology. The fecal abundance of 54 predefined bacterial DNA markers was assessed at inclusion and for a subgroup of IBD patients also after 1-2 years of treatment.

Fecal microbiota profiles could distinguish healthy children from symptomatic patients, both IBD and non-IBD patients had significantly reduced bacterial abundance in 51/54 markers compared to healthy controls. The dysbiotic microbiota profiles in IBD and non-IBD symptomatic patients were similar.

When comparing IBD phenotypes, CD patients with stricturing and/or penetrating disease behavior had a higher abundance of pathobionts such as Proteobacteria, and more extensive disease was associated with more *Ruminococcus gnavus* and *Veillonella*. Patients receiving biologic therapy had lower baseline abundance of Firmicutes and *Mycoplasma hominis*. Poor prognosis, with non-mucosal healing after 1-2 years of treatment and/or later

surgery was associated with high Proteobacteria abundance and low levels of *Faecalibacterium prausnitzii* at baseline. The fecal microbiota abundance remained unchanged after therapy, regardless of treatments and remission status. Our results indicate that baseline microbiota profiles may be of prognostic value and aid in treatment individualization.

8 Methodological considerations

8.1.1 Study design and population

In Norway all suspected cases of PIBD are referred to a tertiary and university hospital for investigations and diagnostic workup. Patients enrolled in the present studies were recruited from the catchment areas of two university hospitals (Akershus University Hospital and Oslo University Hospital-Ullevål) in three population based prospective epidemiological studies of treatment-naïve pediatric IBD; Inflammatory Bowel Disease in South-Eastern Norway (IBSEN-II), Early IBD and the EU IBD Character study. The inclusion period was from 2005 to 2015, with identical protocols and inclusion criteria. All patients referred in the study periods were included after written informed consent and the collection of data was performed on the day of inclusion. Data included demographics, family history, medical history, symptoms, activity scores; PUCAI and PCDAI, pubertal scores and weight and height measurements. During endoscopy, the mucosa was evaluated macroscopically and all changes were noted for all segments of the GI tract. Tissue specimens from EGD and ileocolonoscopy were collected from all gut segments and evaluated by experienced pathologists. The patients were referred to MRI examinations, and all results were recorded. Using a data handling program, Snap Survey, the data were transferred for statistical analyses to SPSS (Spss Inc, Chicago, IL, USA). SPSS and Microsoft Excel were used for storage and handling of data.

Inclusion criteria

Pediatric patients under 18 years, referred during the inclusion periods and believed to have IBD based on symptoms, were included after informed consent. Of note, in these studies we included patients until 18 years of age, not until 17 years of age as in the Paris classification.

Ideally we should have included a healthy control group, but performing endoscopies in general anesthesia in healthy children and adolescents is not considered ethical. In order to create a comparison group to our IBD patients,

children and adolescents presenting with similar symptoms but with negative endoscopies and a normal MRI, were included as non-IBD symptomatic patients. If they were diagnosed with an inflammatory condition other than IBD or an infection, they were excluded. Some of these non-IBD patients may have had other conditions such as disturbances of intestinal permeability, influencing the study results. Most of them were thought to have functional GI disorders such as IBS.

8.1.2 Diagnostic criteria

IBD was diagnosed in accordance with the Porto criteria (10). MRI was used for the investigation of disease location and distribution in the children > 6 years of age where no additional general anesthesia was needed. The Vienna (180), and later the Paris classification (32) was used to characterize the disease distribution and behavior. Reclassifications were made during follow-up, with some patients changing diagnosis to CD from UC and vice versa. Patients who did not meet the diagnostic criteria for IBD, with normal findings on upper and lower endoscopy, and MRI investigations, were included as non-IBD symptomatic controls. At inclusion, and at follow-up for the IBSEN-II patients, the same protocols with registrations, examinations and laboratory tests were performed both in IBD and in non- IBD symptomatic patients.

8.1.3 Study cohorts

IBSEN II

The Inflammatory Bowel Disease of South Eastern Norway II (IBSEN II) study was the basis for paper I and II, and a part of paper III. The inclusion period was from 1st of May 2005 until 31st of December 2007, recruiting a total of 100 children up to 18 years of age (4). After evaluation, 58 treatment- naïve pediatric patients were diagnosed with IBD; 39 with CD, 18 with UC and one with inflammatory bowel disease unclassified (IBDU). Thirty-eight were non-IBD symptomatic patients. Four patients were excluded due to infections or other

diagnoses. The IBSEN-II patients (both IBD and non-IBD symptomatic patients) participated in a prescheduled follow-up within 2 years after diagnosis, where the study protocol from inclusion was repeated; including upper and lower endoscopies and MRI imaging. In paper I, 36 CD patients were included. In paper II, serological and inflammatory markers of 55 IBD (37 CD, 18 UC) and 37 non-IBD patients were compared.

Early IBD study

The Early IBD study, an international multicenter prospective study of inflammatory bowel disease, was conducted between May 2009- 2012 from the same catchment areas and with the same protocols and investigations as in the IBSEN II study. Fifty-nine patients with treatment naïve IBD, and 17 symptomatic non-IBD patients aged less than 18 years were included. Complete sampling was available from 38 IBD patients (29 CD, 9 UC) and 11 symptomatic non-IBD patients, and these patients were part of paper III.

EU IBD Character study

The EU IBD Character study is an international multicenter prospective study of adult and pediatric IBD. The inclusion period was from March 2013 to September 2015. The pediatric patients were included from Akershus and Oslo University Hospital-Ullevål and with the same protocols and investigations as in the IBSEN II and Early IBD studies. IBD was diagnosed in 28 patients, whereas 16 were non-IBD symptomatic patients. Complete sampling was available in 24 patients diagnosed with IBD (18 CD, 4UC and 2 IBDU) and in 13 of the non-IBD symptomatic patients, and these patients were part of paper III.

8.1.4 Non IBD symptomatic patients

Since the non-IBD patients were meticulously examined, we are quite certain that these patients are not misdiagnosed. However, they have not been sub- classified in regard to functional GI disorders, so their diagnosis is not clear, only IBD, inflammation and infections have been ruled out. In the IBSEN-II study, two of the non-IBD patients were diagnosed with celiac disease, one of which also had a

juvenile polyp, and one patient had hypogammaglobulinemia. In the Early IBD study one patient was diagnosed with non celiac gluten sensitivity, one had orofacial granulomatosis and one had gastroesophageal reflux disease. In the IBD Character study three of the non-IBD patients had infectious gastroenteritis; 1 Salmonella and 2 Campylobacter, and one patient was later diagnosed with systemic IgG4 disease. These patients were excluded. The remaining patients displayed no evidence of inflammation during workup indicating functional GI disorders. According to medical records none of the non-IBD symptomatic patients have developed IBD as of December 2018, which gives a follow-up of 3-13 years.

8.1.5 Healthy controls

Altogether 100 healthy children and adolescents between the age 2 and 18 years were recruited from the same catchment areas as the patients in IBSEN-II, Early IBD and IBD Character during the period of 2013-14. They delivered fecal samples and answered a questionnaire regarding their health status. Seventy-five children and adolescents were included as healthy controls. They had no chronic diseases, no IBD in the family, followed a normal diet (children on exclusion diets; gluten-free, cows milk protein-free, vegetarian/vegan, were excluded), had not travelled outside Europe or used antibiotics within the last six months, had no recorded GI complaints, did not use proton pump inhibitors and had normal FC levels.

8.1.6 Data collection and preparation of biologic material

All data was collected on the day of inclusion, by means of filling out forms covering demographic data, family history, medical history, symptoms, activity scores; PUCAI and PCDAI, pubertal scores, weight and height. These data as well as endoscopic histological and radiological findings were entered into a database.

Blood samples

Blood samples were taken on the day of endoscopy and included hemoglobin, hematocrit, complete blood count, CRP, ESR**,** liver enzymes, creatinine, amylase and albumin level. Serum samples from the IBD and non-IBD patients were stored at minus 70 degrees Celsius until analysis of serological markers.

8.1.7 Serological markers

Serum samples were analyzed for the presence and titers of antibodies against ASCA IgA, ASCA IgG, OmpC, CBir1, I2, and for the presence of pANCA. Standardized enzyme-linked immunosorbent assay (ELISA) and indirect immunofluorescence assay were used. All analyses were performed by Prometheus laboratories (San Diego, California, USA). The laboratory was blinded for IBD or non-IBD diagnosis. Antibody levels were expressed by ELISA units (EU/ml), except for pANCA, which was classified as detected or not detected. Reference values (EU/ml, ELISA) for antibodies were: ASCA IgA<8.5, ASCA IgG<17.8, OmpC IgA< 10.9, CBir1 IgG< 78.4 and I2< 368, which were relative to a Prometheus Laboratory Standard, derived from a pool of patient sera with well-characterized disease found to have reactivity towards these antigens (101). Titers above the reference value were considered a positive serologic response. All serologic markers from baseline and follow-up were analyzed at the same time point.

8.1.8 Feces

Feces were sampled at home in three designated containers without additives on the day before endoscopy, before bowel preparation, kept refrigerated or frozen, and brought to the hospital the next day. Feces from one container was analyzed for calprotectin (FeCal-test, Bühlmann, Basel, Switzerland). The second container with feces was investigated for pathogenic bacteria at the microbiology department of the hospitals, and the third container with feces was frozen at -80 degrees Celsius for later microbiota analysis.

The healthy controls (Paper III) received two designated fecal sampling kits at home for handling of samples before deliverance to Genetic Analysis AS, Oslo, Norway. One sample was analyzed for FC (FeCal-test, Bühlmann, Basel, Switzerland), the other was frozen at -80 degrees Celsius and stored for later microbiota analysis. For all samples, the maximum time interval until frozen at -80 degrees Celsius was three days, thereafter the samples were kept frozen and not thawed until analysis.

8.1.9 Biopsies

Biopsies were collected during upper and lower endoscopy. At least two biopsies were taken from the duodenum, ventricle, esophagus, ileum, rectum and all segments of the colon. Additional biopsies were taken if pathologic findings were observed macroscopically during endoscopy. The biopsies were evaluated by experienced senior pathologists. Macroscopic erosions, aphtous lesions and ulcerations were regarded as CD specific lesions of the upper and lower GI tract. Microscopic chronic focal active inflammation and villous atrophy were accounted for, and helicobacter pylori infection and celiac disease were ruled out. The mucosal appearance was scored in accordance with the SES-CD. Mucosal healing was defined as disappearance of ulcerations, multiple erosions, bleeding, and friability. Both complete normalization (grade 0) and light hyperemia and granularity (grade 1) were considered as endoscopic healing (116). Disappearance of ulcerations and erosions, active inflammation and cellular infiltrates were considered as histologic healing as well as light structural changes (114).

8.1.10 Microbiota analysis

The microbiota was analyzed using the GA-map™ technology (Genetic Analysis AS, Oslo, Norway), a PCR and 16SRNA based analysis. The method uses a targeted approach to detect predefined bacteria believed to be important in identifying and characterizing gut bacteria dysbiosis in adults (73). The test measures

relative bacterial abundance based on the fluorescence signal strength (FSS) of bacterial DNA markers. The markers are targeting variable regions V3 to V7 of the bacterial 16S rRNA gene. The method utilizes 54 bacterial markers, covering more than 300 bacteria at different taxonomic levels; 26 species specific, 19 genus specific, and 9 bacteria at higher taxonomic levels (phyla, class and family) (**Supplementary Table 1)**. All samples were analyzed at the same time point. The laboratory was blinded for the diagnosis of IBD, non-IBD or healthy.

8.1.11 Statistical analyses

Data were described with median and observed range (continuous variables) and with frequencies and percentages (categorical variables). Due to a limited sample size, non-parametric methods were used. The Mann-Whitney Wilcoxon test for unpaired samples was used for comparisons between groups with regards to continuous variables. When comparing measurements at baseline and after treatment, Wilcoxon signed rank test was used. Crude associations between pairs of categorical variables were assessed with the Chi-square test. In paper II, univariate and multiple logistic regression models were fitted to estimate odds ratios (OR). The results were also expressed as probabilities for selected values of included variables.

In paper III we performed principal component analysis (PCA) in order to explore the ability of all 54 bacterial markers to distinguish between IBD, non- IBD symptomatic patients, and healthy controls. The FSS from the 54 markers were added for each patient and the sum illustrated a relative abundance, denoted the total fluorescence signal strength.

Areas under the curves (AUCs) were calculated and receiver operating characteristic (ROC) analysis conducted to evaluate the performance of selected bacterial abundances in distinguishing IBD phenotypes and treatments. All tests were two-sided. P-values <0.05 were considered statistically significant. We regarded our analyses as exploratory; therefore, we did not correct for multiple testing. However, in order to validate our results in paper III, each observation

was randomized into a test set or a training set so that the number of observations was equal in both sets. Only the statistically significant differences between selected groups confirmed in the training set were reported. All analyses were performed using SPSS, statistical software version 22 or 24 (SPSS Inc., Chicago, Il, USA) and Stata version 9.

8.1.12 Ethical considerations

Traditionally, medical knowledge from adult patients has been adapted and implemented in the treatment and understanding of diseases in the pediatric population. This is also true regarding IBD. Pediatric populations, however, are not "small adults", and the disease etiology, disease behavior and effect of treatments often differ in children compared to adults. Therefore, we have an obligation to study the pediatric population. The workup of the patients in this project followed guidelines for diagnosing PIBD. The only possible negative consequence for the patients attending was that more biopsies were taken than in the routine setting. Biopsy taking is associated with a less than 5% complication rate, mostly non-clinically significant bleedings. However, no complications after biopsies were noted.

The studies were conducted with informed patients and parental/guardian written consent as appropriate and with full ethical approval, in accordance with the Helsinki declaration, and with approval by the Regional Committee for Medical Research Ethics, South-Eastern Norway, reference no. REK S-04209.

9 General discussion

The incidence of PIBD is rising, and these patients face a chronic disease with the need for lifelong medication as well as a high risk for progressive disease, complications, surgery and cancer. Markers for disease progression, relapse and treatment response are important in the management of IBD. Knowledge about a patient's individual prognosis is warranted in order to guide therapeutic decisions, introducing the optimal treatment at the right time. In this thesis we evaluated clinical factors, inflammatory biomarkers, serological markers and the composition of the gut microbiota in treatment naïve pediatric IBD patients in order to predict prognosis and to individualize and optimize treatment.

9.1.1 Variables associated with TNF blocker therapy in CD and outcome in IBD

Our main aim was to evaluate clinical factors, inflammatory and serological markers and fecal gut microbiota as predictors of outcome and response to treatment. We found short disease duration, widespread disease distribution with upper GI involvement, high levels of the inflammatory markers CRP, ESR and FC at diagnosis, as well as the presence and titers of antibodies to ASCA and no pANCA autoantibodies to be associated with the initiation of early TNF blocker therapy in pediatric CD. Fecal microbial profiles had predictive value. Our PIBD patients who received biologic therapy had lower baseline abundance of Firmicutes and of *Mycoplasma hominis* species than conventionally treated patients. The need for surgery and lack of mucosal healing was associated with higher abundance of Proteobacteria and lower baseline abundance of *Faecalibacterium prausnitzii* compared to non-operated IBD patients and patients with mucosal healing.

Disease location and extension

In adult patients, upper GI involvement has been described to be an independent factor of more complicated disease with more hospitalizations and early surgery

(181). A large pediatric study reported associations between esophageal involvement, higher PCDAI, penetrating disease behavior and perianal involvement (182). A prospective study comparing pediatric CD patients with and without upper GI involvement, reported that patients with upper GI manifestations had more active disease, extraintestinal manifestations and widespread lower GI disease distribution. Patients with upper GI involvement needed immunomodulating therapy and TNF blockers in order to induce and maintain remission more often than patients without GI lesions (183). A paper published in 2018 recognized male sex and young age < 16 years as the main predictors of upper GI involvement. Their observation that these patients are more likely to be treated with TNF blockers is in line with our results.

It is difficult to compare upper GI findings in CD as there is no standardized accepted scoring system. The ImageKids Study recently applied the SES-CD score to the upper GI tract. They found the score to be associated with a more severe disease phenotype but not with disease course (184). Pediatric patients more often exhibit upper GI involvement, underscoring the importance of doing an upper endoscopy as part of the workup even in the absence of symptoms, as upper GI findings seem to have prognostic potential as well as diagnostic implications (2, 185, 186).

It is well known that extensive disease distribution in UC is indicative of a severe outcome. This was confirmed in a pediatric UC study, where children with pancolitis, anemia and low albumin failing to achieve steroid-free remission at 3 months were at higher risk of requiring biologics and/or surgery within 18 months (187). Almost 60% of our UC patients had an extensive or total colitis at diagnosis, which confirms that the pediatric phenotype in UC is more extensive than adult UC. In the first paper we only included CD patients, as none of our UC patients at that time had received TNF blocker treatment. In paper III, 44% of our 27 UC patients had received TNF blocker treatment, and two patients had a colectomy. Disease extension was associated with higher abundance of

Ruminococcus gnavus, both in our UC and CD patients. Upper GI manifestations in CD was associated with more *Veillonella.*

Stricturing and penetrating disease behavior and non-mucosal healing in CD was associated with higher abundance of Proteobacteria compared to patients with an inflammatory disease behavior and who achieved mucosal healing at follow-up.

CRP

Several previous studies have confirmed that CRP may predict disease course and relapse (188). A study of adult CD patients found that elevated CRP at diagnosis was associated with later TNF blocker therapy (189). This finding, in accordance with our results, was replicated in a pediatric study where a baseline elevated CRP was associated with the need for azathioprine and TNF blockers at 1 year (186). A high initial CRP was also more frequent in CD patients with upper GI manifestations, and in stricturing and penetrating phenotypes. Several studies have found CRP to be predictive of surgery in adult IBD (84, 190). A recent pediatric study demonstrated that a PUCAI score > 35 and a positive CRP at diagnosis in UC were important predictive factors for an early relapse within the first year (191). CRP is an easy, cheap and readily available biomarker, and our findings confirm its prognostic potential in PIBD, although an elevated CRP failed to remain statistically significantly associated with TNF blocker treatment in the multivariate analysis in paper II.

ESR

ESR was in our study associated with early TNF blocker treatment in CD, in line with a previous pediatric CD study, where elevated ESR at diagnosis was predictive of early immunomodulator use (192). Consigny et al found ESR > 15 mm to be predictive of relapse in adult CD patients (193). In UC, ESR performs less well, and a recent paper validating clinical predictors of outcomes in pediatric UC found no correlation between initial ESR levels and later disease course (187). In the multivariate analysis in paper II, elevated levels of ESR failed to remain statistically significantly associated with TNF blocker treatment.

FC

A high FC in our CD cohort was associated with early TNF blocker therapy, but as with CRP and ESR, in multivariate analysis in paper II, only pANCA and ASCA status remained independently associated with early TNF blocker treatment. In paper III, FC levels over 1000 mg/kg were associated with subsequent biologic therapy, and these patients had significantly higher abundance of pro-inflammatory Proteobacteria and *Prevotella* than patients with lower levels (<1000mg/kg). In our study, a high FC level over 1000 mg/kg was not associated with later surgery. This is in contrast to a study of adult patients with severe UC, where those requiring a colectomy had significantly higher FC levels than those not needing surgery (194). In severe pediatric acute colitis, the PUCAI has been superior to FC in predicting response after intravenous corticosteroid therapy (169).

Serological markers

In multivariate analysis we found pANCA and/or ASCA status, but not CRP, ESR or FC levels to be associated with early TNF blocker therapy in CD patients. Consequently, being pANCA negative and/or ASCA positive seems to be an indicator of a CD phenotype that warrants early aggressive therapy. PANCA status has previously been found to be associated with distinct phenotypes in IBD. In adult and pediatric populations, pANCA positive CD patients have been reported to have an UC-like phenotype and a more benign disease course. This is in agreement with our results where pANCA positivity was associated with less aggressive therapy (106, 172, 195, 196). In paper II we only had 18 UC patients, and none of them received TNF blocker treatment, as infliximab was not yet standard of care for pediatric UC during the inclusion period of 2005-2007. However, the majority of our UC patients had an extensive phenotype, and all of them were pANCA positive, underlining the prognostic value in UC. Several studies have reported the presence of pANCA to be associated with an aggressive disease course, a higher relapse rate, resistance to therapy as well as more frequent need for early surgery (104, 174, 175, 187).

In both adult and pediatric CD, ASCA positivity is associated with an aggressive disease course with stricturing and penetrating disease behavior, small bowel involvement and the need for early surgery (105- 107, 171, 197). In the "RISK study" of newly diagnosed pediatric CD patients presenting with an inflammatory phenotype, ASCA positive serology and CBir1 antibodies were associated with stricturing and penetrating behavior (198). The strongest effect was seen for CBir1 seropositivity. In our study, the prevalence of CBir1 with 22% was lower than what has been found in other pediatric studies (195, 198). Neither antibodies towards CBir1 nor antibodies against I2 predicted TNF blocker treatment in our patients. I2 status did not differ between UC and CD patients, limiting the ability of anti-I2 to sub-classify IBD. OmpC antibodies were infrequent in our cohort. The predictive value of CBir1, OmpC and I2 antibodies from a clinical perspective seems small, at least in a Norwegian cohort of PIBD patients. In contrast, our study confirmed the ability and usefulness of ASCA and pANCA status in predicting the need for early aggressive therapy with TNF blockers in CD.

Microbiota

We found enrichment of the pro-inflammatory bacteria *Ruminococcus gnavus* in patients with extensive disease distribution; ileocolitis in CD and total colitis in UC, compared to patients with more limited disease. *Ruminococcus gnavus* expresses beta-glucuronidase activity which may cause local inflammation. A study using metagenomic sequencing in monthly stool samples from IBD patients, found an enrichment of *Ruminococcus gnavus*, often co-occurring with increased disease activity (199). This study also found several IBD and *Ruminococcus gnavus* specific genes involved in oxidative stress response, adhesion, iron-acquisition and mucus utilization, indicating that *Ruminococcus gnavus* may be an important member of the dysbiosis found in IBD, adapted to tolerate the higher oxidative stress in the IBD gut.

Veillonella enrichment, as we found in our patients with upper GI involvement, has been reported to be more abundant in pediatric CD patients (76, 198, 200,

201). In a pediatric study, patients not responding to therapy had significantly higher pretreatment fecal abundance of *Veillonella*, adding further evidence to that baseline microbiota abundance may be predictive of disease outcome (201).
Faecalibacterium prausnitzii, a highly abundant human gut microbe, is reported to be consistently reduced in both adult and pediatric patients with IBD and may have utility as a biomarker (64, 199, 200). The bacterium acts as a protective factor for the intestinal mucosa, enhances barrier function and can exert anti-inflammatory effects (202- 204). Our IBD patients who needed surgery and who did not achieve mucosal healing with therapy, as well as patients treated with antibiotics before the IBD diagnosis, had the lowest abundance of *Faecalibacterium prausnitzii*. This is in line with observations that low abundance of *Faecalibacterium prausnitzii* is associated with the use of antibiotics, may predict non- mucosal healing, non-response to anti-TNF therapy in UC, relapse after infliximab termination in CD patients and post-resection relapse if the abundance in the mucosa is low (76, 201, 205- 207).
Proteobacteria are pathobionts; meaning that they may expand as a result of a microbial imbalance and exert pathogenic effects on the host. They are typically reported to be enriched in IBD (177, 208- 210). CD Patients with penetrating or stricturing behavior, who needed surgery or failed to achieve mucosal healing had the highest abundance of Proteobacteria, in accordance with these reports. Proteobacteria enrichment has been associated with early relapse after induction of remission with exclusive enteral nutrition in pediatric CD (211). These findings implicate that Proteobacteria abundance might be a marker of an aggressive disease course with a higher risk of treatment failure and that baseline profiles may aid in stratifying IBD patients.

9.1.2 Safety and efficacy of TNF blocker therapy

A secondary aim was to evaluate if early intervention with biologic treatments in children and adolescents with CD would be safe and efficient in inducing and maintaining remission within the first 1-2 years after diagnosis. In our CD

patients, TNF blocker treatment with infliximab was well tolerated. No serious adverse events were recorded, and 15 (83%) achieved clinical remission, all within six weeks. The response was sustained during the study period of two years for all responders, confirming the ability of TNF blockers to both induce and maintain remission (151). The infliximab treatment was initiated early after diagnosis, usually within the first two months, and we speculate that this may have contributed to the good outcome. Studies have reported a better response to infliximab when given early in the disease course (212, 213). All patients treated with infliximab were also co-medicated with azathioprine, which may have contributed to the sustained treatment effect, whereas the limited treatment period on azathioprine before infliximab initiation is unlikely to have contributed to remission (132, 148).

A majority in the infliximab group had a marked reduction of CD specific upper GI lesions but persistence of unspecific inflammation at follow-up. In our cohort it seemed that infliximab healed ulcerations, aphtous lesions and erosions in the gastric mucosa and duodenum, but that in several patients, unspecific mucosal inflammation remained or developed despite treatment. Some case reports have documented the efficacy of infliximab in treating upper GI CD, but due to small study populations and lack of universally accepted definitions for upper CD characterization, no definitive conclusions can be made (185, 214). There is evidence of microscopic gastric inflammation to be more common in children and adolescents with IBD compared to controls (215, 216). This was confirmed in our study with none of the symptomatic non-IBD controls displaying macroscopic or microscopic evidence of upper GI inflammation. Some of our CD patients had new upper GI lesions detected at follow- up in spite of being asymptomatic and having normalization of CRP, ESR and FC levels. Lesions in the upper GI, like duodenitis, gastritis and esophagitis might be unspecific findings and could for some of our patients have other etiologies than CD. As none of the patients in the non- IBD control group had gastritis or other upper GI lesions detected at follow-up, we nevertheless speculate that the inflammatory findings in the upper GI were

associated with CD. The higher frequency of upper GI lesions in the infliximab group may support this assumption. The prognostic significance of these unspecific upper GI findings is uncertain, and should be evaluated in future studies.

The overall outcome was good as both infliximab and non- infliximab treated patients achieved a clinical remission rate of above 80 % with normalized PCDAI scores. The rate of ileocolonic mucosal healing was high and comparable for both treatment groups, confirming the ability of conventional treatment to achieve endoscopic remission (217). Moreover, it again highlights the importance of individualizing treatment, and that anti TNF treatment is not needed by all patients (218, 219).

9.1.3 Diagnostic value of serological and microbial profiles

We aimed to describe the prevalence of serological markers in newly diagnosed treatment naïve PIBD and their utility in differentiating CD, UC and symptomatic non-IBD patients. A significantly higher prevalence of antibodies against ASCA, pANCA, and I2 was found in IBD patients compared to in non-IBD controls. In line with previous studies, presence of ASCA IgA and IgG autoantibodies was strongly associated with having CD, and ASCA status was useful in differentiating between UC and CD (102). The prevalence of 54% ASCA positivity in our CD patients is similar to what others have reported (220). I2 was a weak indicator of having IBD, with a prevalence of around 40% in our IBD patients, compared to 11 % in the non-IBD controls. This is in accordance with previous reports in pediatric patients (221). I2 was just as frequent in UC and CD patients and could not aid in subtyping IBD patients.

Regarding fecal microbial differences between CD and UC, the literature has been conflicting. We did not find major differences in bacterial profiles between active CD and active UC. This is supported by previous studies (77, 222).

There was a similar dysbiotic profile with reduced microbial abundance in IBD and non-IBD compared to healthy individuals in the present study, thus the

bacterial profiles provided by the GA-map™ technology performed less well than FC in detecting inflammation and discriminating IBD from non-IBD symptomatic patients. However, the finding of dysbiosis in non-IBD symptomatic patients may help these patients in coping and processing their symptoms.

The reduced abundances of beneficial *Eubacterium* and *Bifidobacterium* species in IBD and non-IBD patients compared to in healthy children are in agreement with previous adult and pediatric studies (64, 71, 76, 77, 223, 224). *Eubacteria* and *Bifidobacterium* are known to inhibit the growth of potentially pathogenic species and produce short chain fatty acids (SCFA) through fermentation of dietary fiber (225). The reduction of protective commensal microbes and concomitant loss of their protective function can have an influence on the IBD development and disease course.

The differences in bacterial profiles in IBD versus non-IBD were not as substantial as expected, with considerable overlap within disease categories, and the results provided by the GA-map™ technology were not ideally suited to distinguish IBD from non-IBD symptomatic patients.

9.1.4 Stability of serological markers and microbiota profiles

Serological markers

ASCA has been reported to be relatively stable regardless of disease course, medical or surgical treatment (101, 226- 228). Our study confirmed the stability of ASCA IgA and IgG, as the titers of both markers were stable, regardless of treatment modality. Due to the stability of ASCA antibodies, ASCA status may serve as a prognostic marker even when tested later in the disease course. We observed a non-significant trend towards higher ASCA IgG levels with time in the TNF blocker treated patients. This increase might be an age related phenomenon and not an indicator of increasing disease severity as serologic responses towards ASCA increase with age in children with CD (195).

The stability of pANCA in treated patients has been questioned (173). PANCA prevalence has been reported to be lower when measured after treatment and in

UC patients in longstanding remission (104, 105, 229). In some studies, pANCA status changed after therapy and colectomy, whereas others failed to show an association between disease activity and presence of pANCA (220, 230, 231). In our study, a decline in pANCA titers was demonstrated after treatment, in both CD and UC patients. This difference was only statistically significant in the UC patients. Nevertheless, as some of our patients became pANCA negative after therapy, pANCA status should probably be determined early in the disease course in order to be a reliable prognostic factor.

Microbiota profiles

The IBD patients in our sample with repeated fecal microbiota analysis displayed persistent, unchanged dysbiosis after treatment, regardless of treatment modalities and mucosal healing. Similar results have been reported in another pediatric study, where the dysbiosis improved, but nonetheless persisted despite mucosal healing (201). Lewis et al found that effective EEN and TNF blocker therapy reduced, but failed to eliminate the dysbiosis of pediatric CD patients (200). Others have found the fecal microbiota to become more dysbiotic with dietary treatment such as EEN (232- 234). Perhaps sustained and deep remission requires normalization of the gut dysbiosis, or maybe it is not possible to reverse the dysbiosis once the gut homeostasis is perturbed as fundamentally as it is in IBD. Measuring fecal microbiota abundance might not be an optimal method as it is not suited to determine the effects of dysbiosis. As a prognostic tool fecal microbiota profiles may still be of value, also in established IBD patients on treatment, as the dysbiosis remained despite treatment and remission. However, due to the small number of patients with repeated sampling, firm conclusions cannot be drawn.

9.1.5 Methodological considerations, study design and population

All three studies included in this thesis were longitudinal, prospectively, observational studies of pediatric patients with IBD. Our non-IBD symptomatic patients consisted of pediatric patients admitted to the hospital due to symptoms

and findings suspicious of IBD, but without evidence of inflammation during workup. Some of these patients may have had preclinical/latent IBD or other conditions such as disturbed permeability and motility influencing the study results. We believe most of these non-IBD symptomatic patients had functional gastrointestinal disorders. The fact that there was no visible nor histological evidence of inflammation and that none of these patients have been diagnosed with IBD despite 3 to 13 years of follow-up, makes misclassifications and undiagnosed IBD less likely. Ideally we should have subtyped these patients with the use of Rome criteria for functional disorders (235). However, due to the limited sample size of 50 non-IBD symptomatic patients, further sub-classification would have reduced the statistical power to reveal clinical significant differences between the groups.

All pediatric patients with GI-complaints living in the catchment area of South Eastern Norway were admitted to the two participating hospitals, reducing the selection bias seen when patients are recruited from tertiary care settings. This enabled us to derive a realistic estimate of the frequency of early anti-TNF therapy in CD, to find the presence of serological markers, and to describe fecal microbial profiles in IBD non-IBD patients compared to healthy. The results in our studies can therefore be considered representative for pediatric patients in South Eastern Norway. However, the results may not be generalized to pediatric patients living in other continents. Studies have shown that the microbiota composition differs according to where in the world you live (236).

The healthy controls included in paper III were not investigated in the same manner as the patients, as invasive tests in healthy children are considered unethical. Even though children with GI complaints, those with recent antibiotic exposure and/or elevated FC were excluded from participating as a healthy control, some could nevertheless suffer from conditions that may have influenced the study results, as there is substantial evidence that diseases outside of the GI tract also influence the gut microbiota (237).

With respect to age and gender of our patients, we diagnosed more boys in the IBD patient group and more females in the non-IBD patient group, confirming that pediatric CD is more common in boys and females are more represented in functional GI disease such as IBS (238). Age between the two patient's groups was not statistically different, but our healthy children's group had a lower median age which could have skewed our data.

9.1.6 Strengths and limitations

A strength of the presented studies is that all patients were treatment naïve, allowing us to investigate the early onset of the disease. Because the choice of therapy in all patients was led by the disease severity and following international treatment algorithms, it reflected everyday practice. A major strength of the studies is the extensive characterization and classification of our IBD and non-IBD patients. All non-IBD patients underwent the same procedures as the IBD patients, with upper and lower endoscopies as well as MRI of the small intestine, for those included in the IBSEN II cohort also after 1-2 years of follow-up. The use of a detailed, standardized protocol applied at baseline and at follow up, gave a high diagnostic precision and reduced the likelihood of having missed the diagnosis of IBD.

Sample size

A weakness of our studies was the relative limited number of cases, especially in paper I and II. The limited sample size reduced the ability to detect potential clinically relevant differences of the microbial antigens CBir1, OmpC and I2 as statistically significant. On the other hand, one could argue that when large numbers are needed in order to reveal statistical significance, such differences may not have major clinical implications.

Norway is a small country, and for a pediatric population based study of PIBD, the number of patients in paper III was relatively large. Regarding the healthy controls, a larger sample size would have been preferable to strengthen the significance of our findings. The 75 healthy children came from the same

geographical area in Norway, thus reducing the generalizability towards other parts of Norway and other countries.

Diet

Diet has a profound impact on the microbiota (57). We have limited data on the diet of the IBD and non-IBD children. For the healthy controls, we only know they followed a "normal" Norwegian diet; children on exclusion diets were excluded. Knowing more about the diet of the patients and healthy controls might have given information into disease pathogenesis but was outside the scope of our study. In the next IBSEN III cohort, data on diet and questionnaires regarding eating habits will be included.

Storage and preparation of biological samples

A possible source of error might be the difference in storage time of the fecal samples. Theoretically, the sample quality can deteriorate during the time from collection until frozen. Based on previous experience and in vitro examinations, the microbial material collected in the different cohorts was not considered to be affected (73). Since repeated thawing is known to influence the microbiota, the samples were kept frozen until analysis. We therefore do not believe that the sample handling influenced the results.

Microbiota methodology

The selection of microbes in the GA-map™ technology is based on literature studies, including gut bacteria whose profiles are commonly altered in adult patients with dysbiosis, with the inherent risk of not including bacteria important in PIBD diagnosis and prognosis. Bacterial 16S sequencing of all microbes would give additional results, but is more expensive. The same is true for shotgun metagenomic sequencing, encompassing all genomic bacterial of bacteria, viruses and fungi. Together with an altered bacterial composition, studies have revealed that IBD patients have fungal dysbiosis as well as alterations in the intestinal virome, which we have not investigated in our study (239, 240). Deep sequencing and shotgun metagenomic sequencing methods, which need bioinformatics tools and reference datasets, are still under development and not yet readily available

for clinical practice. The GA-map™ technology provided us with a commercially available and clinically validated tool in adult patients.

We acknowledge that the GA-map™ technology test measures the abundance of bacteria without giving information about the bacteria's functional importance, and high abundant bacteria might not be functionally active (241).

In the present microbiota study we explored the fecal microbiota. One study comparing mucosal associated microbiota with fecal microbiota reported that the ileal mucosa, followed by the rectal mucosa obtained the best performance in classifying CD and that stool samples performed less well (76). But as mucosa associated microbiota must be sampled by invasive methods, we wanted to explore the ability of the fecal microbiota in differentiating patient groups, as it is both non- invasive and easy ascertainable.

Follow-up time

Even if both conventional and infliximab treatment in our CD patients seem to be efficient, a prolonged follow- up is needed to compare the effect on the long-term course of disease between the treatment groups. As CD in 30- 40% of pediatric patients becomes more aggressive with stricturing and fistulizing features over time (19), our follow-up of 1-2 years in paper II is too short to compare the effect of the different treatment modalities on the occurrence of intestinal and extraintestinal complications.

Statistical considerations

Ideally, the use of univariate and multiple logistic regression analyses and the construction of ROC curves would have been preferable in paper I to evaluate the usefulness of clinical and biochemical factors in predicting the initiation of infliximab treatment. However, a much larger data set would have been needed, most likely based on a registry study. In large scale registry studies, however, it is difficult to achieve the same meticulous workup and follow-up as was performed in our cohort. It is challenging to include a large number of PIBD patients as there are fewer cases than in adulthood. A study population of 110 PIBD patients in paper III is a relatively high number compared to what is published on this topic.

Even with lower sample sizes in paper I and II we were able to reveal relevant differences as statistically significant and with clinical implications. Ideally, as mentioned earlier, we should have classified the non-IBD symptomatic patients with the use of Rome criteria for functional disorders (235). However, with the limited sample size, further sub- classification would have reduced the statistical power to reveal clinical significant differences between the non-IBD subgroups and between these subgroups and the IBD patients.

We acknowledge that the limited sample sizes reduce the statistical power to reveal differences as statistically significant and increase the possibility of false negative findings (type II errors). In paper III we did not adjust for multiple testing as we considered the study to be exploratory, increasing the risk of accepting false positive associations (type 1 errors). We validated our results by splitting our data into a training and a test set and most associations estimated in the whole cohort remained statistically significant. The positive relationship between inflammation, increased abundance of pathobionts and concomitant loss of beneficial bacteria found in paper III, is reassuring and in line with previous research reports (242).

10 Conclusions and future perspectives

10.1.1 Conclusions and clinical utility

This project integrated clinical data regarding disease distribution, disease behavior and treatment effects with results from basal research regarding gut microbiota and serological markers in PIBD patients. Extensive disease distribution, high CRP, ESR, FC, positive serology against ASCA/negative pANCA in CD, and severe dysbiosis are indicators of a severe phenotype that warrants early aggressive therapy.

The clinical value and utility of clinical phenotypes, the inflammatory markers CRP, ESR and FC in stratifying PIBD have been reproduced in the present studies. Ascertaining the presence and titers of the serological markers ASCA and pANCA provided additional value in diagnosing and predicting disease course and therapy in PIBD and should be included in the initial assessment of newly diagnosed patients. In contrast, the clinical utility of antibodies towards I2, CBir1 and OmpC in our study was limited.

There were similar dysbiotic profiles with reduced microbial abundance in IBD and non-IBD compared to healthy controls in the present study, and the results provided by the GA-map™ technology performed less well than FC in discriminating IBD from non-IBD symptomatic patients. However, the finding of dysbiosis in non-IBD symptomatic patients may help these patients in processing and managing their symptoms. As diet has a huge impact on the microbiota composition, it may be warranted to suggest dietary alterations as an option to alleviate symptoms (243).

Our findings show promise for microbiota profiles and abundance to risk stratify and individualize treatments in PIBD, but studies validating these preliminary results are needed. In established IBD patients receiving treatment, ASCA serology and microbiota profiles might also be used as a prognostic tool since the levels of microbial antibodies and dysbiosis remained unchanged with treatment. However, due to the small number of patients with repeated sampling in our

study, firm conclusions cannot be drawn and the finding that the dysbiosis persisted needs to be validated in other studies.

Our findings suggest that a combined assay of different markers; clinical, inflammatory, serological and microbial, may improve the diagnostic accuracy as well as the risk stratification of IBD patients and compliment endoscopic and histological examinations. Predicting disease course and prognosis is like a puzzle, and with no single, accurate and precise biomarkers yet available, each variable represent an important piece of the puzzle.

10.1.2 Commercial potential and future perspectives

Detection of alterations in fecal microbiota abundance may distinguish IBD-patients from healthy and diagnose dysbiosis in non-IBD-patients. The same potential exists regarding serological markers. Microbiota profiles and extensive dysbiosis based on findings in this thesis can possibly identify IBD-patients that should be treated early with highly potent, aggressive medications. The technologies used in our analyses are readily available through our commercial partners, and clinical significant results can be implemented in clinical tests offered to the clinicians within reasonable time.

Based on findings in the gut microbiota, targeted therapy with probiotics, special diets or fecal microbiota transplantation may be a treatment option in the future for IBD patients.

The GA-map™ technology uses DNA as study material, and results in the detection of living, dormant and dead microbes. Recent studies reveal that the presence and activity levels of the gut microbiota members do not always correlate (244). In order to detect the active microbiota one must sequence the 16SrRNA transcript, either from feces or mucosal biopsies. We will continue to study the microbiome in PIBD and plan to describe both the standing microbiota with 16S rRNA gene (DNA) targeting as well as the active microbiota with transcript RNA sequencing in PIBD patients in future studies.

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12 Appendix

Supplementary Table 1.

Bacteria number	Phylum	Name
100	Actinobacteria	Actinobacteria
101	Actinobacteria	Actinomycetales
102	Actinobacteria	Atopobium rimae
103	Actinobacteria	Bifidobacterium spp.
201	Bacteroidetes	Alistipes
202	Bacteroidetes	Alistipes onderdonkii
203	Bacteroidetes	Bacteroides fragilis
204	Bacteroidetes	Bacteroides pectinophilus
205	Bacteroidetes	Bacteroides spp.
206	Bacteroidetes	Bacteroides spp. & Prevotella spp.
207	Bacteroidetes	Bacteroides stercoris
208	Bacteroidetes	Bacteroides zoogleoformans
209	Bacteroidetes	Parabacteroides johnsonii
210	Bacteroidetes	Parabacteroides spp.
211	Bacteroidetes	Prevotella nigrescens
300	Firmicutes	Firmicutes*
301	Firmicutes	Anaerotruncus colihominis
302	Firmicutes	Bacilli
303	Firmicutes	Bacillus megaterium
304	Firmicutes	Catenibacterium mitsuokai
305	Firmicutes	Clostridia
306	Firmicutes	Clostridium methylpentosum
307	Firmicutes	Clostridium sp.
308	Firmicutes	Coprobacillus cateniformis
309	Firmicutes	Desulfitispora alkaliphila
310	Firmicutes	Dialister invisus
311	Firmicutes	Dialister invisus & Megasphaera micronuciformis
312	Firmicutes	Dorea spp.
313	Firmicutes	Eubacterium biforme

314	Firmicutes	Eubacterium hallii
315	Firmicutes	Eubacterium rectale
316	Firmicutes	Eubacterium siraeum
317	Firmicutes	Faecalibacterium prausnitzii
318	Firmicutes	Lachnospiraceae
319	Firmicutes	Lactobacillus ruminis & Pediococcus acidilactici
320	Firmicutes	Lactobacillus spp.
321	Firmicutes	Lactobacillus spp. 2
322	Firmicutes	Phascolarctobacterium sp.
323	Firmicutes	Ruminococcus albus & R. bromii
324	Firmicutes	Ruminococcus gnavus
325	Firmicutes	Streptococcus agalactiae and Eubacterium rectale
326	Firmicutes	Streptococcus salivarius ssp. thermophilus and sanguinis
327	Firmicutes	Streptococcus salivarius ssp.thermophilus
328	Firmicutes	Streptococcus spp.
329	Firmicutes	Streptococcus spp. 2*
330	Firmicutes	Veillonella spp.*
331	Firmicutes/Tenericutes/Bacteroidetes species	Firmicutes (various)*
500	Proteobacteria	Proteobacteria
501	Proteobacteria	Acinetobacter junii
502	Proteobacteria	Enterobacteriaceae*
503	Proteobacteria	Pseudomonas spp.
504	Proteobacteria	Shigella spp. & Echerichia spp.
601	Tenericutes	Mycoplasma hominis
701	Verrucomicrobia	Akkermansia muciniphila

13 Errata

14 Papers I-III

Paper III

ORIGINAL RESEARCH

Short running header Fecal microbiota in pediatric IBD

Christine Olbjørn et al

Fecal microbiota profiles in treatment-naïve pediatric inflammatory bowel disease- associations with disease phenotype, treatment and outcome.

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Abstract

Purpose: Imbalance in the microbiota; dysbiosis, has been identified in inflammatory bowel disease (IBD). We explored the fecal microbiota in pediatric patients with treatment-naïve IBD, non-IBD patients with gastrointestinal symptoms and healthy children, its relation to IBD subgroups and treatment outcomes.

Patients and methods: Fecal samples were collected from 235 children below 18 years of age. Eighty had Crohn's disease (CD), 27 ulcerative colitis (UC), 3 IBD Unclassified (IBDU), 50 were non-IBD symptomatic patients and 75 were healthy. The bacterial abundance of 54 predefined DNA markers was measured with a 16S rRNA DNA based test using GA-map™ technology at diagnosis and after therapy in IBD patients.

Results: Bacterial abundance was similarly reduced in IBD and non-IBD patients in 51 of 54 markers compared to healthy ($p< 0.001$). Only *Prevotella* was more abundant in patients ($p< 0.01$). IBD patients with ileocolitis or total colitis had more *Ruminococcus gnavus* ($p=0.02$) than patients with colonic CD or left sided UC. CD patients with upper gastrointestinal manifestations had higher *Veillonella* abundance ($p<0.01$). IBD patients (58%) who received biologic therapy had lower baseline Firmicutes and *Mycoplasma hominis* abundance ($p<0.01$) than conventionally treated. High Proteobacteria abundance was associated with stricturing/penetrating CD, surgery ($p<0.01$) and non-mucosal healing ($p<0.03$). Low *Faecalibacterium prausnitzii* abundance was associated with prior antibiotic therapy ($p=0.001$), surgery ($p=0.02$) and non-mucosal healing ($p<0.03$). After therapy IBD patients had unchanged dysbiosis.

Conclusion: Fecal microbiota profiles differentiated IBD and non-IBD symptomatic children from healthy children, but displayed similar dysbiosis in IBD and non-IBD symptomatic patients. Pre- treatment fecal microbiota profiles may be of prognostic value and aid in treatment individualization in pediatric IBD as severe dysbiosis was associated with an extensive, complicated phenotype,

biologic therapy, and non-mucosal healing. The dysbiosis persisted after therapy, regardless of treatments and mucosal healing.

Keywords: dysbiosis**,** Crohn´s disease, ulcerative colitis, Proteobacteria, biologic therapy, *Faecalibacterium prausnitzii*

Plain English language summary

- Studies have shown a disturbed gut bacterial composition in chronic inflammatory diseases such as inflammatory bowel disease (Crohn's disease and ulcerative colitis).
- In children it might be difficult to diagnose inflammatory bowel disease. Symptoms are often non-specific, such as abdominal pain and altered bowel movements.
- Dr Olbjørn and colleagues investigated whether the bacterial composition from stool samples can help to diagnose and treat inflammatory bowel disease in children.
- They used advanced DNA profiling to identify and quantify bacteria. They compared the bacterial composition in stool from children with inflammatory bowel diseases with healthy children and children with gastrointestinal symptoms but without inflammation.
- The researchers report that the bacterial composition in patients with inflammatory bowel disease was very different than in healthy children. The differences persisted after treatment.
- The bacterial composition in patients with gastrointestinal symptoms but no inflammation was similarly disturbed as in inflammatory bowel disease patients.
- The degree of disturbances in the bacterial composition in children with inflammatory bowel disease correlated with the disease course and later therapy. Patients with higher numbers of "bad "bacteria, such as Proteobacteria, were more likely to need aggressive treatment and surgery.
- In children with inflammatory bowel disease, testing the bacterial composition in the stool before treatment can help physicians in targeting and individualizing treatments.

Introduction

The pathogenesis of the inflammatory bowel diseases (IBD), Crohn's disease (CD) and ulcerative colitis (UC), is not fully understood, but IBD is thought to occur due to an exaggerated immune response to luminal microbial contents in the gastrointestinal tract in genetically susceptible individuals.[1] A rising incidence of IBD, especially in the pediatric population, has been demonstrated, and the influence of environmental changes, including diet and gut microbiota on the disease pathogenesis, are increasingly recognized. [2,3] The gut microbiota is thought to play an important role not only in IBD, but also in functional gastrointestinal disorders such as irritable bowel syndrome which may display similar symptoms representing a differential diagnosis to IBD.[4,5] Studies of the gut microbiota in IBD and functional gastrointestinal disorders have shown an imbalance, dysbiosis, with compositional changes, including decreased bacterial diversity and abundance.[6-8] The shift in the gut microbiota seems to be associated with a depletion of beneficial versus a relative increase of pro-inflammatory bacteria.[9,10] The diagnostic significance of fecal microbiota in children with gastrointestinal symptoms suggestive of IBD, the predictability of IBD phenotypes, need for aggressive therapy and response to treatment are not fully explored.

We hypothesized that the fecal microbiota composition could be helpful in diagnosing pediatric IBD patients and in predicting their prognosis. We aimed to assess differences in the abundance of fecal microbiota in treatment- naïve pediatric IBD patients at the time of diagnosis compared to healthy controls and pediatric non-IBD patients with gastrointestinal symptoms. We further explored the value of microbiota abundance in differentiating between IBD phenotypes, subsequent need of biologic therapy, surgery and treatment outcomes, and whether the microbiota changes with therapy.

Material and methods

IBD patients, non-IBD symptomatic patients and healthy controls

Patients enrolled in the present study were recruited from the catchment areas of two university hospitals in three population based prospective epidemiological studies of treatment-naïve pediatric IBD in South-Eastern Norway (IBSEN II) [11,12], Early IBD (in preparation) and EU IBD Character.[13] The inclusion periods for these three multicenter trials were from 2005 to 2015, all with identical protocols and inclusion criteria. Pediatric patients under 18 years, referred during the inclusion periods and believed to have IBD based on symptoms, were included. IBD was diagnosed in accordance with the Porto criteria.[14] Patients who did not meet the diagnostic criteria for IBD, who had a macroscopically and histologically normal mucosa as well as a normal MRI examination, were included as non-IBD symptomatic controls. Healthy children and adolescents between the age of 2 and 18 years, recruited during the period of 2013-14 from the same catchment areas as the patients, delivered fecal samples and were included as healthy controls. They had no chronic diseases, no IBD in the family, followed a normal diet (children on exclusion diets; gluten-free, cows milk protein-free, vegetarian/vegan, were excluded), had not travelled outside Europe or used antibiotics within the last six months, had no recorded gastrointestinal complaints, did not use proton pump inhibitors and had normal fecal calprotectin levels (<50 mg/kg).[15]

Clinical, endoscopic, radiological and laboratory data

Age, gender, symptoms, disease activity index scores, disease and family history of the IBD and non-IBD symptomatic patients were registered as previously described.[11,12,16] The Paris classification was used to characterize disease distribution and behavior.[17] In patients, feces were sampled at home in three designated containers without additives on the day before endoscopy, kept refrigerated or frozen, and brought to the hospital the next day. Feces from one container was analyzed for calprotectin (FeCal-test, Bühlmann, Basel, Switzerland) the second for pathogenic bacteria and the third container with

feces was frozen at -80 degrees Celsius for later microbiota analysis. The healthy controls received two designated fecal sampling kits at home for handling of samples before deliverance to Genetic Analysis AS, Oslo, Norway. One sample was analyzed for fecal calprotectin (FeCal-test, Bühlmann, Basel, Switzerland), the other was frozen at -80 degrees Celsius and stored for later microbiota analysis. For all samples, the maximum time interval until frozen at -80 degrees Celsius was three days, thereafter the samples were kept frozen and not thawed until analysis.

Microbiota analysis

The microbiota was analyzed using the GA-map™ technology (Genetic Analysis AS, Oslo, Norway), a PCR and 16SRNA based analysis. The method uses a targeted approach to detect predefined bacteria believed to be important in identifying and characterizing gut bacteria dysbiosis.[18] The test measures relative bacterial abundance based on the fluorescence signal strength (FSS) of bacterial DNA markers. The markers are targeting variable regions V3 to V7 of the bacterial 16S rRNA gene. The method utilizes 54 bacterial markers (Supplementary Table 1), covering more than 300 bacteria at different taxonomic levels; 26 species specific, 19 detect genus specific, and 9 bacteria at higher taxonomic levels (phyla, class and family). All samples were analyzed at the same time point. The laboratory was blinded for the diagnosis of IBD, non-IBD or healthy.

IBD treatment

Treatment was decided individually, prospectively, at the discretion of the treating pediatrician. Initial treatment options to induce remission were; exclusive enteral nutrition in CD, corticosteroids and/or 5-aminosalicylic acids in CD and UC patients. Maintenance therapy with azathioprine or methotrexate was in general started simultaneously (Table 1). The indication for surgery or treatment with biologic therapy (tumor necrosis factor (TNF) blockers) was failure to induce remission with conventional treatments or relapse after primary induction.

Statistical analyses

Data were described using counts and percentages for categorical data and medians and ranges for continuous data. To explore the ability of all 54 bacterial markers to distinguish between IBD, non- IBD symptomatic patients, and healthy controls, we performed principal component analysis. The FSS from the 54 markers were added for each patient and the sum illustrated a relative abundance, denoted the total signal strength. Crude comparisons between groups were performed using Mann-Whitney Wilcoxon tests and Wilcoxon signed ranks tests (before and after treatment) for continuous variables and Chi-square tests for categorical data.

Areas under the curves (AUCs) were calculated and receiver operating characteristic (ROC) analysis conducted to evaluate the performance of selected bacterial abundances in distinguishing IBD phenotypes and treatments. All tests were two-sided. P-values <0.05 were considered statistically significant. We regarded our study exploratory; therefore, we did not correct for multiple testing. However, in order to validate our results, each observation was randomized into a test set or a training set so that the number of observations was equal in both sets. Only the statistically significant differences confirmed in the training set are reported. All analyses were performed using SPSS, statistical software version 24 (SPSS Inc., Chicago, Il, USA) and Stata version 9.

Ethical considerations

The study was conducted with informed patients and parental/guardian written consent as appropriate and with full ethical approval, in accordance with the Helsinki declaration, and with approval by the Regional Committee for Medical Research Ethics, South-Eastern Norway, reference no. REK S-04209.

Results

Of the 235 included children and adolescents; IBD was diagnosed in 110 patients, (80 CD, 27 UC and 3 IBDU) (Table 1), 50 patients were included as non-IBD symptomatic patients and 75 healthy children served as controls. None of the non-IBD symptomatic patients have developed IBD as of December 1st 2018. IBD,

non-IBD and healthy controls were comparable concerning all demographic variables except for more females among the non-IBD patients and a slightly lower median age in the healthy controls (Table 2).

The bacterial abundances were compared between the three pediatric groups; healthy controls, IBD patients and non-IBD symptomatic patients, as well as between subgroups of IBD and after treatment in 31 of the IBD patients. To investigate the impact of antibiotics on microbiota profiles of the IBD patients, they were grouped according to whether they had received antibiotics within 3 months prior to diagnosis or not, and analyzed separately. Eight of the 110 IBD patients had received antibiotics and these patients had significantly lower abundance of *Faecalibacterium prausnitzii* ($p=0.001$) compared to IBD patients without prior antibiotic therapy (Figure 1). However, excluding these patients from the statistical analyses did not impact the other results presented in the material.

Microbiota in relation to age

We found significant differences in microbiota abundance when comparing healthy children below (n=38) and above (n=37) 10 years of age. Healthy children aged less than 10 years had lower abundance of *Clostridiales* and higher abundance of *Bifidobacterium*, both $p<0.01$. These differences were not replicated in the patients as we did not find any differences in bacterial profiles between high and low age groups in the IBD and non-IBD symptomatic patient groups. Additional post-hoc analysis with an age matched selection of controls did not influence outcome/ differences between patients and healthy.

Microbiota in IBD and non-IBD versus healthy

In all symptomatic patients, regardless of IBD or non-IBD status, the total signal strength, measured as the sum of the 54 FSSs, was significantly lower compared to healthy controls, illustrating that the patients had lower abundance of the predefined bacterial markers. Patients had reduced bacterial abundances in 51/54 markers, $p< 0.001$ (Figure 2). The only bacterial marker that was more abundant in patients (IBD and non-IBD) compared to healthy controls was

Prevotella (p<0.01). The abundances of *Lachnospiraceae* and *Bacteroides* were similar in all groups. The principal component analysis (PCA) plot visualizes how the microbiota composition differs between IBD, non-IBD and healthy and overlaps between IBD and non-IBD symptomatic patients (Figure 3).

Microbiota in IBD versus non-IBD

The bacterial abundances were similarly dysbiotic in IBD and non-IBD symptomatic patients, however, one marker targeting the Firmicutes phylum was significantly less abundant in IBD patients compared to non-IBD patients (p<0.01), as well as *Eubacterium rectale* (p<0.01), *Eubacterium biforme*/*Streptococcus agalactiae* (p=0.04), *Parabacteroides* and *Bifidobacterium* species (both p=0.02).

Microbiota in IBD patients

The fecal microbiota abundances did not differ between UC and CD, except that CD patients had lower abundance of *Mycoplasma hominis* (p<0.02).

Microbiota related to disease distribution and behavior in IBD patients

IBD patients with extensive disease; ileocolitis in CD or extensive colitis in UC, had higher abundance of *Ruminococcus gnavus*, (p=0.02) compared to CD patients with isolated colonic disease and UC patients with limited disease distribution (left sided colitis or proctitis). CD patients with upper gastrointestinal involvement had higher *Veillonella* abundance (p<0.01) compared to patients without upper gastrointestinal lesions.

CD patients with a high abundance of Proteobacteria were more likely to have complicated disease behavior; stricturing or penetrating disease, compared to patients with lower levels of these bacteria, p<0.01 (Figure 4).

Microbiota and association with treatment

IBD patients who were treated with biologic therapy, 64 (58%), had lower abundance of Firmicutes (p=0.015) and *Mycoplasma hominis* (p=0.009) compared to conventional treated patients (Figure 5). Seventeen (15%) of the IBD patients required surgery, and mucosal healing (assessed by ileocolonoscopy) was not achieved in 40 (36%) of the patients despite medical therapy. Surgery and lack of

mucosal healing was associated with higher abundance of Proteobacteria (p=0.002 and 0.011) (Figure 6) and lower baseline abundance of *Faecalibacterium prausnitzii* (p=0.02 and 0.017) respectively, compared to non-operated IBD patients and patients with mucosal healing.

Of the IBD patients (22 CD and 9 UC) with repeated microbiota analysis at follow-up 18 months after treatment, 15 (48%) patients had received biologic therapy, and 18 (58%) were in remission with mucosal healing. The microbiota composition and bacterial profiles were unchanged for 53 of 54 markers after treatment, regardless of treatment modality received and remission status. One marker targeting *Eubacterium hallii* species was less abundant after treatment, p=0.03.

Microbiota and association with fecal calprotectin

IBD patients with fecal calprotectin levels above 1000 mg/kg (31 CD, 12 UC) had significantly higher abundance of Proteobacteria (p=0.012) and *Prevotella* (p=0.011) than patients with lower levels (<1000mg/kg) of fecal calprotectin (Table 2). Fecal calprotectin over 1000 was associated with subsequent biologic therapy, p=0.001, but not with later surgery.

Discussion

In the present prospective study of newly diagnosed children and adolescents with IBD we demonstrated dysbiosis in both treatment-naïve pediatric IBD and non-IBD symptomatic patients. Their fecal microbiota differed significantly from the microbiota of healthy children with lower bacterial abundances measured with the GA map™ technology. Our non-IBD symptomatic patients consisted of pediatric patients admitted to the hospital due to symptoms and findings suspicious of IBD, but without evidence of inflammation during workup. Some of these patients may have had preclinical/latent IBD or other conditions such as disturbed permeability and motility influencing the study results. We believe most of these non-IBD symptomatic patients have functional gastrointestinal disorders. Ideally we should have further characterized and subtyped these

patients with the use of Rome criteria for functional gastrointestinal disorders. However, due to the limited sample size of 50 non-IBD symptomatic patients, further sub- classification would reduce the statistical power to reveal clinical significant differences between the groups.

There was a similar dysbiotic profile with reduced microbial abundance in IBD and non-IBD compared to healthy individuals in the present study, thus the bacterial profiles provided by the GA-map™ technology performed less well than fecal calprotectin in detecting inflammation and discriminating IBD from non-IBD symptomatic patients. However, the finding of dysbiosis in non-IBD symptomatic patients may confirm the relevance of their symptoms and discomfort. Presence and characterization of dysbiosis enables the physician to diagnose "functional" disease in a positive manner.

Within the group of patients diagnosed with IBD, we found that bacterial abundances at baseline seemed to be associated with disease extension, phenotype, biologic therapy, surgery and mucosal healing. At follow-up, after treatment, the dysbiosis was still present and its status mainly unchanged in IBD patients.

We found reduced abundances of beneficial *Eubacterium* and *Bifidobacterium* species in IBD and non-IBD symptomatic patients compared to in healthy children, in agreement with previous adult [6,19,20] and pediatric studies.[21-23] *Eubacteria* and *Bifidobacterium* are known to inhibit the growth of potentially pathogenic species [24] and produce short chain fatty acids (SCFA) through fermentation of dietary fiber. SCFAs are important energy sources for enterocytes and contribute to homeostasis of colonic regulatory T cell populations.[25] The reduction of protective commensal microbes and concomitant loss of their protective function can have an influence on development of IBD and the disease course. As expected, *Bifidobacterium* was more abundant in healthy children less than 10 years of age than in the healthy adolescents.[26] We found no difference in bacterial abundance between age groups for our IBD and non-IBD symptomatic patients. This may be due to disease state being a stronger driver of the

microbiota composition than age.

Patients with IBD have an expansion of pro-inflammatory bacteria such as *Prevotella* [27,28], *Ruminococcus gnavus* [29] and *Veillonella.*[22,28] *Veillonella* was enriched in our CD patients with upper gastrointestinal involvement. *Ruminococcus gnavus,* a bacterium that expresses beta-glucuronidase activity which may cause local inflammation, was associated with more extensive IBD distribution in our patients. *Prevotella*, *Ruminococcus gnavus* and Proteobacteria have been found to correlate with markers of disease activity and inflammation, [28,30] which was reproduced in the present study. Proteobacteria are pathobionts; meaning that they may expand as a result of a microbial imbalance and exert pathogenic effects on the host, and are consistently reported enriched in IBD.[31-33] Our CD patients with a complicated phenotype had high abundance of Proteobacteria, in accordance with these previous reports. Proteobacteria enrichment has been associated with early relapse after induction of remission with exclusive enteral nutrition in pediatric CD[34], and in our patients, high abundance was associated with the need for surgery and lack of mucosal healing. These findings implicate that Proteobacteria abundance might be a marker for an aggressive disease course with a higher risk of treatment failure.

Faecalibacterium prausnitzii, a highly abundant human gut microbe, is reported to be reduced in both adult and pediatric patients with IBD.[6,13,35,36] It acts as a protective factor for the intestinal mucosa, enhances barrier function and can exert anti-inflammatory effects.[20, 37, 38] Our IBD patients who needed surgery and who did not achieve mucosal healing with therapy, as well as patients treated with antibiotics before the IBD diagnosis, had the lowest abundance of *Faecalibacterium prausnitzii*. This is in line with observations that low abundance of *Faecalibacterium prausnitzii* may predict non-response to anti-TNF therapy in UC[39] and relapse after infliximab termination in CD patients.[40] Studies have found baseline microbiota to be associated with treatment responses[34,36,39], but how the microbiota composition and abundances change with treatment are less studied. The IBD patients in our sample with repeated fecal microbiota analysis displayed

persistent, unchanged dysbiosis after treatment, regardless of treatment modalities and remission status. Similar results have been reported in another pediatric study, where the dysbiosis improved, but nonetheless persisted despite mucosal healing.[41] Lewis et al found that effective exclusive enteral nutrition and TNF blocker therapy reduced, but failed to eliminate the dysbiosis of pediatric CD patients.[42] Others have found the fecal microbiota to become more dysbiotic with dietary treatment such as exclusive enteral nutrition.[43-45] Perhaps sustained and deep remission requires normalization of the gut dysbiosis, or maybe it is not possible to reverse the dysbiosis once the gut homeostasis is perturbed as fundamentally as it is in IBD. Measuring relative fecal microbiota abundance might not be an optimal method as it is not suited to determine the effects of dysbiosis, giving no information about the functional consequences. As a prognostic tool fecal microbiota profiles may still be of value, also in established IBD patients on treatment, as the dysbiosis remained despite treatment and remission. However, due to the small number of patients with repeated sampling, firm conclusions cannot be drawn.

Regarding fecal microbial differences between CD and UC, the literature has been conflicting. Similarly, as in our report, some previous studies did not find major differences in bacterial profiles between active CD and active UC.[23,36]

The strength of our study is the extensive workup, characterization and classification of our IBD patients. All non-IBD symptomatic patients underwent the same procedures as the IBD patients,

with upper and lower endoscopies as well as MRI of the small intestine, for those included in the IBSEN II cohort also after 1-2 years of follow-up. The fact that none of the non-IBD symptomatic patients have been diagnosed with IBD despite several (minimum 3- maximum 13) years of follow-up makes misclassifications and undiagnosed IBD less likely.

The healthy controls were not investigated in the same manner as the patients, as invasive tests in healthy children are considered unethical. Even though children with gastrointestinal complaints, recent antibiotic exposure and elevated fecal

calprotectin were excluded as healthy controls, some could have had conditions that may have influenced the study results, as there is substantial evidence that diseases outside of the GI tract influence the gut microbiota.[46]

Dietary patterns and smoking are known to influence the microbiota [45], therefore we excluded patients on exclusion diets. None of our adolescents admitted to smoking.[47]

The selection of microbes in the GA-map™ technology is based on literature studies and contain gut bacteria whose profiles are known to define dysbiosis in adults, with the inherent risk of not including bacteria that could be important in pediatric IBD diagnosis and prognosis. Bacterial 16S sequencing of all microbes would give additional results, but is more expensive. The same is true for shotgun metagenomic sequencing, encompassing all genomic bacterial of bacteria, viruses and fungi. Together with an altered bacterial composition, studies have revealed that IBD patients have fungal dysbiosis as well as alterations in the intestinal virome, which we have not investigated in our study.[48,49] Deep sequencing and shotgun metagenomic sequencing methods need bioinformatics tools and reference datasets that are still under development and not yet readily available for clinical practice. The GA-map™ technology provided us with a commercially available and clinically validated (in adults) tool.

Our study has several limitations. Firstly, the sample size is limited, reducing the statistical power to detect differences in microbiota composition as statistically significant. We did not adjust for multiple testing as we considered this study to be exploratory, increasing the risk for accepting false positive associations. However, we validated our results by splitting our data into a training and a test set, and most associations estimated in the whole cohort remained statistically significant. The positive relationship between inflammation, increased abundance of pathobionts and concomitant loss of beneficial bacteria, is reassuring as it is in line with previous research reports.[50]

Another limitation is the difference in storage time of the fecal samples which may have influenced outcomes. Also, theoretically, the representativeness of the

samples could have deteriorated during the timespan from collection until frozen. Based on previous experience and in vitro examinations[18], the microbial material collected in the different cohorts was not considered to be affected. Since repeated thawing is known to influence the microbiota, the samples were kept frozen until analysis.

We acknowledge that the GA-map™ technology test measures the abundance of bacteria without giving information about the functional importance, and high abundant bacteria might not be functionally active.[51] Additionally, in the present study we explored the fecal microbiota only. One study comparing mucosal associated microbiota with fecal microbiota reported that the ileal mucosa, followed by the rectal mucosa obtained the best performance in classifying CD and that stool samples performed less well. [22] Mucosa associated microbiota must be sampled by invasive methods. In this study however, we wanted to non-invasive methods to associate microbiota with disease state. Our findings show promise for microbiota profiles and abundance to be used in conjuncture with other prognostic factors and known biomarkers in an attempt to risk stratify and individualize treatments in pediatric IBD.

Conclusions

Fecal microbiota profiles similarly differentiated IBD and non-IBD symptomatic children from healthy children. Microbiota profiles with relative enrichment of Proteobacteria and low abundance of *Faecalibacterium Prausnitzii,* in newly diagnosed pediatric IBD seem to be associated with complicated disease phenotypes, subsequent need of biologic therapy, surgery and non-mucosal healing. The dysbiosis persisted after therapy, regardless of treatments and remission status. The relative abundances of selected bacteria might be of value as prognostic markers in stratifying pediatric IBD into subgroups and aid in patient selection for early aggressive therapy in an effort to prevent a complicated disease course.

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Author contributions

All authors have made substantial contributions in conception and study design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content. All authors have seen and approved the final version of the manuscript submitted to the journal and agree to be accountable for all aspects of the work.

CO and GP contributed in planning the study, collecting data, analyzing and interpreting the results and drafting the article. *MCS, BN, ETE* and *MHV* contributed in planning the study, analyzing and interpreting the results and drafting the article.

Disclosures

Christine Olbjørn is a member of the advisory board of AbbVie, has received speaker honoraria from AbbVie, Nutricia, Norgine, Tillotts Pharma and Mead Johnson. Morten H Vatn has been an advisor for Genetic Analysis and organizer of the International Advisory Board of Genetic Analysis, a member of the advisory board for Tillotts Pharma, and has received speaker honoraria from AstraZeneca, AbbVie, MSD and Falk. Gøri Perminow is a member of the advisory board of AbbVie and is a member of the steering committee in the IBSEN III study. The IBSEN III Study has received an Investigator Initiated Research Grant from Takeda, and non-restricted research grants from Ferring Pharmaceuticals and Tillotts Pharma. Christina Casén and Magdalena K Karlsson are employed by Genetic Analysis. For the remaining authors, none are declared.

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TABLE AND FIGURE LEGENDS

Table 1: Disease extent and behavior in pediatric IBD patients at diagnosis according to the Paris classification.

Table 2. Demographics and laboratory tests of IBD, non-IBD patients and healthy controls at baseline.

Figure 1: *Faecalibacterium prausnitzii* abundance in IBD patients according to whether they had received antibiotics prior to the diagnosis (measured in fluorescence signal strength in 1000 units).

Figure 2: Boxplot illustrating the differences in the total fluorescence signal strength measured in 1000 units between the IBD, non-IBD symptomatic patients and healthy controls.

Figure 3: Principal component analysis, illustrating the distribution of the microbiota abundance of all 54 bacterial probes between IBD, non- IBD symptomatic patients, and healthy controls. Each dot represents one individual. The units represent the total item loadings on each of the extracted factors.

Figure 4: Sensitivity and specificity of Proteobacteria, *Enterobacteriaceae* and *Shigella/Escherichia* abundance in differentiating Crohn's disease phenotypes

(stricturing/penetrating versus inflammatory disease behavior) using the area under the receiver operator characteristics curve (AUROC) analysis.

Figure 5: Sensitivity and specificity of Firmicutes and *Mycoplasma hominis* abundance in differentiating conventional versus biologic therapy treated IBD patients using the area under the receiver operator characteristics curve (AUROC) analysis.

Figure 6: Baseline Proteobacteria abundance in IBD patients according to whether they needed surgery or not (measured in fluorescence signal strength in 1000 units).

Supplementary Table 1. List of phyla and bacterial names of the GA-map™ technology markers

Table 1. Disease extent and behavior at diagnosis according to the Paris classification and treatments in pediatric IBD patients

IBD Diagnosis	**n (%)**
CD	80 (73)
UC	27 (25)
IBDU	3 (3)
CD behavior	
B1. Inflammatory	53 (66)
B2. Stricturing	12 (15)
B3. Penetrating	15 (19)
CD distribution	
L1. Ileal	5 (6)
L2. Colonic	24 (30)
L3. Ileocolonic	47 (59)
L4. Upper gastrointestinal	54 (68)
P. Perianal	17 (21)
UC/IBDU disease extent	
E1. Proctitis	5 (17)
E2. Left sided colitis	8 (27)
E3/E4. Extensive/total colitis	17 (57)
Treatment	
Immunomodulators	12 (89)
Biologic therapy	64 (58)
Surgery	17 (15)

Abbreviations: IBD, inflammatory bowel disease; CD, Crohn's disease; UC, Ulcerative colitis; IBDU, inflammatory bowel disease unclassified

Table 2. Demographics and laboratory tests of IBD, non-IBD patients and healthy controls at baseline

Variable	**CD**	**UC**	**IBD (CD+UC+IBDU)**	**Non-IBD**	**Healthy**
Patients, n (%)	80 (100)	27 (100)	110 (100)	50 (100)	75 (100)
Age in years, median (range)	13 (0.74-17.9)	11.5 (4-17)	12.5 (0.7-17.9)	12 (3.7-18)	10 (2-17.9)
Males, n (%)	43 (54)	11 (41)	56 (51)	18 (36)	34 (45)
PCDAI/PUCAI, median (range)	20 (0-62.5)	40 (0-75)	-	N/A	N/A
Fecal calprotectin mg/kg, median (range)	589 (20-8625)	987 (11-6123)	701	47 (9-1260)	15 (0-50)
Fecal calprotectin >1000 mg/kg, n (%)	31 (39)	12 (48)	43 (40)	2 (4)	0

Abbreviations: IBD, inflammatory bowel disease; CD, Crohn's disease; UC, Ulcerative colitis; IBDU, inflammatory bowel disease unclassified; PUCAI, Pediatric ulcerative colitis activity index; PCDAI, Pediatric Crohn's disease activity index; N/A, not applicable.

Figure 1: *Faecalibacterium prausnitzii* abundance in IBD patients according to whether they had received antibiotics prior to the diagnosis (measured in fluorescence signal strength in 1000 units).

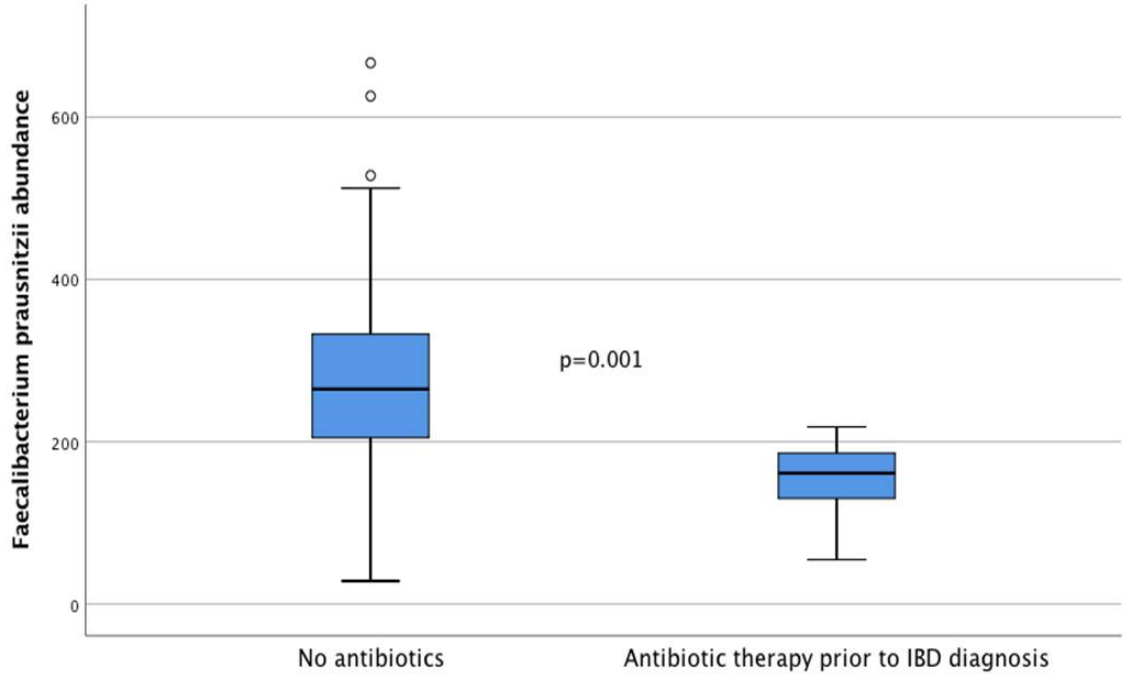
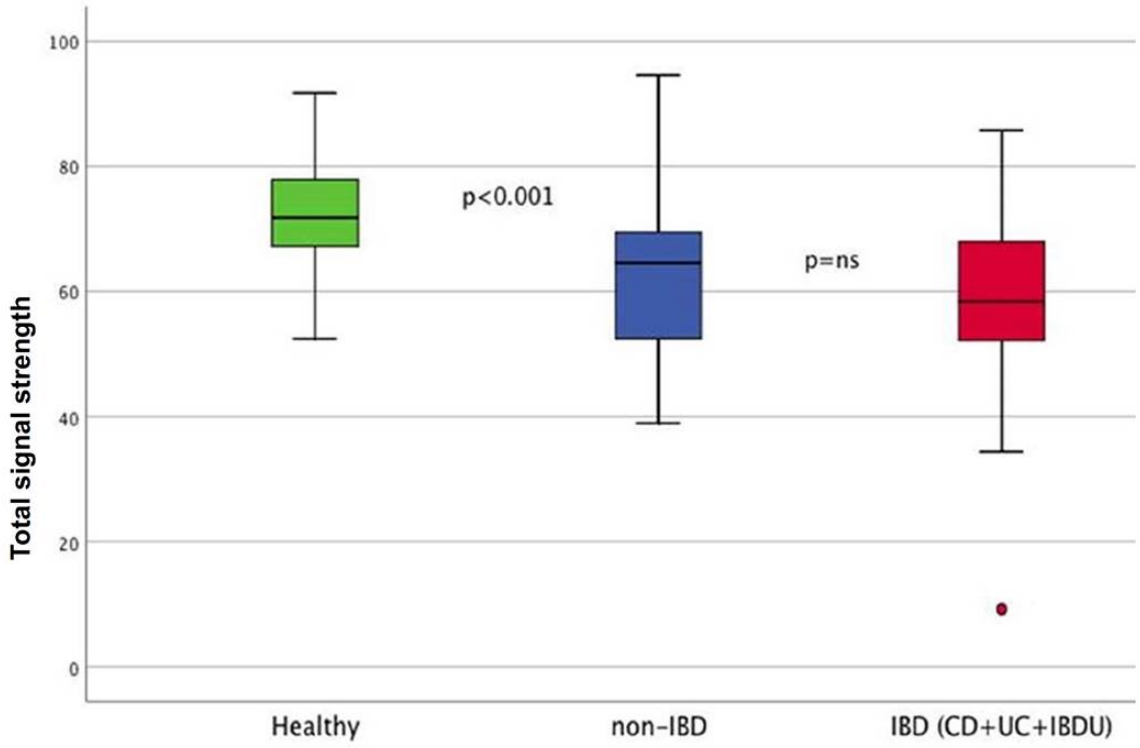
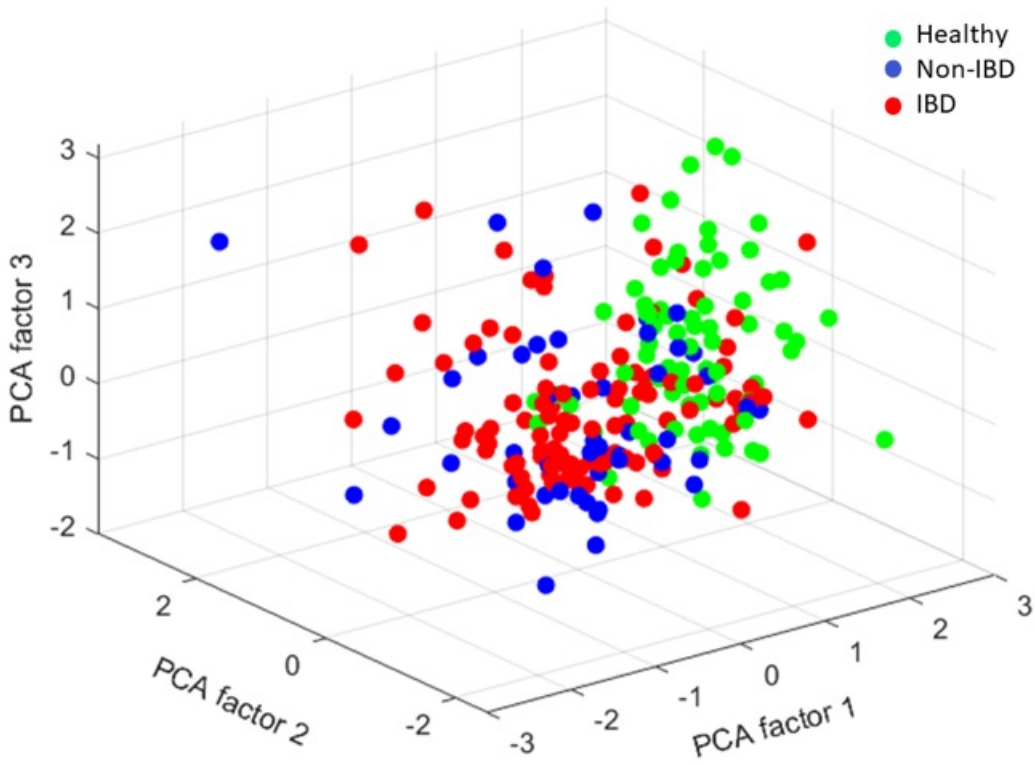


Figure 2. Boxplot illustrating the differences in the total fluorescence signal strength measured in 1000 units between IBD, non-IBD symptomatic patients and healthy controls.



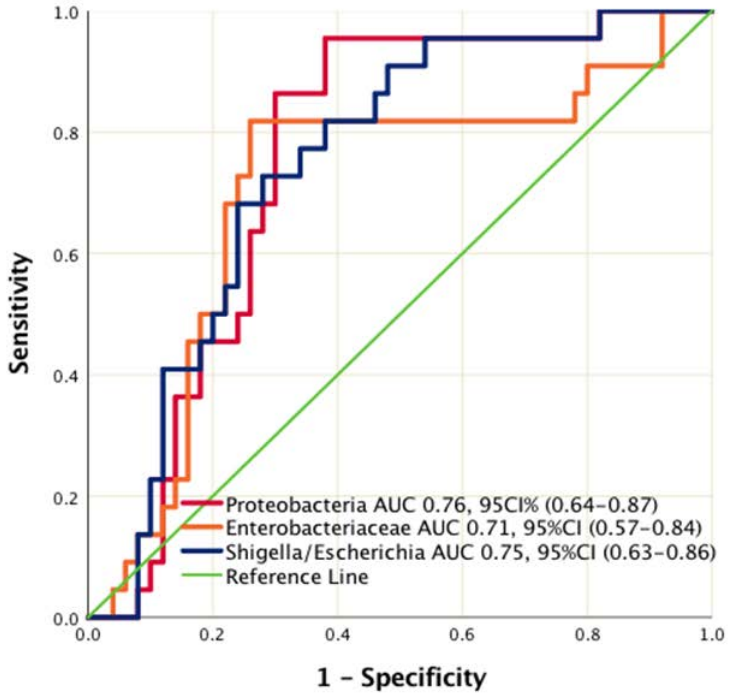
Abbreviations: IBD, inflammatory bowel disease; CD, Crohn's disease; UC, Ulcerative colitis; IBDU, inflammatory bowel disease unclassified; ns, not significant

Figure 3. Principal component analysis, illustrating the difference in microbiota abundance of all 54 bacterial probes between IBD, non- IBD symptomatic patients, and healthy controls. Each dot represents one individual. The units represent the total item loadings on each of the extracted factors.



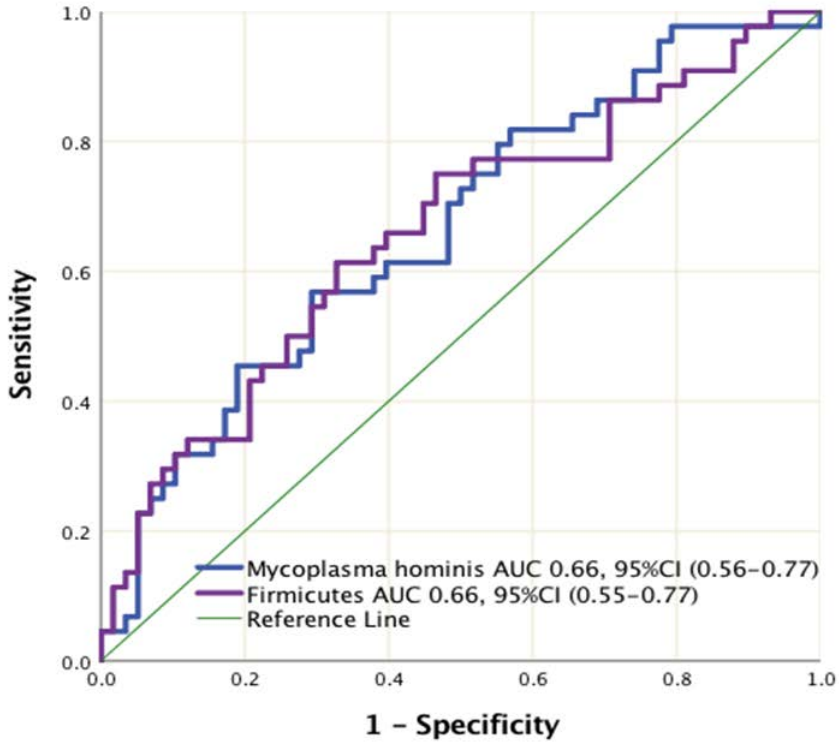
Abbreviations: IBD, inflammatory bowel disease; PCA, principal component analysis

Figure 4: Sensitivity and specificity of Proteobacteria, *Enterobacteriaceae* and *Shigella/Escherichia* abundance in differentiating Crohn's disease phenotypes (stricturing/penetrating versus inflammatory disease behavior) using the area under the receiver operator characteristics curve (AUROC) analysis.



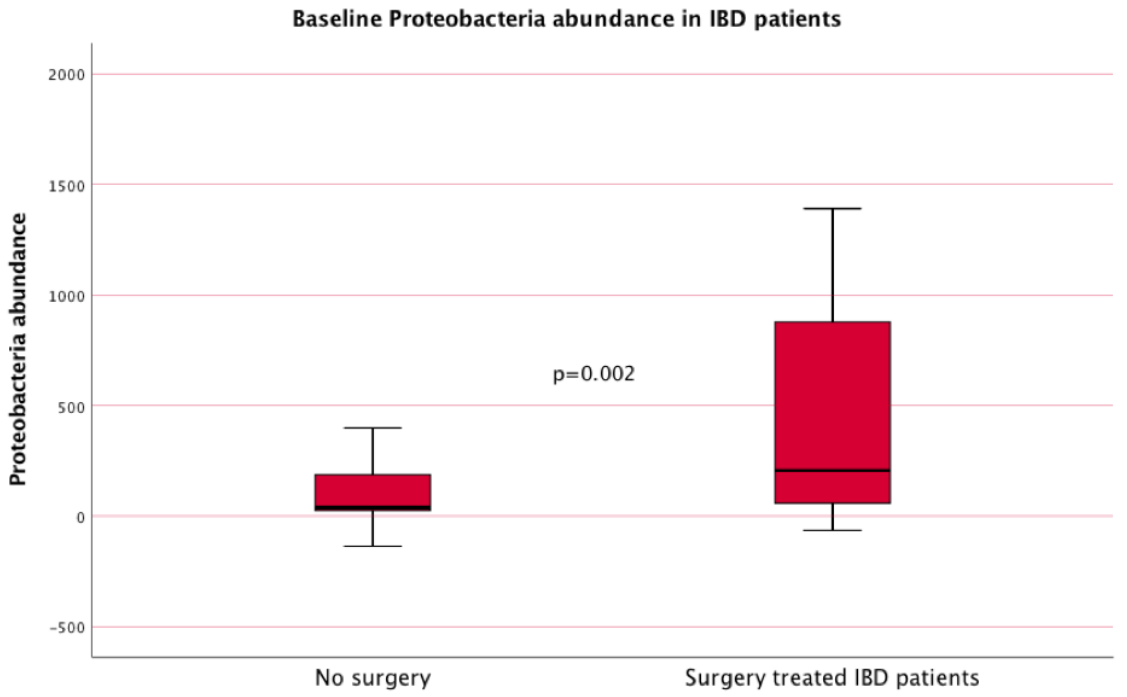
Abbreviations: CD, Crohn's disease; AUC, area under the curve

Figure 5: Sensitivity and specificity of Firmicutes and *Mycoplasma hominis* abundance in differentiating conventional versus biologic therapy treated IBD patients using the area under the receiver operator characteristics curve (AUROC) analysis.



Abbreviations: IBD, Inflammatory bowel Disease; CD, Crohn's disease; AUC, area under the curve

Figure 6: Proteobacteria abundance in IBD patients according to whether they needed surgery or not (measured in fluorescence signal strength in 1000 units).



Supplementary Table 1. List of phyla and bacterial names of the GA-map™ technology markers

Bacteria number	Phylum	Name
100	Actinobacteria	Actinobacteria
101	Actinobacteria	Actinomycetales
102	Actinobacteria	Atopobium rimae
103	Actinobacteria	Bifidobacterium spp.
201	Bacteroidetes	Alistipes
202	Bacteroidetes	Alistipes onderdonkii
203	Bacteroidetes	Bacteroides fragilis
204	Bacteroidetes	Bacteroides pectinophilus
205	Bacteroidetes	Bacteroides spp.
206	Bacteroidetes	Bacteroides spp. & Prevotella spp.
207	Bacteroidetes	Bacteroides stercoris
208	Bacteroidetes	Bacteroides zoogleoformans
209	Bacteroidetes	Parabacteroides johnsonii
210	Bacteroidetes	Parabacteroides spp.
211	Bacteroidetes	Prevotella nigrescens
300	Firmicutes	Firmicutes*
301	Firmicutes	Anaerotruncus colihominis
302	Firmicutes	Bacilli
303	Firmicutes	Bacillus megaterium
304	Firmicutes	Catenibacterium mitsuokai
305	Firmicutes	Clostridia
306	Firmicutes	Clostridium methylpentosum
307	Firmicutes	Clostridium sp.
308	Firmicutes	Coprobacillus cateniformis
309	Firmicutes	Desulfitispora alkaliphila
310	Firmicutes	Dialister invisus
311	Firmicutes	Dialister invisus & Megasphaera micronuciformis
312	Firmicutes	Dorea spp.

313	Firmicutes	Eubacterium biforme
314	Firmicutes	Eubacterium hallii
315	Firmicutes	Eubacterium rectale
316	Firmicutes	Eubacterium siraeum
317	Firmicutes	Faecalibacterium prausnitzii
318	Firmicutes	Lachnospiraceae
319	Firmicutes	Lactobacillus ruminis & Pediococcus acidilactici
320	Firmicutes	Lactobacillus spp.
321	Firmicutes	Lactobacillus spp. 2
322	Firmicutes	Phascolarctobacterium sp.
323	Firmicutes	Ruminococcus albus & R. bromii
324	Firmicutes	Ruminococcus gnavus
325	Firmicutes	Streptococcus agalactiae and Eubacterium rectale
326	Firmicutes	Streptococcus salivarius ssp. thermophilus and sanguinis
327	Firmicutes	Streptococcus salivarius ssp.thermophilus
328	Firmicutes	Streptococcus spp.
329	Firmicutes	Streptococcus spp. 2*
330	Firmicutes	Veillonella spp.*
331	Firmicutes/Tenericutes/Bacteroidetes species	Firmicutes (various)*
500	Proteobacteria	Proteobacteria
501	Proteobacteria	Acinetobacter junii
502	Proteobacteria	Enterobacteriaceae*
503	Proteobacteria	Pseudomonas spp.
504	Proteobacteria	Shigella spp. & Echerichia spp.
601	Tenericutes	Mycoplasma hominis
701	Verrucomicrobia	Akkermansia muciniphila